# THE ROLE OF MATURATION DESICCATION IN REGULATION OF GENE EXPRESSION IN PLANT SEEDS

by

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The role of maturation desiccation in regulation of gene expression in plant seeds

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# Abstract

The final stage of development of most plant seeds is maturation drying, a process often accompanied by a dramatic decline in storage-protein synthesis. When desiccation is imposed prematurely at certain stages prior to the completion of development, a switch in synthetic events is elicited: events unique to development, such as synthesis of storage protein, are terminated, while syntheses of proteins associated with germination and growth are initiated. To determine whether desiccation plays a key role in effecting a decline in expression of genes for storage proteins by acting directly upon the regulatory regions of these developmental genes, the desiccation responsiveness of the 5' and 3' regulatory regions of the genes encoding the pea storage protein vicilin and the *Brassica napus* storage protein napin was tested in transgenic tobacco. Chimeric genes were introduced into tobacco; these genes consisted of the coding region of the reporter gene for β-glucuronidase (GUS) and 5' and/or 3' regions from the vicilin or napin genes. As controls, the same regions derived from constitutively expressed genes, presumed to be desiccation insensitive were used. In transgenic seed expressing the gene constructs containing the vicilin or napin promoters, GUS activities declined during late seed development, and more dramatically, after imbibition of mature dry seed or prematurely dried seed. In contrast, GUS activities increased after seed rehydration when the constitutive 35S CaMV promoter replaced the storage-protein gene 5' region. Transient expression assays support the hypothesis that premature drying down-regulates the expression of the storage protein gene promoter. Following desiccation, this region may become insensitive to positive controlling factors; alternatively, changes to *trans*-acting factors may occur.

The possible interaction between desiccation and abscisic acid (ABA) was also studied at the molecular level using transgenic tobacco seeds. ABA caused a 2-3 fold increase in GUS activity in developing transgenic seed expressing chimeric gene constructs containing the vicilin or napin promoters. However, when these seeds were prematurely dried prior to their culture on ABA, enhancement of GUS activity was virtually abolished. Chimeric gene constructs containing the CaMV 35S promoter did not respond to ABA, whether premature drying was applied or not. Thus, following drying, vicilin and napin chimeric genes, initially sensitive to ABA, become relatively insensitive to the hormone.

The effect of premature drying on the gibberellic acid (GA)-sensitivity of aleurone layer cells of developing barley grain was also investigated. Premature drying of 30 days after anthesis (DAA) developing grain led to the acquisition of GA-responsiveness, resulting in increased α-amylase production and secretion and the induction of mRNA for α-amylase. Transient expression of chimeric gene constructs in aleurone layer cells suggests that maturation drying up-regulates the α-amylase gene promoter in response to GA.

# Dedication

To My Parents, My Aunt, My Grandmother

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## Abbreviations

| | |
|---|---|
| ABA | abscisic acid |
| ABREs | ABA-responsive *cis*-acting elements |
| CaMV | cauliflower mosaic virus |
| DAA | days after anthesis |
| DAF | days after flowering |
| DAI | days after imbibition |
| DAP | days after pollination |
| *Em* | Early methionine-labelled protein in wheat |
| GA | gibberellic acid |
| GUS | β-glucuronidase |
| *LEAs* | late embryogenesis abundant genes |
| *Lux* | luciferase |
| MS | Marashige and Skoog medium |
| MU | methylumbelliferone |
| MUG | 4-methyl umbelliferyl glucuronide |
| Nos | nopaline synthase |
| S | sedimentation coefficients (S values) |
| X-gluc | 5-bromo-4-chloro-3-indolyl glucuronide |

*Chapter 1*

# General Introduction

## Role of Maturation Desiccation in the Transition from Seed Development to Germination

## 1.1. General Events Associated with Seed Development and Germination

Angiosperm embryo development can be divided conveniently into three confluent stages. Initially, during histodifferentiation, the single-celled zygote undergoes extensive mitotic division and the resulting cells differentiate to form the basic plan of the embryo composed of the axis and cotyledons; concurrently, the triploid endosperm is formed. Thereafter, during seed expansion, there is generally no further cell division, the predominant events being cell expansion and deposition of reserves (normally proteins, with lipids or carbohydrates) primarily in the storage tissues (i.e. cotyledons or endosperm). Development of orthodox seeds (i.e. seeds that normally undergo maturation desiccation proceed to germination) is generally 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. Upon imbibition of the mature seed, there is a reactivation of existing metabolic systems, supplemented by syntheses of new components, which leads to renewed cell expansion (elongation of the radicle) and cell division as the seedling becomes established. Seed development and germination are distinct physiological stages of the plant life cycle, in which key metabolic events related to the status of stored reserves contrast markedly. Metabolism during seed development is largely anabolic, being characterized by the massive synthesis and deposition of polymeric reserves within the storage tissues. After germination, mobilization of stored reserves is an essential component of growth, and hence quantitative and qualitative changes in catabolic enzymes, particularly in the storage organs, are commonly observed (Kermode, 1990; Bewley and Black, 1994). The products of these degradative processes are utilized as both the substrate and energy sources for the growing seedling. These distinct metabolic events implicate the

involvement of a 'switch', which effects the transition from development to germination; it is normally triggered in a precise temporal fashion to allow completion of developmental events which are important for successful germination and survival of the young seedling (e.g. the accumulation of sufficient reserves) (Bewley and Black, 1994).

Many important questions are being addressed concerning the control processes involved in embryogeny and germination. There are two major areas of study. (1) What are the important factors or regulatory 'cues' which maintain embryos in a developmental state and prevent them from undergoing premature transition to germinative events? (2) How are these 'developmental' regulatory cues terminated in the mature dispersed progeny (the dry seed), such that a transition to germination events occurs and a continuation of developmental events is prevented? The former area of study has focused upon the role of the seed or maternal environment in maintaining embryos in a developmental mode until they are fully formed and have accumulated sufficient reserves to permit successful germination and subsequent seedling establishment. These studies have implicated two potential regulatory factors: abscisic acid (ABA) and restricted water uptake (reviewed in Kermode, 1990, 1995; Jiang and Kermode, 1994; Skriver and Mundy, 1990; Black, 1991; Galau *et al.*, 1991). There is now substantial evidence that maturation drying is critical in terminating development and overcoming constraints by the maternal environment that maintain seeds in a developmental mode (reviewed in Kermode and Bewley, 1986a, 1987; Kermode *et al*,. 1986, 1989b; Kermode, 1990, 1995; Jiang and Kermode, 1994).

An important experimental approach that has been used by several researchers to define the role of desiccation in terminating development and eliciting germination

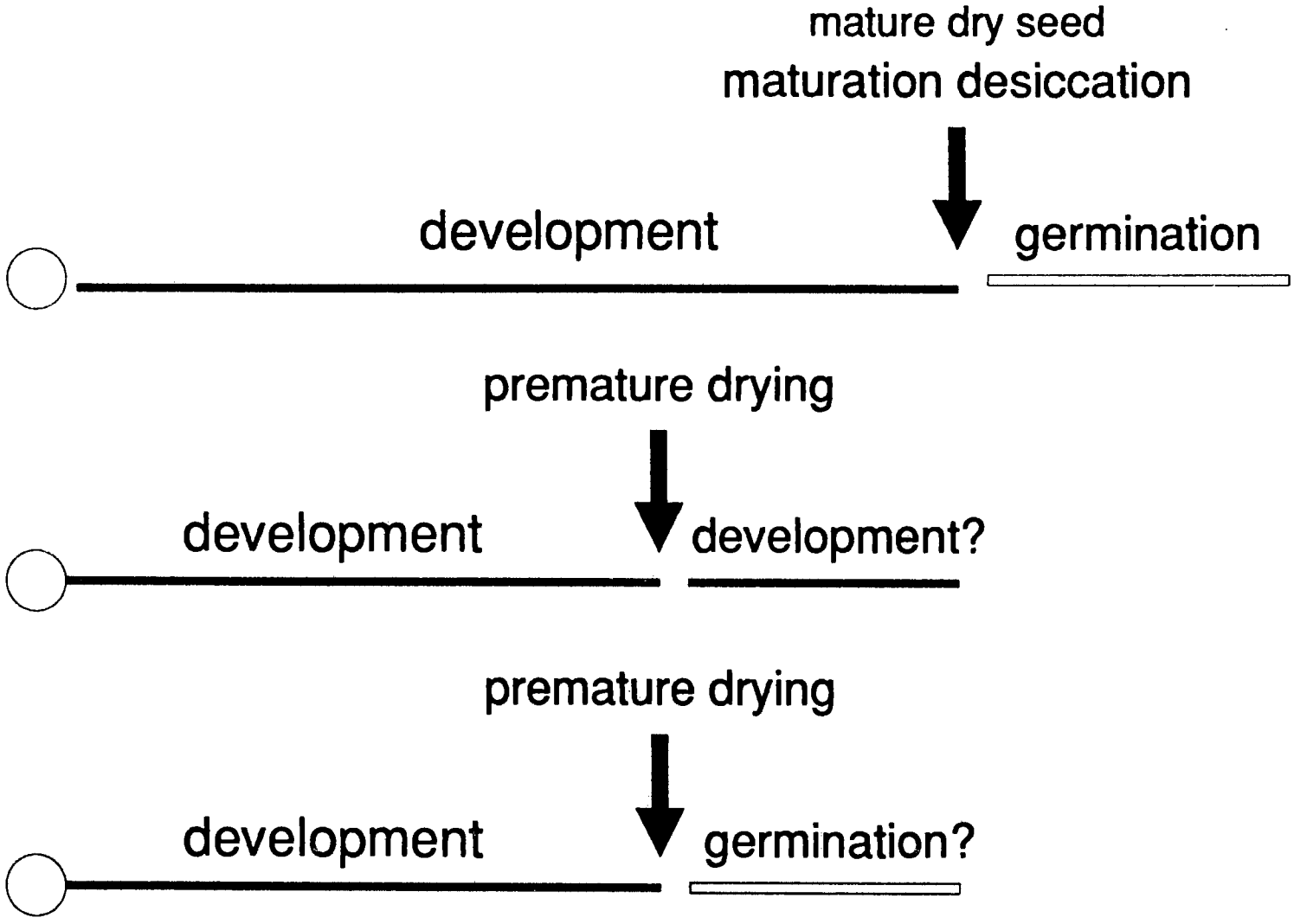
is to subject the developing seed to premature desiccation, i.e. to remove the seed from the mother plant before its normal development is completed and determine the effect of an artificially imposed drying period (Fig. 1.1). The fundamental questions that have been addressed using this approach are: (1) If a seed is dried prematurely, upon subsequent rehydration, is it's metabolism still geared to resume those developmental events that were incomplete at the time of desiccation? (2) Does the prematurely imposed drying period cause the cessation of developmental events and lead, upon subsequent rehydration, to a metabolic program associated exclusively with germination and growth? When desiccation is imposed prematurely at certain stages prior to the completion of development, a switch in metabolic events is elicited: syntheses unique to development, for example, storage-protein synthesis, are terminated, while syntheses associated with germination and post-germinative growth are initiated. In effecting this switch in gene expression, desiccation appears to act primarily at the transcriptional and post-transcriptional levels (see later discussion).

## 1.2. Seed Development and Seed Storage Proteins

Seed development and germination are distinct physiological stages of the plant life cycle, in which key metabolic events related to the status of stored reserves contrast markedly. Generally, development is characterized by the rapid accumulation of storage reserves, including proteins and lipids or carbohydrates. In contrast, germination and subsequent seedling growth is characterized by the mobilization of these stored reserves. Because of their importance as food sources and the fact that developing seeds synthesize large quantities of only a few proteins in a strict temporal sequence and tissue-specific manner, storage proteins have become an unique subject and model system for studying gene expression in developing seeds.

**Figure 1.1.** Premature drying used as an approach to study the role of maturation desiccation. The questions being addressed are: (1) If a seed is dried prematurely, upon subsequent rehydration, is it's metabolism still geared to resume those developmental events that were incomplete at the time of desiccation? (2) Does the prematurely imposed drying period cause the cessation of developmental events and lead, upon subsequent rehydration, to a metabolic program associated exclusively with germination and growth?

# "Premature Drying" vs. Maturation Desiccation



### 1.2.1. Seed Storage Proteins and Their Genes

Seed storage proteins are the major seed proteins. They are synthesized at certain stages of development and are stored in membrane-bound organelles (protein bodies) in storage parenchyma cells of cotyledons or the endosperm. These proteins are rapidly hydrolyzed upon germination to provide nutrient sources for the early stages of seeding growth.

Generally, seed storage proteins can be divided into four major classes based on their solubility in various solutes: they are *albumins*, *globulins*, *prolamins*, and *glutelins*. *Albumins* can be dissolved in water and dilute buffers; *globulins*, which include the legumin group (12S) and the vicilin group (7S), are salt soluble. *Glutelins* and *prolamins*, mainly from monocots, are soluble in dilute acid or alkali solutions and aqueous alcohol, respectively. In maize endosperm, albumins and globulins represent about 6% of the total proteins, while the prolamins and glutelins account for about 50-55% and 30-45%, respectively (Murphy and Dalby, 1971). On the other hand, the major storage proteins of dicotyledonous plants are globulins and albumins (Higgins, 1984), while the prolamins are the major class in most cereal seeds (Bewley and Black, 1994).

Research on seed storage proteins shows that they have several features in common. First, storage proteins are usually encoded by several non-homologous multigene families that vary in size, organization and chromosomal location. For example, there are at least three families and six members of each family of genes encoding the Mr 50,000 subunit of vicilin (a 7S globulin), indicating as many as 18 genes for the Mr 50,000 family (Chandler *et al.*, 1984). Second, their genes are not selectively amplified or structurally altered during seed development. Most storage

proteins are composed of regularly assembled subunits held together noncovalently. Lastly, storage proteins are usually deposited in protein bodies, the special cellular organelles that range in diameter from 0.1 to 25 μm and are surrounded, at least during development, by a single membrane.

Storage proteins of pea and soybean are highly homologous. For example, they share a similar disulfide-bonded subunit structure, undergo similar posttranslational proteolysis of a precursor containing both α- and β-subunits, and possess similar molecular properties (Sengupta *et al.*, 1981; Turner *et al.*, 1983; Gatehouse *et al.*, 1983). This supports the theory of the similarity of storage proteins throughout the Leguminosae and suggests that most major legume storage proteins conform to either a legumin or vicilin type of molecule (Gatehouse *et al.*, 1983).

### 1.2.2. Regulation of Storage Protein Gene Expression

An analysis of the changes in mRNA subsets during embryogenesis indicates that the messenger RNAs detected during seed development and germination can be divided into seven groups (Dure, 1985). 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 mRNA; (5) those which are late embryogenesis-specific (*lea* mRNA); (6) those which are expressed during late embryogenesis and early germination; (7) those which are germination/growth-specific such as isocitrate lyase mRNA. Among them, the seed storage protein genes are the most extensively studied. Some general characteristics are shared by seed storage protein genes in relation to

their expression (Bewley and Black, 1994). These include: (1) Most storage protein genes are either single-copy genes, or there are no more than four or five copies per haploid genome; (2) All storage proteins have a leader sequence for vectoral discharge through the ER membrane; (3) Generally, the expression of storage protein genes is temporally and spatially regulated during seed development and their expression is seed-specific and regulated spatially within embryonic organ systems; (4) Their expression is often cell - or tissue-specific, i.e. localized within specific cells or tissues, such as the embryo and endosperm; (5) Conserved sequences within the 5' upstream regions of genes of similar proteins can be recognized which confer tissue and temporal specificity or hormone sensitivity; (6) Expression of some storage protein genes is regulated by plant hormones (at least *in vitro*), particularly abscisic acid (ABA).

Gene expression in eukaryotes can be regulated at various levels: (1)genomic control (selective loss or amplification of genetic information prior to expression); (2) transcriptional control (selective transcription of genetic information into nuclear RNA); (3) post-transcriptional control (selective processing of transcripts into functional RNA, selective transport of nuclear RNA species to the cytoplasm and/or selective maintenance or degradation of transcripts); (4) translational control (selective translation of cytoplasmic mRNA molecules into polypeptide products); and (5) post-translational control (regulation of polypeptide assembly, biochemical protein modifications, protein stability and activity, and protein targeting).

Studies on expression of storage protein genes *in vivo* and *in vitro* have shown that the primary control of gene expression is at the level of transcription even though a number of cases of post-transcriptional regulation do exist. In addition, there is some evidence which suggests the possible involvement of translational and post-

translational controls over the expression or synthesis of storage protein genes (Kermode, 1990).

### 1.2.2.1. Transcriptional and Posttranscriptional Controls

Seed storage proteins are only found in seeds, which clearly indicates that the regulation of storage protein genes is tissue-specific and temporally regulated. Generally, there are three groups of seed protein genes which are expressed only at specific stage(s) of seed development and germination/growth. They include development-specific storage protein genes, such as vicilin and legumin, late embryogenesis-abundant genes (*lea*) genes and germination/growth-specific genes encoding proteins involved in reserve mobilization, such as genes encoding the α-amylase enzyme in cereal grains.

In general, the amount of mRNA for storage proteins increases during development, to reach a peak at about the time of maximum protein deposition. Thereafter, the amount of mRNA declines, and so does the storage protein synthesis. For example, in pea and soybean, the changing patterns of mRNAs encoding the major storage protein genes parallel the accumulation of these proteins, indicating that regulation of these genes is primarily at the transcriptional level. This pattern of events suggests a simple transcriptional control of protein synthesis: essentially, the amount of storage protein produced is determined by the amount (and stability) of its mRNA (Bewley and Black, 1994).

The accumulation of a protein is a reflection of the equilibrium established between its rate of synthesis and rate of degradation. The rate of storage protein

synthesis largely determines the rate of accumulation since storage proteins are stable during the deposition phase prior to germination (Higgins, 1984). Each mRNA has a characteristic period during which it reaches a maximum level and then declines. In addition, there is a close correlation between the level of mRNA and the rates of synthesis of each of the polypeptides. This evidence indicates that the regulation of many storage protein genes is mainly at the transcriptional level.

The expression of some storage protein genes is also under posttranscriptional control during seed development. Most of the evidence lies on the inability to account fully for changes in steady-state mRNA levels with changes in the transcription rate, or changes in protein levels with changes in mRNA levels. For example, the major and minor legumin genes expressed in pea cotyledons are similar in their transcription rates, but the major legumin (legA) mRNA is inherently more stable than the minor legumin genes (legJ and legS) (Thompson *et al.*, 1989). A similar situation exists with regard to two hordein groups in barley. This suggests that transcripts of certain reserve protein genes are very labile within the developing seeds (Bewley and Black, 1994). Inter-organ differences in mRNA stability also exist. For example, the transcription rate of the soybean lectin gene is 10-fold higher in the embryo than in the root of the parent plant, but the protein in the embryo reaches a concentration about 2000-fold higher than the root (Walling *et al.*, 1986). This suggests that the expression of some storage protein genes is also under posttranscriptional control which involves mRNA stability and turn over.

### 1.2.2.2. Translational Control

The expression of some storage protein genes is also under translational control. For example, in soybean, the subunit composition for the 7S storage protein

(conglycinin) may change during development, in which the β subunit of the 7S protein is only synthesized several days after two of the α subunits, but the mRNAs for all of the α and β subunits of the 7S proteins are present at the earliest time, when only the α subunits are being synthesized (at 20d). This suggests that a translational control is involved in which mRNAs are prevented from being used for β subunit synthesis during early development (Gayler and Sykes, 1981; Bewley and Black, 1994).

Studies in alfalfa somatic embryos suggest that the developmental stage of embryogenesis can influence mRNA selection (Pramanik *et al.*, 1992). During early development of alfalfa somatic embryos, storage protein mRNAs are already present but there is no detectable accumulation of these proteins because these messages are preferentially present in the mRNP (mRNA-protein and ribosomal complex) fraction. In addition, messages for the 11S, 7S, and 2S storage proteins are selectively enriched in the mRNP fraction during the globular, heart, and torpedo stages of somatic embryogenesis, but these messages shift to polysomes by the cotyledon stage. Since mRNPs are an intermediate stage of mRNA translation and represent mRNAs in transit from the nucleus to the polysome, this suggests that translation of storage protein mRNAs is repressed at the early stage of somatic embryo development and that the synthesis of storage proteins does not occur until the embryos are developmentally ready.

As indicated by the above discussion, the expression of seed storage protein genes can be controlled at different levels. Results from *in vivo* and *in vitro* studies suggest that the regulation of seed storage protein genes is primarily under transcriptional and post-transcriptional control. In particular, during the early- to mid-stage of seed development, the primary controlling event in seed storage protein

synthesis is an increase in the transcriptional rate, even though post-transcriptional events such as mRNA stability play an important role in the differential accumulation of the products of a multigene family. Results from analyses of the 5' flanking regions of storage protein genes suggest that this region is responsible for the tissue-specific, temporal and spatial regulation, as well as for quantitative changes of transcriptional activity of the gene.

### 1.2.3. *Cis*-Acting Elements and *Trans*-Acting Factors

Transcriptional controls over storage protein gene expression have been studied via promoter analysis and mutagenesis *in vitro* and *in vivo*. It has been speculated that gene expression is regulated by interactions between the *cis*-acting elements located in the promoter region and the *trans*-acting factors (nuclear binding proteins). The 5' upstream regions of similar genes of different species contain similar sequences, which are important for tissue and temporal expression. For example, each of the legumin, vicilin, and prolamin genes of different species possesses conserved sequences in its 5' upstream region, i.e. the legumin, vicilin, and prolamin boxes, respectively. In addition, analysis of genes including β-conglycinin, β-phaseolin, and legumins have identified both positive and negative *cis*-acting elements determined by the deletion of sequences or introducing specific mutations in promoter fragments and expressing the genes in transgenic plants (Burrow *et al.*, 1992; Bustos *et al.*, 1991; Chen *et al.*, 1986; Fujiwara and Beachy, 1994). DNA-binding factors that recognize some of these important *cis*-acting elements, such as the RY-repeat and the G-box, in β-conglycinin and β-phaseolin genes have also been reported (Allen *et al.*, 1989; Bustos *et al.*, 1991; Kawagoe and Murai, 1992; Lessard *et al.*, 1991; Fujiwara and Beachy,1994). Some of these DNA-binding proteins are present only in seeds and only at times when storage protein gene expression is initiated. Hence, the ultimate

control of expression of these storage protein genes might lie in the production of these appropriate binding proteins at the right time and place (Bewley and Black, 1994).

### 1.2.4. Expression of the Vicilin Gene of Pea

In pea, the principal vicilin-type polypeptide subunits are the convicilin (70 kDa) and vicilin subunits (50 kDa). Vicilin proteins of pea are extensively cleaved into a series of small polypeptides. For example, vicilin subunits (50 kDa) of pea are cleaved into polypeptides of 34, 27, 25, and 12 kDa in size (Higgins, 1984). Pea seed development can be divided into two phases (Gatehouse *et al.*, 1983). The first phase, which occupies one-third of the development of the seed, is characterized by cell division with little synthesis of storage reserves. The second phase is featured by a dramatic increase in protein synthesis, most of this synthesis representing accumulation of the storage proteins. Although small amounts of storage proteins can be detected during the first phase, their rapid synthesis does not occur until about half-way through the development of the seed.

SDS-PAGE and rocket immunoelectrophoresis of protein extracts of developing seeds have been used to study the pattern of protein accumulation over the second phase of pea seed development. Vicilin subunits are present in larger amounts earlier in development than legumin and convicilin, indicating the existence of differential synthesis of different storage protein polypeptides. The accumulation of vicilin peaks at an early stage of the second phase and then decreases after about half-way through this phase (i.e. about 15 days after flowering). One of the vicilin subunits (50 kDa) disappears after the first half of the second phase which must represent a

cessation of synthesis since this subunit undergoes posttranslational proteolysis and will only be present while it is being actively synthesized (Gatehouse *et al.*, 1983).

The pattern of mRNA accumulation has been studied by Northern blot analysis using cDNAs encoding vicilin precursors. The vicilin mRNA is present in large amounts during the early stage of the second phase, corresponding to the period of rapid synthesis of the protein. It is barely detectable at 22 days. Probing with another vicilin cDNA which encodes the 50 KDa subunit showed that a maximum amount of the corresponding mRNA was present earlier in seed development and decreased to a small amount later (Boulter *et al.*, 1987; Beach *et al.*, 1985). These results demonstrate a direct correlation between specific mRNA levels and the rates of individual protein synthesis, i.e. increased rates of vicilin protein synthesis are a direct result of increased amounts of the vicilin mRNAs, suggesting the regulation is most likely at the transcriptional level, even though a direct correlation between transcription of vicilin genes and synthesis of their proteins is needed to support this.

The pea vicilin gene has been transferred and expressed in transgenic tobacco (Higgins *et al.*, 1988). In general, the expression of the introduced vicilin gene in tobacco seed is very similar to that found in pea plants, in terms of seed specificity, time of expression during embryogenesis, initiation site for transcription and susceptibility of the vicilin polypeptide to cleavage. These results suggest that the expression of the pea vicilin gene is correctly regulated in transformed tobacco plants and that the sequences controlling its expression are recognized by regulatory factors present in tobacco cells.

### 1.2.5. Expression of the Napin Gene of *Brassica napus* (oilseed rape)

In *Brassica napus*, there are two major storage proteins known as napin and cruciferin (Crouch *et al.*, 1985). Napin is a family of small, water-soluble proteins, each composed of disulfide bonded 13- and 4-kDa polypeptides, processed from a larger precursor (DeLisle and Crouch, 1989). In *Brassica napus*, napin starts to accumulate from about half-way through seed development (25 DAA) and constitutes about 20-30% of the total seed protein at maturity (60 DAA) (Crouch *et al.*, 1985). Napin mRNA is first detectable at 18 DAA and accumulates to a maximum of 8% of the total cellular mRNA population halfway through development, when the transcription rate is also high. It then declines and becomes nearly undetectable in the mature seed (60 DAA) (DeLisle and Crouch, 1989; Crouch *et al*, 1985). The distribution of napin and its mRNA in developing rape seed embryo has also been studied by immunohistochemical techniques and *in situ* hybridization, which confirm the spatial and temporal regulation of this gene in developing seeds (Hoglund *et al.*, 1992; Fernandez *et al.*, 1991).

ABA modulates the levels of napin storage protein and its mRNAs in cultured embryos. For example, immature embryos excised at the beginning of napin accumulation will continue to synthesize the protein and mRNA at levels comparable to that occuring in the seed developing *in planta* if embryos are cultured in medium with 1-10 μM ABA. The transcription rate of the napin gene also shows a similar increase in the presence of ABA. In contrast, in the absence of ABA, both napin mRNA and protein remain at a low level (Crouch and Sussex, 1981; DeLisle and Crouch, 1989).

Napin is encoded by a gene family of at least sixteen members (Blundy *et al.*, 1991). Genes encoding the 2S napin protein have been isolated and characterized (Baszczynski and Fallis, 1990; Ericson *et al.*, 1991; Josefeeon *et al.*, 1987; Scofield and Crouch, 1987). Some conserved sequences of the napin promoter regions are able to interact *in vitro* with DNA-binding proteins (*trans-acting* factors) from *Brassica napus* (Gustavsson *et al.*, 1991; Ericson *et al.*, 1991). The promoter of the napA gene was also analyzed using transgenic tobacco (Stalberg *et al.*, 1993). A 196-bp fragment between -152 and +44 is the minimum sequence required for seed-specific expression in transgenic tobacco.

### 1.3. Desiccation and the Switch from Seed Development to Germination

Seed development is characterized by the rapid accumulation of storage reserves, while germination is characterized by mobilization of these reserves. These distinct metabolic events implicate the involvement of a switch which effects the transition from development to germination. The final stage of development of orthodox seeds is maturation drying, a process often accompanied by a dramatic decline in storage-protein synthesis. When desiccation is imposed prematurely at certain stages prior to the completion of development, prematurely dried seeds are capable of germinating and a switch in synthetic events is elicited: events unique to development, such as synthesis of storage protein, are terminated, while syntheses associated with germination and growth are initiated.

### 1.3.1. Acquisition of Desiccation Tolerance and Germinability of the Whole Seed during Development

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 (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.) will germinate if dried on the straw, while their fresh counterparts (i.e. those of the same developmental age, 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; Long *et al.*, 1981; Dasgupta and Bewley, 1982; Kermode and Bewley, 1985a; 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. Premature drying of developing seeds during their desiccation-tolerant stage leads to germination upon subsequent imbibition.

The rate at which premature drying is achieved may determine the subsequent germinability of the seed. For castor bean seed, slow desiccation over several days is necessary for their survival soon after acquisition of desiccation tolerance, whereas more rapid drying can be withstood closer to the time of normal maturation drying

(Kermode and Bewley, 1985a). Although the underlying reasons for the differing responses to rapid and slow drying are not understood, rapid drying appears to lead to an increased rate of solute leakage through membranes upon imbibition (Adams *et al.*, 1983), suggesting that damage to cellular membranes is involved. It thus seems that not only is desiccation tolerance achieved at a particular stage of development, but tolerance of harsher drying regimes becomes greater as development proceeds. This is probably a consequence of morphological and physiological changes which take place with time of development, including the synthesis of protective proteins with hydrophilic properties during the later stages. These have been implicated in maintaining the structural integrity of cellular structures during dehydration (Dure *et al.*, 1989). There are some exceptions which must be accounted for, however, since the seeds of some Gramineae appear to be able to withstand rapid desiccation early during their development (Kermode, 1990). Perhaps these seeds undergo whatever changes are associated with the acquisition of desiccation tolerance at early stages in their development. The nature of these changes is probably quite diverse, and they are not simply restricted to the synthesis of specific proteins (reviewed in Kermode, 1990, 1995; Bewley and Oliver, 1992). For example, in addition to some of the proteins such as *LEA*s, other components including sugar and ABA have also been speculated to be important for seed to become desiccation tolerant (Bewley and Black, 1994).

Seeds of many species harvested early in development and allowed to dry are unable to germinate and eventually deteriorate - they are desiccation intolerant. Ultrastructural studies indicate that the inability of prematurely dried embryonic axes of *Phaseolus vulgaris* to germinate at the intolerant developmental stage is associated with rapid, irreversible damage to cellular membranes during drying, including mitochondrial and nuclear membranes (Dasgupta *et al* 1982). Correspondingly, desiccation-intolerant seeds fail to recover full metabolic activity upon imbibition.

Polysome recruitment and protein synthesis are both diminished. Similar damage is not apparent in rehydrated axes of desiccation-tolerant seeds.

In addition to the positive effects of desiccation on the germinability of whole seeds (at the desiccation-tolerant stages), in many cases premature drying also promotes the seedling's capacity for normal post-germinative growth (reviewed in Kermode, 1990).

Although developing whole seeds are usually incapable of germinating unless first dried, the developing embryos will germinate when isolated and placed in culture or on a liquid medium. It is likely, therefore, that drying overcomes an imposition on the embryo by the surrounding structures (the storage tissues, seed coat and maternal environment) releasing it from these constraints. Thus, desiccation causes the decline and/or negates the effect of regulatory cues which encourage development (e.g. ABA), thus permitting germination. However, the switch from a developmental to a germinative mode, both morphologically and metabolically, can be brought about in some seeds by treatments other than complete desiccation, such as partial drying, embryo isolation, and washing (Cornford *et al*,. 1986, 1987a; Kermode and Bewley, 1989; Garcia-Maya *et al*,. 1990). However, the quantitative and qualitative responses are sometimes different from those induced by drying. While maturation drying is normally expected to overcome the constraints on germination imposed on the developing seed/embryo, other treatments may reduce these constraints and thus trigger the switch.

'Orthodox' seeds undergo a period in a dry state which is interpolated between development and germination, but the seeds of some species can germinate without being dried (e.g. recalcitrant seeds, viviparous caryopses of mutant cereals, and

certain weed seeds). In recalcitrant species, dehydration subsequent to seed shedding leads to rapid loss of viability; the extent to which desiccation can be tolerated differs among plant species (Farrant *et al.*, 1988). The term recalcitrant emphasizes the difficulties inherent in the storage and handling of these seeds. In recalcitrant mangrove (Sussex, 1975) and other *viviparous* seeds, e.g. *viviparous* maize mutants (McDaniel *et al.*, 1977; Smith *et al.*, 1978; Robichaud *et al.*, 1980), reduction in sensitivity to, or in the amount of, ABA, may explain why these seeds pass from a developmental to a germinative mode without an intervening quiescent period.

### 1.3.2. Changes in Protein Synthesis in Response to Desiccation

Desiccation (when imposed during the tolerant phase of development) evokes a germination and growth response upon subsequent rehydration. Studies over the past decade in several laboratories have clearly demonstrated that this morphological response induced by premature drying is accompanied by a normal switch in the seed's metabolism, i.e. cessation of reserve synthesis and the onset of syntheses associated with germination and post-germinative growth. This is well illustrated in the endosperm of castor bean (*Ricinus communis* L.), where premature drying leads to a change in the polypeptide profile, as demonstrated using SDS-PAGE and *in vivo* protein synthesis, to one which is identical to that in germinating seeds and seedlings after normal maturation drying. After rehydration of prematurely dried seeds, soluble proteins characteristic of development (including the soluble lectin and albumin storage proteins) are no longer synthesized in detectable amounts (after 5 h), while others associated with germination and post-germinative reserve mobilization are induced (Kermode and Bewley, 1985b, 1986b; Kermode *et al.*, 1985). A similar change in direction of protein synthesis as a consequence of premature drying during

the desiccation-tolerant stages of development has also been demonstrated in other seeds, e.g. bean (*Phaseolus vulgaris*) (Dasgupta and Bewley, 1982; Misra and Bewley, 1985). Drying of developing P. *vulgaris* seed during the desiccation-intolerant stages of development, not surprisingly, leads to reduced protein synthesis upon rehydration and the eventual demise of the seed. Interestingly, though, the pattern of protein synthesis exhibited upon rehydration indicates a failure to complete a switch in direction of metabolism; instead, there is synthesis of proteins characteristic of both germination and development (Dasgupta and Bewley, 1982). This suggests that the competence of the seed to switch the mode of its synthetic events completely in response to drying is acquired simultaneously with the acquisition of desiccation tolerance.

Premature desiccation also elicits the production of enzymes essential to the post-germinative phase of seedling development. Adams *et al.* (1983) demonstrated that immature soybean seeds cannot produce malate synthetase and isocitrate lyase activities unless the seeds are first subjected to a slow-drying regime. Such is also the case in the castor bean endosperm. Desiccation, prematurely imposed at 40 DAP, causes an increase in activity of isocitrate lyase (an enzyme of the glyoxylate cycle involved in gluconeogenesis of lipid reserves) and LeuNAase (a proteolytic enzyme) to levels comparable to those produced after germination of the mature (60 DAP) seed (Kermode and Bewley, 1985b). In the absence of a drying period at 40 DAP, seeds with the capsule and testa removed, but maintained in the hydrated state, do not show an increase in the activity of the two enzymes.

Drying is critical to effect a clear transition of protein synthesis from a developmental to a germinative mode in maize embryos (Oishi and Bewley, 1992). Embryos isolated from developing kernels at 35 DAP germinate when incubated on

water; they also produce some developmental proteins during germination. Likewise, fluridone-induced *viviparous* germination of whole kernels is accompanied by the simultaneous synthesis of both developmental and post-germinative proteins (Oishi and Bewley, 1992), as occurs in some *viviparous* mutants of maize (Kriz *et al.*, 1990). Only premature desiccation (e.g. imposed at 35 DAP) elicits a pattern of protein synthesis upon rehydration which is similar to that in germinated embryos from mature (naturally dried) kernels (Oishi and Bewley, 1992).

Competence of the aleurone layer of cereals to produce α-amylase in response to GA is normally not acquired until late development; however, it can be induced prematurely after imposed drying, e.g. in wheat, triticale and maize (Evans *et al.*, 1975; King *et al.*, 1979; Armstrong *et al.*, 1982; Oishi and Bewley, 1990). This has been attributed to an increase in sensitivity of the aleurone layer to GA; a mechanism requiring desiccation-induced membrane changes and alterations to integral hormone receptors has been invoked (Norman *et al.*, 1982, 1983).

Other treatments (most of which do not result in substantial water loss) are also capable of inducing GA responsiveness in aleurone layers of developing cereal grain. These include temperature treatments (Norman *et al.*, 1982, 1983), maintenance in an atmosphere of high relative humidity (Cornford *et al.*, 1986, 1987a) and preincubation in water or a buffered-medium (immediately after detachment from the parent plant) for a period prior to the addition of the growth substance (Cornford *et al.*, 1986, 1987a). Thus, drying may not be the sole requirement for the acquired GA sensitivity of cereal aleurone layers; however, a mechanism simply involving seed detachment from the parent plant and a concomitant decline in the supply of sucrose (Nicholls, 1979, 1986) does not adequately explain the experimental results (reviewed in Kermode, 1990). For

example, the various treatments in which immature grain are detached and held moist are not equally effective in inducing a GA-responsive state and none is as effective as premature drying. Frequently, they do not elicit a 'normal' (full) response to GA, i.e. one similar in pattern and extent of an increase in α-amylase activity to that exhibited by the aleurone layer of mature grain which has undergone normal maturation drying (Cornford *et al.*, 1986, 1987a).

Speculations about what may be responsible for the change in sensitivity of the aleurone layer after drying in respect of α-amylase production include a decline in the concentration of growth regulator which is antagonistic to GA (i.e. ABA) (King, 1982; Cornford *et al.*, 1987a) and/or a decline in sensitivity to ABA.

### 1.3.3. Protein Synthesis Associated with Germination and Seedling Growth

Although the mature seed is metabolically quiescent, resumption of metabolism during germination commences within minutes of the introduction of water to the dry seed. It is generally acknowledged that early metabolic events utilize and depend upon components present within the dry seed, which are replaced as normal turnover events proceed during germination. For example, protein synthesis during very early hydration of mature seed utilizes some of the conserved mRNA (which persists during desiccation and is stored in the mature dry seed) ( reviewed in Bewley and Black, 1994; Kermode, 1990; Lane, 1991). However, within a few hours, new mRNAs are synthesized and as the conserved ones are degraded, protein synthesis leading to a completion of germination, probably becomes increasingly dependent on the newly synthesized messages. Some of the newly synthesized mRNAs may code for the same proteins as conserved messages, whereas others are for different products, perhaps proteins essential for the commencement of radicle

elongation and/or differentiation during early seedling formation (Bewley and Black, 1994; Lalonde and Bewley, 1986; Datta *et al.*, 1987). However, such proteins remain to be identified. Gene expression during germination and early seedling growth shows precise temporal and spatial regulation (Datta *et al.*, 1987; Allen *et al.*, 1988; Harada *et al.*, 1988; Kermode, 1990; Comai *et al.*, 1992). Regulatory controls over the synthesis of post-germinative proteins (e.g. those involved in reserve mobilization) appear to differ among species, and have one or more components, i.e. transcriptional (where *de novo* synthesis occurs after germination), translational (where stored messages are accumulated during development, but not translated until seedling growth) and post-translational (where mRNA and protein are present during development, but enzyme activity is not detected until post-germinative growth) (Marcus and Rodaway, 1982; Harada *et al.*, 1988; Krishna and Murray, 1988).

The ability of the seed to synthesize mRNAs for developmental proteins is retained during drying, although the rates at which their genes are transcribed decline with increasing water loss. It is upon rehydration that developmental genes are no longer transcribed, and only then is the germinative and post-germinative program of gene expression initiated (Comai and Harada, 1990).

Simple 'on-off' models of gene expression during development and germination are oversimplistic; genes also appear to be subject to an 'up/down' modulation (Weintraub and Izant, 1984; Dure, 1985). A residual pattern of developmental protein synthesis may continue for several hours during germination prior to the expression of a pattern unique to germination and growth (Kermode *et al.*, 1985, 1989c; Kermode and Bewley, 1986b). Presumably, the residual mRNAs present in the dry seed are utilized to direct this synthesis. Alternatively, some developmental genes may retain a transient 'transcriptional competence' (even while in a state of

'down-regulation', in which their full expression is suppressed), a lag being required for complete cessation of their expression during subsequent imbibition. In cotton, mRNA sequences for storage proteins can be detected in mRNA from 24-h-germinated cotyledons, albeit at about 1/1000 of their maximum in developing embryo cotyledons (Galau and Dure, 1981). Thus, genes that are expressed during development may be 'down-modulated' during desiccation and subsequent germination and growth, yet not returned to the 'architecturally off' mode. Some genes expressed during seed development can be induced in the vegetative plant, often under stress conditions, e.g. *Lea* genes (for late-embryogenesis-abundant proteins). In a similar fashion, genes once presumed to be unique to germination and growth may in fact be activated during embryogeny, although their full expression does not occur at this time (Harada *et al.*, 1988; Hughes *et al.*, 1988). In *Brassica napus* (rape), for example, spatially regulated post-germinative-abundant mRNAs (e.g. those involved in cotyledonary reserve mobilization) are also detected (albeit in low amounts) at distinct embryonic stages (Harada *et al.*, 1988).

### 1.3.4. Molecular Level of the Switch

Although there are important exceptions, changes in the extent of synthesis of storage proteins during seed development appear to be related to the amount of appropriate mRNAs available (Goldberg *et al.*, 1981a, 1981b), which is characteristic of regulatory processes effected primarily at the level of transcription (mRNA synthesis) and mRNA stability (reviewed in Higgins, 1984; Muntz, 1987; Goldberg *et al.*, 1989; Bewley and Marcus, 1990). The changes in protein synthesis from a developmental to a germinative/growth mode brought on by premature drying are indicative of a switch in genome activity. This results in a permanent suppression of developmental protein synthesis and induction of germination- and growth-related

proteins. The molecular level of control at which desiccation effects this switch in metabolic activity has been investigated (Misra and Bewley, 1985; Cornford *et al.*, 1986; Kermode *et al.*, 1989c) by examining the effects of premature desiccation and rehydration upon the fate and stability of the mRNA populations in the seed. A switch in message population is induced by premature desiccation and rehydration of *P. vulgaris* axes (at a tolerant stage of development). For example, the mRNAs coding for germination proteins are available for utilization in these rehydrated axes, messages which were not present either before drying or in the dry state (Misra and Bewley, 1985). Desiccation of *P. vulgaris* axes at 22 DAP (the intolerant stage) does not lead to any significant change in their complement of translatable messages. The mRNAs present upon rehydration code largely for developmental proteins; germination messages are not produced in detectable amounts. Thus, when the seed is still desiccation intolerant, drying neither elicits 'germination-gene' activity nor leads to a decline in developmental messages after rehydration.

In aleurone-layer cells from immature wheat grain, premature desiccation and rehydration induce the accumulation of mRNA coding for the post-germinative enzyme α-amylase in response to GA (Cornford *et al.*, 1986). However, this induction of GA responsiveness in immature aleurone-layer cells does not extend to the production of other enzymes associated with normal post-germinative growth, including acid phosphatase, protease and ribonuclease. This suggests that there is a differential sensitivity of the aleurone layer to desiccation, with sensitivity increasing as development progresses, because after normal maturation drying all these enzymes are synthesized post-germinatively.

Premature desiccation and rehydration of castor bean seeds induce several quantitative and qualitative changes in the translatable mRNA complement of the

endosperm (Kermode *et al.*, 1989c). In particular, messages coding for developmental proteins (putative storage-protein precursors) decline substantially during premature desiccation of developing seeds. Others increase in the endosperm during natural or imposed desiccation. After rehydration of the prematurely dried seed, the pattern of proteins synthesized *in vitro* by extracted mRNAs is similar to that carried out by messages from mature seed endosperms during and following germination. Most of the messages associated with development, which persist in the endosperm following natural or imposed desiccation, decline during early imbibition. During germination and growth of mature and prematurely dried seeds, the same complement of mRNAs arises in the endosperm, in a similar temporal sequence. A loss of some developmental (storage protein) mRNAs occurs when developing seeds are detached from the parent plant but prevented from undergoing desiccation by maintenance on water. This may suggest that a cessation of vascular supply from the parent plant contributes to the decline in storage-protein mRNAs during late maturation. Seed detachment in the absence of water loss does not elicit any changes in mRNAs which are indicative of a switch from a developmental to a germinative mode (Kermode *et al.*, 1989c).

To summarize, it is clear that drying ultimately affects transcription (i.e. mRNA synthesis) after desiccation, be it premature or during the final stages of maturation. The production of messages for developmental proteins is suppressed, whereas mnessages for germinative and growth proteins increase upon subsequent rehydration. Thus, desiccation effects an off- or down- regulation of developmental messages and an on-regulation of messages for germination and growth. However, it is equally clear that all of the changes in the message population, from those characteristic of development to those specific to germination or growth, do not occur during drying *per se* (Kermode *et al.*, 1989c: Kermode 1990, 1995). Messages for

germination appear after hydration of the dry seed (after a lag of a few to several hours), and the loss of (or substantial decline in) developmental messages is largely completed at this time. Thus, while most developmental messages may decline during drying itself (which may be due to a direct effect of water loss on the rate of transcription of these genes), hydration appears to be the crucial event leading to the loss of any residual developmental messages that remain stable throughout desiccation. These messages are presumably degraded upon rehydration by the normal turnover processes and are not replaced, because their genes have been off- or down-regulated.

### 1.3.5. Cessation of Developmental Protein Synthesis: A Key Role for Desiccation?

It appears that premature desiccation leads to a cessation of developmental protein synthesis and effects qualitative and quantitative changes at the mRNA level upon subsequent rehydration. In some species, the natural drying period may play a similar role (*in situ*), precipitating the decline in developmental (storage protein) synthesis during late maturation. Clearly, desiccation may not play a causative role in all species; in some dicots, for example, deposition of storage protein ceases prior to major water loss from the seed (perhaps as a consequence of the attainment of a specific physiological age and/or the cessation of maternal vascular supply) (Galau *et al.*, 1987). Nonetheless, drying may be a critical regulatory cue for some seeds, and the various ways in which it may terminate developmental protein synthesis have been speculated upon (reviewed in Kermode, 1990).

A decline in the synthesis of storage protein mRNAs during drying could be due to a diminished capacity to initiate their transcription and/or to a reduction in the rate of transcription. Transcriptional regulation of storage-protein genes is primarily a function of the 5' upstream promoter regions (Brown *et al.*, 1986; Chen *et al.*, 1986; Goldberg, 1986). Thus, changes to the 5'-flanking sequences of storage-protein genes (or to *trans*-acting factors required for *cis*-sequence activation) may occur as a result of drying, modulating the rate of transcription or its initiation. More specifically, after seed desiccation, the 5' upstream region could become unresponsive to positive controlling *trans*-active factors; alternatively, the factors themselves could become destabilized or their production terminated. These developmental genes would presumably remain suppressed, i.e. 'transcriptionally silent' or 'down-modulated' after subsequent hydration. Additional control may be exerted at the post-transcriptional level; for example, a reduction in the stability of storage protein mRNAs may occur as a consequence of drying. If desiccation acts directly upon developmental genes in effecting a decline in their transcription and/or reduced stability of their corresponding mRNAs, it is pertinent to ask where the desiccation-responsive sequences are located, e.g. are they in the 5' upstream, the 3' down-stream, or the coding regions?

If, indeed, drying is acting in some seeds to down-regulate expression of storage protein genes, we cannot rule out the possibility that drying may cause changes to positive *trans*-acting factors which interact with the *cis*-acting elements of storage-protein genes. In addition to possible effects of drying upon *trans*-acting factors, it could also act through changes in the content of hormones (or their receptors) involved in the expression of storage-protein genes (e.g. ABA). Whether this hormone acts (*in planta*) specifically to increase the production of storage protein mRNAs during the maturation or seed-expansion phase - when maximum synthesis

occurs - remains to be determined. Some evidence supports this contention, and, at the transcriptional level, some of the *cis*-regulatory sequences responsive to ABA within developmental genes (e.g. the sunflower helianthinin gene) have been identified (Thomas *et al.*, 1991, Thomas, 1993). Hence the maturation drying period, by effecting changes in the content of, or sensitivity to, ABA may partially or wholly remove the effects of this 'positive modulator' of developmental gene expression.

## 1.4. Possible Interactions Between Desiccation and ABA

An important area of study in seed development is focused upon the role of seed or maternal environment in maintaining embryos in a developing mode until they are fully formed and have accumulated sufficient reserves to permit successful germination and subsequent seedling establishment. These studies have implicated two potential regulatory factors: abscisic acid (ABA) and restricted water uptake (Kermode, 1990, 1995). In many cases, germination and growth of immature/developing embryos can be elicited precociously by removal from the surrounding seed tissues, thus bypassing the later stages of development. Abscisic acid (ABA) has been implicated in the control of a diverse range of physiological process in higher plants, such as inhibition of precocious germination of embryos, acquisition of desiccation tolerance, and enhancement of expression of storage protein genes, which are dependent upon the levels of ABA and the sensitivity of the competent tissue to ABA (Kermode, 1990). There is some evidence suggesting that both desiccation and ABA play an important role in regulating the expression of storage protein genes, and possibly, their interaction may be the crucial factor for such an effect.

### 1.4.1. Desiccation, ABA Changes, and Seed Development

Desiccation is the normal terminal event in seed development and may itself promote, or lead to, a loss of control by tissues surrounding the embryo. Such a loss of control may result from a reduction in the content and/or flow of ABA from the mother plant or surrounding seed tissues. There is some evidence that desiccation alters the hormonal balance of the seed. In some cases, there is a temporal correlation between the onset of maturation drying and the precipitous decline in seed ABA (accompanied by a corresponding increase in its metabolites) (McWha, 1975; King, 1976). However, there are exceptions: in field-grown wheat (cv. Cappelle), the decline in ABA precedes the onset of water loss by days (Radley, 1976).

Soybeans can be induced to germinate precociously by slow drying, which depletes the pool of endogenous ABA (Ackerson, 1984). Similarly, premature drying of immature cereal grain elicits a decline in ABA and enhances degradation of the applied hormone (King, 1976, 1979). It also leads to inducibility of production of post-germinative α-amylase in response to added GA (Evans *et al.*, 1975; Armstrong *et al.*, 1982; Oishi and Bewley, 1990). The rate and extent of premature desiccation may be critical, since limited desiccation (i.e. mild water stress imposed during development) can lead to an increase in endogenous ABA (e.g. within barley embryos) (Robertson *et al.*, 1989).

The substantial decline in endogenous ABA in the mature dry (non-dormant) seed allows its germination upon imbibition. Concomitant with the decrease in ABA during the late stages of seed development, there is evidence for a decrease in tissue sensitivity to ABA. For example, in wheat, the levels of ABA alone cannot account for dramatic differences in germination between non-dormant and dormant types of

seeds, suggesting that sensitivity differences account for such responses. Thus, control of embryo sensitivity to ABA may be the physiological basis for the difference in germination characteristics between non-dormant (less sensitive) and dormant (more sensitive) wheat seeds (Walker-Simmons, 1987).

A decline in endogenous ABA is not always correlated with the ability of seeds to germinate and drying can also be effective at stages when ABA content is low (Black, 1983, Kermode *et al.* 1989a). In developing castor bean seed, premature drying leads to a major decline in endogenous ABA content within the embryo and endosperm; a reduction in embryo sensitivity to ABA is also evident following drying, the latter perhaps being more crucial for effecting the 'switch' to a germination/growth program (Kermode and Bewley, 1987; Kermode *et al.*, 1989a). However, the reduction in ABA within the embryo and surrounding endosperm may not be sufficient in itself to elicit a switch to germination and growth. A strong negative correlation between ABA content and germination capacity of the whole seed is not found. For example, partial water loss from the seed at 35 DAP, which like natural and premature desiccation leads to subsequent germination upon return of the seed to full hydration, causes a much smaller decline in ABA. In contrast, ABA declines substantially in the undried (hydrated) control at 35 DAP, but the seeds do not germinate. Drying also appears to reduce the sensitivity of the embryo to ABA, resulting in a loss of competence to respond to this hormone at physiological concentrations (Kermode *et al.*, 1989a). The diminished ABA responsiveness is correlated with the capacity of the whole seed to germinate. In castor bean embryos, there is about a tenfold reduction in sensitivity to ABA after natural or imposed seed drying (Kermode *et al.*, 1989a) (as measured by the capacity of exogenous ABA to block embryo germination), which cannot be attributed to the period of seed detachment from the mother plant required to impose premature drying. Likewise,

reduced ABA uptake leading to an apparent decline in sensitivity is unlikely, since uptake would be expected to be greater in dried tissues than in their undried (i.e. fresh or hydrated) counterparts. Similarly, in wheat, alfalfa, rape and soybean embryos, the decline in ABA during late development is accompanied by a corresponding decrease in tissue sensitivity to exogenous ABA, as measured by its capacity to inhibit germination and to maintain or enhance expression of developmental storage-protein genes (Eisenberg and Mascarenhas, 1985; Finkelstein *et al.*, 1985 Williamson *et al.*, 1985; Quatrano, 1986; Xu and Bewley, 1991).

It is not known whether this change in response to exogenous ABA is a direct result of drying, e.g. via changes in receptor contents or conformation. However, the reduced sensitivity does not appear to be due to a reduction in ABA content, since embryos of partially dried castor bean seeds, which retain the high contents of ABA characteristic of those in seeds at 35 DAP developing *in situ* on the mother plant, also undergo a subsequent decline in sensitivity to exogenous ABA (Kermode *et al.*, 1989a). Another possibility is that desiccation enhances the ability of these dried or partially dried embryos to metabolize the exogenously supplied ABA. For example, somatic embryo suspension cultures of white spruce (Dunstan *et al.*, 1992) are only capable of metabolizing the natural isomer of ABA, the (+)-ABA isomer, but not the (-)-ABA form.

### 1.4.2. Desiccation and Seed Storage Protein Gene Expression

As mentioned previously, in effecting a switch in gene expression, desiccation appears to act primarily at the transcriptional and posttranscriptional levels (Misra and Bewley, 1985; Cornford *et al.*, 1986; Kermode *et al.*, 1989c; Jiang and Kermode, 1994). At the molecular level, drying may ultimately result in the off- or down-

regulation of development-related genes and the on-regulation of germinative and post-germinative genes. For example, the effects of maturation and premature drying on the expression of a full β-phaseolin gene has been compared in transgenic tobacco seed (Oliver *et al.*, 1993); the results suggest that the β-phaseolin promoter is directly down-regulated by desiccation during maturation and is not active upon rehydration or imbibition. Following drying, this region may become insensitive to positive controlling factors; alternatively, changes to *trans*-acting factors important for gene expression may occur as a result of drying (Oliver *et al.*, 1993). In addition to possible effects of drying on *trans*-acting factors, it could also act through changes in the content of hormones (or their receptors) involved in the expression of storage protein genes. ABA has been implicated in the regulation of storage protein synthesis and some of the *cis*-acting sequences responsive to ABA within developmental genes have been identified (Thomas, 1993)

### 1.4.3. ABA and Seed Storage Protein Gene Expression

During mid to late stages of seed development, messenger RNAs for certain storage proteins accumulate in seeds at times when the endogenous ABA level is high. When these seeds are isolated at earlier developmental stages and isolated embryos are exposed to exogenous ABA, some of the mRNAs for storage proteins are precociously accumulated, including β-phaseolin of bean (*Phaseolus vulgari*), napin and cruciferin of rape seed (*Brassica napus*), β-conglycinin of soybean, and helianthinin of sunflower (Crouch and Sussex, 1981; Bray and Beachy, 1985; Nunberg *et al.*, 1994; Quatrano, 1986). In addition, some other proteins such as *LEAs* (Late Embryenesis Abundant proteins) from cotton and wheat (*Em*) are also induced precociously by exogenous ABA treatment in developing seeds. Thus, the response of

reproductive tissue (seed) to increased levels of ABA involves a change in the levels of specific mRNA transcripts for storage proteins (Skriver and Mundy, 1990).

The expression of storage protein genes in response to exogenous ABA can be regulated at various levels. For example, even though the responsiveness of the napin and cruciferin genes of *Brassica napus* to ABA is under transcriptional control (DeLisle and Crouch, 1989), the accumulation of *Em* transcripts in mature wheat embryos in response to ABA is also controlled at a posttranscriptional level (Williamson and Quatrano, 1988). In addition, controls could also be operative at the translational level, for example, *Em* transcripts are present in seedlings but no *Em* protein was detected in the same tissue (Berge *et al.*, 1989). The expression of storage protein genes in response to exogenous ABA is also dependent upon the developmental stage of the embryo or seed. For example, when developing transgenic tobacco seeds (expressing a helianthinin 5'-GUS chimeric gene) are incubated on a basal medium containing 1 μM ABA, they exhibit enhanced GUS activities, as compared to control seeds placed on a basal medium minus ABA. However, the responsiveness of GUS expression to ABA declines as seed development progresses. For example, cultured seeds at 14 DAA show a four-fold enhancement of GUS activity when treated with ABA for 5d, but this enhancement in GUS activity declines to only 1.5-fold in seeds cultured at 24 DAA (Thomas *et al.*, 1991; Nunberg *et al.*, 1994).

ABA induces the transcription of responsive genes through *cis*-acting promoter sequences known as ABA-responsive *cis*-acting elements (ABREs) (Skriver *et al.*, 1991; Marcotte *et al.*, 1989). This element, comprised of the consensus sequence ACGTGG, is conserved in a variety of ABA-regulated genes and comprises a subset of the G-box (CACGTG) family of *cis*-acting elements (Williams *et al.*,

1992). In the case of the *Em* gene, a basic leucine-zipper transcription factor binds to the G-box, which is required for ABA-inducible expression (Hetherington and Quatrano, 1991). On the other hand, a calcium-binding protein, GF14, which has homology to a 14-3-3 kinase regulatory protein and is located in both the cytoplasm and nucleus, has been shown to be a component of the DNA-protein complexes that bind to G-box elements, including the ABRE (Lu *et al.*, 1994; Rock and Quatrano, 1994). Phosphorylation of leucine-zipper transcription factors *in vivo* and *in vitro* has been shown to modulate their nuclear localization and alter their DNA-binding properties, respectively (Harter *et al.*, 1994).

Another fruitful approach to examining the ABA signal transduction pathway in regulating gene expression has been through the analysis of the *viviparous* mutants of maize (Robertson, 1955) and the ABA-insensitive mutants of *Arabidopsis* (Koornneef *et al.*, 1984). The *vp*-1 and *ABI3* loci have been cloned (Giraudat *et al.*, 1992; McCarty *et al.*, 1991) and are proposed to encode novel transcription factors. The relationship between the gene products of these loci and the DNA-binding protein (*trans*-acting factor) identified by Guiltinan *et al.* (1990) still remains to be investigated.

### 1.4.4. Desiccation and ABA Sensitivity

A change in sensitivity to ABA after natural drying is suggested by several studies that reveal distinct differences between the metabolic responses of developing and mature embryos to ABA. Such is the case even when both are inhibited from germinating by ABA. For example, in cotton, synthesis of malate synthetase (an enzyme associated with both late embryogeny and post-germinative seedling growth) can either be stimulated or inhibited by ABA, depending on the physiological

maturity and water status of the isolated embryo (Choinski *et al.*, 1981). A difference in response to ABA between immature and mature wheat embryos in relation to production of α-amylase (α-$AMY_2$ isozyme) has also been noted (Cornford *et al.*, 1987b). Expression of storage-protein genes remains responsive to ABA in precociously germinating rape embryos, but not in mature tissue, which lacks this response to ABA (Crouch *et al.*, 1985). Similarly, in the absence of a desiccation treatment, isolated immature alfalfa embryos that have completed germination and initiated seedling growth, still remain responsive to ABA, which causes the cessation of postgerminative activities and the recommencement of synthesis of storage proteins (Xu and Bewley, 1994). However, when the isolated developing embryos are desiccated, imbibed in water and then cultured on media containing ABA, storage protein synthesis is not restored. Thus, it appears that desiccation permanently switches the embryo from development to germination, but in its absence the capacity for developmental synthesis is retained even after germination. Interestingly, fluridone-induced viviparous germination of whole maize kernels is accompanied by the simultaneous synthesis of both developmental and postgerminative proteins (Oishi and Bewley, 1992). This indicates that a decline in ABA content (induced by the fluridone treatment), while sufficient for a morphological switch to germination, is not sufficient for a metabolic switch, accompanied by a cessation of storage protein gene expression. In conclusion, ABA alone may not be sufficient to regulate activity of the storage-protein genes; desiccation may be a prerequisite for cessation of their expression. This may be an oversimplistic view, since some developmental events remain responsive to ABA, even after drying (whether natural or imposed). However, this appears to be the case only when isolated embryos are exposed to very high concentrations of ABA, i.e. those well above maximum physiological concentrations during development, under normal (i.e. non-stress) conditions.

The protein synthesis response of castor bean embryos to $10^{-4}$M ABA after premature desiccation exhibits both similarities to, and differences from, the response of mature dry embryos (Kermode and Bewley, 1987; Kermode *et al.*, 1989a). Premature desiccation is an interruption of the normal developmental processes; upon rehydration in $10^{-4}$M ABA, germination is suppressed and the synthetic capacity for some developmental proteins in the cotyledons is retained. However, the full range of developmental responses is not maintained by even this concentration of ABA because some of the developmental proteins are hydrolysed upon subsequent rehydration - as occurs in the embryos of mature dry seeds imbibed on ABA. Hence, some developmental events appear to have retained a sensitivity to promotion by $10^{-4}$ M ABA after the premature drying process, whereas others have not, leading to an 'intermediate' response to the hormone. When isolated embryos are exposed to ABA at concentrations closer to the physiological concentration present in the seed at the mid-maturation stage of development (i.e. $10^{-5}$M ABA), the scenario changes. Both natural drying and premature drying of developing seed at 35 DAP reduce the sensitivity of embryos to $10^{-5}$M ABA and permit germination. Upon rehydration in this concentration of ABA, germinative and post-germinative metabolism proceeds in prematurely dried and mature embryos, albeit at a somewhat reduced rate (Kermode *et al.*, 1989a). Thus, the *relative* insensitivity of the embryo to ABA, with respect to its capacity to inhibit germination after drying, is indeed reflected at the level of protein metabolism.

Under some conditions, embryos isolated from mature seeds (which have undergone the natural drying process) may still be capable of responding to ABA in a 'developmental' manner, i.e. by enhancing the synthesis of storage protein or other developmentally associated proteins. For example, high concentrations of exogenous ABA (i.e. $10^{-4}$M) can maintain concentrations of *Em* mRNA (primarily via its

stability) and increase the amount of globulin mRNA (via its synthesis) in cultured mature embryos of wheat (Williamson *et al.*, 1985; Quatrano, 1986; Quatrano *et al.*, 1986; Williamson and Quatrano, 1988; Berge *et al.*, 1989). This lends support to the contention that some developmental genes are not completely 'turned off' during late development, but are simply 'down-regulated' indirectly, or directly, as a consequence of desiccation. This retained 'transcriptional competence' is expressed upon rehydration of the embryos in high concentrations of ABA. Of course, the extent to which genes of the developmental program are subject to 'up-down' (as opposed to 'on-off') regulation is not known; a complex pattern of expression may emerge where individual genes differ in this respect. Some genes expressed during development (e.g. late-embryogenesis-abundant genes such as *Em*) show more flexible expression, being responsive to environmental cues (and high concentrations of endogenous ABA) in seedlings - i.e. following the transition to a germination and post-germination program.

It has been suggested that ABA (at $10^{-5}$M) reinduces expression of storage-protein genes in mature embryos of mustard (Fischer *et al.*, 1987). However, the possibility that ABA maintains or increases the stability of conserved messages for storage protein (i.e. those which survive desiccation and are present in mature dry seed) was not evaluated. A subsequent study (Croissant-Sych and Bopp, 1988) found no reinduction of storage-protein synthesis (in embryos exposed to $10^{-6}$M ABA); post-germinative events (e.g. reserve catabolism) were initiated, but delayed by 2 d.

Thus, it is evident from the above-mentioned studies that the decline in embryo sensitivity to ABA after natural or imposed drying is relative, not absolute. It is not known whether the 'switch' from processes associated with development to those associated with germination and growth, brought about by premature or normal

drying of whole seeds, is mediated solely through this altered sensitivity or response of the embryo to ABA, but, in any event, a decline in endogenous ABA may be required.

A major objective of this thesis is to examine the role of desiccation in the cessation of developmental gene expression at the molecular level. For example, in Chapter 2, I present evidence that premature drying may down-regulate storage protein gene expression upon rehydration by acting directly upon the 5' upstream regions of these developmental genes. As will be discussed, following drying, the 5' upstream region of the developmental gene may become insensitive to positive controlling factors; alternatively, changes to *trans*-acting factors important for gene expression may occur as a result of drying (Jiang and Kermode, 1994; Jiang *et al.*, 1995). In addition to possible effects of drying on *trans*-acting factors, it could also act through changes in the content of hormones (or their receptors) involved in the expression of storage protein genes. A further objective of this thesis is to examine the hypothesis that the maturation drying period, by effecting changes in the content of, or sensitivity to, ABA, partially or wholly removes the effects of this 'positive modulator' of developmental gene expression.

## 1.5. Summary

Desiccation, whether natural or imposed prematurely, plays an important role in switching seeds from a developmental mode to one that is essential to promote germination. Premature drying not only redirects metabolism from a developmental to a germination program, but does so permanently, thus effecting a normal temporally regulated 'switch'. However, this switch cannot be brought about at all stages of

development; the seed must first develop competence to withstand the drying treatment - a process which presumably involves various metabolic and/or structural changes. One can only speculate that this competence is achieved simultaneously by the genome.

Drying leads to the suppression of synthesis of developmental proteins and the induction of synthesis of those for germination and growth. There may be some direct action of drying upon the 5' regulatory regions of developmental genes, effecting a decline in their expression, but the precise nature of the effects caused by drying needs to be elucidated. Other changes at the cellular level may also be operative.

## 1.6. The Present Study: Research Objectives

The primary objective of the present study was to investigate events at the molecular level caused by drying, which ultimately result in the off- or down-regulation of development-related genes and the on-regulation of germinative and post-germinative genes. This was addressed, using molecular approaches, involving the expression of chimeric genes in transgenic tobacco. A major question being addressed was whether desiccation acts directly upon the regulatory regions of developmental storage protein genes in effecting a decline in their transcription and/or reduced stability of their corresponding mRNAs? And, if so, where are the desiccation-responsive sequences located (e.g. are they in the 5' upstream, the 3' downstream or the coding region)? Chapter 2 addresses these questions. In Chapter 3, the cellular action of desiccation is investigated. In particular, it examines the possible interactions between desiccation and ABA at the molecular level, by asking if premature drying reduces the ABA-sensitivity of storage protein gene expression in

developing transgenic tobacco seed. The final objective was to investigate the molecular action of desiccation in the induction of a germinative and post-germinative metabolic program. More specificially, I investigated the role of desiccation in the acquisition of GA-responsiveness of developing barley aleurone layer cells in respect of α-amylase gene expression (see Chapter 4).

*Chapter 2*

# The 5' Flanking Regions of Vicilin and Napin Storage Protein Genes Are Down-Regulated by Desiccation in Transgenic Tobacco

## 2.1. Introduction

Seed development and germination are distinct physiological stages of the plant life cycle, in which key metabolic events related to the status of stored reserves contrast markedly; this is reflected in the mRNA subsets prevalent within the seed at these distinct stages. In particular, storage protein synthesis which is prevalent during mid- to late-stage embryogenesis does not occur during germination. Residual mRNAs for storage proteins present in the dry seed are degraded upon subsequent imbibition (Dure, 1985; Bewley and Marcus, 1990; Kermode, 1990). Early metabolic events utilize and depend upon components present within the dry seed. After a short lag, new sets of messages are synthesized during germination (Sanchez-Martinez *et al.*, 1986; Lane, 1991) and also during seedling growth (Harada *et al.*, 1988) when the enzymes necessary for mobilization and utilization of stored reserves are produced.

These changes in mRNA subsets which underlie the distinct metabolic events associated with development and germination/growth implicate the involvement of a "switch", which effects the transition from development to germination; it is normally triggered in a precise temporal fashion to allow completion of developmental events which are important for successful germination and survival of the young seedling (e.g., the accumulation of sufficient reserves). There may be several regulatory cues which result in the switch from seed development to germination (Galau *et al.*, 1991; Kermode, 1990; 1995). In many cases, germination and growth of the immature/developing embryo can be elicited precociously by its removal from the surrounding seed tissues, thus bypassing the later stages of development. However, for most seeds, a period of desiccation is the normal terminal event in embryo development and there is now substantial evidence that maturation drying is critical in

terminating development and overcoming constraints by the maternal environment that maintain seeds in a developmental mode (reviewed in Kermode and Bewley, 1986a, 1987; Kermode *et al.*, 1986, 1989b; Kermode, 1990, 1995; Jiang and Kermode, 1994). This results in a switch in cellular activities from an exclusively developmental program to an exclusively germination/growth-oriented program.

An important experimental approach that has been used by several researchers to define the role of desiccation in terminating development and eliciting germination is to subject the developing seed to premature desiccation, i.e. to remove the seed from the parent plant before its normal development is completed and determine the effect of an artificially imposed drying period. When desiccation is imposed prematurely at certain stages prior to the completion of development, a switch in metabolic events is elicited: syntheses unique to development, for example storage-protein synthesis, are terminated, while syntheses associated with germination and post-germinative growth are initiated (Kermode and Bewley, 1985b, 1986b; Kermode *et al.*, 1985; Misra and Bewley, 1985; Dasgupta and Bewley, 1982; Adams *et al.*, 1983; Rosenberg and Rinne, 1987; Cornford *et al.*, 1986; Oishi and Bewley, 1992; Evans *et al.*, 1975).

In effecting this switch in gene expression, desiccation appears to act primarily at the transcriptional level, but post-transcriptional controls may also be operative (Kermode *et al.*, 1989c; Cornford *et al.*, 1986; Misra and Bewley, 1985). For example, within the castor bean endosperm storage protein messages present during development (e.g., ricin D and LEG B MAT1 mRNAs) decline after natural and imposed drying and are not resynthesized upon subsequent rehydration (Kermode *et al.*, 1989c; Kermode, Hughes, Galau, and Bewley, unpublished data). On the other hand, messages associated with post-germinative activities from 24 h onwards, and

that are not abundant during development, are induced after rehydration as a consequence of premature drying. Thus, desiccation effects an off- or down-regulation of developmental messages and an on-regulation of messages for germination and growth. However, whereas most developmental messages decline during drying, the ability of the seed to synthesize mRNAs for developmental proteins is retained, although the rates at which their genes are transcribed decline with increasing water loss. It is upon rehydration that developmental genes are no longer transcribed, and only then is the germinative and post-germinative program of gene expression initiated (Comai and Harada, 1990). In addition, it is the subsequent hydration phase that appears to be the crucial event leading to the loss of any residual developmental messages that remain stable throughout desiccation. These messages are presumably degraded upon rehydration by the normal turnover processes and are not replaced, because their genes have been off- or down-regulated.

One of the objectives of this work was to examine events at the molecular level caused by drying, which ultimately result in the off- or down-regulation of development-related genes and the on-regulation of germinative and post-germinative genes. Does desiccation act directly upon the regulatory regions of developmental genes in effecting a decline in their transcription and/or reduced stability of their corresponding mRNAs? For example, after seed desiccation, the regulatory regions may become unresponsive to positive controlling *trans*-active factors or the factors themselves may be destabilized or their production diminished (Oliver *et al.*, 1993; Kermode 1990, 1995; Jiang and Kermode, 1994). These developmental genes would presumably remain suppressed, i.e. "transcriptionally silent" or "down-modulated" after subsequent hydration. Additional control may be exerted at the post-transcriptional level; for example, a reduction in the stability of storage protein mRNAs may occur as a consequence of drying. Further, if desiccation acts directly on

developmental genes, it is pertinent to ask where the desiccation-responsive sequences are located (e.g., are they in the 5' upstream, the 3' downstream or the coding region)?

The developmental genes under study are those encoding the 7S vicilin storage protein from pea seed and the 2S napin storage protein from *Brassica napus* seed. As an initial step toward understanding the effect of desiccation on the expression of genes for storage proteins, and to investigate the responsiveness of the 5' or 3' regions of these genes, seven chimeric genes were introduced into tobacco. The chimeric genes consist of 5' and/or 3' regions from the genes under study (which were being tested for desiccation sensitivity); or, as controls, the same regions derived from constitutively expressed genes which are presumed to be desiccation insensitive--those from the cauliflower mosaic virus (35S) and nopaline synthase (Nos) genes. All constructs contain the coding region of a reporter gene encoding an easily assayable enzyme: $\beta$-glucuronidase (GUS). The fate of GUS gene expression from these various constructs has been monitored (at the protein level) within seeds of transgenic tobacco after hydration of the mature (naturally dried) seed and after desiccation/rehydration (and control) treatments imposed during seed development. The hypothesis being tested here is that expression of the GUS reporter gene will be sensitive to desiccation (and therefore will be down-regulated) in the presence of the 5' or 3' regulatory region(s) from the storage-protein genes, but not when under control of regulatory (5' or 3') regions that have no known sensitivity to this perturbation.

## 2.2. Materials and Methods

### 2.2.1. Construction of Vicilin and Napin Chimeric Genes

The chimeric gene constructs generated for expression in transgenic tobacco are shown in " Results". All procedures for DNA manipulation were carried out according to standard procedures of Maniatis *et al.* (1982). A vicilin gene (pEN 2) described earlier (Higgins *et al.*, 1988) was modified by *in vitro* mutagenesis (Amersham Kit, Amersham, Arlington Heights, IL) so that unique restriction sites were introduced into the DNA near the cap site at +2 (*Cla I*), at the ATG encoding the initiator methionine (*Nco I*), and at a site corresponding to the penultimate amino acid of vicilin (*Xho I*). The plasmid pCW 50 (containing 135 bp of 5' flanking sequence and the entire vicilin coding sequence and 3' flanking region in pUC 118, minus its polylinker) (Wandelt *et al.*, 1992) was digested with *EcoR I* and *Nco I* to release a 3.17-kb fragment consisting of 135 bp of vicilin 5' flanking sequence (-100 to +1 and the vicilin 5' untranslated region) and pUC 118. A second fragment (1.87 kb) consisting of the GUS gene coding region was generated by cutting plasmid pRAJ 275 (CLONTECH Laboratories, Inc., Palo Alta., CA) with *EcoR I* and *Nco I*. Ligation of these two fragments produced plasmid pLJ-1 containing the partial vicilin 5' upstream region and the GUS gene coding region. pLJ-1 was then digested with *EcoR I* and *Cla I* to generate a 1.905-kb fragment consisting of the vicilin 5' untranslated region or leader sequence (+2 to the ATG encoding the initiator methionine) and the GUS gene coding region. The above fragment was then ligated to a 5.005-kb fragment containing the entire 2.4-kb vicilin 5' flanking region (to +2) and pUC 8, the latter fragment generated by digesting plasmid pAK 11 (the intact vicilin gene in pUC

8) (AR Kermode, unpublished data) with the same enzymes. This produced pLJ-2 consisting of the entire vicilin 5' flanking region and the GUS gene coding region. In order to link the Nos 3' region onto the terminus of the GUS gene coding region, a 1.77-kb fragment (consisting of the Nos 3' end and 1.47 kb of GUS gene coding region) was produced by digesting pBI 221 (CLONTECH Laboratories, Inc., Palo Alto, CA) with *EcoR I* and *SnaB I*. The fragment was then ligated to a 5.401-kb fragment generated by cutting pLJ-2 with *EcoR I* and *SnaB I*; this created pLJ-3 consisting of the entire 2.4-kb vicilin 5' flanking region (including the 5' untranslated sequence), the GUS gene coding region and the Nos 3' end in pUC 8 (**Vic-GUS-Nos**).

Cloning of **35S-GUS-Nos** into the plant transformation vector, pGA 492 (An, 1986) to create pLJ-4 was achieved by digesting pBI 221 (CLONTECH Laboratories, Inc., Palo Alto, CA) with *EcoR I* and *Hind III* to generate a 3-kb fragment consisting of the 35S 5' flanking region, the GUS gene coding region, and the Nos 3' region. This fragment was then ligated into pGA 492 using the same restriction sites. Cloning of **Vic-GUS-Nos** into pGA 492 to create pLJ-5 was achieved by digesting pAK 22 (AR Kermode, unpublished data) with *Cla I* and *EcoR I* to create a fragment consisting of the vicilin 5' flanking region (to +2) and pGA 492. This was ligated to an insert consisting of the GUS gene coding region and Nos 3' end, generated by treating pLJ-3 with the same enzymes.

To replace the Nos 3' region with the vicilin gene 3' region, plasmid pCW 50 was digested with *Xho I*, followed by blunt ending, and subsequent digestion with *EcoR I* to generate a 0.356-kb vicilin 3' fragment. This fragment was then cloned into pBI 221 by digesting the pBI 221 plasmid with *Sst I*, followed by blunt ending, and subsequent *EcoR I* treatment; ligation created the plasmid pLJ-6, which consists of the 35S 5' flanking region, the GUS gene coding region, and the vicilin 3' region in

pUC 19. Cloning of **35S-GUS-Vic** into pGA 492 to create pLJ-7 was achieved by digesting pLJ-6 with *EcoR I* and *Hind III* to generate a 2.8-kb fragment consisting of the 35S 5' flanking region, the GUS gene coding region, and the vicilin 3' region, and ligating into pGA 492 using the same restriction sites. To insert **Vic-GUS-Vic** into pGA 492, pLJ-5 was partially digested with *EcoR I* and *SnaB I* to generate a 14.63-kb vector fragment consisting of the vicilin 5' flanking region, 400 bp of the GUS gene coding region, and the pGA 492 vector; another 1.8-kb insert consisting of the remaining GUS gene coding region and the vicilin 3' region was generated by digesting pLJ-6 with *EcoR I* and *SnaB I*. The ligation of these two fragments created pLJ-8, which is **Vic-GUS-Vic** in pGA 492. The orientation of the vicilin 3' region with respect to the promoter was confirmed by restriction mapping and DNA sequencing.

The napin gene used for making the chimeric gene constructs was cloned as described in Baszczynski and Fallis (1990). DP 1744 contains the **Nap-GUS-Nos** chimeric construct in the binary vector; the chimeric gene contains 800 bp of napin 5' flanking region (including the 5' untranslated region), the GUS gene coding region, and the Nos 3' end (Baszczynski, unpublished). A second plasmid, pTZESnap 2 was used to isolate the napin 3' end; this plasmid contained 990 bp of napin 5' flanking region, the napin coding region (540 bp) and a 201-bp napin 3' flanking sequence (Baszczynski, unpublished). A *Sst I* site was created at position 1534 of pTZESnap 2, corresponding to the beginning of the napin 3' flanking region by site-directed mutagenesis (using the Altered Sites ™ *in vitro* mutagenesis system from Promega, Madison, WI). A 311-bp fragment consisting of 110 bp of napin coding region and the 201-bp napin 3' flanking region containing the new *Sst I* site was then generated by digesting the mutated pTZESnap 2 plasmid with *Pst I* and *Hind III*; this fragment was then cloned into pUC 19 using the same restriction sites to create plasmid pUC

19S/B1. A 2.7-kb fragment consisting of the CaMV 35S 5' flanking region and the GUS gene coding region was generated by digesting pBI 221 (CLONTECH Laboratories, Inc., Palo Alto, CA) with *Pst I* and *Sst I*. This fragment was then ligated into pUC 19S/B1 using the same restriction sites to create pUC 19/B2, which contained the 35S 5' flanking region, the GUS gene coding region, and the napin 3' flanking region in pUC 19. The **35S-GUS-Nap** fragment was then removed from pUC 19/B2 and ligated into pGA 492 using the *EcoR I* and *Hind III* restriction sites.

The napin 5' flanking fragment (800 bp) was isolated by digesting DP 1744 with *Xba I* and *BamH I*; this fragment was subsequently inserted into pUC 19S/B2 to replace the 35S 5' flanking region, creating pUC 19S/B3, consisting of the napin 5' flanking region, the GUS gene coding region, and the napin 3' region. The **Nap-GUS-Nap** construct was isolated from pUC 19/B3 by digesting the plasmid with *Xba I* and *Hind III*; subsequently this fragment was inserted into pGA 492 using the same restriction sites. The fidelity of the site-directed mutagenesis was confirmed by DNA sequencing.

### 2.2.2. Transfer of Engineered Genes into Tobacco and Growth of Transgenic Tobacco Plants

Constructs were ligated into the *Agrobacterium tumefaciens/E. coli* shuttle vector pGA 492 (An, 1986) as outlined above. Plasmids were transferred from *E. coli* HB 101 to the *Agrobacterium* strain LBA 4404 by triparental mating using the conjugate strain of *E. coli*, RK 2013 as helper (Ditta *et al.*, 1980). Tobacco (*Nicotiana tabacum* cv. Winsconsin 38) leaf pieces were infected with the new *Agrobacterium* strains as described earlier (Higgins *et al.*, 1988), with the modification that only a 5 min incubation in *Agrobacterium* was carried out. Kanamycin resistant plantlets were

transferred to a soil : peat mixture (3:1) and grown to maturity in a greenhouse (26 $^{o}$C, 16-h photoperiod). Flowers were tagged at anthesis just as the petals opened and began to turn a pink color. Seeds were collected at different days after anthesis (DAA) and at maturity (30 DAA). Stable transformation of chimeric genes into the tobacco genome was confirmed by several levels of screening, including plantlet regeneration from callus on medium containing kanamycin; rooting of plantlets on medium with kanamycin; GUS assays on seeds from plants grown in greenhouse; and germination of $F_2$ seeds on medium containing kanamycin.

### 2.2.3. Seed Isolation, Desiccation, and Hydrated Control Treatments

Transgenic tobacco seed used for the developmental profile study were isolated in the fresh state from pods harvested at the stated days after anthesis (DAA) and analyzed immediately by Western blotting or GUS assay. For drying studies, pods were harvested at different developmental stages, the seed removed and then air dried for 5 d at 26 $^{o}$C. Partial drying was achieved by placing seeds excised from the pod for the same length of time in a moist atmosphere (a sealed 10 X 50 mm Petri dish containing a few drops of water on the lid). For each treatment, about 500-1000 seeds were used with 3-4 replications. The germination percentage (at 26 $^{o}$C) was determined after a minimum of 10 d following transfer of fresh, partially-dried or air-dried seeds onto Petri dishes containing 1% bacterial agar with no nutrients or hormones. Air-dried seeds at a desiccation tolerant stage were used for subsequent analyses to determine the effects of premature drying and rehydration on GUS activities in transgenic seed. Prematurely dried seed and mature dry seed were rehydrated by transferring surface sterilized seed onto Petri dishes containing 1% bacterial agar and maintaining them at constant temperature (26 $^{o}$C, 16-h photoperiod). Seed at different times after rehydration were used for subsequent

analyses. For the hydrated control treatment, freshly harvested developing seed (10-12 DAA) were placed immediately onto Petri dishes containing 1% bacterial agar (for a period equivalent to the air-drying treatment, (i.e. 5d) prior to analysis at the appropriate time.

For the biolistic studies, fresh or dried developing castor bean seed at 35 days after pollination (DAP) were used. Fresh seed excised from capsules harvested at 35 DAP were either used immediately for biolistics or were first dried slowly to known percentage water contents by placing them in a desiccator over stirred saturated salt solutions (McLean and Cook, 1941; Schonbeck and Bewley, 1981). The drying regime used for 35 DAP seed (5d) was as described in Kermode and Bewley (1985a). Following the drying, seeds were rehydrated for 48 h by placing them in Petri dishes containing a filter paper and sterile water. A hydrated control treatment consisted of placing freshly harvested 35 DAP seed in water for 7d (a period equivalent to the drying and rehydration treatment).

### 2.2.4. Assays for GUS Activities in Control and Transgenic Tobacco Seed

Procedures for extraction and measuring GUS activity by the fluorimetric assay were essentially as described in Jefferson (1987). Enzyme extracts were prepared by grinding seed from control (W38) or transgenic tobacco plants in GUS extraction buffer (50 mM sodium phosphate, pH 7.0, 10 mM $Na_2EDTA$, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100 and 25 µg/ml PMSF) and pelleting the cell debris by centrifugation in a microfuge (16,000 xg, 4 $^{o}C$, 10 min). The enzyme reaction was initiated by adding extract (20-50 µl) to 1 ml of pre-warmed assay buffer (1 mM methylumbelliferyl $\beta$-D-glucuronide in extraction buffer), vortexing, then incubating at 37 $^{o}C$. Reactions were terminated by removing (at timed intervals)

successive 200 µl aliquots into test tubes containing 1.8 ml of stop buffer (0.2 M $Na_2CO_3$). Fluorescence of each sample was measured by a Hoefer Fluorimeter, model TKO-100. Enzyme activities were calculated as a rate (pmol methylumbelliferone/min) per µg seed protein. Protein concentrations were determined by the Bradford assay (Bradford, 1976), using reagents from Bio-Rad (Bio-Rad Laboratories, Hercules, CA). Average GUS activities were calculated on the basis of 2 or 3 replicates.

### 2.2.5. Western Blot Analysis for Detection of GUS Protein

Protein extracts were generated as described above, using GUS extraction buffer. Protein assays (Bradford, 1976) were carried out and the extracts containing equal amounts of protein (40 µg) were diluted with 30 µl SDS-PAGE sample buffer (0.125 M Tris-HCl, pH 6.7, 2% SDS, 10% glycerol, 0.003% bromophenol blue and 5% $\beta$-mercaptoethanol). The mixture was heated for 10 min at 95 $^{o}$C, cooled and then fractionated by SDS-PAGE. After electroblotting onto nitrocellulose membrane, the GUS protein was detected with rabbit anti-GUS serum (CLONTECH Laboratories, Inc., Palo Alto, CA) (diluted 1/700 before use), followed by goat anti-rabbit IgG (Promega, Madison, WI) (diluted 1/3000) to which was conjugated alkaline phosphatase. The accuracy of protein loading was confirmed by staining replicate SDS-gels with Coomassie blue.

### 2.2.6. RNA Isolation and Dot Blot Analysis

Tobacco seeds of various stages and treatments were frozen immediately in liquid $N_2$ and kept at - 80 $^{o}$C until used for RNA extraction. Total RNA was extracted from seeds according to the method of Higgins *et al.* (1988). Briefly, after

powdering in liquid $N_2$, samples were extracted in Tris buffer (1M Tris, pH 9.0 with 1% β-mercaptoethanol), followed by phenol/chroloform extraction. RNA was then percipitated in 4M NaCl (final concentration) at 4 $^{o}$C overnight. 5 to 10 µg of total RNA was tranferred onto Hybond-N membrane and then probed with a dioxigen-labelled 1.87 kb fragment (the GUS coding region); detection was achieved using chemifluorescence as outlined by the Amersham instructions.

### 2.2.7. Biolistics Studies and Detection of Transgenic GUS Expression

For the transient gene expression assays, two chimeric gene constructs were used. One contained the vicilin 5' upstream region, the GUS gene coding region, and Nos 3' region (Vic-GUS-Nos); in the other, the CaMV 35S 5' upstream region replaced the vicilin 5' upstream region (35S-GUS-Nos). Both gene constructs were cloned into the high copy plasmid, pUC 18. The Helium Biolistic gene transformation system (Du Pont, Model PDS/Helium) was used to deliver the chimeric GUS gene constructs into the cotyledons of 35 DAP castor bean seeds in the fresh state, following premature desiccation and following desiccation/rehydration. For each delivery, 1 µg of purified plasmid DNA coated onto 1.6 µm gold particles was used; a resultant pressure of 1300 psi was optimal for delivery of particles into the cotyledons. Prior to bombardment, seeds treated in the appropriate manner were bissected in a mid-frontal manner and placed on Petri dishes containing Murashige and Skoog (MS; Murashige and Skoog, 1962) basal medium; the inner surface, containing the thin cotyledons and underlying endosperm, was the target site subjected to DNA delivery. After particle bombardment, Petri dishes were sealed with parafilm and incubated for 24 h at 26 $^{o}$C in the dark. The GUS histochemical assay was then performed by incubating the seeds in 1 mg/ml X-Gluc substrate solution at 37 $^{o}$C overnight (Jefferson, 1987). The experiment was replicated 4-5 times.

## 2.3. Results

### 2.3.1. Acquisition of Desiccation Tolerance and Germinability of the Seed during Development

Premature drying of developing seeds during their desiccation-tolerant stage leads to germination upon subsequent imbibition. Under our growth conditions, development of tobacco seed was completed by approximately 30 d after anthesis (DAA); however, seeds subjected to slow drying (resulting in partial drying) (Kermode, 1990) or to air drying, germinated at 10-12 DAA (Fig. 2.1). Germinability of freshly harvested seed occurred slightly later during development at 15 DAA. Seeds younger than 10 DAA were not tolerant of premature drying and did not germinate upon subsequent rehydration.

### 2.3.2. Chimeric GUS Gene Constructs Expressed in Transgenic Tobacco

The two genes under study encoding vicilin and napin storage proteins exhibit normal tissue-specific and temporal regulation in transgenic tobacco (Higgins et al., 1988; Baszczynski, unpublished). As an initial step toward understanding the effect of desiccation on the expression of genes for storage proteins and to investigate the responsiveness of the 5' or 3' regions of these genes, a number of chimeric GUS genes were made and introduced into tobacco. As shown in Fig. 2.2 A, the chimeric genes consisted of 5' and/or 3' regions from the genes under study (which were being tested for desiccation sensitivity); or, as controls, the same regions derived from constitutively expressed genes (which are presumed to be desiccation insensitive)--those from the cauliflower mosaic virus (CaMV) 35S and nopaline synthase (Nos) genes. All constructs contained the coding region of a reporter gene encoding the

easily assayable enzyme: $\beta$-glucuronidase (GUS). The hypothesis being tested here is that expression of the GUS reporter gene will be sensitive to desiccation in the presence of the 5' or 3' regulatory regions from the storage protein genes (those from the vicilin and napin genes), but not when under the control of regulatory (5' or 3') regions that have no known sensitivity to this perturbation (Fig. 2.2 B).

### 2.3.3. GUS Activities in Transgenic Tobacco Seed at 20 Days after Anthesis

Activities of GUS in transgenic tobacco seed at 20 DAA expressing gene constructs containing the vicilin (*Vic*) or the napin (*Nap*) 5' upstream regions (Table2.1; Vic-GUS-Nos, Vic-GUS-Vic, Nap-GUS-Nos, and Nap-GUS-Nap) were generally 4-7 times higher than in seed expressing chimeric gene constructs containing the constitutive viral (35S) 5' upstream region (Table 2.1; 35S-GUS-Nos, 35S-GUS-Vic, and 35S-GUS-Nap). The 3' flanking region of the chimeric genes had little influence on the GUS activities exhibited by transgenic seed, although slightly lower GUS activities were observed from seed expressing the chimeric gene containing the napin 3' region (Table 2.1). No GUS activity (above background) was detected in vegetative tissues (e.g. leaves and stems) of transgenic tobacco plants expressing gene constructs containing either the vicilin or napin promoter (data not shown). A fidelity of seed-specific expression directed by vicilin and napin gene promoters in transgenic tobacco has also been shown in other studies (Higgins *et al.*, 1988; Baszczynski, unpublished data).

**Figure 2.1.** Germination of fresh, air-dried, or partially dried intact seed of tobacco harvested at various times (days) after anthesis (DAA).

## Germinability of Fresh vs. Dried Seeds

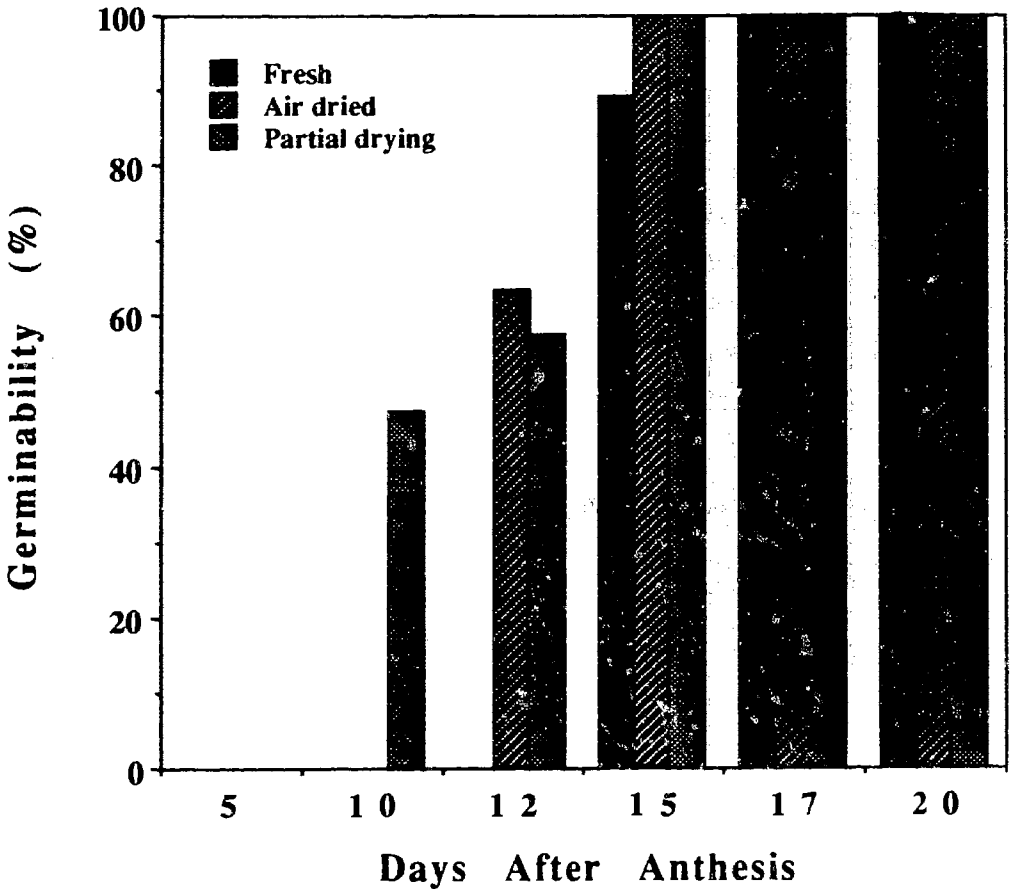
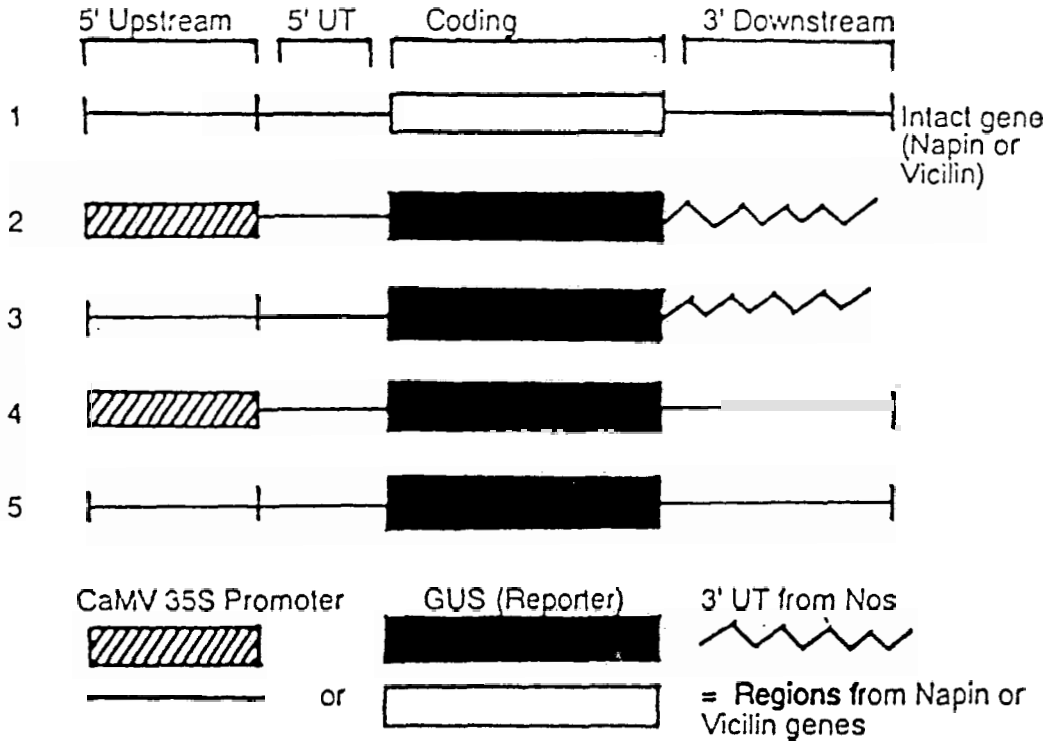


**Figure 2.2. A.** Chimeric gene constructs introduced into tobacco to determine the desiccation-responsive regions in vicilin and napin storage-protein genes. All chimeric genes contain the coding region of the bacterial reporter gene for $\beta$-glucuronidase (GUS). CaMV: cauliflower mosaic virus; UT: Untranslated. **B,** Hypothesis being tested by this study and predicted results.

A. Where are the desiccation-responsive sequences in napin and vicilin genes?




B. GUS expression:

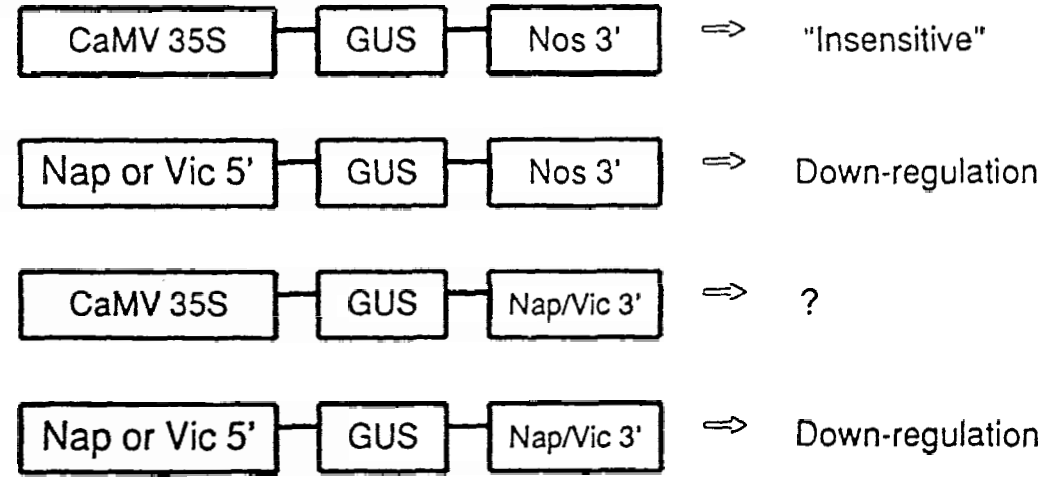


**Table 2.1.** Activity of β-glucuronidase (GUS) in developing seed at 20 d after anthesis of transgenic tobacco plants.

| Gene construct[a] | No. of plants assayed | Plants with high activity[b,c] | Average activity[c] | Range of activity[c] | Activity of highest expressor[c] |
|---|---|---|---|---|---|
| 35S-GUS-Nos | 12 | 7 | 296 | 166-425 | 425 |
| Vic-GUS-Nos | 17 | 6 | 1173 | 74-2445 | 2445 |
| Nap-GUS-Nos | 14 | 11 | 1517 | 410-2862 | 2862 |
| 35S-GUS-Vic | 18 | 6 | 292 | 150-457 | 457 |
| 35S-GUS-Nap | 16 | 10 | 162 | 89-206 | 206 |
| Vic-GUS-Vic | 15 | 8 | 1422 | 89-2610 | 2610 |
| Nap-GUS-Nap | 12 | 10 | 1044 | 601-1426 | 1426 |

[a] Vic, vicilin; Nap, napin; Nos, nopaline synthase; 35S, Cauliflower mosaic virus 35S promoter.

[b] Transgenic plants were scored as having "high" GUS activity if it was at least 120 times higher than that in seeds of untransformed (W38) tobacco plants.

[c] GUS activities are expressed as specific activities (pmol methylumbelliferone $min^{-1}$ $\mu g^{-1}$ protein).

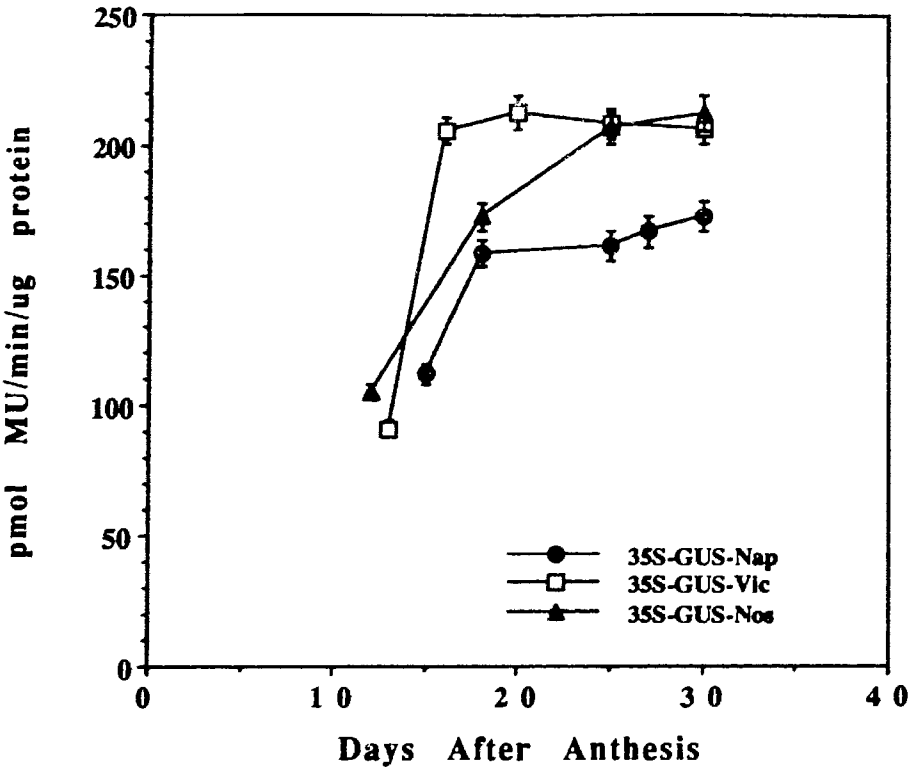
### 2.3.4. Profiles of GUS Activities and Protein during Seed Development

Generally, GUS activities were not detected in developing transgenic seed at stages younger than 10 DAA. Activities in seed expressing the gene constructs containing either the vicilin or the napin promoter increased dramatically from 12 to 20 DAA, and gradually decreased thereafter, up to seed maturity at 30 DAA (Fig. 2.3 B). However, since GUS activities were expressed as a specific activity (pmol methylumbelliferone $min^{-1}\mu g^{-1}$ protein), the decline during late development may in part be due to an increase in total seed protein. GUS activities in seed expressing constructs containing the 35S promoter increased from 12 to 18 DAA and remained fairly constant thereafter (Fig. 2.3 A). The 3' flanking region of the chimeric gene constructs affected absolute GUS activities, but had little influence on the patterns of changes in GUS activities exhibited by transgenic seed during seed development (Fig. 2.3).

Changes in GUS protein during seed development were also monitored using the technique of Western blot analysis (Fig. 2.4 A). Very little GUS protein was detected in seed expressing the two chimeric genes (Nap-GUS-Nos or 35S-GUS-Nos) at stages younger than 14 DAA (data not shown). On the Western blot, the largest change in the amount of GUS protein occurred between 15 and 20 DAA (for Nap-GUS-Nos) or between 15 and 25 DAA (for 35S-GUS-Nos); thereafter (between 25 and 30 DAA), there was little change (Fig. 2.4 A). Thus, GUS protein was maintained at a fairly constant amount during late development in transgenic seed, regardless of the 5' upstream region of the chimeric gene being expressed. However, this may be a consequence of a lower sensitivity of Western blot analysis as compared to the fluorimetric assay for GUS activity; the amount of protein used for Western blot

**Figure 2.3.** Activity of GUS in transgenic tobacco seed during seed development. **A,** GUS activities in developing seed expressing gene constructs containing the 35S promoter: **35S-GUS-Nos** (▲), **35S-GUS-Vic** (□), **35S-GUS-Nap** (●). **B,** GUS activities in developing seed expressing gene constructs containing either the vicilin or napin gene promoters: **Vic-GUS-Nos** (○), **Vic-GUS-Vic** (●), **Nap-GUS-Nos** (□) and **Nap-GUS-Nap** (■). Each point is an average ± SD of two to three determination.

A. CaMV 35S Constructs




B. Vicilin and Napin Constructs

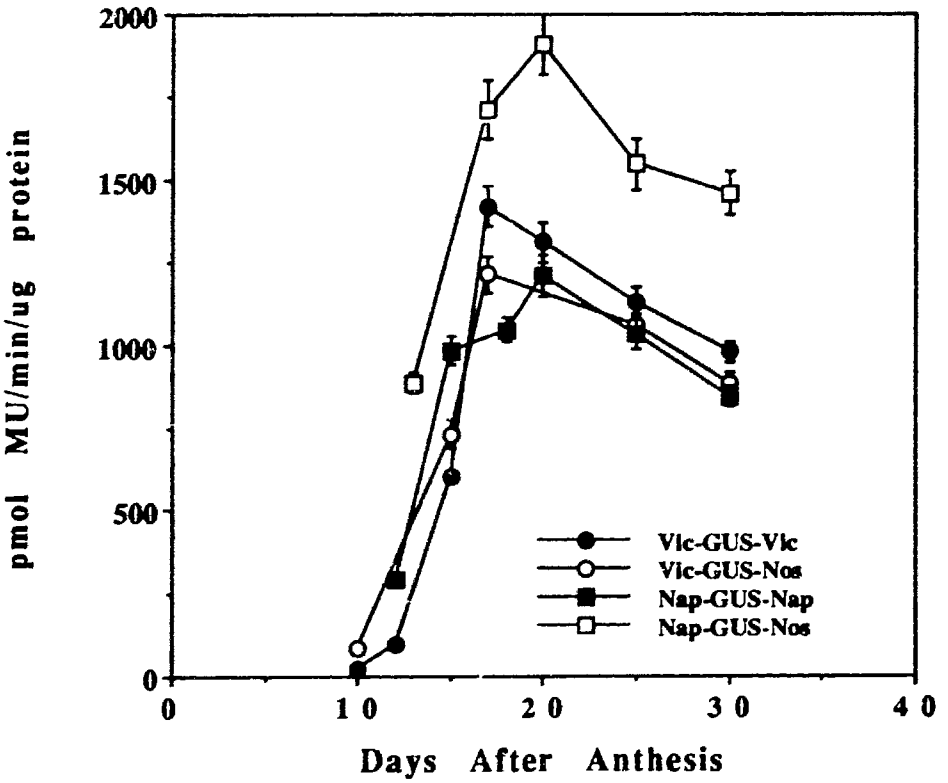
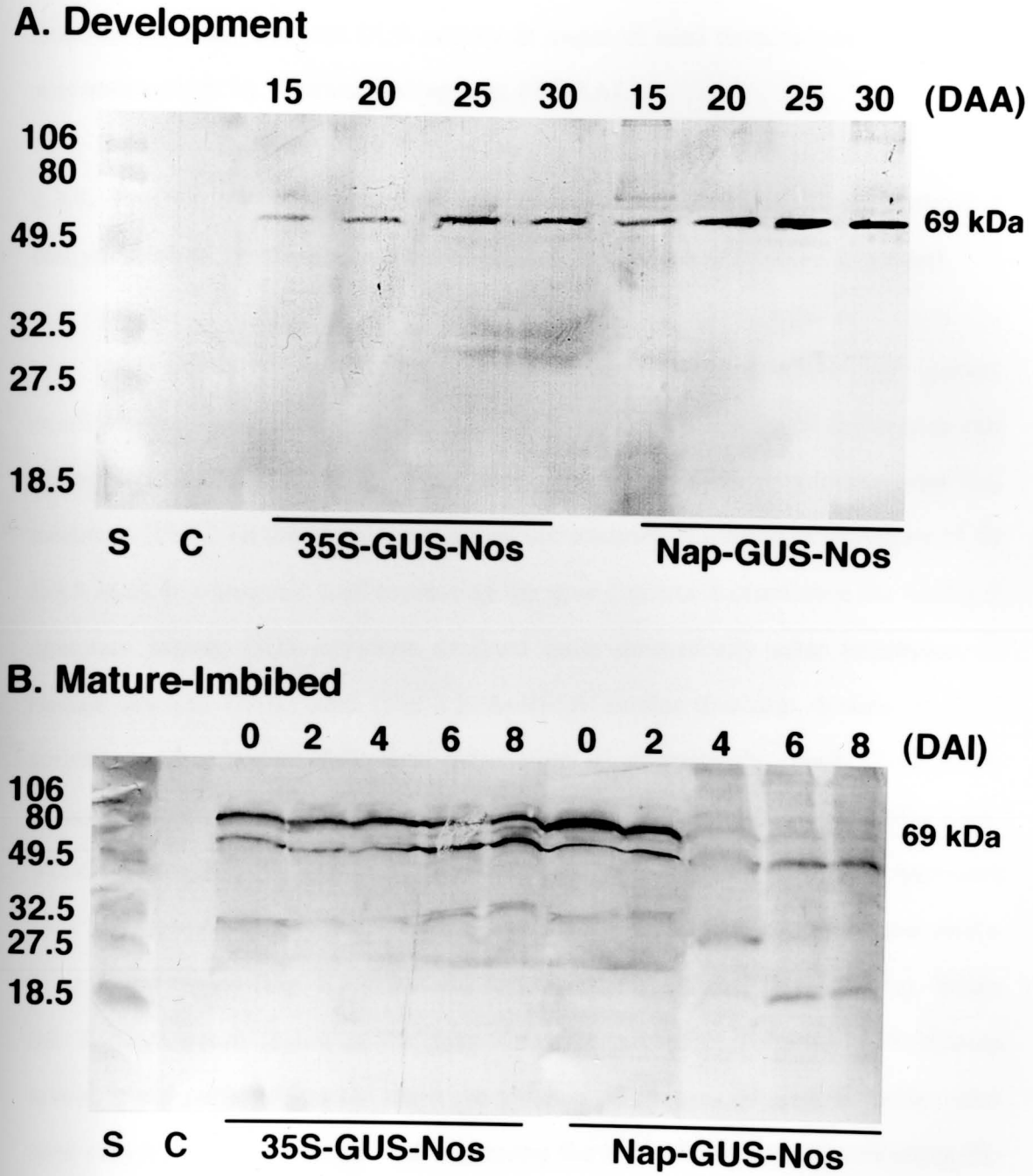


**Figure 2.4.** Western blot analysis of GUS protein in transgenic tobacco seed expressing gene constructs containing either the napin 5' upstream region (Nap-GUS-Nos) or the 35S 5' upstream region (35S-GUS-Nos) during seed development (15-30 DAA) and following imbibition of mature (30 DAA) seed. A, Developing seed (15-30 DAA); B, Mature seed (30 DAA) following imbibition (0, 2, 4, 6 and 8 days after imbibition; DAI). (C, Control W38 untransformed seed; S, Molecular weight standards.


A. Development
15 20 25 30 15 20 25 30 (DAA)
106
80
49.5
32.5
27.5
18.5
69 kDa
S C
35S-GUS-Nos
Nap-GUS-Nos
B. Mature-Imbibed
0 2 4 6 8 0 2 4 6 8 (DAI)
106
80
49.5
32.5
27.5
18.5
69 kDa
S C
35S-GUS-Nos
Nap-GUS-Nos

analysis was 10-fold higher than that used for fluorometric analysis. Further, the fluorometric assay detected GUS activity at stages of seed development when GUS was undetectable by Western blotting (e.g. 10 DAA).

### 2.3.5. Profiles of GUS Activities and Protein in Transgenic Seed Following Rehydration of Prematurely Dried Seed and Imbibition of Mature Dry Seed

As noted previously, development of tobacco seed under our growth conditions was completed by approximately 30 DAA. A tolerance of desiccation (air drying) was acquired at 12 DAA and seeds germinated upon rehydration after this treatment (Fig. 2.1); therefore, we confined our analyses of premature drying to 12-14 DAA seed. In transgenic seed expressing the gene constructs containing the vicilin 5' upstream region, GUS activities declined quite dramatically after imbibition of mature dry (30 DAA) seed (Fig 2.5 A+B). A similar dramatic decline in GUS activities was apparent following rehydration of prematurely dried 12-14 DAA tobacco seed which mirrored the pattern of decline in mature-imbibed seed (Fig 2.5A: Vic-GUS-Nos and Fig. 2.5B: Vic-GUS-Vic). In contrast, GUS activities increased after seed rehydration when the constitutive viral (35S)promoter replaced the vicilin 5' upstream region (Fig. 2.5A: 35S-GUS-Nos and Fig. 2.5B: 35S-GUS-Vic). While the 3' downstream region of the gene construct did affect absolute GUS activity levels, it did not significantly affect the patterns of changes in GUS activities after seed rehydration. Analysis of seed expressing the chimeric constructs containing the napin gene 5' and/or 3' regulatory regions revealed similar differences in the fate of GUS activity (upon imbibition or rehydration) when the 35S promoter replaced the storage protein gene promoter (Fig. 2.5C: Nap-GUS-Nos vs. 35S-GUS-Nos and Fig. 2.5D: Nap-GUS-Nap vs. 35S-GUS-Nap). Although the general pattern of changes was similar, a much sharper and more dramatic decline of GUS activity occurred

following hydration of mature and prematurely dried seed expressing chimeric constructs containing the napin 5' upstream region as compared to those containing the vicilin 5' upstream region [compare Nap-GUS-Nos (Fig. 2.5C) vs. Vic-GUS-Nos (Fig. 2.5A) and Nap-GUS-Nap (Fig. 2.5D) vs. Vic-GUS-Vic (Fig. 2.5B)]. This may indicate a greater sensitivity of the napin promoter to desiccation/rehydration. Regardless of the gene construct expressed, the pattern of changes in reporter enzyme activities after rehydration of mature vs. prematurely dried seed were strikingly similar (Fig. 2.5).

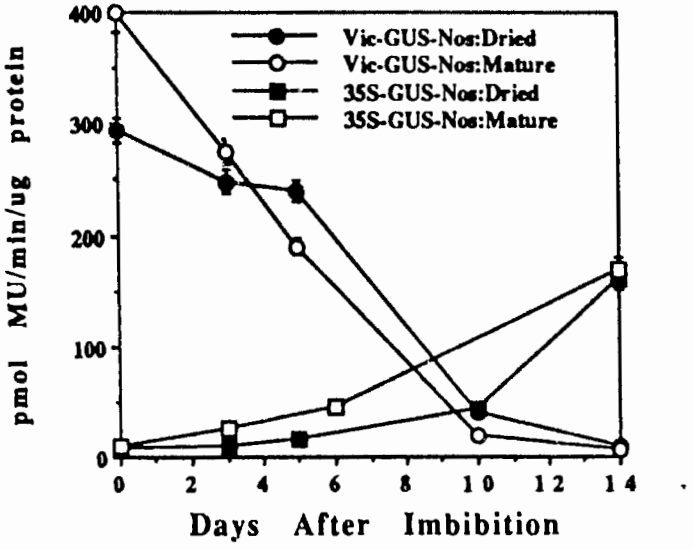
Changes in GUS protein following imbibition of mature seed were also monitored by Western blot analysis (Fig. 2.4 B). The amount of GUS protein (69-kD) detected in seed expressing the chimeric gene containing the 35S 5' flanking region (35S-GUS-Nos) remained unchanged during imbibition (0-8 d). In contrast, there was a decline in the amount of GUS protein in seed expressing the chimeric gene containing the napin 5' flanking region, particularly after the second day of imbibition; no GUS protein (at 69 kD) was detected at day 8. The lower molecular weight bands on the Western blot (at 4-8 days after imbibition) may represent degradation products of GUS protein.

Thus, the dramatically different effect of desiccation/rehydration upon the fate of GUS reporter gene expression driven by the storage protein gene promoter vs. the 35S promoter, which was evident from the changes in GUS activity upon imbibition or rehydration, was also revealed at the level of detectable GUS protein.

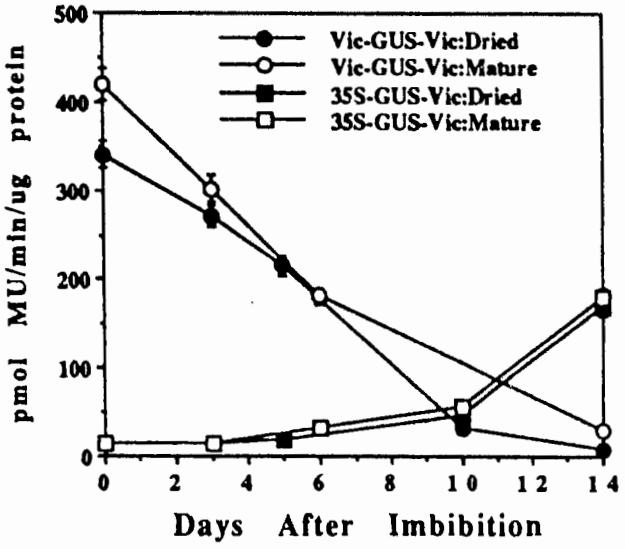
It is noteworthy that in Figures 2.3 to 2.5 changes in GUS reporter were monitored in seed from a high-expressing plant. However, the patterns of changes in GUS activity or protein across the seven chimeric constructs (during development or

**Figure 2.5.** Activity of GUS in transgenic tobacco seed after rehydration of prematurely dried seed 12 to 14 DAA and imbibition of mature seed 30 DAA in seed expressing the chimeric gene constructs: **A**, Vic-GUS-Nos (mature (○) or prematurely dried (●) seed) or 35S-GUS-Nos (mature (□)or prematurely dried (■) seed); **B**, Vic-GUS-Vic (mature (○) or prematurely dried (●) seed) or 35S-GUS-Vic (mature (□) or prematurely dried (■) seed); **C**, Nap-GUS-Nos (mature (○) or prematurely dried (●) seed) or 35S-GUS-Nos (mature (□) or prematurely dried (■) seed); **D**, Nap-GUS-Nap (mature (○) or prematurely dried (●) seed) or 35S-GUS-Nap (mature (□) or prematurely dried (■) seed). All at 0-14 DAI or days after rehydration. Each point is an average of 2-3 determinations ± s.d.

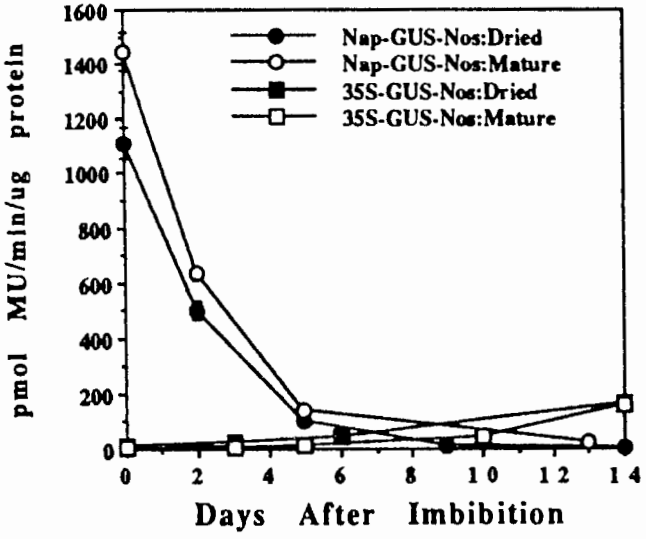
A. 35S-GUS-Nos vs. Vic-GUS-Nos



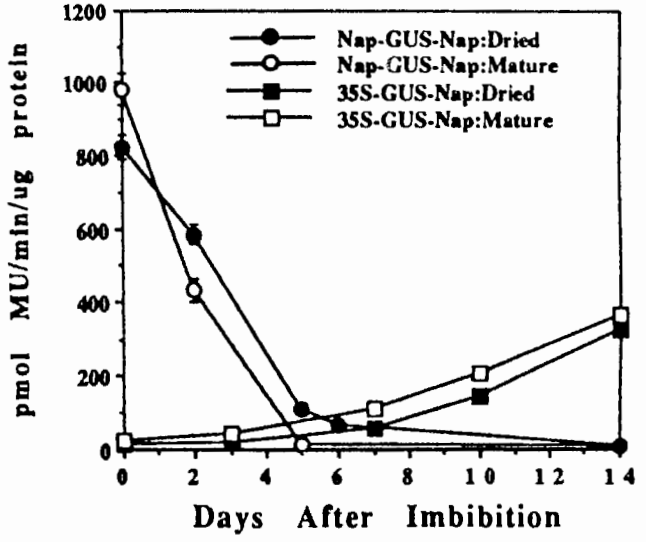

B. 35S-GUS-Vic vs. Vic-GUS-Vic




C. 35S-GUS-Nos vs. Nap-GUS-Nos




D. 35S-GUS-Nap vs. Nap-GUS-Nap



following imbibition/rehydration) were very similar, regardless of the absolute amount of GUS expression (i.e. whether seeds were from a high-expressing plant or a lower-expressing plant) (data not shown).

When developing fresh tobacco seeds were not dried, but maintained on a simple agar medium after excision from the capsule (Fig. 2.6), the GUS activities either increased (e.g. in transgenic seed expressing the constructs containing the vicilin 5' upstream region) or remained fairly constant (e.g. in transgenic seed expressing the constructs containing the 35S 5' upstream region). GUS activities in seed expressing chimeric constructs containing the napin 5' upstream region increased in a manner similar to those in seed expressing constructs in which the vicilin 5' upstream region was present (data not shown). Thus, only desiccation (and not simply seed detachment from the parent plant) is able to down-regulate GUS gene expression in the presence of the storage protein gene 5' upstream region.

### 2.3.6. GUS mRNA Profiles during Seed Development and after Premature Drying

Since GUS is an extremely stable enzyme, the effect of desiccation on the amount of GUS mRNA in transgenic tobacco seed was also determined using RNA dot blot analysis (Fig. 2.7). The amount of GUS mRNA gradually increased during development from 5 to 20 DAP in transgenic tobacco seed expressing the chimeric gene construct containing the vicilin 5' upstream region (Fig. 2.7, Vic-GUS-Nos). Transgenic seed expressing the chimeric gene construct containing the viral (35S) 5' upstream region showed a fairly constant amount of GUS mRNA during development from 5 to 15 DAP, although the amount of message appeared to increase between 15 and 20 DAP. After premature desiccation at 20 DAP, GUS mRNA decreased

**Figure 2.6.** Activity of GUS in developing transgenic tobacco seed at 10-12 DAP during a hydrated control treatment for 5d: **A,** Vic-GUS-Nos; **B,** Vic-GUS-Vic; **C,** 35S-GUS-Nos; **D,** 35S-GUS-Vic. Developing seeds were placed on Petri dishes containing 1% bacterial agar and the GUS activity was determined. MU, Methylumbelliferone.

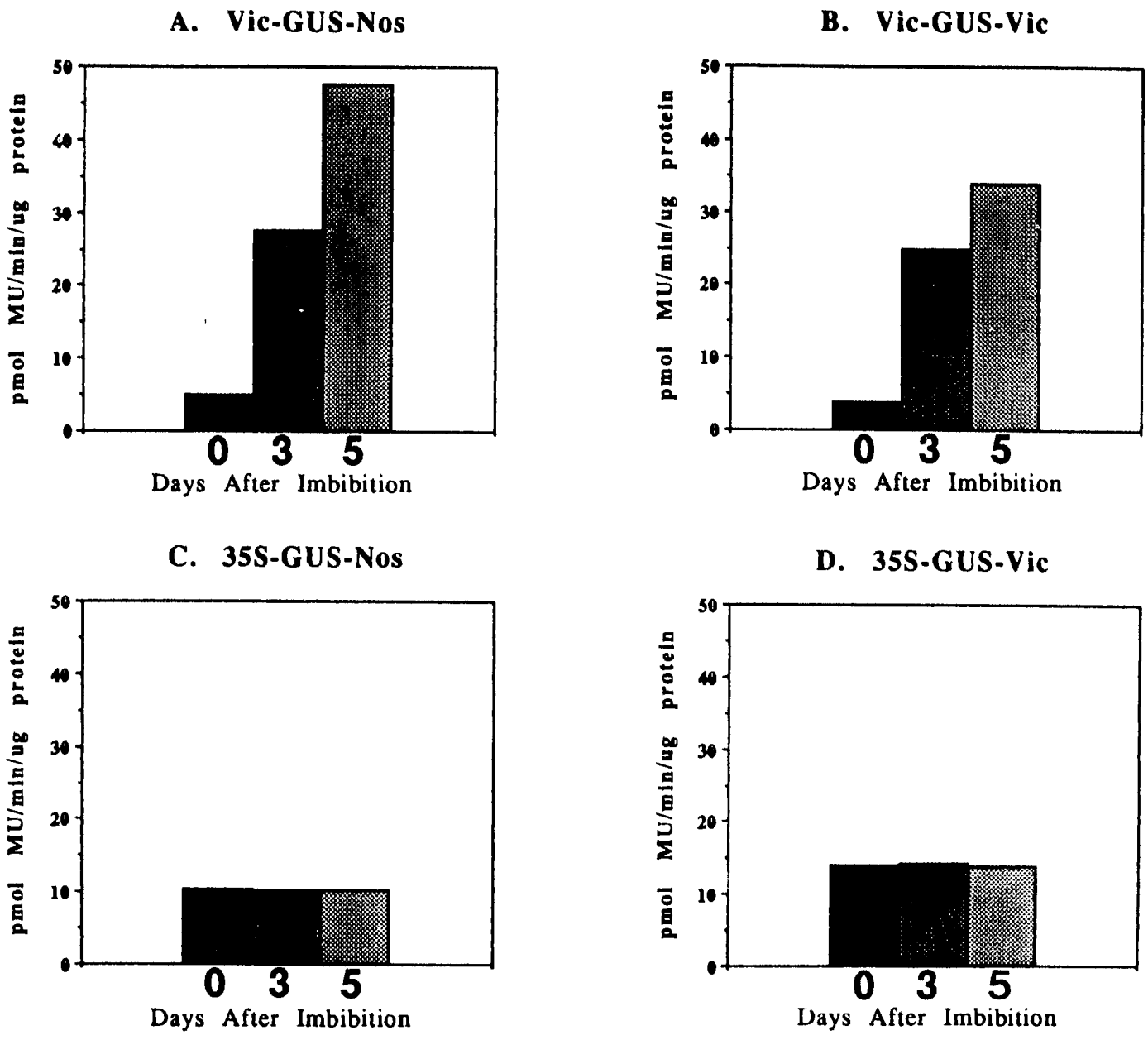
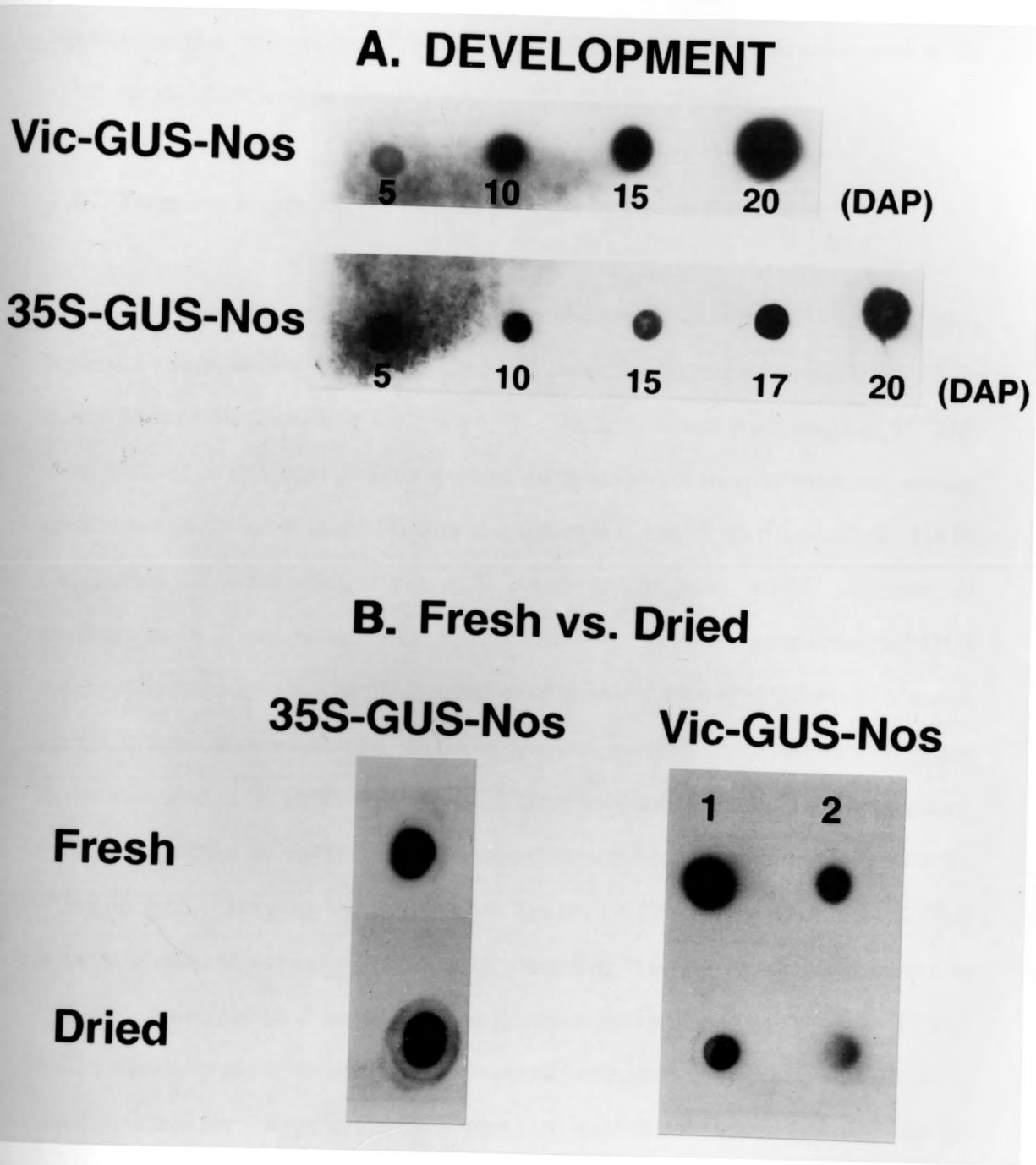

A. Vic-GUS-Nos
B. Vic-GUS-Vic
C. 35S-GUS-Nos
D. 35S-GUS-Vic
pmol MU/min/ug protein
0
10
20
30
40
50
0 3 5
Days After Imbibition

**Figure 2.7.** RNA dot blot analysis of GUS mRNA in developing fresh or dried transgenic tobacco seed at 20 DAP expressing the chimeric constructs 35S-GUS-Nos or Vic-GUS-Nos: A, at developmental stages from 5 to 20 DAP; B, effect of premature drying in developing seed at 20 DAP. The 1.87 kb fragment for the GUS coding region was used as probe to do the detection.

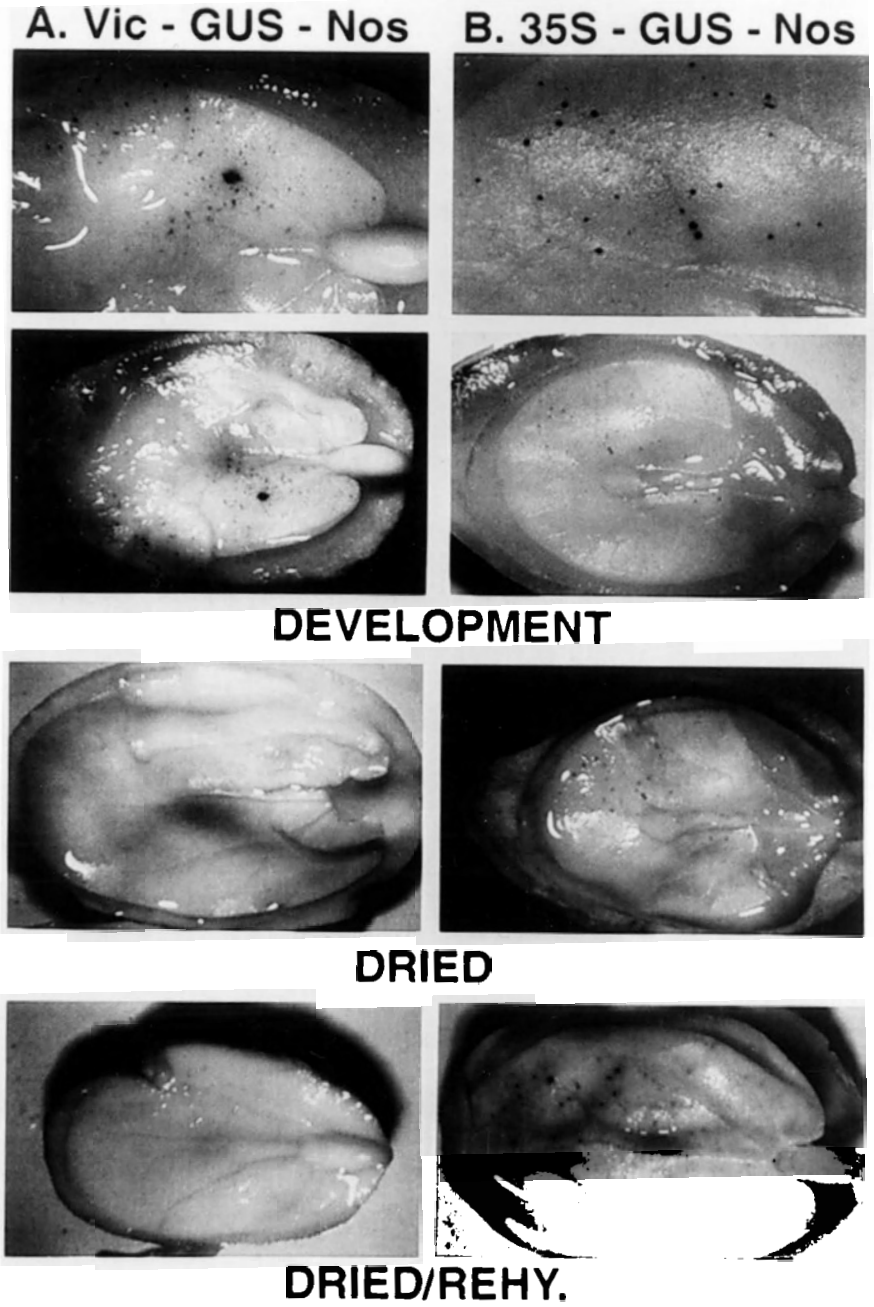

A. DEVELOPMENT
Vic-GUS-Nos
5
10
15
20
(DAP)
35S-GUS-Nos
5
10
15
17
20
(DAP)
B. Fresh vs. Dried
35S-GUS-Nos
Vic-GUS-Nos
Fresh
Dried
1
2

substantially, but only in transgenic seed expressing the gene construct containing the vicilin 5' upstream region (Fig.2.7 B, Vic-GUS-Nos, 1 and 2); when the 35S 5' upstream region was present, the amount of GUS mRNA in transgenic seed at 20 DAP remained unchanged after drying (Fig. 2.7 B, 35S-GUS-Nos).

### 2.3.7. Transient Expression of Chimeric Genes in Castor Bean Seed

The chimeric genes containing either the vicilin or viral (35S) 5' upstream regions (Vic-GUS-Nos and 35S-GUS-Nos) were also expressed transiently within castor bean cotyledons using biolistics (Fig. 2.8). Cotyledons from seeds at 35 DAP were utilized; at this time of development, seeds are desiccation-tolerant and storage protein is rapidly synthesized (Kermode and Bewley, 1985b; Kermode *et al.*, 1985). Cotyledons of developing fresh (35 DAP) castor bean seeds subjected to microprojectile bombardment with the chimeric Vic-GUS-Nos gene exhibited GUS activity (detected by histochemical assay) prior to desiccation (Fig. 2.8A). In contrast, no GUS activity was detected by histochemical staining in cotyledons of either desiccated seed at 35 DAP, or those at 35 DAP rehydrated for 48h after desiccation (Fig. 2.8A). However, castor bean cotyledons subjected to biolistic treatment with the chimeric gene containing the 35S 5' upstream region (35S-GUS-Nos) showed GUS activity whether or not desiccation was imposed (i.e. in cotyledons from fresh seed at 35 DAP, desiccated seed at 35 DAP and desiccated/rehydrated seed at 35 DAP; Fig. 2.8B). These results were confirmed by several replications. Castor bean seeds at 35 DAP hydrated for 7 days in the fresh state (i.e. immediately after excision from the capsule) did not survive the hydration treatment and rapidly deteriorated; the cotyledons of these seeds were incapable of expressing GUS activity regardless of the gene construct used for biolistics (data not shown).

**Figure 2.8.** Histochemical assay after biolistic treatments to examine the effect of desiccation and desiccation/rehydration on the activity of GUS within developing castor bean cotyledons at 35 days after pollination (DAP). Biolistic treatment using (**A**) the Vic-GUS-Nos chimeric gene and (**B**) the 35S-GUS-Nos gene. The top two panels are developing fresh seed at 35 DAP. The GUS histochemical assay was performed 24h after biolistic treatment by incubating the seeds in 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) substrate solution overnight at 37 $^{o}$C.


A. Vic - GUS - Nos
B. 35S - GUS - Nos
DEVELOPMENT
DRIED
DRIED/REHY.

## 2.4. Discussion

Taken together, these results suggest that premature desiccation may down-regulate expression of storage-protein genes upon rehydration by acting directly upon the 5' upstream region of these genes. Distinct differences in expression of GUS reporter in response to desiccation/rehydration were observed in transgenic seed expressing chimeric genes containing either the vicilin or the napin gene promoter vs. a promoter derived from a constitutively expressed gene, the 35S promoter of cauliflower mosaic virus. Moreover, the effects of premature desiccation and subsequent rehydration mimicked those occurring after natural desiccation (i.e. in mature seed after imbibition). Results from a hydrated control treatment indicated that only desiccation (and not simply seed detachment from the parent plant) is able to down-regulate GUS gene expression in the presence of the storage protein gene 5' upstream region.

The 3' flanking region of a gene is often associated with post-transcriptional controls such as the regulation of mRNA stability. Very little effect upon the general patterns of changes in GUS expression (at least at the protein activity level) was detected, whether the chimeric gene contained this region from the storage-protein genes (vicilin or napin) or the constitutively expressed *Agrobacterium* T-DNA gene (Nos). However, more information is needed regarding changes in GUS mRNA following desiccation/rehydration in transgenic seed expressing the various chimeric genes containing different 3' regions. Further, because all the chimeric gene constructs contain the GUS coding region, we cannot fully address the role of regulation at the post-transcriptional level.

The fate of GUS mRNA during development and following premature drying was determined for two of the gene constructs (Vic-GUS-Nos and 35S-GUS-Nos) (Jiang and Kermode, 1994). After premature desiccation at 20 DAA, GUS mRNA decreased substantially, but only in transgenic seed expressing the gene construct containing the storage protein (e.g., vicilin) 5' upstream region; when the 35S 5' upstream region was present, the amount of GUS mRNA in transgenic seed at 20 DAA remained unchanged after drying (Fig. 2.7.; Jiang and Kermode, 1994). I am presently investigating the effect of premature desiccation imposed at an earlier developmental stage (e.g., at 12 DAA) and whether the substantial amount of GUS mRNA preserved after drying is rapidly lost upon subsequent rehydration. Oliver *et al.* (1993) demonstrated a down-regulation of phaseolin mRNA production following desiccation (in transgenic seed expressing an intact $\beta$-phaseolin gene); however, it was clear that the rehydration phase following natural or imposed drying was the critical event leading to an mRNA decline. In this study, I did not systematically compare difference in the 5' untranslated region of the chimeric genes; however, no differences in desiccation responsiveness were observed between two $\beta$-phaseolin gene constructs in which the natural 5' untranslated region of the intact $\beta$-phaseolin gene was replaced with an alfalfa mosaic virus 5' untranslated region (Oliver *et al.*, 1993).

Does desiccation (maturation drying) act *in planta* as a "cue" for eliciting the decline of storage-protein synthesis during late seed development? Clearly, desiccation may not play a causative role in all species--particularly in seeds in which deposition of storage protein ceases prior to major water loss. Here, the attainment of a specific physiological age and/or the cessation of maternal vascular supply may be operative (Galau *et al.*, 1987). Within pea seeds, messages for the major storage proteins (including vicilin) decline slightly earlier than the onset of maturation drying

(Chandler *et al.*, 1984; Boulter *et al.*, 1987). In contrast to changes in vicilin mRNA within the pea seed, the decline in napin mRNA within *B. napus* seed correlates well with the onset of maturation drying (DeLisle and Crouch, 1989). The present results in transgenic tobacco and in castor bean seed suggest that the vicilin gene (particularly the vicilin 5' upstream region) is responsive to desiccation; however, they also indicate that the napin 5' upstream region may be more responsive to desiccation/rehydration than the vicilin 5' upstream region. Transient or stable expression of the vicilin and napin chimeric genes within seeds of the homologous plant hosts (i.e. the hosts from which the vicilin and napin genes were isolated), pea and *B. napus*, respectively, may help to elucidate the role of desiccation *in planta*.

If indeed, drying is acting in some seeds to down-regulate expression of storage protein genes, it may mediate this effect by causing a reduction in the synthesis, stability and/or binding ability of positive *trans*-acting factors which interact with *cis*-active elements of storage protein genes. Experiments to address this latter possibility are underway.

In addition to possible effects of drying upon *trans*-acting factors, desiccation could also act through changes in the content of hormones (or their receptors) involved in the expression of storage-protein genes (Kermode *et al.*, 1989a). ABA has been implicated in the regulation of storage protein synthesis; whether this hormone acts (*in planta*) specifically to increase the production of storage protein mRNAs during the maturation or seed-expansion phase--when maximum synthesis occurs--remains to be determined. Some evidence supports this contention, and at the transcriptional level, some of the *cis*-regulatory sequences responsive to ABA within developmentally regulated genes (e.g. the sunflower helianthinin gene) have been identified (reviewed in Thomas, 1993). In chapter 3, I investigate the hypothesis that

the maturation drying period, by effecting changes in the content of, or sensitivity to, ABA may partially or wholly remove the effects of this "positive modulator" of developmental gene expression (see Chapter 3).

*Chapter 3*

# Desiccation Causes a Decline in the Sensitivity of Vicilin and Napin Storage Protein Gene Promoters to ABA in Transgenic Tobacco Seed

## 3.1. Introduction

An important area of study in seed development is focused upon the role of the seed or maternal environment in maintaining embryos in a developing mode until they are fully formed and have accumulated sufficient reserves to permit successful germination and subsequent seedling growth. These studies have implicated two potential regulatory factors: abscisic acid (ABA) and restricted water uptake (Kermode, 1990, 1995). In many cases, germination and growth of the immature/developing embryo can be elicited precociously by its removal from the surrounding seed tissues, thus bypassing the later stages of development. However, for most seeds, a period of desiccation is the normal terminal event in embryo development and there is now substantial evidence that maturation drying is critical in terminating development and in overcoming constraints by the maternal environment that maintain seeds in a developmental mode (Kermode and Bewley, 1986a, 1987; Kermode et al., 1986, 1989b; Kermode 1990, 1995; Jiang and Kermode, 1994). This results in a switch in cellular activities from an exclusively developmental program to an exclusively germination/growth-oriented program.

Premature drying has been used as an important experimental approach to define the role of desiccation in switching seed from development to germination. In effecting this switch in gene expression, desiccation appears to act primarily at the transcriptional and posttranscriptional levels (Misra and Bewley; Cornford et al., 1986; Kermode et al., 1989c; Jiang and Kermode, 1994; Jiang et al., 1995). I am interested in examining events at the molecular level caused by drying, which ultimately result in the off- or down-regulation of development-related genes and the on-regulation of germinative and post-germinative genes. The 5' upstream regions of

vicilin and napin storage protein genes are responsive to desiccation in transgenic tobacco seed (Jiang and Kermode, 1994; Jiang *et al.*, 1995). For example, GUS gene expression is sensitive to premature drying and is down-regulated upon subsequent rehydration in the presence of the 5' regulatory regions of the storage protein gene, but not when under the control of the same region from the cauliflower mosaic virus 35S promoter. Thus premature desiccation may down-regulate storage protein gene expression upon rehydration by acting directly upon the 5' upstream regions of these genes. Following drying, this region may become insensitive to positive controlling factors; alternatively, changes to *trans*-acting factors important for gene expression may occur as a result of drying (Jiang *et al.*, 1995; Oliver *et al.*, 1993).

In addition to the possible effects of drying on *trans*-acting factors, it could also act through changes in the content of hormones (or their receptors) involved in the expression of the storage protein genes. ABA has been implicated in the regulation of storage protein synthesis and some of the *cis*-acting elements responsive to ABA within the developmentally regulated genes, such as the sunflower helianthinin gene, have been identified (reviewed in Thomas, 1993). A major objective is to test the hypothesis that a maturation drying period, by effecting changes in the content of, or sensitivity to, ABA may partially or wholly remove the effects of this 'positive modulator' of developmental gene expression. In developing castor bean seeds, premature drying leads to a major decline in ABA content; a reduction in embryo sensitivity to ABA is also evident following drying, the latter perhaps being more crucial for effecting the 'switch' (Kermode and Bewley, 1987; Kermode *et al.*, 1989a).

As an initial step toward understanding the possible interactions between desiccation and ABA at the molecular level, I examined the enhancement of GUS

activity in transgenic tobacco by exogenous ABA before and after premature drying. The chimeric genes constructs described in chapter 2 were used to address several questions. For example, are the 5' flanking regions of the storage protein genes responsive to ABA in the developing seed? Further, is this responsiveness diminished following premature drying? And if so, is the reduced sensitivity due to desiccation *per se*, or a period of seed detachment from the parent plant? This has yielded preliminary results with vicilin and napin chimeric genes suggesting that these developmentally regulated genes, initially responsive to ABA, become relatively insensitive to the hormone following premature drying. Premature drying did not lower the content of endogenous ABA in developing tobacco seed; however, upon subsequent rehydration, the amount of ABA declined dramatically. A decline in ABA sensitivity may be an important factor in the cessation of storage protein gene expression during late seed development or upon subsequent rehydration following natural or imposed drying.

## 3.2. Materials and Methods

### 3.2.1. Generation of Transgenic Tobacco Seeds

Generation of chimeric gene constructs and transgenic tobacco plants used for this study is described in Jiang *et al.* (1995 and chapter 2). The gene constructs used in this study are shown in Figure 3.1. The chimeric gene constructs consisted of the coding region of the reporter gene for GUS and 5' and/or 3' regions from vicilin and napin genes; or as control, the same regions derived from constitutively expressed genes (CaMV 35S and nopaline synthase), presumed to be non reponsive to

desiccation or ABA. Transgenic tobacco plantlets have been maintained by subculturing in MS (Murashige and Skoog, 1962) medium. Plantlets were transferred to a soil:peat mixture (3:1) and grown to maturity in the greenhouse (24-26 $^{o}$C during daytime and 20-22 $^{o}$C at nighttime, and 16-h photoperiod). Flowers were tagged at the time of anthesis just as the petals opened and began to turn pink. Seeed were collected at different days after anthesis (DAA) and maturity (30 DAA).

### 3.2.2. ABA Treatments of Developing, Prematurely Dried and Hydrated Control Seeds

To test the effects of exogenous ABA on GUS activities in transgenic tobacco seed, three sources of ABA were used: a racemic mixture (Sigma, St. Louis, MI) and the two isomers of ABA (the natural form, (S)-(+) ABA and the unnatural form, (R)-(-)ABA). These two isomers of ABA were prepared by resolution of the methyl ester of racemic ABA, and then hydrolyzed to the acid as described in Dunstan *et al.* (1992).

Developing tobacco seed (12 DAA) expressing the various gene constructs were harvested and either used immediately in the fresh state or were air-dried for 5d prior to the ABA treatments. Seeds isolated from pods were placed in Petri dishes containing 1% bacteriological agar (GIBCO, BRL) or 1% agar (Sigma) supplemented with ABA. The racemic ABA mixture used was 10-40 μM, while the optically pure ABA isomers were used at 1-40 μM. Seeds were incubated at 25 $^{o}$C with 16h photoperiod. Samples were collected at 0, 3, and 5d after incubation and extracts prepared for GUS assays. As a control, fresh 12 DAA transgenic seeds were maintained for 5d (a period equivalent to the drying regime) on a 1% bacterial agar medium prior to the incubation treatment. Procedures for preparing extracts and

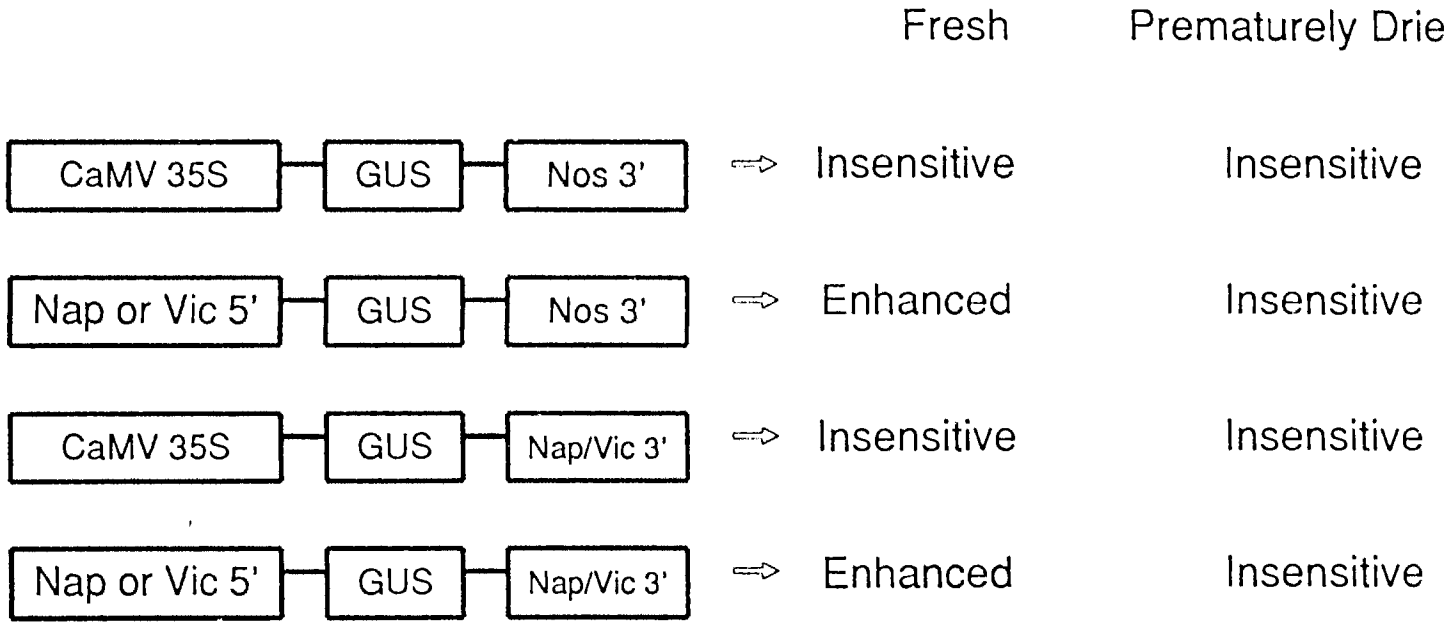
measuring GUS activity by the fluorimetric assay are as described in Jefferson (1987) and Jiang *et al.* (1995; Chapter 2). GUS activity was expressed as pmol methylumbelliferone per min per µg of seed protein. Protein concentrations were determined by the Bradford assay (Bradford, 1976).

### 3.2.3. ABA quantitation via immunoassay

To measure changes in endogenous ABA content in tobacco seed during development, seeds of various stages were harvested, frozen immediately in liquid $N_2$, and kept at -80 $^{o}$C prior to use. To examine the effect of premature drying on ABA content, seeds were harvested at 12 DAA and subjected to a 5-d air-drying treatment. For a hydrated control treatment, pods were maintained at 100% relative humidity for 5d. To monitor any changes in ABA upon rehydration following natural and premature drying, 12 DAA prematurely-dried seeds and mature dry seeds (30 DAA) were imbibed in water for 3-5d. Following all treatments, seeds were frozen in liquid $N_2$ and kept at - 80 $^{o}$C prior to ABA quantification. For ABA analysis, seeds were ground to a fine powder in liquid $N_2$ and extracted with water or buffer essentially as described in Weiler (1980) and Quarrie *et al.*, (1988) with some modifications. Water has previously been shown to be as effective as aqueous methanol and acetone in extracting the ABA from plant materials (Quarrie *et al.*, 1988). The amount of free ABA in the extraction was determined by using an ABA immunoassay kit from Idetek (Sunnyvale, CA) as per the manufacturer's instructions. Briefly, seed powder (equivalent to 200 to 400 mg fresh weight) was transferred into 5 ml of extraction buffer (25 mM Tris-HCl, pH 7.5 or water), mixed well and the extraction processes carried out for 72h at 4 $^{o}$C with gentle shaking in the dark. After centrifugation (13,000 rpm for 10 min) in a microfuge, the supernatant (100 µl) was

**Figure 3.1**. Chimeric gene constructs introduced into tobacco to determine the interaction between desiccation and the ABA responsiveness of the 5' flanking regions of vicilin and napin storage-protein genes. All chimeric genes contain the coding region of the bacterial gene for GUS. CaMV 35S, cauliflower mosaic virus 35S promoter; Nos, nopaline synthase. Also the hypothesis being tested.

Enhancement of GUS Expression by ABA in Developing Seed



used in triplicate for ABA assays. For recovery determination, 50 ng of ABA was added into the extraction buffer; the average percent recovery was 82%.

## 3.3. Results

### 3.3.1. Effect of Desiccation on the ABA-Sensitivity of Storage Protein Gene Promoters

The GUS activities in fresh and prematurely dried seed at 12 DAA after 5d of culture on basal medium containing no ABA or 10 μM racemic ABA are shown in Figure 3.2. Developing fresh seed expressing the chimeric gene constructs containing the vicilin 5' upstream region (Fig. 3.2 A, Vic-GUS-Nos and C, Vic-GUS-Vic) showed about a doubling of GUS activity when cultured in the presence of ABA. After desiccation of these developing seeds, GUS activity decreased; moreover, there was no enhancement of GUS activity by ABA after drying (Fig.3.2, A and C, Dried (D)). This decline in ABA response after drying is not due to the period of seed detachment from the mother plant required to effect water loss. Enhancement of GUS activity by ABA in hydrated control seeds (seeds at 12 DAA previously maintained on a 1% bacterial agar for 5d, a period equivalent to the drying treatment) was similar to that exhibited by developing fresh seed (data not shown). In contrast, developing fresh seed expressing the chimeric gene constructs containing the CaMV 35S promoter showed no enhancement of GUS activity by ABA, either before or after desiccation (Fig. 3.2, B and D).

### 3.3.2. Effect of ABA Isomers on GUS Gene Expression in Developing and Prematurely Dried Seeds

Using a racemic mixture of ABA (10 μM), developing fresh seed expressing the chimeric gene constructs containing the napin promoter (Nap-GUS-Nos) showed an enhancement of GUS activity by ABA of about three-fold (data not shown). Application of the optically pure ABAs (1 μM) revealed that the enhancement in GUS activity in developing fresh seed expressing the construct containing the napin promoter was largely due to the natural (S)-(+) form of ABA rather than the unnatural (R)-(-) ABA isomer (Fig. 3.3). Following premature drying of developing seed, the enhancement of GUS activity effected by exogenous application of the natural ABA isomer was completely abolished. In contrast, in developing seed expressing the gene construct containing the CaMV 35S promoter, both ABA isomers of the same concentration had little effect on GUS activities, whether or not premature drying was applied (Fig.3.3, C and D). These results suggest that vicilin and napin 5' flanking regions, initially responsive to ABA, becomes *relatively* insensitive to the hormone after desiccation.

It is also possible that drying enhances the metabolism of ABA; we are presently examining this hypothesis. Only the (+)-ABA isomer, but not the (-)-ABA, is capable of being metabolized by somatic embryo suspension cultures of white spruce (Dunstan *et al.*, 1992).

### 3.3.3. Changes in ABA during Tobacco Seed Development and Following Premature Drying and Control Treatments

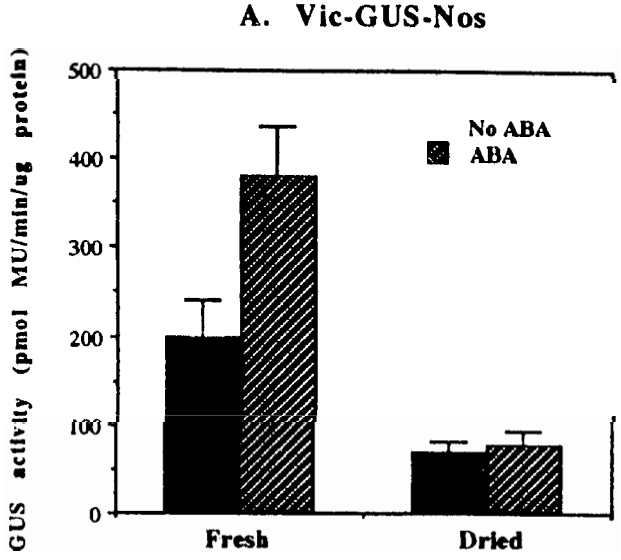
As an initial step toward determining the effects of premature desiccation on ABA metabolism, we examined changes in ABA content within the seed during normal tobacco seed development and following premature desiccation of 12 DAA seeds. In addition, it was necessary to determine the effects of a control treatment in

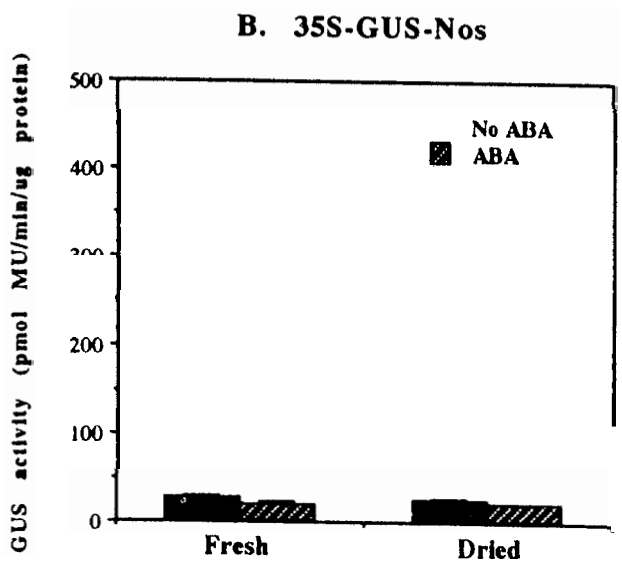
which pods were detached from the parent plant but prevented from undergoing water loss. As shown in Figure 3.4 A, the amount of endogenous ABA (expressed as ng ABA/mg fresh weight and pg ABA/seed) increased during seed development to a maximum around 13-14 DAA; thereafter, it decreased dramatically. Following imbibition of the mature dry (30 DAA) seed, ABA decreased even further (Fig. 4C).

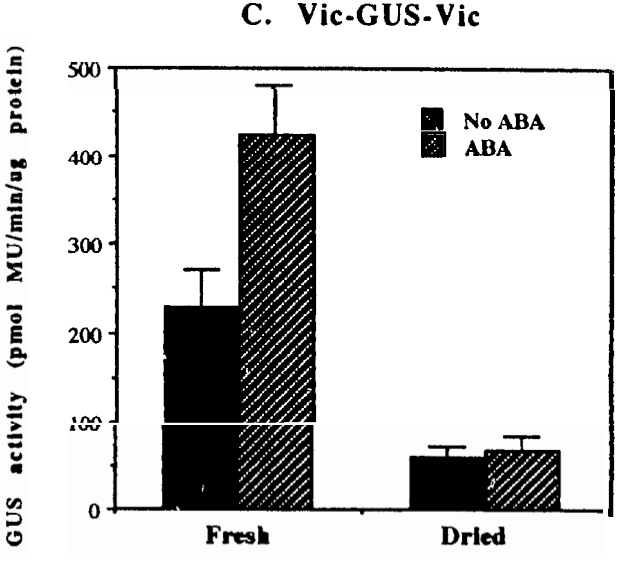
A similar profile of changes in ABA was observed in seed of different (individual) tobacco plants, wild type (untransformed) and transgenic, even though some variability was observed in absolute amounts during the developmental stages at which ABA was at a maximum (13-14 DAA). Further, little difference was observed between seed of transgenic and untransformed (W38) tobacco plants (data not shown).

Premature drying did not lower the endogenous ABA of developing 12 DAA seed, as compared to its nondried counterpart; however, upon subsequent rehydration, the amount of ABA declined dramatically (Fig. 3.4B; compare F,D and R). The 5-d hydrated control treatment had very little effect on the ABA content in developing 12 DAA seed (Fig. 3.4B; compare F and C). Thus, premature drying may lead to a decline in the ABA-promotion of developmental events, without effecting a decline in the amount of endogenous ABA.

**Figure 3.2.** Effect of desiccation on the subsequent response to ABA (10 μM) of the vicilin storage protein gene promoter, as measured by activity of GUS in developing 12 DAA transgenic tobacco seeds after 5d of culture. A, GUS activities in seeds expressing Vic-GUS-Nos gene construct after various treatments: fresh=12 DAA developing seeds, dried=prematurely dried 12 DAA seeds, no ABA=culture without ABA, ABA=culture with ABA. B, C, D, as A but different gene constructs.


A. Vic-GUS-Nos
GUS activity (pmol MU/min/ug protein)
500
400
300
200
100
0
No ABA
ABA
Fresh
Dried


B. 35S-GUS-Nos
GUS activity (pmol MU/min/ug protein)
500
400
200
100
0
No ABA
ABA
Fresh
Dried


C. Vic-GUS-Vic
GUS activity (pmol MU/min/ug protein)
500
400
300
200
0
No ABA
ABA
Fresh
Dried

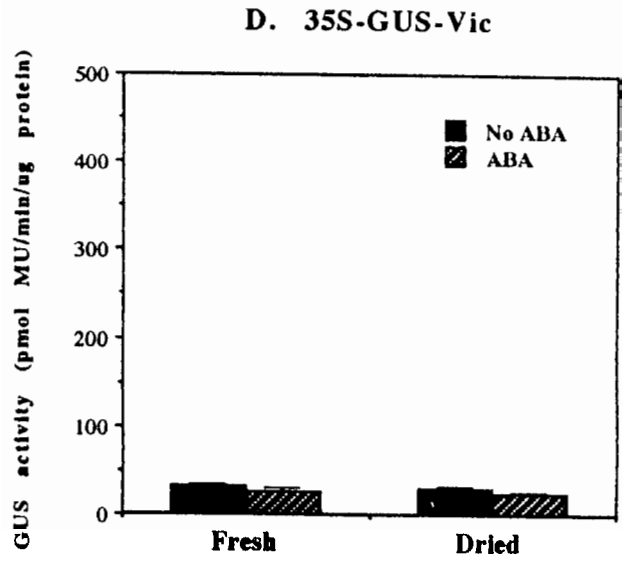
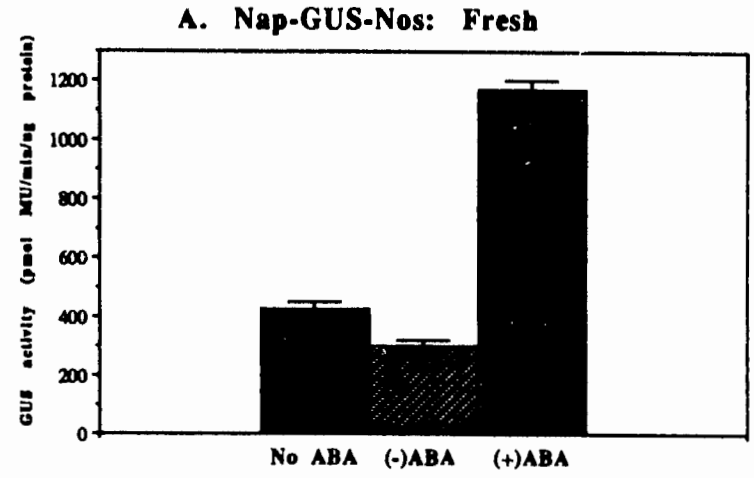
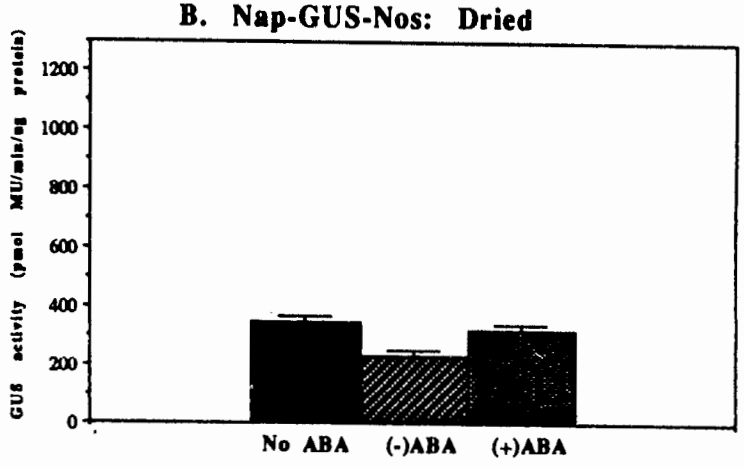
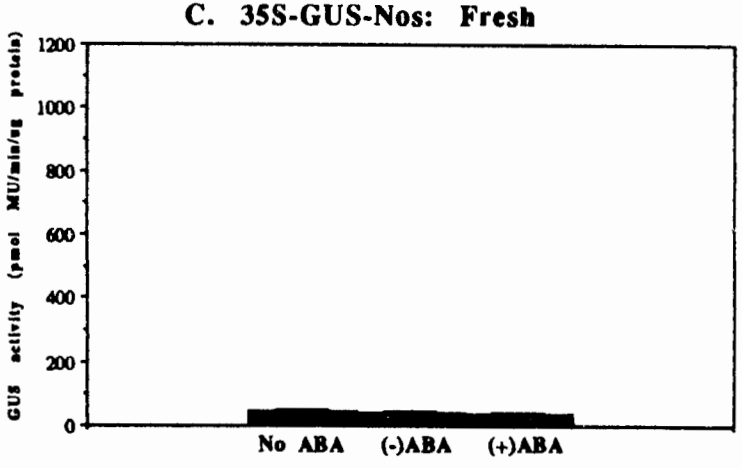

D. 35S-GUS-Vic
GUS activity (pmol MU/min/ug protein)
500
400
300
200
100
0
No ABA
ABA
Fresh
Dried

**Figure 3.3**. Effect of desiccation on the subsequent response of the napin and viral 5' upstream regions to optically pure ABA isomers (1 μM), as measured by activity of GUS in developing 12 DAA transgenic tobacco seeds after 5d culture. A, GUS activities in developing seeds (Fresh, 12 DAA) expressing Nap-GUS-Nos gene construct after cultures in the presence of natural form of ABA (+ ABA) or unnatural form of ABA (- ABA) or without ABA (No ABA); B, as A but in prematurely dried seeds at 12 DAA; C and D, as A and B, but in seeds expressing 35S promoters.


A. Nap-GUS-Nos: Fresh
GUS activity (pmol MU/min/ug protein)
1200
1000
800
600
400
200
0
No ABA
(-)ABA
(+)ABA


B. Nap-GUS-Nos: Dried
GUS activity (pmol MU/min/ug protein)
1200
1000
800
600
400
200
0
No ABA
(-)ABA
(+)ABA


C. 35S-GUS-Nos: Fresh
GUS activity (pmol MU/min/ug protein)
1200
1000
800
600
400
200
0
No ABA
(-)ABA
(+)ABA

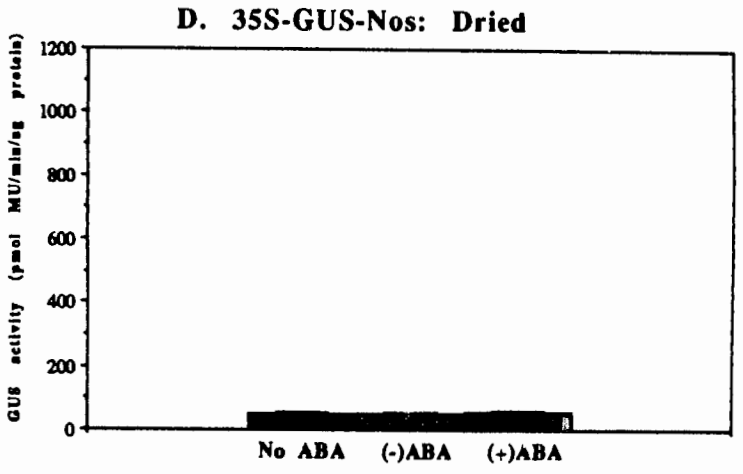
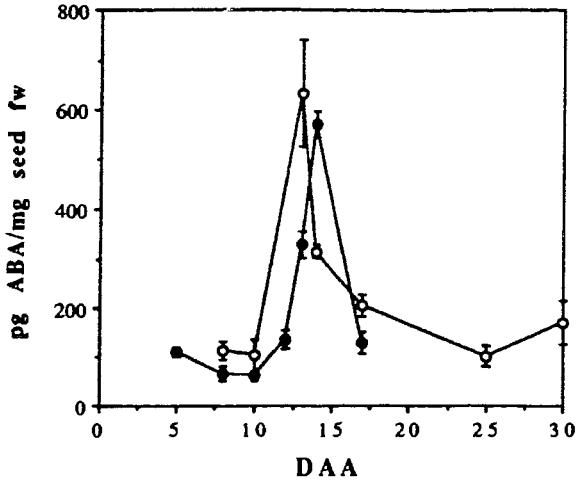
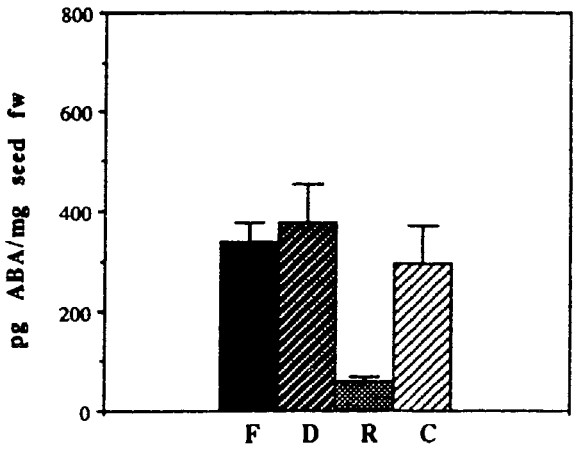

D. 35S-GUS-Nos: Dried
GUS activity (pmol MU/min/ug protein)
1200
1000
800
600
400
200
0
No ABA
(-)ABA
(+)ABA

**Figure 3.4.** **A**, Profile of endogenous ABA contents in whole seed during seed development in tobacco, from 5-30 d after anthesis (DAA), as measured from two individual plants (O and •); **B**, Endogenous ABA contents in fresh seed at 12 DAA (F) and following premature drying for 5d (D) and subsequent rehydration for 3d (R), and after hydration control for 5d (C); **C**, ABA contents in mature seed (30 DAA) and following imbibition for 3 and 5d.

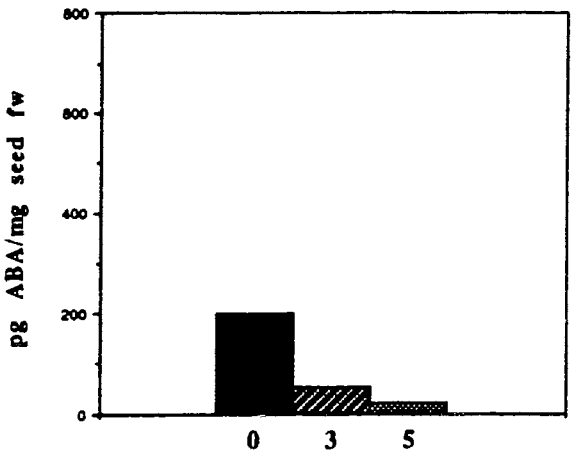
A. Changes in ABA during Seed Development



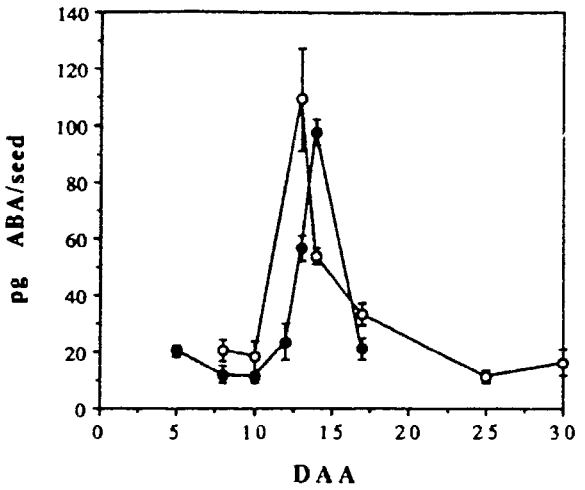

B. Effect of Drying on ABA in 12 DAA Seeds



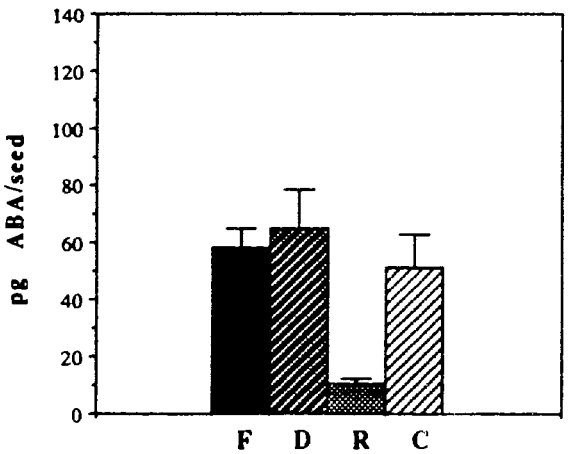

C. Changes in ABA following Imbibition of Mature Seeds



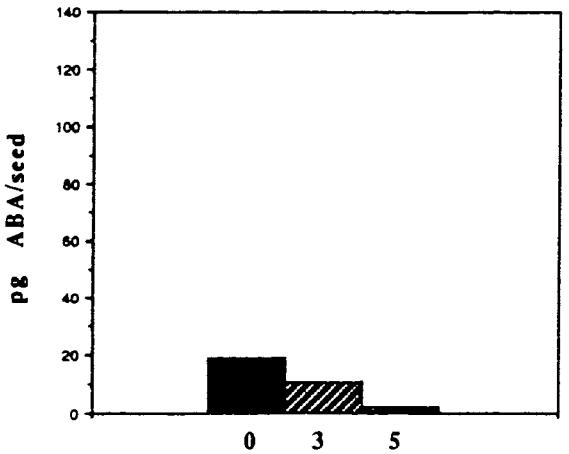
A. Changes in ABA during Seed Development




B. Effect of Drying on ABA in 12 DAA Seeds




C. Changes in ABA following Imbibition of Mature Seeds



### 3.4. Discussion

An important field of study in the area of seed development is focused upon the role of the seed or maternal environment in maintaining embryos in a developing mode until they are fully formed and have accumulated sufficient reserves to permit successful germination and subsequent seedling establishment. These studies have implicated two potential regulatory factors: abscisic acid (ABA) and restricted water uptake (Kermode, 1990, 1995). The question also arises as to how 'developmental' regulatory cues are terminated in the mature dispersed progeny (the dry seed), such that a transition to germiantion events occurs and a continuation of developmental events is prevented. Desiccation is the normal terminal event in seed development and may itself promote, or lead to, a loss of control by the maternal plant or seed environment (i.e. the tissues surrounding the embryo). Such a loss of control may result from a reduction in the content and/or flow of ABA from the mother plant or surrounding seed tissues. Another possibility is that drying decreases embryo sensitivity to ABA. A large component of growth or maturation of the developing embryo is associated with the laying down of reserves; a role for ABA in this respect has been suggested, particularly in promoting the synthesis of storage proteins (Kermode, 1990, 1995). Whether this hormone acts (in planta) specifically to increase the production of storage protein mRNAs during the seed expansion phase (when maximum synthesis occur) remains to be determined. Some evidence lends support to this contention, and at the transcriptional level, some of the *cis*-regulatory sequences responsive to ABA within developmentally regulated genes (e.g. the sunflower helianthinin gene) have been identified (reviewed in Thomas *et al.*, 1991; Thomas, 1993; Nunberg *et al.*, 1994).

The previous studies (Chapter 2) show that the 5' upstream regions of vicilin and napin storage protein genes are responsive to desiccation in transgenic tobacco seed (Jiang and Kermode, 1994; Jiang *et al.*, 1995). For example, GUS gene expression is sensitive to premature drying and is down-regulated upon subsequent rehydration in the presence of the 5' regulatory regions of the storage protein genes, but not when under control of the same region from the cauliflower mosaic virus 35S gene. A major objective of the present study was to test the hypothesis that the maturation drying period, by effecting changes in the content of, or sensitivity to, ABA partially or wholly removes the effects of this 'positive modulator' of developmental gene expression. As an initial step toward understanding possible interactions between desiccation and ABA at the molecular level, I examined the enhancement of GUS reporter activities in transgenic tobacco by exogenous ABA before and after premature drying. This has yielded preliminary results suggesting that the promoters of these developmental genes are responsive to ABA, but become *relatievly* insensitive to the hormone after premature drying. Reduced ABA uptake in prematurely dried seed leading to an apparent decline in sensitivity is unlikely, since uptake would be expected to be greater in dried tissues than in their undried (i.e. fresh or hydrated) counterparts.

In developing castor bean seeds, premature drying leads to a major decline in ABA content within the embryo and endosperm; a reduction in embryo sensitivity to ABA is also evident following drying, the latter perhaps being more crucial for effecting the "switch" to a germination/growth program (Kermode and Bewley, 1987; Kermode *et al.*, 1989a). Similarly, in rape, alfalfa, wheat, and soybean embryos, the decline in endogenous ABA during late development is accompanied by a corresponding decrease in tissue sensitivity to exogenous ABA, as measured by its capacity to inhibit germination and to maintain or enhance expression of

developmental storage protein genes (Eisenberg and Mascarenhas, 1985; Finkelstein *et al.*, 1985; Williamson *et al.*, 1985; Quatrano, 1986; Xu and Bewley, 1991). When developing transgenic tobacco seeds (expressing a helianthinin 5'-GUS chimeric gene) are incubated on a basal medium containing $10^{-6}$ M ABA, they exhibit enhanced GUS activities, as compared to control seeds placed on a basal medium minus ABA (Thomas *et al.*, 1991; Nunberg *et al.*, 1994). However, the responsiveness of GUS expression to ABA declines as seed development progress. For example, cultured seed at 14 DAA show a 4-fold enhancement of GUS activity when treated with ABA for 5d, but declines to only a 1.5-fold enhancement in seeds cultured at 24 DAA.

It is not known whether the changes in response to exogenous ABA are a direct result of drying, e.g. via changes in the synthesis, turnover or conformation of receptors. Another possibility is that desiccation enhances the ability of the embryo/seed to metabolize exogenously applied ABA. Somatic embryo suspension cultures of white spruce (Dunstan *et al.*, 1992) are only capable of metabolizing the natural isomer of ABA, the (+)-ABA, but not the (-)-ABA form. The present studies indicate that ABA enhancement of GUS expression from chimeric genes directed by the napin storage protein gene promoter was largely due to the (+) form of ABA. The effects of desiccation on ABA metabolism are being investigated by using ABA analogs that differ in their rate of conversion to physiologically inactive forms.

There is some evidence that desiccation alters the hormone balance of the seed. In some cases there is a temporal correlation between the onset of maturation drying and the precipitous decline in seed ABA (accompanied by a corresponding increase in its metabolism) (McWha, 1975; King, 1976, 1979). In the present study, the regime for drying tobacco seeds consisted of air-drying seeds in pods for 5d, a

treatment that led to about a 50% loss in fresh weight. Premature drying did not lower the content of endogenous ABA in developing tobacco seed (on a per fresh weight basis); however, upon subsequent rehydration of the prematurely dried seed, the amount of ABA declined dramatically, as it did within the mature seed following imbibition. Since it was necessary to use the whole seed for ABA determinations, I cannot comment on any ABA changes within the embryo or endosperm.

While more investigation is required, a decline in sensitivity of the developing seed to ABA, resulting in a loss of competence to respond to the hormone at physiologial concentrations, may be an important factor in the cessation of storage protein gene expression. If this is the case, more information is needed on how regulatory cues such as ABA and drying interact to effect changes in gene expression, including events at the molecular level, as well as the various events that comprise the signal transduction process. For example, does seed drying alter embryo or seed sensitivity to ABA via changes in receptor level or conformation? If the receptor(s) to ABA is membrane-associated (Gilroy and Jones, 1994), do changes in membranes (e.g., during drying or upon subsequent rehydration) play a pivotal role, such that receptor sites become incorporated and hence unavailable? If there is some direct effect of drying upon the 5' upstream region of developmental genes, how does this manifest itself such that transcription is no longer affected by ABA?

*Chapter 4*

# Premature Drying Increases the GA-Responsiveness and Up-Regulates the Expression of the α-Amylase Gene Promoter in Developing Aleurone Layers of Barley (*Hordeum vulgare* L.) Grain.

## 4.1. Introduction

Seeds of most plant species undergo maturation drying on the parent 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. Premature drying has been used as an important experimental approach to define the role of desiccation in terminating development and eliciting germination. Here the seed is removed from the parent plant at some time before its normal development is completed and the effects of an artificially imposed drying period are determined (reviewed in Kermode, 1990, 1995; Jiang and Kermode, 1994). When desiccation is imposed prematurely at certain stages prior to the completion of development, a switch in metabolic events is elicited: syntheses unique to development are terminated, while those associated with germination and growth are initiated (Kermode and Bewley, 1985b, 1986b; Kermode *et al.*, 1985; Dasgupta and Bewley, 1982; Adams *et al.*, 1983; Rosenberg and Rinne, 1987; Oishi and Bewley, 1992; Evans *et al.*, 1975). In effecting this switch in gene expression, desiccation appears to act primarily at the transcriptional and posttranscriptional levels, i.e. desiccation effects an off- or down-regulation of developmental messages and an on- or up-regulation of messages for germination and growth (Cornford *et al.*, 1986; Jiang and Kermode, 1994; Jiang *et al.*, 1995; Kermode *et al.*, 1989c; Kermode, 1990, 1995; Misra and Bewley, 1985). The 5' upstream regions of napin and vicilin storage protein genes are responsive to desiccation in transgenic tobacco seed (Jiang and Kermode, 1994; Jiang *et al.*, 1995). For example, GUS gene expression is sensitive to premature desiccation and is down-regulated upon subsequent rehydration in the presence of the 5' regulatory regions of the storage protein genes, but not when under control of the same region from the cauliflower mosaic virus (CaMV) 35S gene. Thus, premature desiccation may down-regulate storage protein gene expression upon rehydration by acting directly upon the 5'

upstream regions of these genes. Following drying, this region may become insensitive to positive controlling factors; alternatively, changes to *trans*-acting factors important for gene expression may occur as a result of drying (Jiang *et al.*, 1995; Oliver *et al.*, 1993).

Premature desiccation is also necessary for the induction of enzymes involved in reserve mobilization during the postgerminative phase of seedling development. In dicot seeds, Adams et al. (1983) demonstrated that immature soybean seeds cannot produce malate synthetase and isocitrate lyase activities unless the seeds are first subjected to a slow drying regime. Such is also the case in the castor bean endosperm in which premature drying induces isocitrate lyase and LeuNAase (a proteolytic enzyme) to levels comparable to those produced following germination of mature seed (Kermode and Bewley, 1985b).

The present study focuses on α-amylase, an enzyme synthesized and secreted by the aleurone layer cells of germinated cereal grains in response to gibberellin (GA) produced by the embryo. The enzyme is largely responsible for starch hydrolysis in the endosperm and represents as much as 70% of the newly synthesized protein in GA-treated barley aleurone cells, the tissue of study (Jones and Jacobsen, 1991). A positive role for desiccation in the acquisition of a competence of the aleurone layer to produce α-amylase in response to GA has been demonstrated in a number of cereals. This competence is normally not acquired until late development; however, it can be induced prematurely following imposed drying, e.g., in wheat, barley, triticale and maize (Evans *et al.*, 1975; Armstrong *et al.*, 1982; King *et al.*, 1979; Oishi and Bewley, 1990). Interestingly, in wheat, the induction of GA responsiveness in immature aleurone layer cells does not extend to the production of other enzymes associated with normal postgerminative growth, including acid phosphatase, protease

and ribonuclease (Cornford *et al.*, 1986). More recently, it has become evident that other treatments (most of which do not result in substantial water loss) are also capable of inducing some degree of GA responsiveness in aleurone layers of developing cereal grain. These include temperature treatments (Norman *et al.*, 1982), maintenance in an atmosphere of high relative humidity (Cornford *et al.*, 1987a) and preincubation in water or in a buffered medium (immediately following detachment from the parent plant) for a period of time prior to the addition of the growth regulator (Cornford *et al.*, 1987a). However, these treatments are not equally effective in inducing a GA-responsive state and none are as effective as premature drying. Frequently, they do not elicit a normal (full) response to GA, i.e. one similar in pattern and extent of an increase in α-amylase activity to that exhibited by the aleurone layer of mature grain which has undergone normal maturation drying.

The objective of the present study was to investigate the role of natural and premature drying in controlling α-amylase synthesis and secretion in response to GA, particularly in regard to changes that occur within the aleurone layer of barley grain (cv. Golden Promise) following the transition from a developmental to a germinative program. Toward this end, I examined the effects of desiccation and other treatments on the acquisition of a competence of barley aleurone layer cells to respond to GA and hence express their full potential for α-amylase production and secretion. Results indicate that only desiccation is capable of inducing a GA-responsive state characteristic of the aleurone layer of mature grain. Further, results of Northern blot analyses and transient expression of chimeric gene constructs in immature aleurone layer cells of half-grains suggest that drying may act, in part, by up-regulating the α-amylase gene promoter in response to GA. In immature barley aleurone cells, premature desiccation was able to induce GUS reporter gene expression from a chimeric gene containing a truncated α-amylase promoter consisting of the minimal

promoter elements required for normal hormonal regulation of this gene. These studies will serve as a prelude to an examination of the complex and interrelated events that constitute, coordinate and control the synthetic and secretory response of the barley aleurone layer to the hormones ABA and GA and the regulatory role of desiccation.

## 4.2. Materials and Methods

### 4.2.1. Plant Materials

Barley plants (*Hordeum vulgare* L.) of the spring cultivar Golden Promise were grown in growth chambers under a 16-h photoperiod at 12 $^{o}$C, with a humidity of 60-80% and light intensity of 350-400 μE. Plants were fertilized with Osmocote (Sierra, 17-6-12 plus minors) at the time of planting and then fertilized weekly with 0.02% verdi (Peter's 20-20-20). Under these conditions, development of barley grains was completed by approximately 65d after anthesis (DAA).

### 4.2.2. Grain Treatments

Grains of various developmental stages were collected and either used in the fresh state or subjected to various treatments for 3d prior to their incubation in 5 μM GA or water. These treatments included: air drying of grain at room temperature, preincubation of grain on water and maintenance of grain at 100% relative humidity. Following the various treatments, grains were surface sterilized as described in Jones and Varner (1967). Briefly, grains were first soaked in 5% bleach (v/v) for 20 min with vigorous shaking. After rinsing four times with distilled water, the grains were

neutralized with 0.1N HCl for 10 min, followed again by four rinses with distilled water. Half-grains were then generated by deembryonating grains prior to incubation in water or 5 μM GA.

### 4.2.3. Grain Germination

Developing fresh and prematurely dried grains were tested for germinability. After dehulling and surface sterilization, fresh grains of various stages and prematurely dried grains were placed in Petri dishes containing filter paper moistened with distilled water or in Petri dishes containing 1% agar (Sigma) and incubated at 25 $^{o}$C in the dark for 5d before assessing germinability. Grains having roots of at least 1 cm long were counted as germinated.

### 4.2.4. Incubation and Assay for α-Amylase Secretion

After surface sterilization, half-grains were generated by deembryonating grains under sterile conditions and incubating them in a 25-ml flask containing sterile distilled water or 5 μM GA (Sigma, St. Louis, MO). Ten half-grains were incubated in 1.7 ml water or 5 μM GA at room temperature in the dark with gentle shaking (50 rpm). Following 24-, 48-, and 72-h incubation periods, the medium was mixed well, collected and used immediately for assay to determine the α-amylase activity secreted as described in Jones and Varner (1967). Alternatively, the medium was frozen in liquid $N_2$ and kept at -80 $^{o}$C until use for Western blot analysis. At the end of the incubation, the aleurone layers were isolated and extracted for assay of non-secreted α -amylase remaining in the cells according to Sticher and Jones (1992), with the modification that no purification by affinity chromatography was carried out.

### 4.2.5. Western Blot Analysis

The culture medium collected after 72h of incubation from different treatments was used for Western blot analysis. The medium from different treatments was centrifuged (13,000 rpm for 5 min) and the supernatants were mixed with 5X SDS-PAGE sample buffer (0.625 M Tris-HCl, pH 6.7, 10% SDS, 50% glycerol, 0.015% bromphenol blue, and 50 mM DTT) and boiled for 10 min. Samples containing equal volumes (20 µl) were then fractionated by SDS-PAGE using 10% gels. After electroblotting onto nitrocellulose membrane, the α-amylase was detected with rabbit anti-total-α-amylase serum (diluted 1/1000 in TBST [1.211 g/l Tris-HCl, pH 8.0, 8.766 g/l NaCl, 0.05% Tween-20] containing 5% milk powder), followed by anti-rabbit IgG coupled to peroxidase (Sigma) (diluted 1/1000 in TBST containing 5% milk powder). Peroxidase activity was visualized with 4-chloro-1-naphthol and $H_2O_2$, according to the manufacturer's instructions (Sigma). As a positive control, 1 µg of α-amylase powder (Sigma) was included as a sample for Western blot analysis. Replicate SDS gels were stained with Coomassie blue to show profiles of extant proteins.

### 4.2.6. RNA Isolation and Northern Blot Analysis

Sterilized half-grains subjected to the various treatments were incubated in sterile distilled water or 5 µM GA for 24h in the dark with gentle shaking. Aleurone layers were then isolated at the end of the incubation and immediately frozen in liquid $N_2$. The isolated layers (30-50 layers for each treatment) were kept at -80 $^{o}$C until used for RNA isolation. Isolation of bulk RNA was carried out according to Deikman and Jones (1985). Briefly, aleurone layers were ground to a fine powder with a

mortar and pestle in liquid $N_2$ and resuspended in lysis buffer (0.5M NaCl, 50 mM Tris, 50 mM EDTA, pH at 8.5 and 10 mM β-mercaptoethanol). This was followed by extraction in buffer (6% butanol, 1% tri-isopropylnaphtalene disulfonate, 2% para-aminisalicylate and 1% SDS). After extracting three times with phenol/chloroform, total RNA was precipitated with 8M LiCl at -20 $^{o}$C overnight. Bulk RNA was then dissolved in sterile distilled water and kept at -80 $^{o}$C. For Northern blot analysis, equal amounts of RNA (10 or 15 μg, as determined by a Spectrophotometer) were fractionated on 0.8% agarose gels in the presence of formaldehyde using 1X MOPS (0.04 M Mops, 10 mM sodium acetate, 1 mM EDTA, pH 7.0) running buffer. RNA was then transferred overnight onto Hybond-N membrane (Amersham, Arlington Heights, IL) using 10X SSPE (1.5 M NaCl, 0.1 M Phosphate, 10 mM EDTA, pH 8.0). After UV cross-linking to fix the RNA on the membrane, the membrane was baked at 80 $^{o}$C for 1 h and then used for prehybridization.

Two cDNA clones were used as probes: clone E is a clone for the low-pI α-amylase of barley (Rogers and Milliman, 1983), while 1-28 is a clone for the high-pI enzyme (Deikman and Jones, 1985). To ensure equal loading of RNA, clone pTA71 containing a complete rRNA repeat from barley (Gerlach and Bedbrook, 1979) was used to reprobe the membrane. cDNA probes were isolated from agarose gels after electrophoresis and purified by a GeneClean kit (Bio-RAD, Hercules, CA). Probes were labeled with $^{32}$P using a random primer labeling kit (Promega, Madison, WI) and purified using columns from Stratagene (La Jolla, CA) before use in hybridization. The hybridization was carried out at 42 $^{o}$C in a solution containing 50% formamide, 2X Denhardt's, 5X SSPE, 0.3% SDS, and 100 μg/ml denatured salmon sperm DNA in an Hybaid oven for 20h. After two washes in 2X SSPE and two washes in 0.2X SSPE, 0.1%SDS at 65 $^{o}$C (30 min per wash), the membranes were exposed to X-ray film at room temperature for autoradiography.

Autoradiograms were quantified by scanning with an LKB Bromma Ultrascan XL enhanced laser densitometer. Peak integration values were normalized within each gel to the most dense band. Individual bands were standardized by dividing the integration value against the relative amount of rRNA detected by the densitometer.

### 4.2.7. Transient Gene Expression using Particle Bombardment

The following chimeric gene constructs were used for biolistics: Am2.1 TGN, ML022, and pDO432. Am2.1 was a gift from John V. Jacobsen (CSIRO, Plant Industry, Canberra, Australia) and is the high-pI α-amylase gene promoter fused to GUS with a nopaline synthase (Nos) 3' end (Jacobsen and Close, 1991). ML022 was a gift from John Rogers (Dept. Biochemistry, Univ. of Missouri, Columbia, MO) and is a 331-bp α-amylase gene promoter fused to GUS. This construct exhibits a strong response to GA, even stronger than the entire 1.4-kb α-amylase gene promoter (Lanahan *et al.*, 1992). pDO432 is the CaMV 35S promoter fused to *Lux* (the luciferase gene) with a Nos 3' end (Ow *et al.*, 1986), which is constitutively expressed in barley aleurone cells and is not responsive to GA (Huttly and Baulcombe, 1989; Skriver *et al.*, 1991). The CaMV 35S promoter is also unresponsive to desiccation in dicot seeds (Jiang *et al.*, 1995). The activity of GUS indicates the activity of the α-amylase gene promoter, while the activity of luciferase indicates the efficiency of transformation. The ratio between the GUS activity and luciferase activity was used to standardize the transformation efficiency (Bruce *et al.*, 1989; Lanahan *et al.*, 1992).

Plasmids were purified on CsCl/EtBr gradients as described in Maniatis *et al.* (1982). Microprojectile bombardment was carried out essentially as outlined in Lanahan *et al.* (1992) with some modifications. Briefly, barley grains were dehulled and surface sterilized and then deembryonated. Half-grains were imbibed in distilled

water for 2d and the pericarp/testa layers were removed prior to bombardment. The α-amylase-GUS and 35S-Lux constructs were mixed in a ratio of 2:1 in molar amounts and precipitated onto gold particles (1.0 μm in diameter) using $CaCl_2$ and spermidine (free base). For each bombardment, 3 μg of DNA mixture was delivered into 10 deembryonated half-grains. Following particle bombardment, 5 half-grains were incubated in Petri dishes containing water and the other 5 half-grains were incubated in 5 μM GA for 40h in the dark at 25 °C. Aleurone layers were then isolated from the half-grains and homogenized at 4 °C using a plastic pestle and a 1.5-ml microfuge-tube in 400 μl extraction buffer (100 mM $NaPO_4$, pH 7.8, 10 mM DTT, 1mM EDTA, 5% glycerol). After centrifugation at 13,000 rpm for 10 min, the supernatants were used for enzyme assays.

Luciferase assays were carried out immediately after the extraction. Fifty-μl extracts were mixed with 100 μl of reaction buffer (50 mM Hepes at pH 7.8, 20 mM $MgCl_2$, 0.5 mg/ml BSA, 10 mM ATP). To start the reaction, 100 μl of luciferin (1 mM) was injected and the emitted photons were counted for 30 sec in a Luminometer (FlowTech Engineering, Model 3010). Data from counting was discarded if samples had less than a 5-fold activity against the background (determined from samples of untransformed cells). These samples were excluded from the analyses of GUS activity. For GUS assay, 50-μl of extract was mixed with 450 μl of extraction buffer and the reaction carried out at 37 °C. GUS assays were carried out as described in Jefferson (1987), using a Luminescence Spectrometer LS30 (Perkin Elmer Corp., San Jose, CA). To stop the reaction (e.g. at 0,10, 30, 60 min), 100 μl of the reaction mixture was transferred into 900 μl stop buffer (0.2 M $Na_2CO_3$) to ensure a linear range of GUS activity. GUS activity was expressed as units of fluorescence (minus the control background value) generated in 1h at 37 °C. The relative GUS activity was standardized by dividing the units of fluorescence against the luciferase value for

a particular sample, which indicates the efficiency of transcription for the α-amylase gene promoter as compared to that of the 35S promoter (Lanahan *et al.*, 1992). Relative GUS activity was calculated using 4-6 replicate bombardments per gene construct.

## 4.3. Results

### 4.3.1. Germinability of Fresh and Dried Grains

In order to determine when barley grains acquire desiccation-tolerance and germinability during development, we tested the ability of immature grain to germinate in the fresh state and upon subsequent rehydration following a 3-d air-drying treatment. Under our growth conditions, development of grains was completed by approximately 65 DAA. Grains at 30 DAA were not capable of germinating when placed on water in the fresh state; however, following premature drying at this stage of development, 20-40% germinated upon subsequent rehydration. Grains at stages earlier than 25 DAA were unable to germinate following drying. In contrast, germinability of the fresh seed was not acquired until around 40-50 DAA, with a percentage of about 50-65%. We confined our studies of the effects of premature drying to grains at 30 and 40 DAA.

### 4.3.2. Acquisition of GA-Responsiveness of the Aleurone Layer during Barley Grain Development: Secretion of α-Amylase Activity into the Medium

Half-grains of developing fresh and prematurely dried grain were assessed for their ability to secrete α-amylase into the medium when incubated in water or 5 μM

GA. In aleurone layers responsive to GA, secretion of α-amylase began at 48-72h after addition of the hormone (Fig. 4.1). As barley matured, the aleurone layers of half-grains became progressively more responsive to GA as measured by their ability to secrete α-amylase into the medium. An increasing responsiveness occurred between 30 DAA and maturity; aleurone layers at stages 25 DAA or younger did not respond to the hormone. Thus, the ability of the aleurone layer to secrete α-amylase in response to GA (albeit a reduced response) was acquired before the onset of germinability of the fresh grain. In contrast to the aleurone layers of fresh (nondried) grain, those of immature grains at 30 or 40 DAA subjected to premature drying exhibited the same extent of GA-responsiveness upon rehydration as mature grain (65 DAA). Following drying and subsequent rehydration, the aleurone layers of these grains secreted a similar amount of α-amylase into the medium as mature-imbibed grain at 48 and 72h following the addition of GA (Fig. 4.1 B,C). Aleurone layers of half-grains incubated for 72h in GA were also analyzed to determine the amount of unsecreted enzyme. Only about 10% of the α-amylase produced by the aleurone layers of barley grains (whether fresh or dried) represents unsecreted enzyme at 72h (Fig.4.1, compare C and D; secretion at 72h vs. total α-amylase at 72h).

### 4.3.3. Effectiveness of Control Treatments in Inducing GA-Responsiveness in Aleurone Layers of Immature (30 DAA) Grains

The effectiveness of two treatments (preincubation and maintenance of grain at high relative humidity) in inducing a GA-responsive state of the aleurone layer of 30 DAA barley grain (Fig. 4.2 A and B) was studied. In contrast to premature drying at this stage (Fig. 4.1), which elicited a maximal GA-responsiveness of the aleurone layer in respect of enzyme secretion, the two control treatments were of limited effectiveness, eliciting only a small amount of α-amylase secretion over a 72h period

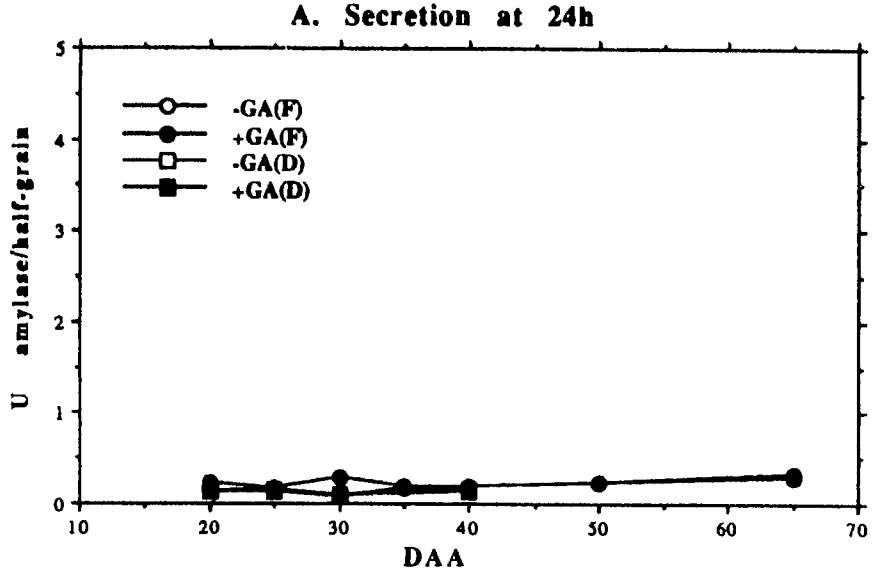
following the addition of GA. Furthermore, this limited amount of enzyme secretion by the aleurone layer (comparable to that of the fresh 30 DAA grain at 72h; see Fig. 4.1) cannot be attributed to an increased responsiveness of the aleurone tissue to exogenous GA; approximately the same amount of enzyme secretion occurred when GA was absent from the incubation medium (Fig. 4.2, compare +GA vs. -GA).

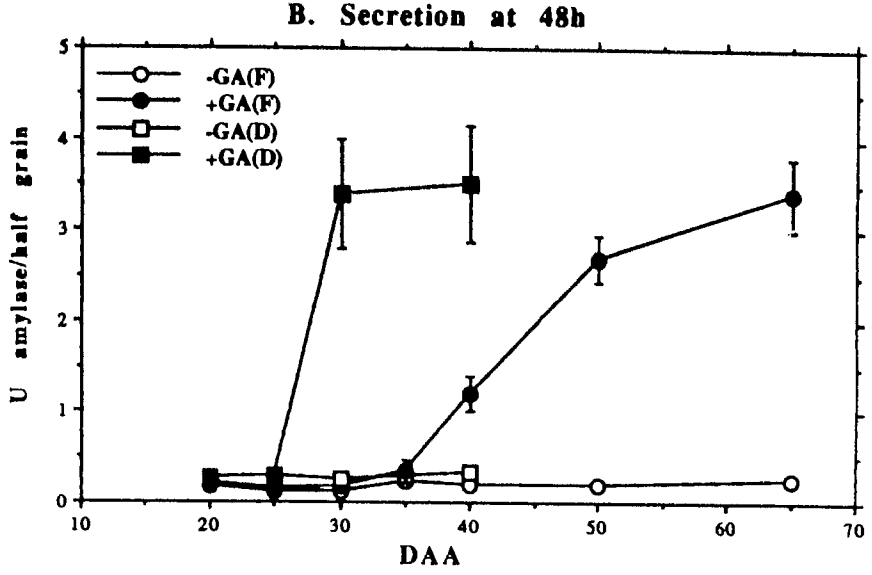
The ability of aleurone layers to secrete α-amylase in response to GA following desiccation and control treatments of developing grain was also determined by Western blot analysis using extracts derived from the 72-h incubation medium (Figs. 4.3 and 4.4). The 43-kDa α-amylase protein was not detected in the incubation medium from fresh 30 DAA half-grains incubated with GA for 72h; however, it was detected following GA-incubation of more mature (40 and 50 DAA) half-grains, the amount of enzyme secretion becoming more pronounced with increasing grain maturity. Grains at 30 DAA, detached from the parent plant and maintained at 100% RH, for a period equivalent to the drying treatment (3d), exhibited some α-amylase secretion over 72h in contrast to the developing fresh 30 DAA half-grain. However, similar to the enzyme activity studies (Fig. 4.2), the same amount of enzyme was secreted by the aleurone layers of these half-grains in the absence of GA (Fig. 4.3; 100% RH, compare + vs. -), indicating that the production of enzyme was not due to an increased responsiveness of the aleurone layer to exogenous GA. I was unable to detect any α-amylase secretion following a preincubation treatment of detached 30 DAA grain prior to the introduction of GA (Fig. 4.4). This is somewhat surprising, given the enzyme activity results (Fig. 4.2) showing enzyme secretion equivalent to that from grains subjected to the high humidity treatment. However, this may reflect the inability of grain detachment alone to effectively elicit GA-responsiveness and α-amylase production by the immature aleurone layer. In developing wheat grain, the various treatments that do not promote extensive water loss are not equally effective

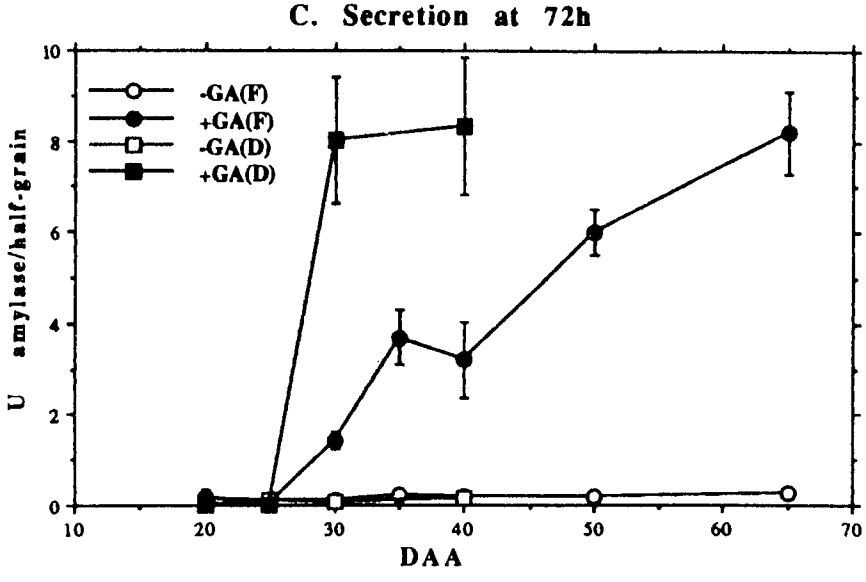
in eliciting α-amylase production and often are not themselves consistently effective (Cornford *et al* , 1986, 1987a). Furthermore, in barley grain, any increased production of α-amylase as a result of the control treatment does not appear to be due to an increased responsiveness or sensitivity to exogenous GA. In contrast, premature drying of 30 or 40 DAA grain resulted in a dramatic induction of α-amylase synthesis and secretion, which only occurred when exogenous GA was included in the medium (Fig. 4.4). The Western blots revealed a number of bands having molecular weights lower than the 43-kDa band corresponding to α-amylase. It is not clear whether these represent breakdown products of α-amylase; they were observed only on blots derived from fresh grain at more mature stages (Fig. 4.3; 50 DAA) and prematurely dried 30 and 40 DAA grain (Fig. 4.4).

The stained gels show several low molecular weight proteins in the culture media from all samples (Figs. 4.3 B and 4.4 B); this may be due, in part, to some protease activity during the incubation period. Similar protein profiles were observed following incubation of isolated aleurone layers (instead of half-grains) derived from mature grains of two barley cultivars (Golden Promise and Himalaya).

**Figure 4.1.** α-Amylase activity following incubation of half-grains in media containing GA (5 μM) or in media lacking GA. A-C: Activity in culture medium at 24, 48, and 72h after the start of incubation. D. Total α-amylase activity at 72h. Activity is expressed as units per half-grain; each point is an average of 4 determinations ± SE. F, fresh grain; D, prematurely dried 30 DAA grain.


A. Secretion at 24h
-GA(F)
+GA(F)
-GA(D)
+GA(D)
U amylase/half-grain
DAA


B. Secretion at 48h
-GA(F)
+GA(F)
-GA(D)
+GA(D)
U amylase/half grain
DAA


C. Secretion at 72h
-GA(F)
+GA(F)
-GA(D)
+GA(D)
U amylase/half-grain
DAA

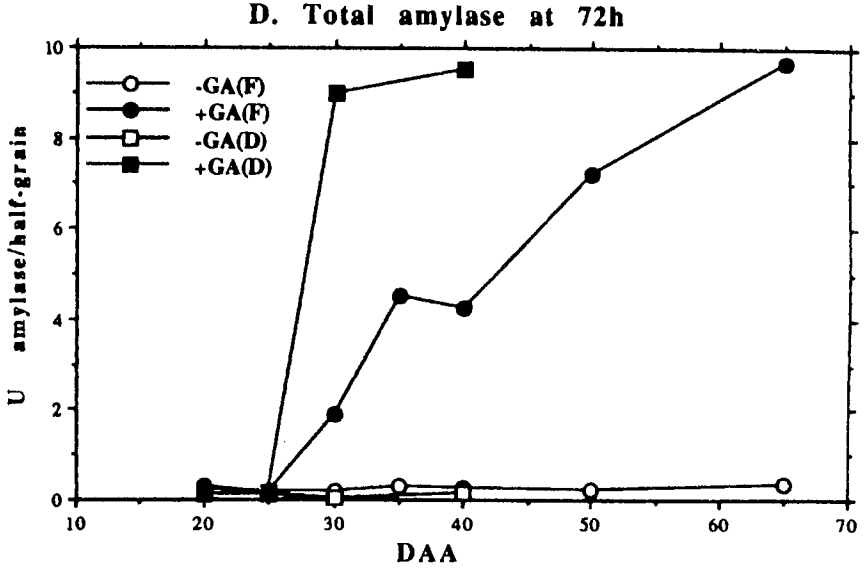
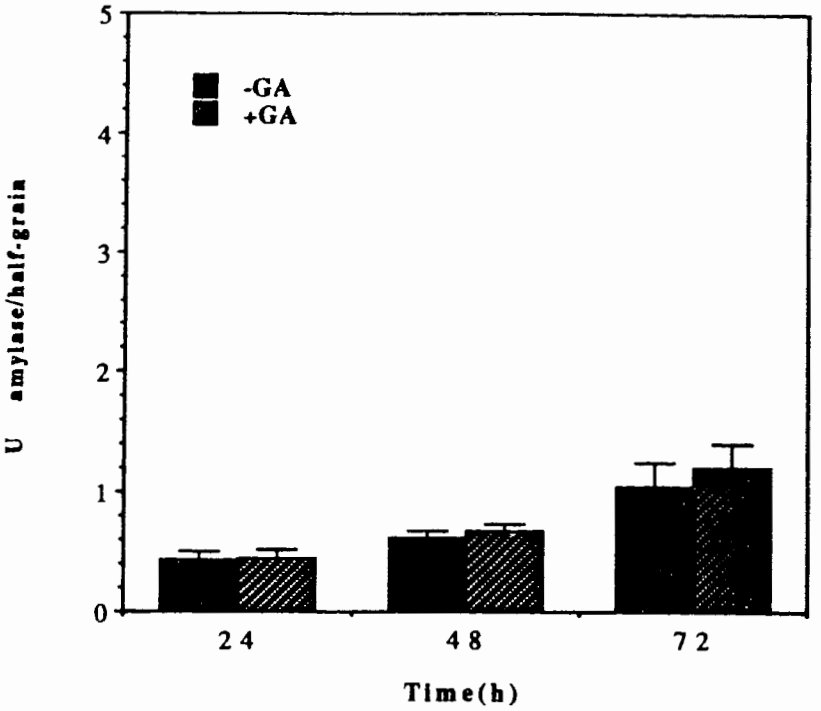

D. Total amylase at 72h
-GA(F)
+GA(F)
-GA(D)
+GA(D)
U amylase/half-grain
DAA

**Figure 4.2.** Secretion of α-amylase from immature (30 DAA) half-grains following preincubation and high humidity treatments. Treated grains were placed in media containing GA or lacking hormone and the enzyme activity in the media determined at 24-72h. Each point is an average of 4 determinations ± SE.

A. Secretion from 30DAA grain (Pre-incubation)




B. Secretion from 30DAA grain (100% RH)

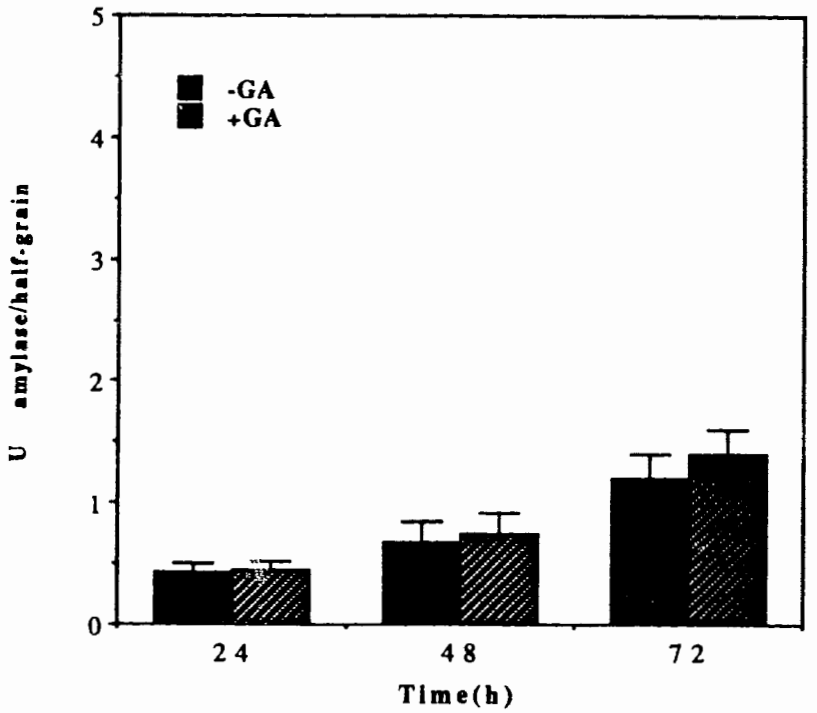
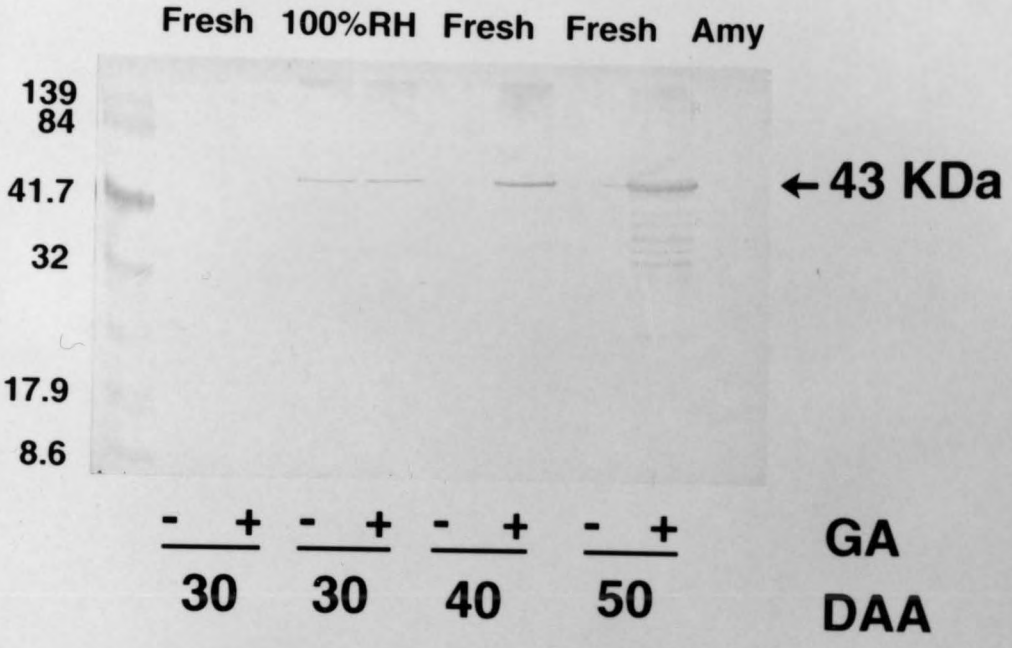


**Figure 4.3.** Western blot analysis of α-amylase protein secreted from developing fresh grain at different stages and from immature (30 DAA) grain following a high humidity treatment. Fresh and treated grains were placed in media containing GA or lacking hormone and the α-amylase secreted into the media after 72h determined by Western blot analysis. **A.** Western blot. **B.** Coomassie stained gel of protein in culture medium. DAA, d after anthesis; Amy, α-amylase protein.

# A. Development




# B. Stained Gel

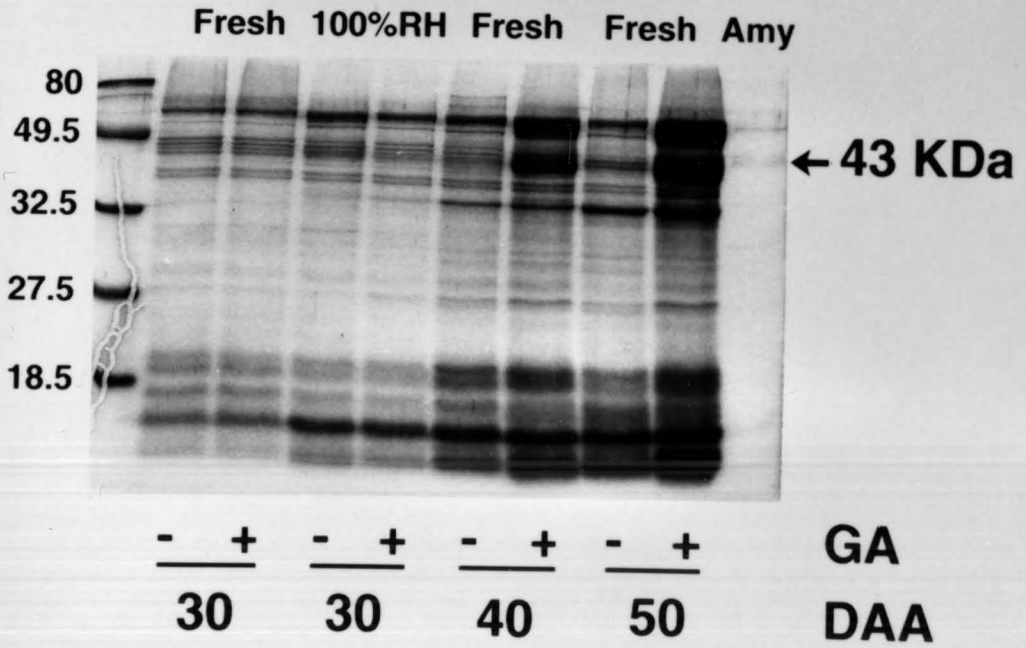
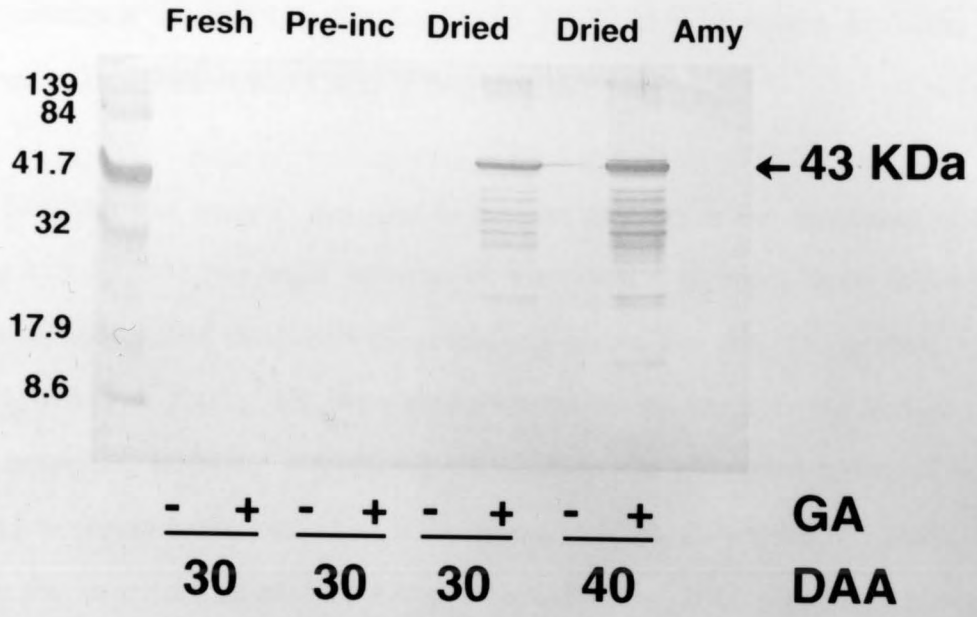
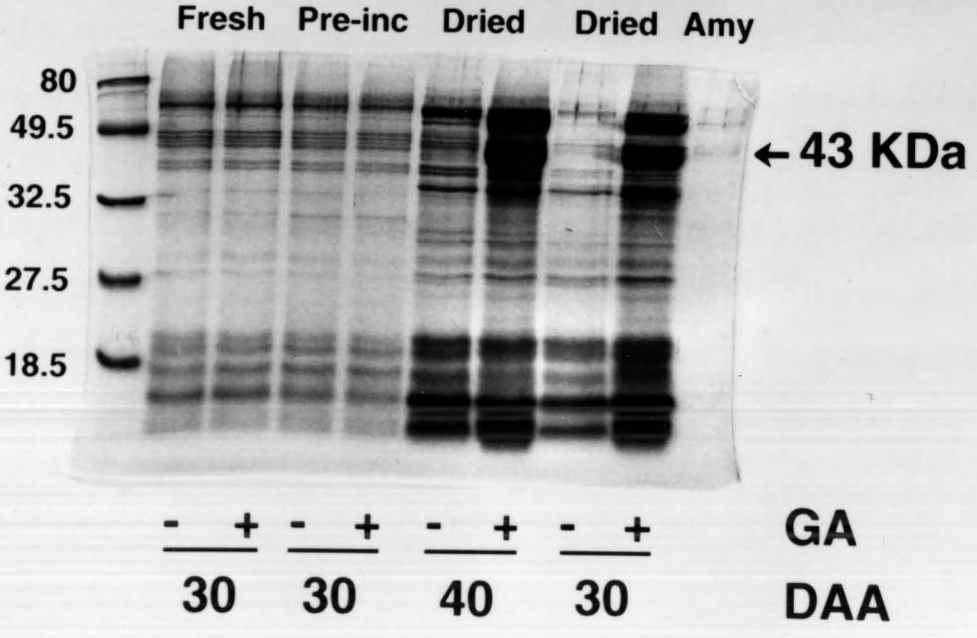


**Figure 4.4.** Western blot analysis of α-amylase protein secreted from fresh immature grain, from immature grain subjected to premature drying and from immature grain subjected to a preincubation treatment. Fresh and treated grains were placed in media containing GA or lacking hormone and the α-amylase secreted into the media after 72h determined by Western blot analysis. **A.** Western blot. **B.** Coomassie blue stained gel of protein in culture medium. DAA, d after anthesis; Amy, α-amylase protein; Pre-inc, preincubation.

# A. Fresh vs. Dried




# B. Stained Gel



### 4.3.4. Induction of mRNAs for Low and High pI α-Amylase Isoforms in Response to Desiccation and Control Treatments

Northern blot analysis was used to monitor changes in the abundance of the mRNAs encoding the two major isoforms of α-amylase in aleurone layers following desiccation and control treatments of developing grains. For this, two probes, 1-28 (Fig. 4.5) and clone E (Fig. 4.6), were used to detect the messages for the high-pI and low-pI α-amylase isoforms, respectively. In addition, the ribosomal probe, pTA71, was used to ensure equal loading of RNA across samples. As shown in Figures 4.5 and 4.6, premature drying of 30 DAA grain induced the aleurone layer to produce mRNAs for both the high and low pI forms of α-amylase when GA was included in the incubation medium. The amount of message produced in aleurone layers of prematurely dried/rehydrated grain after 24h of incubation in GA was somewhat lower than that produced by aleurone layers of mature-imbibed grain. The two control treatments were ineffective or only partially effective, inducing very low amounts of α-amylase mRNAs in 30 DAA grains detected by overexposure of the blots (data not shown). The blots were further analyzed by densitometry (Fig. 4.7) in which band intensity corresponding to the α-amylase mRNA was expressed relative to the amount of rRNA on the control blot. As shown in Figure 4.7, the amount of GA-induced α-amylase mRNA in prematurely dried/rehydrated grain was about 2-fold and 3-fold lower than that produced in response to GA in mature-imbibed grain (for the high-pI and low pI isoforms, respectively). Also evident is the relative ineffectiveness of the two control treatments in inducing a GA-responsive state of the aleurone layer in respect of eliciting α-amylase mRNAs.

Thus, the increased GA-responsiveness of the aleurone layer following premature drying that results in α-amylase synthesis and secretion upon rehydration is preceded by increased α-amylase mRNA, which may indicate a primary control by drying at the transcriptional and post-transcriptional levels.

### 4.3.5. Transient Expression of α-Amylase-GUS Gene Constructs

Premature drying may elicit α-amylase production in developing aleurone layers by causing an increased responsiveness of the α-amylase gene promoter to GA, thus affecting its transcriptional activity. As an initial step toward understanding the effect of desiccation on α-amylase gene expression, I carried out transient expression of chimeric gene constructs in the aleurone layers of half-grains via biolistics. I used two chimeric gene constructs, one consisting of the high-pI α-amylase gene promoter (2.1 kb) fused to the coding region of the reporter gene, β-glucuronidase (GUS) (Fig. 4.8, Am2.1) and the other with a truncated (331-bp) α-amylase gene promoter (from the Amy b32 gene of barley) linked to GUS (Fig. 4.8, ML022). In aleurone layers of barley, the latter construct is expressed at a 2-fold higher level and is more responsive to GA than a construct (JR248) containing the whole gene promoter (1.4 kb) (Lanahan *et al.*, 1992). I used a control construct (pDO432), the CaMV 35S promoter/firefly luciferase reporter construct as an internal standard (Ow *et al.*, 1986), which when introduced with the α-amylase promoter-GUS test constructs, allowed standardization of transformation efficiency, enabling reproducible comparisons between levels of expression in aleurone layers of differently-treated grain. Expression from the CaMV promoter is constitutive in barley aleurone cells and is unresponsive to GA (Huttly and Baulcombe, 1989; Skriver *et al.*, 1991); further, it is unresponsive to desiccation and to ABA in dicot seeds (Jiang and Kermode, 1994;

Jiang *et al.*, 1995). In aleurone layers of mature-imbibed grain, GA caused a 10-12 fold increase in GUS activity from both chimeric constructs containing the α-amylase gene promoter or the truncated promoter (Fig. 4.8 A,C). Likewise, premature drying of 30 or 40 DAA grains resulted in an increased responsiveness of the α-amylase gene promoter; GUS activity in aleurone layers was increased 7-12 fold in the presence of GA (Fig. 4.8 A,B). In contrast, treatment of developing 30 DAA grain by preincubation or maintenance at high relative humidity, was relatively ineffective in causing an increased responsiveness of GUS activity to GA in aleurone layers, and in some cases, even led to a slight negative response (Fig. 4.8 C,D). The lack of GA-induced GUS expression following biolistics in aleurone layers of untreated 30 DAA half-grains (Fig. 4.8 B) was also confirmed using PEG-transformation of aleurone protoplasts with the Am2.1 gene construct (data not shown).

Thus, only desiccation (and not simply grain detachment from the parent plant) leads to GA-induction of GUS gene expression from the α-amylase gene promoter in developing aleurone layers. Further, desiccation is effective in inducing expression of the reporter gene when it is directed by a truncated α-amylase promoter containing all the elements necessary for high levels of GA-dependent transcription.

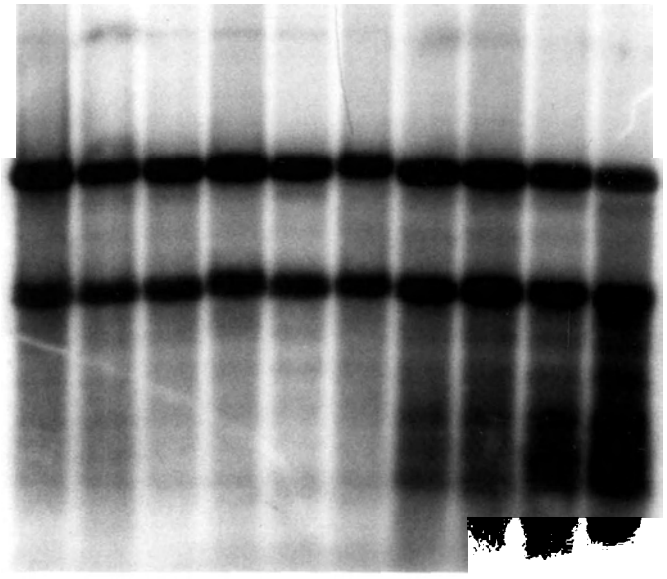
**Figure 4.5.** Northern blot analysis to detect mRNA encoding the high pI α-amylase following desiccation and control treatments of immature 30 DAA grain. Bulk RNA was extracted from mature 65 DAA grain, developing fresh 30 DAA grain and developing 30 DAA grain subjected to premature drying, high humidity or preincubation treatments following 24h of incubation in water or 5 μM GA. Gels were loaded with 10 μg total RNA and the blots probed with the 1-28 clone to detect mRNA for the high pI α-amylase (A) or a control probe (pTA71, a ribosomal RNA probe) to ensure equal loading of RNA across samples (B). F, fresh; pre-inc, preincubation; 100%RH, 100% relative humidity; Dried, prematurely dried; mature, mature (65 DAA) grain.

# A. High-pI Amy (1-28)

- + - + - + - + - + GA

Fresh Pre-inc 100%RH Dried Mature

# B. Ribosomal RNA (pTA71)

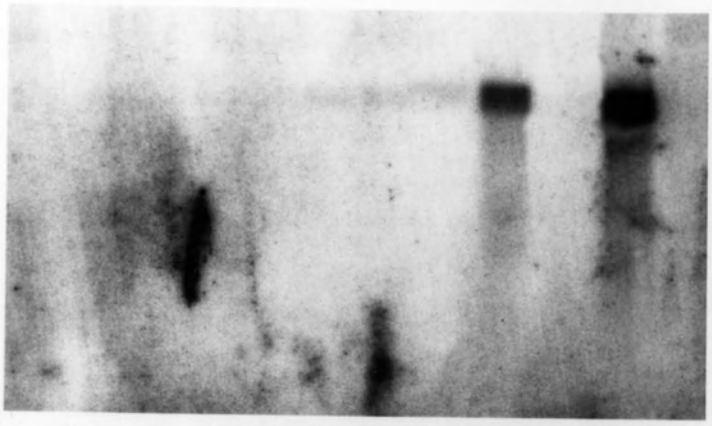


- + - + - + - + - + GA

Fresh Pre-inc 100%RH Dried Mature

**Figure 4.6.** Northern blot analysis to detect mRNA encoding the low pI α-amylase following desiccation and control treatments of immature 30 DAA grain. Bulk RNA was extracted from mature 65 DAA grain, developing fresh 30 DAA grain and developing 30 DAA grain subjected to premature drying, high humidity or preincubation treatments following 24h of incubation in water or 5 μM GA. Gels were loaded with 10 μg total RNA and the blots probed with the clone E to detect mRNA for the low pI α-amylase (A) or a control probe (pTA71, a ribosomal RNA probe) to ensure equal loading of RNA across samples (B). Pre-inc, preincubation; 100%RH, 100% relative humidity; Dried, prematurely dried; Mature, mature (65 DAA) grain.

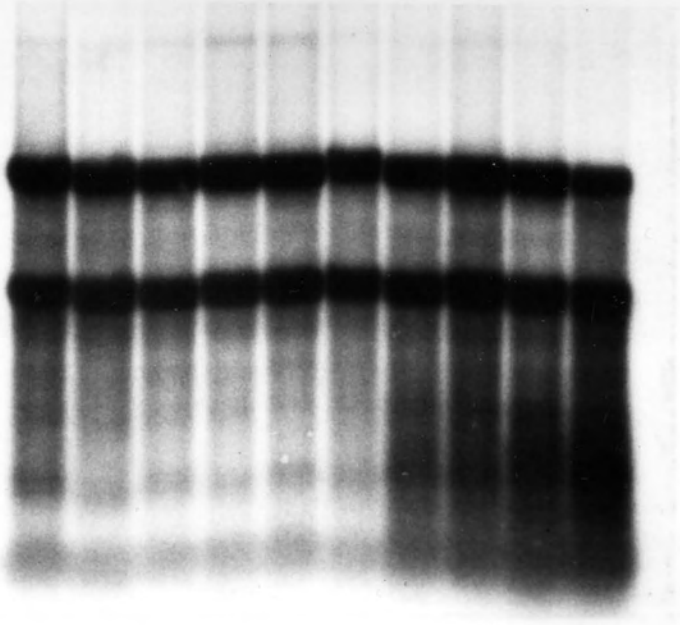
## A. Low-pI Amy (clone E)



- + - + - + - + - + GA

Fresh Pre-inc 100%RH Dried Mature

## B. Ribosomal RNA (pTA71)

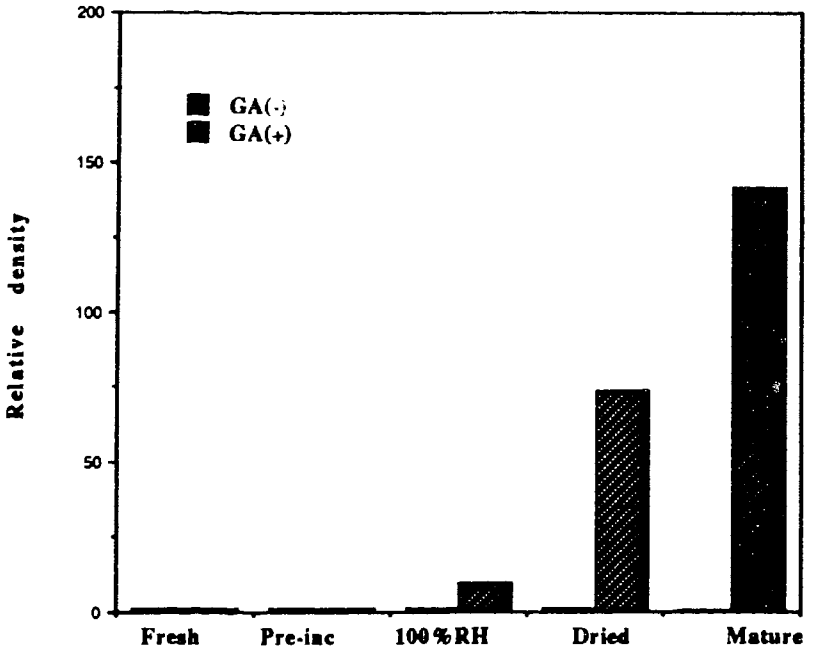


- + - + - + - + - + GA

Fresh Pre-inc 100%RH Dried Mature

**Figure 4.7.** Quantitation of Northern blots of mRNAs encoding high and low pI α-amylase (Figures 4.5 and 4.6) by laser densitometry. Peak integration values were normalized within each gel to the most dense band. Band intensity corresponding to α -amylase mRNA is expressed relative to the amount of rRNA on the control blot. Pre-inc, preincubation; 100%RH, 100% relative humidity; Dried, prematurely dried; Mature, mature (65 DAA) grain.

A. High-pI Amy (1-28)




B. Low-pI Amy (clone E)

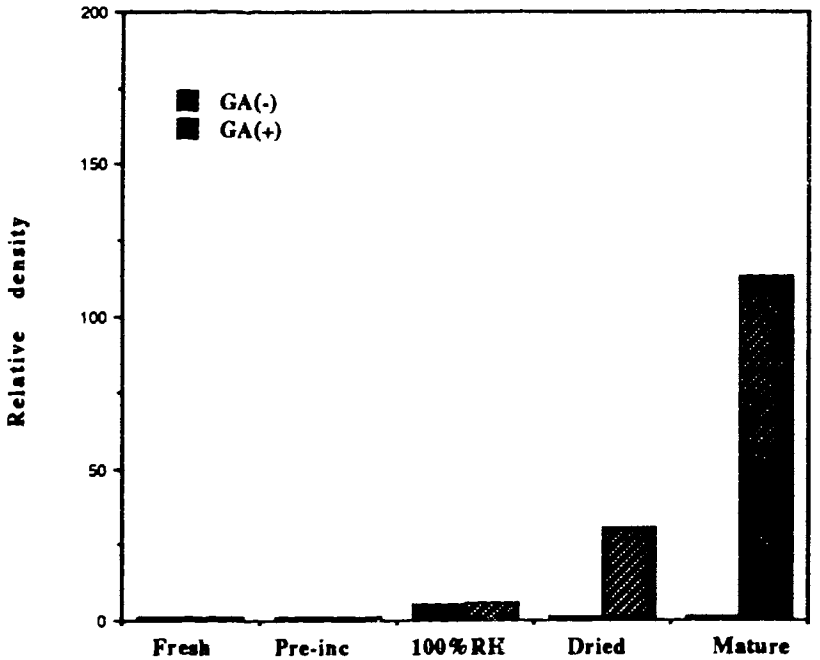
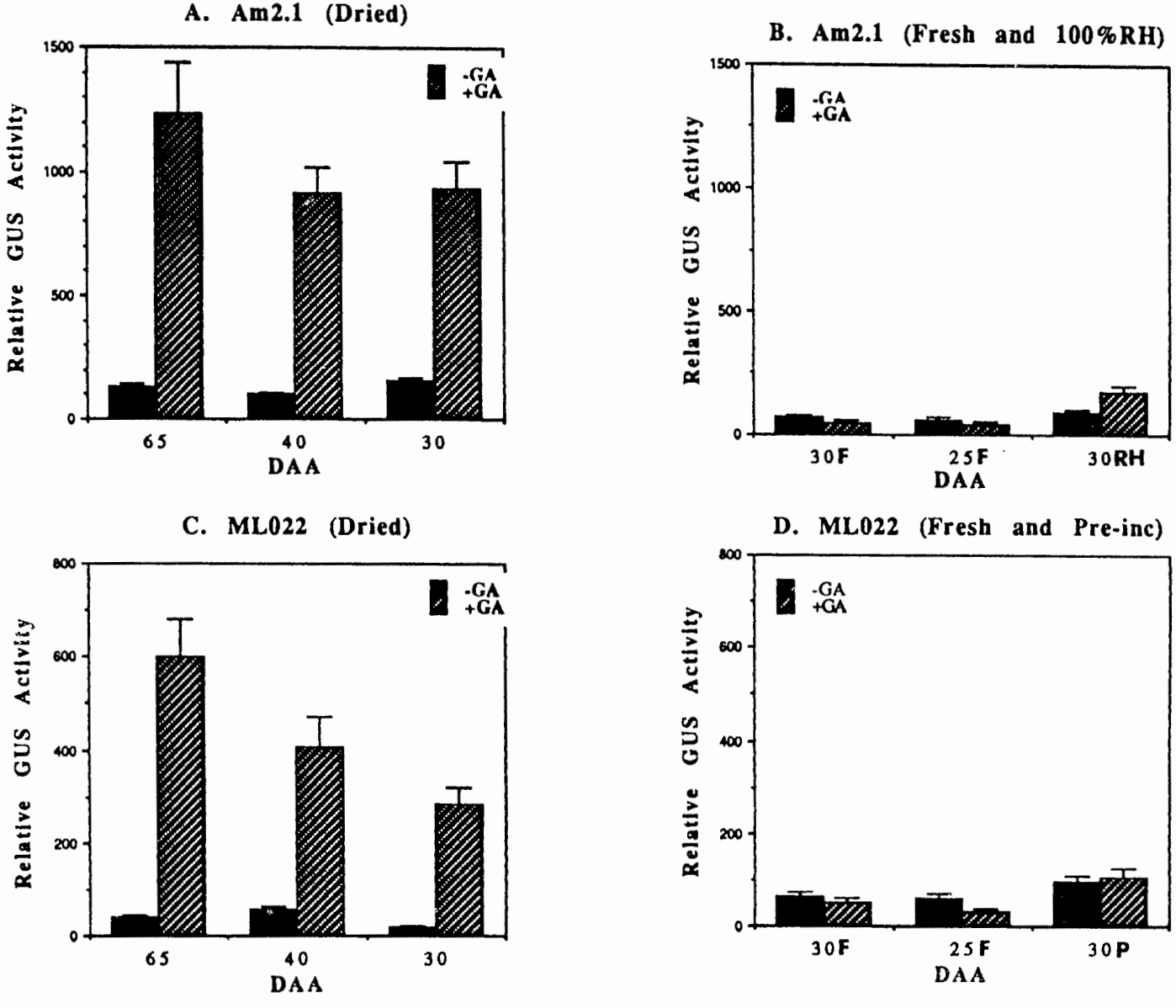


**Figure 4.8.** Effects of premature desiccation and control treatments on transient GUS expression directed by the α-amylase gene promoter in response to GA in aleurone layers of developing and mature grains. After surface sterilization, half-grains were generated from developing fresh (25 and 30 DAA) grain, treated 30 DAA grain or mature 65 DAA grain and imbibed in water for 2d. The pericarp and testa were removed prior to bombardment. Following particle bombardment, 50% of the half-grains were incubated in water and 50% were incubated in GA (5 μM) for 40h at 25 $^{o}$C. The average relative GUS activities (with SE bars) in the aleurone layer obtained when half-grains were bombarded with either Am2.1 (A and B) and ML022 (C and D) are presented. The relative GUS activity was standardized by dividing the units of fluorescence against the luciferase value for a particular sample, which indicates the efficiency of transcription for the α-amylase gene promoter as compared to that of the CaMV 35S promoter. Relative GUS activities were calculated using 4-6 replicate bombardments per gene construct. F, fresh; RH, 100% relative humidity treatment; P, preincubation.


A. Am2.1 (Dried)
Relative GUS Activity
1500
1000
500
0
-GA
+GA
65
40
30
DAA
B. Am2.1 (Fresh and 100%RH)
Relative GUS Activity
1500
1000
500
0
-GA
+GA
30F
25F
30RH
DAA
C. ML022 (Dried)
Relative GUS Activity
800
600
400
200
0
-GA
+GA
65
40
30
DAA
D. ML022 (Fresh and Pre-inc)
Relative GUS Activity
800
600
400
200
0
-GA
+GA
30F
25F
30P
DAA

## 4.4. Discussion

The present results indicate that premature drying is effective in inducing developing barley aleurone layer cells to become responsive to GA with regard to α-amylase synthesis and secretion. It has been argued that desiccation *per se* is not the causative factor leading to a GA-responsive state; rather a mechanism involving seed detachment from the parent plant and a concomitant decline in the supply of sucrose is operative (Nicholls, 1979; 1986). Therefore, I also examined the effectiveness of two control treatments (which do not result in substantial water loss from the grain) for their ability to induce developing aleurone layer cells to produce and secrete α-amylase. These included maintenance of developing grain in an atmosphere of high relative humidity and preincubation of detached grains in water (for a period equivalent to drying regime) prior to the addition of GA. However, these treatments were found to be relatively ineffective in barley as compared to premature drying. These results are comparable to those found by others, particularly in wheat (Cornford *et al.*, 1986, 1987a). Here, various treatments where immature grain are detached and held in a moist state are not equally effective in inducing a GA-responsive state; moreover, they do not elicit a "normal" (full) response to GA (i.e., one similar in pattern and extent of an increase in α-amylase activity to that exhibited by the aleurone layer of mature grain which has undergone normal maturation drying) (Cornford *et al.*, 1986; 1987a). Preincubation of immature wheat grain in a simple buffered medium prior to the addition of GA becomes increasingly effective in eliciting a GA-responsive state in aleurone layer tissue when imposed at progressively older stages (>20 to 25 DAA), particularly when the treatment is extended to 4d. However, it is unlikely that its effectiveness is due solely to seed detachment and diminished sucrose supply (see later). Interestingly, preincubation of wheat grains of a

certain age (25 to 28 DAA) leads to α-amylase production (albeit in low amounts) even in the absence of exogenously applied hormone. I found a similar occurrence in 30 DAA barley grains subjected to the preincubation treatment. This may indicate that the cells become capable of responding to their endogenous GA (Cornford *et al.*, 1986, 1987a).

The GA-induced synthesis and secretion of α-amylase within the aleurone layer following premature drying/rehydration of 30 DAA barley grain was preceded by an increase in mRNAs for both high- and low-pI isoforms of α-amylase. The present studies indicated an onset of α-amylase secretion between 24 and 48h of incubation of the half-grains in GA. Although I did not examine the time course of accumulation of α-amylase mRNAs following incubation in GA, in both mature-imbibed grain and in prematurely dried/rehydrated 30 DAA grain, substantial amounts of α-amylase mRNAs were present in the aleurone layer tissue at 24h. The control treatments were relatively ineffective in inducing the α-amylase mRNAs in 30 DAA grain; however, a small amount of mRNA was detected in GA-treated grains which had previously been subjected to the high humidity treatment.

Although other controls may be operative, induction of α-amylase synthesis and secretion as a result of premature drying is likely due to controls primarily at the transcriptional and/or posttranscriptional levels, resulting in increased production of translatable α-amylase mRNA in the aleurone layer. The transient expression studies using chimeric constructs with the α-amylase gene promoter suggest that the induction of α-amylase and its corresponding mRNA following premature drying is associated with increased transcriptional activity of the α-amylase gene promoter in the presence of GA. Controls by the hormones GA and ABA in respect of induction or suppression of α-amylase synthesis and secretion by aleurone layers appear to be

exerted in part at the level of transcription. The 5' upstream *cis*-acting sequences responsive to ABA and GA have been identified in α-amylase genes and the *trans*-acting factors (DNA binding proteins) potentially involved in regulating gene expression are being characterized (Skriver *et al.*, 1991; Jacobsen and Close, 1991; Gubler and Jacobsen, 1992; Lanahan *et al.*, 1992; Rogers *et al.*, 1994 and references therein). A regulatory mechanism may involve hormone dependent interaction between *cis*-acting promoter elements and *trans*-acting factors that are either induced *de novo* in the presence of GA or undergo hormonal dependent activation or relocation (Jones and Jacobsen, 1991). Desiccation may enhance the perception of GA by aleurone cells, which in turn, may lead to induction of synthesis of *trans*-acting factors or the activation of preexisting factors that interact with GA-responsive *cis*-acting elements of the α-amylase gene. The ability of the aleurone layer to respond to GA and thus produce α-amylase may not only depend upon a balance of endogenous hormones (GA and ABA) but also the relative sensitivity of the tissue to these two hormones. It is tempting to speculate that desiccation may sensitize the aleurone layer to GA by causing changes in hormone receptors (for GA and/or ABA), for example, their levels, availability and/or conformation (and thus the capacity to bind the hormone). Identification of GA and ABA receptors has not yet been achieved. However, recent studies have yielded convincing evidence that GA and ABA are perceived on the external face of the plasma membrane (Gilroy and Jones, 1994; reviewed in Allan and Trewavas, 1994). For example, barley aleurone protoplasts respond to GA and ABA applied extracellularly but not to hormone microinjected into the cytosol of the cell (Gilroy and Jones, 1994); membrane impermeant GAs have biological activity (i.e., are capable of eliciting α-amylase synthesis) in oat protoplasts, also indicative of a surface receptor for GA action (Hooley *et al.*, 1991). However, changes in GA perception may not be fully responsible for the changes in sensitivity of the aleurone layer following drying

(Armstrong *et al.*, 1982; King, 1982); an alternative mechanism (which gains some support) is a decline in the level of (or sensitivity to) a hormone which is antagonistic to GA (i.e., ABA) (Cornford *et al.*, 1986; King, 1982). Premature drying of immature wheat grain causes a concomitant decline in grain ABA content (King, 1976), possibly due to its enhanced breakdown as a direct result of water loss (King, 1979, 1982). Most (if not all) of the treatments capable of inducing some GA sensitivity in immature aleurone layers of wheat are dependent on both temperature and time -- consistent with a possible leakage or breakdown of ABA (Cornford *et al.*, 1986). However, treatment of developing maize kernels with fluridone (an ABA biosynthesis inhibitor), which enhances their germination capacity, only slightly increases the ability of the aleurone tissue cells to produce α-amylase (Oishi and Bewley, 1990). Instead, the kernels must be dried. Both fluridone and drying cause a decrease in the ABA content within developing maize kernels; thus a decline in endogenous ABA levels is not the only determinant in the regulation of α-amylase production and alone appears to be insufficient to permit an increase in α-amylase activity (Oishi and Bewley, 1990). For the aleurone tissue to achieve its full potential to produce α-amylase, it must not only be free of the inhibitory effects of ABA but also be competent to respond to GA. There may be changes in ABA sensitivity of the aleurone tissue following drying that are important for this latter event. In castor bean embryos, there is about a ten-fold reduction in ABA sensitivity following natural or imposed seed drying (as measured by the capacity of exogenous ABA to block germination) (Kermode *et al.*, 1989a). Further, this reduced sensitivity is not due to the period of seed detachment from the parent plant required to effect premature drying. Likewise, in developing transgenic tobacco seeds, ABA enhancement of GUS activities (from chimeric gene constructs driven by promoters of storage protein genes) is substantially reduced following premature drying (Jiang *et al.*, 1995; Jiang and Kermode, 1994). In dormant cereal grain, the germination process is blocked; this

state may be associated with both a reduced sensitivity to GA, and an enhanced responsiveness to ABA. Increased responsiveness of aleurone protoplasts to $GA_3$ is correlated with a loss of embryo dormancy in oat seed (Hooley, 1992). A comparison of dormant and nondormant isogenic barley shows that both the embryos and aleurone layers of dormant grain have an increased responsiveness to ABA as compared to their nondormant counterparts (van Beckum *et al.*, 1993). Thus, the acquisition of a competence of the aleurone layer to respond to GA may require a decline in sensitivity to endogenous ABA.

These studies will serve as a prelude to an examination of the regulatory mechanisms by which desiccation causes changes in the responsiveness of the barley aleurone layer to the hormones ABA and GA, thus controlling the synthetic and secretory response of this tissue upon the transition to a germination and growth program.

## General Summary and Future Prospects

By using molecular approaches, I have studied the role of desiccation (maturation drying) in the control of expression of storage protein genes (vicilin and napin) in transgenic tobacco seed. I further studied the possible interaction between desiccation and abscisic acid in controlling the expression of vicilin and napin storage protein genes in transgenic tobacco seed. In addition to examining the role of desiccation in the cessation of developmental gene expression, a further goal was to determine how drying may promote the transition to a germinative and growth program. More specifically, the expression of α-amylase and induction of GA responsiveness of developing aleurone layer cells of barley was studied. General conclusions can be made from the above studies and are summarized as follows:

**(1)** The regulatory role of desiccation on the expression of storage protein genes was studied using a molecular approach, in which chimeric gene constructs containing different regulatory regions (5' and/or 3' regions) of the vicilin and napin genes, or control regions from constitutively expressed genes were analyzed in seed of transgenic tobacco plants:

a) The vicilin and napin gene promoters were temporally and spatially regulated in transgenic tobacco seed, i.e. their expression is seed-specific and developmentally regulated;

b) Generally, GUS activities in seed expressing gene constructs containing the vicilin and napin promoters were 4-8 times higher than that expressing gene constructs containing the CaMV 35S promoter;

c) Following imbibition of mature or prematurely dried developing seed expressing gene constructs containing the vicilin or napin gene promoters, GUS activities declined dramatically. This decline was a result of drying and rehydration, and not due to seed detachment from the parent plant. GUS activities increased in mature or prematurely dried seed expressing the CaMV 35S promoter. Thus, the constitutive promoter is not down-regulated by desiccation/rehydration;

d) The 3' regions of the tested genes (vicilin, napin, and nopaline) had little effect on the patterns of changes in GUS activities in seed expressing gene constructs containing the same promoter;

e) GUS mRNA also declined in developing seed expressing gene constructs containing the vicilin and napin promoters as a result of drying. This decline in GUS mRNA did not occur in seed expressing the CaMV 35S promoter;

f) Western blot analysis of GUS protein supported the GUS activity results;

g) Transient expression of chimeric gene constructs in castor bean cotyledons supported the hypothesis that desiccation down-regulates the storage protein gene promoter;

h) The above studies suggest that desiccation down-regulates the 5' flanking regions of vicilin and napin storage protein gene promoters. Thus, desiccation may act directly on the 5' upstream regions of developmental genes and down-regulate the expression of these genes during late seed development.

(2) A further goal was to examine whether the maturation drying period, by causing changes in the content of, or sensitivity to, ABA, partially or wholly removes the effects of this positive modulator of developmental gene expression:

GUS activities were enhanced by exogenous ABA in developing seed expressing gene constructs containing vicilin and napin gene promoters. This enhancement of GUS activity by ABA was abolished by premature drying, i.e. the vicilin and napin promoters were responsive to ABA in developing seed, but lost their sensitivity to ABA after premature drying. Measurements of endogenous ABA levels in tobacco seed suggest that drying can reduce the ability of storage protein gene promoters to respond to ABA, without causing a decline in the amount of endogenous ABA.

(3) In relation to promoting the transition to a germinative and growth program, premature drying plays a key role in increasing the GA-responsiveness of developing aleurone layer cells in respect of α-amylase synthesis and secretion in barley. Further, desiccation may up-regulate the expression of α-amylase gene promoter in response to GA.

The results of the present study will serve as a prelude to study the precise regulatory mechanism of desiccation, in regulating the expression of developmental and postgerminative genes. Many questions can be addressed in future research and can be summarized as follows:

(1) The possible desiccation-responsive region(s) or sequences in the napin gene promoter should be localized via two approaches: a) Analysis of promoter deletion mutants in transgenic tobacco seed or in a transient expression system. These approaches can be used to narrow down the desiccation-responsive region(s) in the promoter; b) The precise sequences responsive to desiccation can subsequently be analyzed via gel shift assays and DNA-footprinting analysis; and such desiccation responsive sequence(s) identified via *in vitro* techniques can be further confirmed by "gain-of-function" analysis, i.e. expressing chimeric gene construct containing only that sequence(s) linked to a minimal 35S promoter;

(2) If the desiccation-responsive sequences are identified, a long term research goal is to investigate any effects of desiccation on *trans*-acting factors (DNA binding proteins) that interact with storage protein genes. If the *trans*-acting factor is specifically regulated by desiccation, cloning of its corresponding gene will allow in-depth investigation on the regulatory mechanism of desiccation.

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