AN ABSTRACT OF THE THESIS OF

Estela B. Thomann for the degree of Master of Science in Botany and Plant Pathology presented on November 30, 1994, Title: Expression of a Group 3 LEA Protein During Maturation of Zea mays L. Embryos

Abstract approved: Redacted for Privacy

Several different types of proteins that are modulated by abscisic acid (ABA) accumulate in developing embryos of maize (Zea mays L). Some of these proteins are specific to the developing seed, such as the storage globulin, GLB1, while others are involved in general responses to water deficit. Here, I report the isolation and characterization of a very distinct family of proteins, named MLG3.

MLG3 (Maize Lea Group 3) are low molecular weight proteins that become abundant around the onset of desiccation tolerance in maize embryos. Like other proteins of this class, MLG3 polypeptides are ABA-responsive. They are found in maturing seeds and in dehydrating plant tissues.

Antigenically related proteins are found in other cereals. MLG3 proteins decline in abundance after germination, but increasing levels accumulate when seedlings are grown under conditions of water stress.

To distinguish the regulation of developmentally-programmed ABA responses from those that are environmentally induced, a comparison was made between the ontological pattern and accumulation requirements of MLG3 polypeptides and the previously described results for GLB1 protein.

GLB1 accumulation begins early in maturation phase and specifically requires high levels of ABA and the
participation of the *Viviparous-1 (VP1)* gene product. *VP1* is required for other ABA-modulated events in maize seed development as well. Experiments using *vp1* mutants and mutants deficient in ABA synthesis (*vp5* mutation), show that MLG3 accumulation also is dependent upon ABA, but it occurs much later in embryogenesis, coincident with the onset of dehydration. In contrast to GLB1, MLG3 proteins can be induced by *de novo* ABA synthesis in response to culturing in high osmoticum, and MLG3 has no specific requirement for the *Vp1* gene product.
Expression of a
Group 3 LEA Protein During Maturation
of Zea mays L. Embryos

by

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A THESIS
submitted to
Oregon State University

in partial fulfillment of
the requirements for the
degree of
Master of Science

Completed November 30, 1994
Commencement June 1995
Master of Science thesis of Estela B. Thomann presented on November 30, 1994

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Dean of Graduate School

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Estela B. Thomann
DEDICATION

To my family, for their understanding, love and support.
AKNOWLEDGEMENTS

I wish to express sincere appreciation to my major professor, Dr. Carol Rivin, for her support, and to Drs Donald Armstrong, Machteld Mok, and Mark Daeschel for serving on my committee.

I wish to thank Dr. Connie Bozart (Oregon State University, Corvallis) for assisting me with the production of the antibody and Dr. Timothy Close (California, Riverside) for providing me with the dehydrin antiserum.

A very special thanks to my friend, John Sollinger for his personal and professional support.
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<tr>
<td>ABA,</td>
<td>Abscisic acid</td>
</tr>
<tr>
<td>D.T,</td>
<td>Desiccation Tolerance</td>
</tr>
<tr>
<td>D.I,</td>
<td>Desiccation intolerant</td>
</tr>
<tr>
<td>DAP,</td>
<td>Days after pollinations</td>
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<td>ECL,</td>
<td>Enhanced Chemiluminescence</td>
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<td>LEA,</td>
<td>Late Embryo Abundant</td>
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<td>MLG3,</td>
<td>Maize LEA Group 3</td>
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<td>vp</td>
<td>viviparous</td>
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Expression of a Group 3 LEA Protein During Maturation of Zea mays L. Embryos

1. INTRODUCTION

Angiosperm embryo development can be conveniently divided into three stages: histodifferentiation, maturation and dormancy. During histodifferentiation, the single cell zygote undergoes extensive mitotic division, and the resultant cells differentiate to form the basic body plan of the embryo. Thereafter, maturation occurs largely in the absence of further cell division and is characterized by cell expansion and the deposition of storage proteins, along with lipids and carbohydrates in the storage tissues. Maturation is generally terminated by some degree of drying, which results in a gradual reduction in the rate of metabolism as water is lost from the seed tissues and the embryo enters a period of physiological dormancy or quiescence (Kermode, 1990).

Many different types of proteins accumulate during seed maturation and desiccation in developing embryos of maize (Zea mays L.). Some are seed storage proteins, like the globulins, GLB1 and GLB2. Others are believed to play a role in seed desiccation, and are often referred to as the late embryonic abundant (LEA proteins or genes) (Dure et al., 1989). Because these LEA genes are regulated by abscisic acid (ABA) or tissue dehydration, some attempts have been made towards understanding the molecular mechanism of hormonal regulation (Marcotte et al., 1988; McCarthy et al., 1991).

The main objective of this research was to identify proteins in the maturation phase of corn that were correlated with the acquisition of desiccation tolerance.
Here I describe a maize protein family, named MLG3 (Maize LEA Group 3) found in maturing seeds and in dehydrating plant tissues. I studied its developmental accumulation during embryo development and early germination as well as its expression and tissue specificity.

In order to uncouple the regulation of developmentally programmed ABA responses from those that are environmentally induced, I compared the ontological pattern and accumulation requirements of MLG3 proteins with GLB1 polypeptides. Unlike GLB1, MLG3 accumulates much later in embryogenesis, coincident with the onset of dehydration. The specific requirements for the expression and regulation of both polypeptides are discussed.

1.1 ROLE OF DESICCATION

Desiccation plays two critical functions in seed biology. The first is to induce and maintain physiological dormancy. Physiological dormancy is controlled by changes in the availability of water, oxygen, and by temperature. In other words, the chemical and physical environment must be favorable to allow germination and the establishment of a new plant. In this context, the purpose of dormancy is associated with a life strategy: protection of the embryo during environmental stresses and dispersal of seeds in time and space (Bewley et al., 1985). According to Virginia Walbot "embryo development must lead to a seed system in which dispersal is facilitated and a high capacity of germination and growth is retained under optimal conditions" (Walbot, 1978). In some plants there are additional requirements for maintaining dormancy following imbibition (embryo dormancy, secondary dormancy, genetic control, inhibitors) (reviewed in Bewley, 1985).
Desiccation also appears to play a developmental role: A number of researchers have presented evidence suggesting that desiccation acts as a developmental switch, closing events associated with embryogenesis and maturation and inducing those associated with germination (reviewed by Kermode, 1990). In some species hydrated immature embryos do not germinate without desiccation. For example, immature seeds of some legumes and cereal grains will not germinate on water when removed from the mother plant in the fully hydrated state, but will germinate only after drying (Evans et al., 1975; Long et al., 1981; Dasgupta et al., 1982). Furthermore, postgermination events such as hydrolase production by the aleurone layer of barley (Evans et al., 1975) and wheat (Armstrong et al., 1982) will not occur unless the grain has been subjected to drying.

Molecular evidence that drying plays an important role in switching seeds from a developmental regime to a germination regime comes from studies in *Phaseolus vulgaris*. Analysis of the pattern of proteins synthesized after seeds have been desiccated shows that polypeptides synthesized during development are no longer produced, while germination polypeptides are. This change in protein synthesis is indicative of changes in gene expression in *Phaseolus vulgaris*. For example, the mRNAs for several major storage proteins (like phaseolin) are degraded during early rehydration following premature drying. New mRNAs, coding for germination proteins, are present for utilization in the rehydrated axes. Desiccation of *Phaseolus vulgaris* axes while the seed is still intolerant to desiccation does not lead to any change in their complement of translatable messages. That is, after rehydration, the mRNAs still code for developmental proteins (Dasgupta et al., 1982; Misra et al., 1985).

A similar situation has been found in the endosperm of *Ricinus communis* L. seeds. Drying, whether occurring naturally or imposed prematurely, results in the permanent suppression
of transcription of most developmentally related messages and, upon subsequent rehydration, the induction of those associated with germination and growth. Some residual mRNAs for developmental proteins which are present in the dry seed are selectively degraded during germination (Kermode et al., 1985).

That drying is important for the acquisition of germinability has been well documented for cereals. Air-dried grains of wheat not only germinate at an earlier stage of development than undried grains, but at later stages, they also germinate at a faster rate (reviewed in Mitchell et al., 1978.)

Virginia Walbot (1978), introduced the idea that normal development will proceed directly from histodifferentiation to germination if certain physical and environmental restraints were not placed on the embryos. Maturation and dormancy could be bypassed if the right conditions for success of seedling establishment are encountered by the embryo. Normal development in the intact seed is accomplished by suppressing germination related events and promoting the stimulation of maturation events.

1.1.1 Germination in the Absence of a Drying Period

The fact that isolated embryos of some species are capable of germinating any time after a certain point in development contradicts the idea that normal development demands maturation and drying. Excised immature embryos of rape (Crouch et al., 1981), french bean (Long et al., 1981), cotton (Ihle et al., 1972), wheat (Triplett et al., 1982), soybean (Ackerson, 1984) can be germinated in culture, either immediately or after a suitable lag period, without the need for desiccation. Another evidence is provided by
embryos that avoid maturation, and they are known as viviparous. This condition is characterized by germination of the developing embryo within the fruit of the mother plant. There is an uninterrupted progression from embryogenesis to germination with little or no intervening cessation of growth (quiescence) and, in most cases, little or no dehydration (Sussex, 1975; Robichaud et al., 1980).

1.1.2 Acquisition of Desiccation Tolerance

For most seeds, a period of desiccation is the normal terminal event in embryo development. In several dicot species it has been shown that seeds became tolerant of desiccation well before maturity and that when these dry seeds are imbibed they germinate at a much faster rate than fresh seeds of the same age (Bewley et al., 1985; Long et al., 1981; Kermode et al., 1985). Seeds are not able to tolerate drying at all stages of development; they usually go from a desiccation intolerant (DI) to a desiccation tolerant (DT) stage half way through development. For example, seeds of *Phaseolus vulgaris* desiccated at 22 days of development fail to germinate after rehydration and deteriorate. At 32 days, these seeds became desiccation tolerant and capable of germination after premature drying (Long et al., 1981; Dasgupta et al., 1982). In castor bean seeds, the acquisition of desiccation tolerance to slow drying occurs around 25 days after pollination (DAP) (Kermode et al., 1985). A similar situation is found in maize seeds. Desiccation tolerance is acquired between 20-25 DAP, well before seed maturation is achieved. Before the onset of desiccation tolerance, embryos are not able to survive drying (Bochicchio et al., 1988). The transition to a desiccation-tolerant state approximately midway through
development is also characteristic of other seeds: soybean (Adams and Rinne, 1981), barley (Bartels et al., 1988), *Agrostemma githago* L. (deKlerk, 1984).

In many of the examples just mentioned, the seed acquires a competence to germinate after premature desiccation, even though the major developmental events such as reserve deposition are far from completed. For example, castor bean seeds at 25 DAP had only reached 20% of the maximum dry weight gained during development (Kermode et al., 1986). In relation to natural drying and germinability, fresh harvest seeds of *Phaseolus vulgaris* and castor bean will germinate only after achievement of maximum dry weight, which is just subsequent to the beginning of water loss *in situ* (Dasgupta et al., 1982; Kermode and Bewley, 1985).

The prerequisites for desiccation tolerance are unknown. Several theories exist in the literature to explain how embryos are able to cope with desiccation. At the cellular level, membranes have been suggested to play a role in the transition from desiccation intolerant to a desiccation tolerant state. Changes in fatty acid and phospholipids appeared to be related to the acquisition of desiccation tolerance (Dasgupta et al., 1982; Misra and Bewley, 1985). Membranes can also be stabilized by carbohydrates. A role for sucrose and raffinose in the preservation of membranes during drying has been suggested by some researchers (Leopold and Vertucci, 1986). It was mentioned before that the acquisition of desiccation tolerance during maturation takes place well before the completion of reserve deposition. It has been suggested that a critical level of reserves in vacuoles and storage bodies is required before the seed can stand desiccation (Kermode and Bewley, 1986).

More recently, a highly abundant set of hydrophilic proteins have been implicated in desiccation tolerance. First identified in cotton these are known as LEAs for Late
Embryo Abundant (Galau et al., 1987; Galau & Hughes, 1987). LEA mRNA and proteins accumulate in embryonic tissue during seed maturation (Dure et al., 1981). LEA mRNAs and proteins can also be induced by the application of abscisic acid (Galau et al., 1986) or by tissue dehydration in cereal (Chandler et al., 1988). A protective role for these proteins during plant tissue dehydration that could be mediated by ABA has been proposed (Close et al., 1989; Dure et al., 1989; Koster & Leopold, 1988). Expression of these polypeptides takes place during early mid-development and is highest at incipient desiccation. The timing of their expression, as well as their general physical characteristics provide indirect evidence that they may play a decisive role during desiccation tolerance of the developing seed (Gomez et al., 1988; Mundy and Chua, 1988; Close et al., 1989).

More direct evidence of the protective role comes from studies done in barley and corn seedlings. For example, seedlings pretreated with ABA can survive rapid desiccation (up to 50% decrease in fresh weight), while untreated tissues die (Close et al., 1989; Chandler et al., 1988). Bartels and Salamini have also identified a set of polypeptides in maize and barley embryos which are synthesized de novo just before the onset of desiccation tolerance (Bochicchio et al., 1988; Bartels et al., 1988).

1.1.3 Loss of Desiccation Tolerance

During germination, seeds remain tolerant of desiccation, but at some stage after axis elongation there is generally a loss of this tolerance. This phenomenon can be explained by the loss of the protective substances accumulated during the maturation phase. Koster and Leopold
found, for example, that the loss of desiccation tolerance in germinating axes of soybean, pea and corn was correlated with changes in the soluble sugar content (Koster and Leopold, 1988). Loss of desiccation tolerance corresponded to the loss of oligosaccharides, which may serve to prevent sucrose crystallization. In the non-crystalline form, sucrose may interact with membrane surfaces, possibly replacing water in the maintenance of the membrane structure (Caffrey et al., 1988).

1.2 ROLE OF ABA

During embryogenesis, ABA plays a major role in the seed strategy not only preventing precocious germination of the embryo, but promoting the maturation pathway, which includes the synthesis and accumulation of storage reserves and proteins that may have a protective function during long periods of seed desiccation.

1.2.1 Inhibition of Precocious Germination

Early in embryogenesis embryos complete organogenesis and acquire two developmental potentials: germination and maturation. Within the seed environment, the germination potential of the embryo is inhibited and maturation occurs. However, if removed from the seed, many types of embryos can germinate and develop into normal seedlings (Sheridan, 1988).

Embryos of wheat, rice, rapeseed, corn, bean and many others have been studied in this regard. It is therefore
assumed that some factor(s) present in the seed environment, prevents precocious germination in vivo, while allowing the rest of the embryo development to occur. Two possible regulators have been implicated in the non-toxic inhibition of precocious germination: 1) High levels of ABA and 2) Osmotic stress.

Changes in ABA content during development and maturation of fruit, seeds or embryos have been followed in several species. Most of them show a similar pattern: a fairly steep rise during development followed by a sharp drop as the seed matures (Walton, 1981; Black, 1983).

These changes in endogenous ABA during seed development are consistent with its proposed role which emerged from in vitro experiments, i.e. as inhibitor of precocious germination and promoter of embryo maturation. In many species, precocious germination is inhibited if embryos are removed at the begining of maturation and cultured in the presence of ABA (Ackerson, 1984a; Ackerson, 1984b; Finkelstein et al., 1985; Triplett and Quatrano, 1982).

Leaching and drying treatments, which deplete endogenous ABA in soybean embryos, induces precocious germination (Ackerson, 1984a). Levels of ABA during development of the corn embryo change drastically. It increases rapidly during the early phases of development and is highest just prior to maturity. At the same time, the embryo moisture content and sugar uptake rate are diminishing. During the grain filling period, the ABA concentration in the embryo is higher than that found in any other kernel components (Jones & Brenner, 1987; Neill et al., 1987).

Since ABA has been implicated in the regulation of embryo maturation and precocious germination, the use of mutants with ABA deficiency provides a valuable approach to investigate the role of ABA and its importance in embryo development. Mutants with reduced sensitivity to ABA, or, altered ABA metabolism resulting in abnormal tissue levels
of ABA have been studied in maize (Neill et al., 1986; Robertson, 1955), Arabidopsis (Koornneef et al., 1982; 1984), tomato (Taylor, 1984), pea (Wang et al., 1984) and potato (Quarrie, 1982). The viviparous (vp) mutants of maize show precocious germination while still on the ear. Most of these mutants are ABA-deficient, although ABA levels in the vpl mutant are similar to the wild type (Neill et al., 1986). Rivin et al. (1991) studied the effect of ABA over precocious germination by comparing the behavior of wild type and viviparous mutants of maize. Wild type embryos and mutants that cannot synthesize ABA (vp5) were inhibited from germination. In contrast, ABA had no effect over mutant embryos insensitive to the hormone (vpl).

Koornneef et al. (1982) working in Arabidopsis, isolated single-gene mutant lines which exhibited symptoms of withering, increased transpiration, and a lowered ABA content in mature seeds and leaves. Dormancy of the mature seeds of ABA mutants was strongly reduced, and in high humidity vivipary was observed (Koornneef et al., 1982). Other types of mutants, called response mutants, exhibit a decreased response to exogenous ABA, in Arabidopsis the mutants abi-1, abi-2 and abi-3 belong to this category, as does the vpl mutant of maize. All of them show reduced seed dormancy, yet they contain ABA levels similar to the wild type (Neill et al., 1986; Robertson, 1955). Various viviparous mutants of corn have been tested for the ability to accumulate and respond to ABA during kernel development. Vivipary in maize has been described in detail by Robertson (Robertson, 1955). Since two mutants were utilized in this study, I will describe them in more detail.
1.2.2 \textit{vpl} Mutants:

\textit{vpl} mutant seeds are viviparous and lack anthocyanin in the aleurone. Viviparous seeds can be rescued and will grow to make a normal appearing plant. Embryo ABA and carotenoids levels are normal in \textit{vpl} homozygotes (Neill et al., 1987; 1986), but much higher levels of ABA are necessary to inhibit both germination and seedling growth, indicating relative insensitivity to ABA (Robichaud et al., 1980). McCarthy et al. have isolated the \textit{Vp1} gene by transposon tagging and shown that it is a single-copy gene with 2500 nucleotide, seed specific transcript (McCarthy et al., 1989). Also, \textit{Vp1} action is limited to the embryo and aleurone tissues (Dooner, 1985). McCarthy et al. have proposed that VP1 acts as a transcriptional activator (McCarthy et al., 1991).

1.2.3 \textit{vp5} Mutants:

The lack of carotenoids gives this mutant a pale endosperm in a yellow genetic background and albino seedling lethal leaves due to photooxidation of chlorophyll.

Homozygous mutants seedlings are therefore lethal. Carotenogenesis is blocked at early stages in this mutant so that xanthophylls are absent and the ABA content of the embryo is substantially reduced to 6 to 16\% of that in the corresponding wild type (Neill et al., 1986).

Carotenogenesis and ABA biosynthesis share some early steps, so it seems consistent that \textit{vp5} mutants that accumulate low amounts of ABA are also those that show reduced levels of carotenoids (Neill et al., 1986; Dooner, 1985). When an inhibitor of carotenoid synthesis,
fluoridone, is applied to wild type ears at an early time during embryogeny, a phenocopy of \( \text{vp}^5 \) mutants is obtained. Application of exogenous ABA to fluoridone treated ears was effective in reducing vivipary (Fong et al., 1983).

High osmolarity can substitute for ABA in suppressing precocious germination and allowing embryo maturation to continue. In many plant tissues, ABA levels rise as a result of water deficit (Zeevart and Creelman, 1988) and this has been shown to modulate the levels of mRNAs encoding proteins associated with water stress (Chandler et al., 1988; Galau et al., 1987; Neill et al., 1987; Gomez et al., 1988; Mundy and Chua, 1988). Culture of soybean cotyledons on high osmoticum increases the ABA level by about 2.5 fold compared with that detected in cotyledons cultured on basal medium. However, the endogenous levels are 30 to 50 fold lower in these cultured cotyledons than in those from embryos developing \textit{in situ} (Bray and Beachy, 1985). In young embryos, the rise of endogenous ABA levels by itself appears to inhibit water uptake (Schopfer & Plachy, 1985), and the regulation of gene expression may be a result of water limitation, rather than a direct effect of ABA (Finkelstein et al., 1986). Culturing immature embryos of maize in high osmoticum causes only a slight increase in ABA content (Neill et al., 1987), and leads to the accumulation of only a subset of the embryo-specific storage polypeptides induced by ABA treatment (Rivin, 1988).

1.2.4 Regulation of Gene Expression

Culturing in ABA not only inhibits precocious germination but induces the expression of proteins characteristic of maturation. Prominent among these are the storage proteins. Evidence for these comes mainly from \textit{in}
vitro studies in french bean (Sussex and Dale, 1974), wheat (Triplett and Quatrano, 1985), rapeseed (Finkelstein et al., 1985), corn (Rivin, 1988) and soybean (Bray and Beachy, 1985), where enhanced or maintained expression of specific storage protein genes correlates very well with the high endogenous levels of ABA (reviewed in Kermode, 1991). However, there are exceptions. ABA does not play a role in the expression of storage proteins genes in cotton, either in vivo or in vitro. No synthesis of the major storage proteins occurs in isolated immature cotton embryos treated with ABA, although some embryonic protein synthesis associated with late development occurs (Galau et al., 1986; 1987).

The previous examples show a strong correlation between high levels of ABA and gene products in planta and in culture conditions, but not all proteins that normally accumulate during mid and late maturation phase are equally affected by ABA. The alpha and alpha-subunit of soybean conglycinin (Bray and Beachy, 1985), and napin are normally synthesized earlier than the increase in levels of ABA (Finkelstein et al., 1985). Both ABA and the VPI gene product are required to initiate synthesis and accumulation of globulins storage proteins in maize. Globulin storage proteins do not accumulate in mutants unable to synthesize embryo ABA(vp5). They are induced by culturing with exogenous hormone, but not with high osmoticum. GLB1 encoded proteins are also absent from vp1 mutant embryos that do not respond to ABA (Rivin and Grudt, 1991).

That ABA is involved in the enhanced expression of GLB1 was visualized by analysing amount of transcript produced. In vp1, GLB1 transcripts were absent, while vp5 had reduced amounts (Kriz et al., 1990). These differences in GLB gene expression, particularly the absence of detectable gene products in vp1 embryos, strongly suggest regulation of the GLB genes by ABA. Another class of abundant seed proteins and their corresponding mRNAs regulated by exogenous ABA were first identified in cotton. This group of proteins
(termed late embryonic abundant or LEA), present during late maturation (incipient desiccation) have been detected in mature embryos of cotton (Dure et al., 1981).

A great proportion of the mRNA coding these LEA proteins (13 out of 18) could be precociously induced by the addition of ABA at concentrations that inhibited precocious germination. Also these polypeptides are rapidly degraded 24 to 36 hrs after germination. These results suggested a relationship between LEA genes, ABA and perhaps seed/dormancy and desiccation (Galau et al., 1986). Further studies have shown that these proteins are abundant in the seeds of many higher plants and probably universal in occurrence in plant seeds. LEA mRNA and proteins can be induced to appear at other stages in the plant life cycle besides embryo development, by desiccation stress and/or treatment with the hormone ABA (Berge et al., 1989; Baker et al., 1988).

Three groups of LEA proteins have been identified by sequence homology (Dure et al., 1989). Group I: cotton LEA D19 (Baker et al., 1988) and the wheat Em protein (Litts et al., 1987), Group II: cotton LEA D11 (Baker et al., 1988), the rice RAB 21 (Mundy and Chua, 1988) and dehydrins (DHN) in barley and maize (Close et al., 1989); Group III: cotton LEA D7 (Baker et al., 1988) and a LEA protein from barley (Hong et al., 1988) and rape (Harada et al., 1989), Dc-3 (Seffens et al., 1990) and Dc-8 from carrot (Hatzopoulos et al., 1990), and the two recently discovered in maize (Thomann et al., 1992) and wheat (Curry et al., 1991). As opposed to the storage proteins, the LEA polypeptides are not seed specific and can be detected in young embryos treated with ABA and in roots, shoots and leaves of seedlings subjected to environmental stresses such as water deficit, cold, salt and sugar (reviewed in Mundy and Skriver, 1990). One of the predominant features of the LEA proteins is a tandemly repeated 11 amino acid motif that is
thought to form an amphiphilic alpha helix (Dure et al., 1989).

The molecular levels at which ABA maintains or enhances storage protein synthesis/accumulation in isolated embryos are various and complex. One of the most recent approaches is to look at the role of transcript activation by ABA. When the Em-5 regulatory region was linked to the reporter gene, beta-glucoronidase (GUS), and used in transient (Marcotte et al., 1988) and transgenic (Marcotte et al., 1989) assays, a 646 bp region that was essential for response to ABA was identified. Within this region, a 50 bp ABA response element (ABRE) was discovered, capable of conferring ABA inducibility upon a minimal cauliflower mosaic virus (CaMV) promoter. Two elements (Em1 and Em2) within this 50 bp ABRE are conserved in other ABA-regulated promoters (Marcotte et al., 1989), including the rice Rab (Yamaguchi et al., 1990) and the cotton LEA gene families (Baker et al., 1988).

A plant leucine zipper protein (EmBP-1) that recognizes the ABRE has been isolated and cloned. The sequence of this binding factor is conserved in other ABA-response promoters and in promoters from plants that respond to signals others than ABA, like wounding and light. The hypothesis that EmBP-1 belongs to a highly conserved family of proteins that recognize a core sequence found in regulatory regions of various genes is discussed by Guiltinan (1990).

McCarthy et al. (1991) were able to show that \( Vp1 \) encodes a novel protein with a transcriptional activator function. In order to address the function of VP1 protein, they utilized Em promoter-GUS reporter as a target gene in maize suspension culture protoplasts. They found that VP1, specifically transactivated the Em promoter. Different hypotheses of how VP1 might be involved in ABA-regulated gene expression are discussed by the authors.
(McCarthy et al., 1991). Understanding the function of the $Y:p/\gamma$ gene will be an important step in identifying key steps in the signal transduction pathway linking ABA stimulation to changes in gene expression.
2. MATERIALS AND METHODS

2.1 MAIZE STOCKS:

Maize (*Zea mays* L.) varieties and viviparous mutants were obtained from several sources. Gaspe Flint from J. Beckett; W22 from J. Kermicle (U. Wisconsin, Madison). The viviparous mutants *vpl* and *vp5* from D. Robertson (Iowa State University). The genetic background for the *vpl* stock is chiefly W22. All lines were propagated in Corvallis, Oregon. Due to the lack of carotenoid synthesis, mutant *vp5* kernels appear white in a segregating ear while their wild type sib kernels are yellow in color. Homozygous *vp1* kernels can be identified on a segregating ear because they fail to produce anthocyanins in the aleurone layer, unlike their wildtype homozygous and heterozygous sibs. Due to the fact that aleurone pigmentation is not visible in early development, homozygous *vp1* seedlings were grown in the greenhouse and later transferred to the field. From these plants stage 1 and 2 embryos were recovered. Seed of *Hordeum vulgare* cv. Himalaya was obtained from Andrew Kleinhofs at Washington State University, Pullman WA.

2.2 EMBRYO CULTURE:

Ears were harvested 12 to 50 days after controlled pollinations. They were surface sterilized with 2.5% bleach for 20 min. and rinsed two times with sterile deionized H₂O. Embryos were dissected free of maternal and endosperm tissue under sterile conditions and staged morphologically by
examining the development of the shoot apex according to the classification scheme of Abbe and Stein (1954).

For growth in tissue culture, 20 embryos were placed scutellum down, in petri dishes containing Murashige and Skoog mineral nutrients (Murashige and Skoog, 1962), supplemented with 3% (w/v) sucrose, 100 mg/L myo-inositol and 0.4 mg/L Thiamine-HCl. To study the effect of abscisic acid, ABA (Sigma mixed enantiomers) was added at 10<sup>-5</sup> M. For experiments with high osmoticum, the concentration of sucrose was raised to 20% (w/v). Embryos were incubated in a growth chamber in the dark at 26° C and the media were changed daily. When treatment was completed, embryos were rinsed briefly in deionized H<sub>2</sub>O, blotted dry, frozen in liquid N<sub>2</sub> and stored at -80° C.

2.3 GERMINATION EXPERIMENTS:

Mature seeds of W22 and Gaspe Flint were surface sterilized as above and imbibed for 48 h at 4° C. The seeds were placed on top of sterile Whatmann filter paper and incubated in the dark at 26° C. After one day of incubation, the first sample was removed. The rest of the time points were taken at one day intervals after radicle emergence, which usually occurred two days after culturing. The embryos were dissected free of endosperm tissue and frozen for protein extraction.
2.4 PROTEIN EXTRACTION

Embryos were crushed in liquid N$_2$ and ground to a powder with a mortar and pestle using 50mM TRIS buffer [pH 6.8] and 1mM PMSF. This slurry was heated for five min in a 65° C bath, vortexed, and heated again at 95° C for two min. Centrifugation was carried out at 35,000 g for 15 min to separate cellular debris and polysaccharides. The supernatant was removed and centrifuged again for 10 min for further purification. The protein content was determined using a Bradford protein micro-assay (Bio Rad), using BSA (SIGMA) as a standard. In general, the yields were around 0.03 ug protein/ug of starting material. When necessary, protein extracts were concentrated by adding four volumes of ice-cold acetone with 10 mM 2-B-mercaptoethanol. The pellet was dried in a speed vacuum and resuspended in the extraction buffer.

2.5 PROTEIN ANALYSIS:

Proteins were analyzed by SDS-PAGE using 15 % acrylamide mini gels. Reagents and gel preparation for SDS-PAGE were done according to Laemmli protocols (Laemmli, 1970). Gels were stained with Coomassie brilliant blue and photographed. Duplicate gels were run for western blots transferred to Immobilon-P membrane (Millipore) using an LKB-NOVABLOT.
2.6 MLG3 ANTIBODY PRODUCTION:

Rabbits were pre-bled in order to supply pre-immune serum as a control. Preparation of the antigen was done according to Hurn and Chantler protocol (Hurn and Chantler, 1980): 1mm thick 18% SDS-PAGE gels were run to isolate MLG3 (29 kD abundant protein band) using embryo protein isolated from mature seed of the W22 genotype. BioRad low molecular weight markers were included in all preparative gels in order to locate the desired band. The gel was stained with Coomassie Brilliant Blue to locate the band of interest. Afterwards the band was carefully excised and part of it was used to run a control gel to make absolutely sure I had picked the correct band. The purified protein was repurified by SDS-PAGE twice more. All the excised bands were diced, then ground in 0.15 M NaCL using a mortar and pestle, and homogenized to a smooth paste. 1ml of Freunds complete adjuvant (SIGMA) was added and when the paste was homogeneous, the mixture was poured into a 10 ml disposable syringe with a 21 gauge needle. The mixture was pushed through the syringe a couple of times in order to get an emulsion.

Two New Zealand white rabbits were injected with 0.5 ml of the protein solution intramuscularly in each leg and 1ml subcutaneously at 10 different locations. After six weeks, the same procedure was repeated. A week after the second booster, a cardiac puncture was performed and 20 ml of blood were drawn to titer the serum. The tubes were left in the refrigerator overnight in order to separate the red blood cells from the serum. Tubes were spun at high speed in a table top centrifuge in the fridge for 20-30 min. Thimerosal was added at a concentration of 0.02 % (w/v), aliquoted into microfuge tubes and kept at -80° C.
2.7 ANTIBODY TITER:

Protein blots were used to determine the amount of serum to use for experiments. A preparative gel was run, loading 300 µg of mature W22 protein extract. After blotting onto nitrocellulose, the western blot was cut lengthwise in strips. Different dilutions were tried, for one hour incubations, and a dilution of 1:200 of the primary antibody gave a very sharp resolution.

2.8 DETECTION OF MLG3 AND DEHYDRIN POLYPEPTIDES:

Maize Lea group 3 (MLG3) polypeptides were detected using polyclonal antibodies raised in rabbits with proteins isolated from SDS-PAGE. Anti-MLG3 binding was detected using ECL Kit from Amersham following manufacturer’s directions with the following modifications: 5% (w/v) dry milk was used only in the blocking solution and removed thereafter to reduce background problems.

Different dilutions of anti-MLG3 primary polyclonal antibodies were used for detecting MLG3 polypeptides, and from these trials it was established that one hour of primary antibody at 1:200 dilution, combined with 1/2 hour of secondary antibody at a 1:400 dilution gave a nice and clean reaction in the westerns.

Dehydrin (DHN) polypeptides were detected using polyclonal antibodies raised in rabbits to a conserved synthetic peptide from the carboxyl terminus of barley dehydrin. This serum was kindly provided by Timothy Close.
Anti-MLG3 and anti-DHN binding was observed using the Vectastain ABC horseradish peroxidase kit (Vector Laboratories, Inc). The manufacturer’s directions were followed except for the detection step when the ECL chemiluminescent reagent (Amersham) was utilized to obtain greater sensitivity.
3. RESULTS

When embryos move from embryogenesis through maturation, they accumulate a new set of low molecular weight polypeptides. Prior studies have shown that during normal development, the products of the genes GLB1 and GLB2, encoding storage proteins of 63,000 and 45,000 kD, respectively, accumulate in maize embryos as early as 20 DAP (Kriz et al., 1990; Rivin et al., 1991). In late stage 4 (about 30 DAP), a new set of low molecular weight proteins becomes very abundant in maize embryos.

Results presented in FIG 1 show the protein profiles from young embryos (stage 1, 15 DAP) until kernel maturity (stage 6, 60 DAP). The accumulation of new low molecular weight polypeptides can be observed as embryos enter the late maturation phase. Due to the fact that these low molecular weight proteins became abundant around the onset of desiccation tolerance in maize embryos (Bochicchio et al., 1988), I decided to study the relationship between desiccation tolerance and the expression of this class of proteins. An antiserum was raised to a late abundant protein of 29 kD from W22 embryos. The serum was tested on western blots with mature embryo proteins of several maize varieties FIG 2A and B, detecting a small family of polypeptides termed MLG3 for Maize LEA Group 3.

The size of MLG3 polypeptides varies among maize strain (observation of John Sollinger). In W22, Gaspe Flint, and the vg1 heterozygote stock, the major band is 29 kD; in Ky21, B37, and the vg5 heterozygote stock, the major band detected is 27 kD. To analyze further the source of this variation, F1 hybrids and F2 individuals were studied.
### FIG 1: Low molecular weight protein profile from Gaspe Flint embryos.

Equal amounts of proteins extracted from developmentally staged embryos were subjected to SDS-PAGE in gels containing 15% acrylamide. The gels were subsequently stained with Coomassie brilliant blue, demonstrating the accumulation of several low molecular weight polypeptides. The migration of molecular weight markers is also indicated.
FIG 2: Allelic variants of MLG3 polypeptides.
A: Coomassie-stained gel of mature embryo proteins from: a, Gaspe Flint; b, W22; c, vp1/++; d, vp5/++; e, B37; f, Ky21. The arrow shows the accumulation of the 29 kD band excised for antibody production from w22.

B: Western blot of duplicate gel using anti-MLG3.

C: Western blot of MLG3 proteins in mature embryos of: a, q, Ky21; b, r, W22; c, Ky21 x W22; d-o, F2 individuals from Ky21 x W22 self-pollination; p, W22 x Ky21.
FIG 2C shows the MLG3 pattern of Ky21xW22 cross, 12 F2 individuals from that hybrid, and the reciprocal F1. Both F1s show a codominant pattern (29 kD and 27 kD bands of each parent is present) and the F2s segregate for parental or F1 patterns in the ratios expected for a single gene or a complex of closely linked genes. A weaker, cross-reacting band of 31 kD in all lines was detected except in Ky21. In the hybrids and F2 individuals shown in FIG 2C, this band appears to segregate as the product of another, unlinked locus. Also a faint band of 24 kD is seen in all lines having the major 29 kD band, while those lines having the major 27 kD MLG3 each has a weak band of 21 kD. The amount corresponding to the smaller bands shows a wide variation between individual seeds of the same genotype, so it is not possible to interpret their apparent segregation in F2 individuals with confidence.

3.1 DEVELOPMENTAL ACCUMULATION

Having identified this family of low molecular weight proteins, I proceeded to examine their accumulation profiles during embryo development and seedling growth. Two varieties of maize were used for these experiments: W22, a midwestern dent line, and Gaspe Flint, a northern flint strain.

As shown in FIG 3A, the 29 kD MLG3 band polypeptide can be detected at low levels in W22 embryos in the early maturation phase(20 DAP). Moreover, the high sensitivity of the enhanced chemiluminescence detection method (ECL) made it easy to detect amounts 20-fold less in prematuration embryos FIG 3B. The MLG3 polypeptides accumulated slowly during early maturation phase and they increased
FIG 3: Accumulation of MLG3 proteins during maturation of W22 and G.F. embryos.

Equal amounts of protein from embryos of different stages were separated by SDS-PAGE, blotted, and reacted with anti-MLG3 serum.

A: MLG3 proteins were detected in young W22 embryos from 20 DAP and throughout maturation phase (60 DAP). An abrupt increase in the level of the 29 kD and 27 kD band is observed between 29 and 31 DAP.

B: Detection of MLG3 proteins in prematuration Gaspe Flint stage embryos. 12-16 DAP: 13.5 ug/lane. 60 DAP: .125, .25 ug/lane.
dramatically at approximately 30 DAP. It should be emphasized that around this time the onset of dehydration and the acquisition of desiccation tolerance takes place in maize embryos TABLE 1a and 1b. The dramatic increase in MLG3 polypeptides was seen in the two lines studied and in ears with different pollination dates.

3.2 LOSS OF MLG3 DURING GERMINATION

After germination, the MLG3 polyptides declined in abundance and were not detected in the germinated seedlings five days after radicle emergence FIG 4. A low molecular weight band appears to accumulate as the 29 kD MLG3 dissapears, suggesting that it may be a breakdown product. Comparable results were observed for Gaspe Flint materials.

3.3. TISSUE SPECIFICITY AND ASSOCIATION WITH DESICCATION

The timing of expression and accumulation profiles of MLG3 polypeptides suggested that MLG3 might be among the class of proteins that accumulate upon water deficit both in seeds and plant tissues (Close et al., 1989; Gomez et al., 1988; Mundy and Skriver, 1990). To test this hypothesis, I looked for the accumulation of MLG3 in dehydrating vs well-watered seedlings.
Table

Embryo Growth and Acquisition of Desiccation Tolerance in W22 and Gaspe Flint: Embryos were dissected, staged and weighed. Desiccation was carried out at 30°C for 24 h.

1a: W22 Embryo Growth and Desiccation Tolerance.

<table>
<thead>
<tr>
<th>DAP</th>
<th>Stage</th>
<th>Fresh Wt.</th>
<th>H₂O* Tolerance.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg</td>
<td>%</td>
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<tr>
<td>15</td>
<td>1</td>
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</tr>
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<td>18</td>
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<td>72</td>
</tr>
<tr>
<td>28</td>
<td>4</td>
<td>15.1</td>
<td>65</td>
</tr>
<tr>
<td>31</td>
<td>5</td>
<td>19.1</td>
<td>62</td>
</tr>
<tr>
<td>35</td>
<td>5-6</td>
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<tr>
<td>46</td>
<td>6</td>
<td>22.4</td>
<td>51</td>
</tr>
</tbody>
</table>

1b: Gaspe Flint Embryo Growth and Desiccation Tolerance.

<table>
<thead>
<tr>
<th>DAP</th>
<th>Stage</th>
<th>Fresh Wt.</th>
<th>H₂O* Tolerance.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td>50</td>
<td>6</td>
<td>56.7</td>
<td>43</td>
</tr>
</tbody>
</table>

* Percentage based on sample sizes of 40-60 embryos.

> Refers to the percentage of immature embryos initiating germination (signified by radicle elongation) between 24 h and 48 h after imbibition in GM medium, as occurs in mature (60 DAP) embryos.
FIG 4: Turnover of MLG3 polypeptides during germination of W22 embryos.

Proteins were isolated from embryos of mature seeds after 24 h of imbibition (I), and at 1-day intervals following radicle emergence.

Mature seeds of Gaspe Flint and W22 were surface sterilized in 2.5% bleach, rinsed twice in deionized H$_2$O with Captan fungicide and imbibed for 24 h at 40$^\circ$ C. The kernels were planted in well-watered vermiculite, in the dark at 26$^\circ$ C for five days to allow the maturation phase accumulation of MLG3 to turn over. After this growth period, half of the germinated seedlings were transplanted to dry vermiculite and the remainder were returned to the fully hydrated condition. The seedlings were allowed to germinate for four extra days, harvested, measured, separated into shoots and roots, quick frozen in liquid N$_2$ and stored at -80$^\circ$ C. The western blot, FIG 5 shows the profile of MLG3 proteins of the two sets of roots and shoots.
Dehydrated seedlings, especially roots, accumulated the 29 kD and 24 kD polypeptides that were observed in dehydrating embryos, as well as lower molecular weight bands. None of these proteins were detected in well-watered roots and little 29 kD protein was observed in well-watered shoots. All the MLG3 polypeptides were shown to be heat stable by the criterion of Close et al. (1989), remaining soluble in water at 100° C for ten minutes.

3.4 IDENTITY OF MLG3 POLYPEPTIDES

My data indicate that the regulation and temporal pattern of MLG3 accumulation follows that of LEA proteins that have been found to be associated with embryo and plant dehydration in many species (Reid et al., 1988; White and Rivin, unpublished; Chandler et al., 1988; Dure et al., 1989). Dure et al. (1989) have divided the LEA proteins in three groups based upon cDNA homologies from a variety of plants. The ability of the different classes of LEA proteins to recognize maize embryo proteins on western blots was tested and these patterns were compared with that created by anti-MLG3 serum.

Group 1 identity was checked using a monospecific antibody to the Em protein of wheat. This serum reacted with a single maize embryo protein of less than 14 kD, the same size as in wheat embryo (Ralph Quatrano, personal communication). Group 2 LEA homology was tested using an antiserum raised to a barley dehydrin (DHN) synthetic peptide. Studies done by Vilardell showed that in maize, dehydrin is found as a 23-25 kD phosphorylated polypeptide (Vilardell et al., 1990), similar to the sizes of MLG3 proteins.
FIG 5: Western blot showing expression of MLG3 proteins in dehydrated seedlings.

Proteins were isolated from roots and shoots of 8-day old Gaspe Flint seeds that had been grown for 4 days in either dry (D) or well-watered (W) vermiculite. Equal amounts of these proteins were separated on SDS-PAGE, along with an equal amount of protein from mature dry embryos as a control. The gel was blotted and reacted with anti-MLG3 serum.

To test whether MLG3 and DHN are distinct families, embryo and endosperm proteins from mature maize and barley seeds were run on duplicate SDS-PAGE, blotted and reacted either anti-MLG3 serum, or anti-DHN synthetic peptide. The results are shown in FIG 6 MLG3 antiserum detects strong bands at 29 kD and 27 kD and a weak band at 24 kD in Gaspe Flint embryos. Embryo proteins from W22 show these bands, a strong 31 kD band, plus an additional weak band at about 45 kD. Both lines showed the same protein pattern in endosperm protein from barley seed, and with 23 kD and 31 kD. A duplicate blot reacted with anti-DHN strongly detects maize proteins of 24 kD in Gaspe Flint, 24 and 45 kD in W22.
FIG 6: Western blot of embryo and endosperm proteins of maize and barley reacted with anti-MLG3 and anti-DHN sera.

Embryo and endosperm (ENDO) proteins from mature maize seed and total barley seed proteins were separated by SDS-PAGE and electroblotted. Duplicate blots were reacted with anti-MLG3 and anti-DHN sera. G: Gaspe Flint, W: W22, H: Himalaya barley.

Anti-DHN does not detect 29 kD or 27 kD proteins of maize. In the barley sample, anti-DHN detects a strong band at 29 kD, along with several much weaker bands.

My data show that the 29/27 kD MLG3 and DHN are distinctive proteins. MLG3 antiserum does not detect barley dehydrin, although it does strongly react with another barley seed protein of 27 kD, and DHN antiserum does not detect the two major MLG3 bands. The cross reaction of anti-MLG3 and anti-DHN regarding the 24 kD and 45 kD polypeptides appears to be coincidental or may be due to an antigenic determinant shared by these proteins. LEA proteins are known to share conserved domains (Dure et al., 1989).
FIG 7: Western blot of embryo proteins of maize and wheat reacted with Group 3 LEA antiserum. Embryo proteins from mature maize and wheat seed were separated by SDS-PAGE, electroblotted, and reacted using antiserum to a wheat LEA group 3 fusion protein (see Materials and Methods). a, W22; b, Ky21; c, Ky21 x W22; d-g, F2 individuals of Ky21 X W22; h, wheat.

Group 3 LEA antibody derived from a fusion protein using the wheat cDNA (Curry et al., 1991; Reid and Walker-Simmons, 1991), strongly recognizes a 29 kD maize embryo protein, and reacted more weakly with the 24 kD protein. Proof that these were the same proteins detected by anti-MLG3 was obtained by showing that the wheat antibody detects the same 29 kD to 27 kD polymorphisms as anti-MLG3 in a western blot of F2’s from Ky21xW22 FIG 7. Abundant proteins of 29 kD and 27 kD were detected in the wheat seed sample, along with fainter bands.
3.5 FACTORS THAT REGULATE MLG3 ACCUMULATION

ABA and dehydration have been suggested to be important developmental factors in seed development, and both may be involved in modulating embryo proteins (Finkelstein and Crouch, 1986). The role played by ABA and dehydration in the accumulation of MLG3 polypeptides was investigated by comparing the proteins of wild-type embryos with those of vp mutants that are deficient in either ABA synthesis (vp5 homozygotes) or ABA response (vp1 homozygotes). The mutants and wild types were compared for the proteins that accumulate during development on the ear, and those that can be induced in embryo culture by exogenous ABA or a high osmoticum (20% sucrose).

Stage 2 (18 DAP) and stage 3 (23 DAP) embryos from vp5 segregating ears, vp1 homozygous and wild type (W22) ears were isolated under sterile conditions. While samples of mutants and wild type were frozen immediately after dissection, stage 2 embryo samples of each phenotype were also dissected and cultured for five days in a growth medium supplemented with either 10⁻⁵ M ABA or high osmoticum.

These treatments were chosen based on previous research (Rivin et al., 1988), as capable of blocking precocious germination of wild type and vp5 homozygous embryos, but not in vp1 homozygous embryos that germinate in the presence of ABA. It should also be mentioned that embryos of all genotypes germinate within a few days when cultured in growth medium alone. At least 40 embryos of each genotype and treatment sample were used to prepare protein extracts for western blot analysis.
Results presented in FIG 8 show that precocious accumulation of the Maize Group 3 LEA proteins detected by anti-MLG3 can be induced in wild-type embryos by either exogenous ABA or high osmoticum. Precocious accumulation of the 27 kD and 21 kD proteins was observed when the 18 DAP wild-type embryos were placed in culture with either ABA or sucrose. These proteins are characteristic of mature wild-type embryos of this stock. Also, ABA induces an intermediate size band that is not seen in mature embryos. Embryos cultured in unsupplemented growth medium germinated and failed to accumulate any of these proteins.

The contrasting behavior of the cultured vp5 homozygous embryos indicates that ABA, rather than dehydration, is specifically required for the accumulation of MLG3 polypeptides in isolated embryos. Stage 2 mutant embryos cultured with ABA accumulated the 27 kD and 21 kD MLG3. However, no accumulation of MLG3 was observed when embryos were cultured in high osmoticum, a non-lethal treatment that blocks precocious germination. Unlike their wild siblings, vp5 mutant embryos are unable to synthesize ABA in response to water deficit (Neill et al., 1987). These results suggest that ABA, provided exogenously or synthesized de novo in response to high osmoticum, is a specific requirement for MLG3 accumulation.
FIG 8: Induction of MLG3 accumulation by ABA and high osmoticum.

Wild-type and vp5 homozygous embryos were excised from segregating ears at 18 and 23 DAP. Proteins were isolated from 18 and 23 DAP freshly dissected embryos, and from 18 DAP embryos that were cultured for 5 days in a hormone free medium (GM), in GM + 10 µM ABA (ABA), or in GM + 20% sucrose (OS). Equal amounts of protein from each sample were run on SDS-PAGE and blotted. Detection was carried out using anti-MLG3 serum.
3.6 MLG3 REGULATION DOES NOT REQUIRE THE Vp1 GENE PRODUCT

Other ABA-regulated activities of the maize seed have been shown to require the participation of Vp1 gene, including suppression of precocious germination on the ear and the accumulation of the globulins storage proteins (Kriz et al., 1990; Rivin and Grudt., 1991; McCarthy et al., 1991).

The role of Vp1 in the accumulation of MLG3 proteins was tested in a similar experiment. As shown in FIG 9, the stage 2 (18 DAP) wild-type embryos that were frozen after dissection or cultured in growth medium gave no detectable signals. When cultured with ABA, the 29 kD, 27 kD and 24 kD proteins typical of W22 accumulated to levels comparable to those observed in the freshly dissected 60 DAP embryos. The 27 kD band was lacking from those embryos cultured in high osmoticum.

Comparisons of the vpl homozygous mutants with wild-type siblings or vp5 mutants revealed a different behavior. Culturing 18 DAP vpl homozygous in ABA resulted in precocious germination, and no MLG3 accumulation was observed. Supplementing the medium with high osmoticum prevented germination of vpl mutants at the same time that the 29 kD and 24 kD polypeptides accumulated.

Previous research, demonstrated that vpl homozygous embryos are not capable of initiating synthesis of storage proteins (Rivin and Grudt, 1991). For this reason, it was important to test if Vp1 was involved in the synthesis of MLG3. To confirm this idea that non-germinating vpl embryos are capable of MLG3 accumulation, vpl embryos that remained ungerminated on the ear were analyzed. The timing of vpl precocious germination is variable and late season pollinations frequently show more delayed germination.
FIG 9: Role of vpl in the induction of MLG3 accumulation.

Embryos were isolated from W22 ears at 18 and 23 DAP, and from vpl homozygous ears at 18 and 23 DAP. Proteins were isolated from freshly dissected embryos (18, 23, 60), and from 18 DAP embryos that were cultured for 5 days in a hormone free medium (GM), in GM + 10 uM ABA (ABA), or in GM + 20 % sucrose (OS). Equal amounts of protein from each sample were run on SDS-PAGE and blotted. The proteins were detected using anti-MLG3 serum.
FIG 10: Accumulation of MLG3 proteins in ungerminated vpl embryos, contrasts with the absence of globulins.

Proteins from ungerminated 35 DAP of vpl were compared on western blots with protein of 35 DAP and mature embryos of W22. Duplicate blots were reacted with anti-GLB1 and anti-MLG3 sera. Top of figure shows accumulation of globulins and bottom shows accumulation of MLG3 proteins.

Results presented in FIG 10 compare the protein profiles of mature and 35 DAP (stage 5) wild type embryos with those of ungerminated 35 DAP (stage 5) vpl embryos. The sibling vpl embryos on these ears went on to precociously germinate. MLG3 polypeptides accumulate in 35 DAP vpl embryos in amounts comparable to wild type, although the 31 kD polypeptide is more prominent. No accumulation of GLB1 was detected in the vpl mutant. These data, supports the idea that suppression of precocious germination or dehydration is a prerequisite for MLG3 accumulation, whereas VP1 is not directly required to accumulate these polypeptides.
4. DISCUSSION and CONCLUSIONS

The protein synthesis pattern of the maize embryo has been shown to change drastically during seed development. Three sets of expressed polypeptides have been characterized as following: an embryonic set with seven members expressed in young (20 DAP) and in mature (50 DAP) embryos, a maturation set with 44 members present in embryos from 31 DAP to 50 DAP and a germination set with 13 members appearing during the first hours of germination (Sanchez-Martinez et al., 1986). Also the mRNAs corresponding to these polypeptides have been found stored in the dry embryos, although the majority of them disappear quickly after imbibition or germination (reviewed in Kermode, 1990).

To study proteins that accumulate late in embryo development, antibodies were raised to a 29 kD protein that is prominent in mature maize embryos of W22. The antiserum detects a Group 3 LEA protein family, named MLG3 (Maize LEA Group 3). The size of the major MLG3 proteins varies slightly between different inbred lines, and this can be explained by the presence of different alleles due to a single gene or a closely linked gene complex. The MLG3 proteins have characteristics of the LEA proteins reported in other species (Dure et al., 1989). They increase during embryogenesis before the seed begins to dehydrate and prior to the acquisition of desiccation tolerance and they remain soluble in water at high temperatures. A gradual decline in abundance is observed during germination, but high levels accumulate when seedlings are grown under conditions of water stress.

The early work on LEAs was done by Dure et al. (1981) by analyzing classes of mRNA appearing in late development in cotton. Further studies showed that these mRNAs coded for very unusual polypeptides: they are highly hydrophilic; no tryptophan or cysteine is present in them; and they seem to
form amphiphilic helical structures. Based on these unusual characteristics a possible role might be to alleviate the physiological problems encountered in the plant cytosol during desiccation (Baker et al., 1988).

The possibility that MLG3 is regulated by ABA was investigated, because several lines of evidence point to a key role for ABA in modulating gene expression of some of the LEA proteins in plant tissues undergoing water stress:

1- Increasing the levels of ABA either by endogenous production or by exogenous application, resulted in an increase in the levels of LEA mRNA’s in cereals. For example, in shoots and roots of dehydrated barley and corn these mRNA are very conspicuous, while the transcripts are absent in the well watered controls (Close et al., 1989). A parallel situation can be found in rice embryos, which accumulate RAB-21 mRNA by the addition of ABA or under water stress conditions (Mundy and Chua, 1988). White and Rivin have shown that the expression of MLG3 mRNA is induced by ABA and osmotic stress in maize (unpublished results).

2- Blocking ABA synthesis genetically or with inhibitors prevents LEA accumulation during water stress: These polypeptides can be induced by ABA in wild type as well as in viviparous embryos, but fail to accumulate when leaves of these mutants are subjected to water stress (Pla et al., 1989).

3- Exogenous application of ABA stimulates accumulation in the absence of water stress: Young embryos of maize accumulate the 23 kD and 25 kD proteins in the presence of ABA (Pla et al., 1989). Similar of results have been reported for rice (Mundy and Chua, 1988), cotton (Dure et al., 1989) and wheat (Morris et al., 1991).

During this investigation it was found that ABA supplied exogenously to immature embryos does stimulate the accumulation of MLG3, but from this result alone it can not be concluded that the hormone regulates the appearance of these proteins during normal development. For example,
cotton embryos exposed to exogenous ABA induce the expression of the LEA mRNAs, but in vivo only a subset of these genes appears to be ABA regulated. Moreover, Galau et al. (1989) favor the idea that LEA transcript accumulation could be regulated by factors associated with low embryo water potential or the cessation of the vascular flow from the mother plant. One possibility is that exogenous ABA is responsible for altering the water relations of the embryo. It has been very difficult to elucidate the roles played by ABA and restricted water uptake, because ABA may change water relations and drought stress may lead to an increase in ABA levels (Zeevart and Creelman, 1988; Schopfer and Plachy, 1985).

Indication of a specific ABA requirement for MLG3 accumulation in embryos was obtained by comparing the response of wild type and vp5 embryos to exogenous ABA or high osmoticum. The data shows that ABA was effective in inducing these proteins in vp5 mutant embryos, but high osmoticum was not. Neill et al. (1987) presented evidence that wild type maize embryos cultured in high osmoticum showed a small increase in ABA level.

Because the vp5 embryos are blocked in ABA synthesis, I interpret my results to mean that exogenous ABA, or ABA synthesized de novo in response to water limitation, can stimulate MLG3 accumulation, but water stress in the absence of ABA is not a sufficient inducer. No MLG3 proteins were detected in young vp mutant embryos developing on the ear, suggesting that ABA is directly or indirectly involved in the developmental accumulation of these polypeptides. However, because the germination of vp5 embryos occurs in early stage 4, it was not possible to compare wild-type and mutant embryos at times when MLG3 was very abundant.

The accumulation patterns of MLG3 polypeptides are in interesting contrast to another abundant ABA-regulated embryo protein, GLB1. This storage protein begins to accumulate much earlier in development (approximately 20 DAP)
and peaks in abundance 30 DAP to 35 DAP (Kriz, 1989; Rivin and Grudt, 1991; Kriz et al., 1990). The idea of ABA modulation for GLB1 is an attractive one mainly because its synthesis initiation is correlated with the sharp increase in maize embryo ABA that takes place around 16-18 DAP (Neill et al., 1987). The most interesting difference between GLB1 and the MLG3 polypeptides is that the \( Vp1 \) gene product is a specific requirement for GLB1, but not for the others. Kriz et al. (1990) were able to show that no protein or mRNA could be found in \( vpl \) mutants \textit{in vivo}. Comparison of proteins profiles from precocious germinating embryo mutants with those of normally germinating embryos revealed substantial differences. The GLB1 protein and its precursors were barely detected in \( vpl \) mutants and present in reduced amounts in \( vp5 \) embryos. To extend this observation further, a cDNA clone corresponding to GLB1 was used as probe in Northern blot analysis of embryo RNA. No transcript was detected for \( vpl \) embryos, while \( vp5 \) embryos showed a slight reduction compared to wild type.

The evidence presented here shows that homozygous \( vpl \) embryos that remain ungerminated on the ear or are blocked from germination in culture by high osmoticum do accumulate MLG3. This result was unexpected because \( Vpl \) controls multiple developmental responses associated with the maturation phase of seed development (Robertson, 1955; Dooner, 1985; Rivin and Grudt, 1991; McCarthy et al., 1991).

More recent studies show that \( Vpl \) encodes a novel protein that may function as a transcriptional activator for the expression of Group 1 LEA proteins in maize. The expression of a functional protein in response to ABA can be regulated at many different levels, as has been demonstrated for the Em gene in wheat (Williamson and Quatrano, 1988; Berge et al., 1989). The Em-promoter fragment fused to the bacterial glucuronidase (GUS) gene was used as a reporter gene in electroporated maize protoplasts. \( Vpl \) protein was able to transactivate the
presumptive target gene in maize, providing additional functional evidence that VPI is a transcription factor (McCarthy et al., 1991). My conclusion is that ABA alone is sufficient to modulate accumulation of Group 3 LEAS in maize, whereas other proteins require ABA and VPI to act in concert. Finally, another scenario for how VPI might be involved in ABA-regulated gene expression is that some other activating factor may be important in late maturation, when the level of VPI expression declines (McCarthy et al., 1991).
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