Experiments were performed to assess regulatory factors governing maize embryo maturation and vivipary. Both visual and molecular markers of embryo development were used to examine the roles of the hormones abscisic acid (ABA) and gibberellins (GAs), as well as water stress in governing transit from early embryogeny to maturation-phase development. A differential screen identified cDNAs whose expression is impaired in maize viviparous mutants which fail to undergo maturation and instead precociously germinate. The cDNAs isolated in this screen absolutely required both ABA and the Viviparous1 (Vp1) gene product for expression both in vivo and in vitro. Two novel clones were isolated: a maize homologue of the wheat metallothionein gene Ec and a second clone which may encode a novel seed storage protein of maize. In a separate screen, a maize cDNA encoding a Lea group 3 protein was isolated. Like many maturation-associated genes, maize Lea 3 was shown to ABA-inducible but is also expressed in response to water stress in the absence of ABA or the Vp 1 gene.

We examined whether gibberellins might also be a factor modulating precocious germination. Gibberellin inhibitors applied to cultured wildtype embryos suppressed precocious germination and enhanced anthocyanin accumulation in a
developmentally specific manner. These behaviors mimicked the effect of ABA and they were reversed by the addition of exogenous GA$_3$. Vivipary in vivo resulting from diminished ABA levels could be suppressed by either chemical or genetic reduction of GA levels in immature kernels and resulted in desiccation-tolerant seed. In contrast, reduction of endogenous gibberellins did not suppress vivipary of the ABA-insensitive mutant vp1. Temporal analysis of gibberellin accumulation in developing kernels revealed the accumulation of two bioactive species (GA$_1$ and GA$_3$) during a developmental window just prior to peak ABA levels. It is suggested that these species stimulate a developmental program leading to vivipary in the absence of sufficient levels of ABA and that reduction of GA levels reestablishes a hormone balance appropriate for suppression of germination and induction of maturation in ABA-deficient kernels. The failure to suppress vivipary via reduction of GA levels in the ABA-insensitive mutant vp1 suggests that the wildtype gene product functions downstream of the sites of GA and ABA action in regulation of maturation versus germination.
Molecular and Physiological Aspects of Maize Embryo Maturation

by

Constance N. White

A THESIS

submitted to

Oregon State University

In partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

Completed January 13, 1995
Commencement June 1995
I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.
ACKNOWLEDGMENTS

I am grateful to a long list of people for their aid and encouragement during the last eight years (and before). My father, for instilling in me a love of science. My mother, for making me love words and the people who use them well. Uncle John for stepping in whenever and wherever needed. Carol, for giving me a scientific home and offering me resources and encouragement (albeit always with one eyebrow slightly raised). The members of my committee, Drs. Tom Wolpert, Terri Lomax, Dallice Mills, Michael Burke, who have variously offered very useful scientific insights, excitement, and instruction in meiosis. A very long list of colleagues, both here and at the University of Oregon, who taught me science by excellent example. My peers and professors at Oregon State College of Veterinary Medicine who have provided me with a wonderful new home. And Patty.
CONTRIBUTION OF AUTHORS

Dr. Carol Rivin provided both intellectual and technical assistance in the design and execution of each set of experiments. All manuscripts herein were written by the first author but heavily edited by Dr. Rivin. The author is grateful to Alan Kriz for use of his cDNA library and to M.K. Walker-Simmons for the gift of the wheat Lea 3 probe. Malin Masreliez provided valuable aid in sequence analysis. David Stein assisted in many of the cloning experiments. Dr. Bill Proebsting and Fawn Tran assisted with preparation of plant materials for hormone analysis. Dr. Peter Hedden and members of his laboratory undertook the hormone quantitation and identification assays.
Maize embryo maturation: an overview .................................. 1
LITERATURE CITED ......................................................... 14

Cloning and characterization of cDNAs of maturing maize embryos ...... 19
ABSTRACT ........................................................................ 20
INTRODUCTION ................................................................ 21
MATERIALS AND METHODS ............................................. 23
RESULTS .......................................................................... 27
DISCUSSION ..................................................................... 45
LITERATURE CITED ............................................................ 55

Sequence and expression of the message encoding a Lea Group 3
protein of maize (Zea mays L.) ................................................ 59
ABSTRACT ........................................................................ 60
INTRODUCTION ................................................................ 60
MATERIALS AND METHODS ............................................. 62
RESULTS AND DISCUSSION ............................................. 63
LITERATURE CITED ............................................................ 78

Evidence for a role for gibberellins in the precocious
germination of maize (Zea mays L.) embryos ......................... 81
ABSTRACT ........................................................................ 82
INTRODUCTION ................................................................ 83
MATERIALS AND METHODS ............................................. 86
RESULTS .......................................................................... 91
DISCUSSION ..................................................................... 103
LITERATURE CITED ............................................................ 107
Effects of altered gibberellin metabolism on viviparous maize kernels developing on the ear: correlations of endogenous gibberellin levels with precocious germination in vitro and in vivo: 110

ABSTRACT ................................................................. 111
INTRODUCTION .......................................................... 112
MATERIALS AND METHODS .......................................... 114
RESULTS ................................................................. 116
DISCUSSION ............................................................ 133
LITERATURE CITED .................................................. 137

Physiological and molecular aspects of maize embryo maturation revisited:
A model for the ontogeny and regulation of seed maturation: 139
LITERATURE CITED .................................................. 147

BIBLIOGRAPHY .......................................................... 148
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>II.1</td>
<td>Accumulation of 5 maturation-associated mRNAs in developing wild type embryos</td>
</tr>
<tr>
<td>II.2A</td>
<td>Expression of maturation messages in stage 4 (28-30 DAP) wildtype and <em>viviparous</em> embryos</td>
</tr>
<tr>
<td>II.2B</td>
<td>Expression of maturation messages in wildtype and <em>viviparous</em> embryos assayed by RNA slot blot</td>
</tr>
<tr>
<td>II.3</td>
<td>Expression of Globulin 2 in embryos carrying the <em>vp1mc</em> allele</td>
</tr>
<tr>
<td>II.4</td>
<td>Globulin 2 message levels in embryos with mutations in anthocyanin regulatory genes</td>
</tr>
<tr>
<td>II.5</td>
<td>Maturation message levels in different plant tissues</td>
</tr>
<tr>
<td>II.6</td>
<td>Expression of maturation messages in cultured immature embryos</td>
</tr>
<tr>
<td>II.7A</td>
<td>Sequence of pM44 insert</td>
</tr>
<tr>
<td>II.7B</td>
<td>Alignment of maize putative pM44 translation product with polypeptide encoded by wheat Ec cDNA</td>
</tr>
<tr>
<td>II.8</td>
<td>Southern blot of W22 genomic DNA probed with pM44</td>
</tr>
<tr>
<td>II.9</td>
<td>Sequence of pM69</td>
</tr>
</tbody>
</table>
LIST OF FIGURES (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>II.10A</td>
<td>Alignment of clone pM69 with <em>Pisum sativum</em> cvcA gene for convicilin polypeptide (PEACVCA)</td>
<td>50</td>
</tr>
<tr>
<td>II.10B</td>
<td>Alignment of clone pM69 with <em>G. hirsutum</em> (cotton) storage protein (beta-globulin = COTSPB)</td>
<td>51</td>
</tr>
<tr>
<td>II.10C</td>
<td>Alignment of clone pM69 with Maize embryo globulin S allele (7S-like) cDNA (MZEGLB1SA)</td>
<td>52</td>
</tr>
<tr>
<td>II.10D</td>
<td>Alignment of clone pM69 with Barley embryo globulin (BEG1) cDNA (BLYBEG1)</td>
<td>53</td>
</tr>
<tr>
<td>II.10E</td>
<td>Alignment of clone pM69 with <em>Pisum sativum</em> legJ gene for minor legumin (PEALEGJ)</td>
<td>54</td>
</tr>
<tr>
<td>III.1</td>
<td>Nucleotide sequence of pMLg3</td>
<td>65</td>
</tr>
<tr>
<td>III.2</td>
<td>Alignment of maize MLG3 with Lea 3 proteins from wheat and barley</td>
<td>66</td>
</tr>
<tr>
<td>III.3</td>
<td>Southern blot of maize genomic DNA (W22 inbred) probed with pMLg3</td>
<td>68</td>
</tr>
<tr>
<td>III.4</td>
<td>Accumulation of MLG3 polypeptide during wildtype embryo development</td>
<td>71</td>
</tr>
<tr>
<td>III.5</td>
<td>Northern analysis of maize tissues probed with pMLg3</td>
<td>72</td>
</tr>
<tr>
<td>III.6</td>
<td>Accumulation of maize Lea 3 protein and mRNA in cultured embryos</td>
<td>74</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>III.7</td>
<td>Endogenous protease activity in cultured embryos</td>
<td>76</td>
</tr>
<tr>
<td>IV.1</td>
<td>Germination of wildtype embryos isolated at successive developmental stages when cultured in the presence and absence of growth regulators and inhibitors</td>
<td>90</td>
</tr>
<tr>
<td>IV.2</td>
<td>Effects of exogenous GA$_3$ on the germination of paclobutrazol-treated embryos</td>
<td>91</td>
</tr>
<tr>
<td>IV.3</td>
<td>Effects of exogenous GA$_3$ on the germination of wildtype embryos in culture</td>
<td>92</td>
</tr>
<tr>
<td>IV.4</td>
<td>Germination of <em>viviparous</em> mutant embryos in culture</td>
<td>95</td>
</tr>
<tr>
<td>IV.5</td>
<td>Germination of cultured embryos carrying <em>vp1</em> (viviparous) and <em>vp1-mcwhirter</em> (dormant) alleles</td>
<td>96</td>
</tr>
<tr>
<td>IV.6</td>
<td>Effects of developmental stage and growth regulators on anthocyanin accumulation</td>
<td>100</td>
</tr>
<tr>
<td>IV.7</td>
<td>Anthocyanin accumulation in cultured embryos</td>
<td>101</td>
</tr>
<tr>
<td>IV.8</td>
<td>Anthocyanin accumulation in cultured embryos requires the <em>R-sc</em> allele</td>
<td>102</td>
</tr>
<tr>
<td>V.1</td>
<td>Hypothetical GA biosynthetic pathway for maize</td>
<td>117</td>
</tr>
<tr>
<td>V.2</td>
<td>Suppression of fluridone-induced vivipary by treatment</td>
<td>120</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>V.3</td>
<td>Suppression of vp5-induced vivipary by treatment with GA biosynthesis inhibitors</td>
<td>121</td>
</tr>
<tr>
<td>V.4</td>
<td>Restoration of vivipary to vp5 inhibitor-treated kernels with GA₃ treatment</td>
<td>122</td>
</tr>
<tr>
<td>V.5</td>
<td>vp5 segregants from d1 ear</td>
<td>125</td>
</tr>
<tr>
<td>V.6A</td>
<td>Germinating vp5 d1 double mutant kernels</td>
<td>127</td>
</tr>
<tr>
<td>V.6B</td>
<td>Germinating ancymidol-treated vp5 kernels</td>
<td>128</td>
</tr>
<tr>
<td>V.6C</td>
<td>Germinating paclobutrazol-treated vp5 kernels</td>
<td>129</td>
</tr>
<tr>
<td>V.7</td>
<td>GA and ABA levels in developing wildtype W22 embryos</td>
<td>131</td>
</tr>
<tr>
<td>VI.1</td>
<td>Schematic of ABA, Vp1, and GA function in modulating seed development</td>
<td>143</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>II. 1</td>
<td>Estimated sizes of maturation-associated transcripts</td>
</tr>
<tr>
<td>V.1</td>
<td>vivipary of <em>vp1</em>/<em>d1</em> mutants</td>
</tr>
<tr>
<td>V.2</td>
<td>GC-MS quantitation of gibberellins and abscisic acid in developing W22 kernels</td>
</tr>
</tbody>
</table>
Chapter One

Maize embryo maturation: An overview
Introduction

Angiosperm embryo development appears to be separated into two sequential developmental programs: an initial phase characterized by rapid growth and histodifferentiation is followed by a maturation period in which storage macromolecules are vigorously synthesized and desiccation tolerance is acquired [reviewed by Kermode, 1990]. In addition to these processes, mature embryos also undergo the phenomenon of developmental arrest. The severity of this arrest may range from mere quiescence, in which any conditions favorable to germination will result in growth, to true dormancy which may require special conditions, such as vernalization or light, in order for germination to occur. Regulation of seed dormancy is of particular agronomic importance since a variety of pernicious weed species may exist as dormant seed banks which pose problems for eradication. In addition, the problem of preharvest sprouting remains a considerable source of economic loss to cereal producers.

Seed maturation is an essential component of angiosperm terrestrial adaptation. Two key features of maturation, the accumulation of storage reserves and the acquisition of desiccation tolerance are important factors in allowing embryos to tolerate dry conditions and to initiate growth and development prior to initiation of significant photosynthetic energy transformations. The desiccation tolerance of seeds is unique. Dry seeds from a variety of species may contain as little as 10 percent water as a percent of fresh weight. How cellular integrity is maintained under such conditions is a matter of intense scrutiny. It has been suggested that both the accumulation of storage reserves and the increased synthesis of hydrophilic macromolecules preserves cellular solvation, by either binding or replacing water [Baker et al., 1988; Kermode, 1990; Skriver and Mundy, 1990].
A role for ABA in regulating seed maturation

It is nearly axiomatic that processes leading to mature seed must be highly regulated and conserved in order that such an essential program is initiated and maintained correctly. The elucidation of such regulation poses a variety of problems. Two approaches have had wide currency: The first involves the explantation of embryos into tissue culture and manipulation of culture conditions to induce explants to undergo maturation processes; the second method involves the use of mutants which fail to undergo maturation in vivo. Both of these strategies have uncovered a vital role for the hormone abscisic acid (ABA) in inducing maturation-associated events. In experiments using cultured embryos from rape, cotton, wheat, and maize (as well as a number of other species) exogenous ABA can both suppress germination and induce precocious expression of a number of maturation-associated genes [Robichaud et al., 1980; Triplett and Quatrano, 1982; Hendrix and Radin, 1984; Ackerson, 1984; Finkelstein et al., 1985; Eisenberg and Mascarenhas, 1985; for reviews see Quatrano, 1987; Zeevart and Creelman, 1988]. In addition, a number of ABA-deficient mutants which perturb embryo maturation have been isolated from maize (vp2, vp5, vp7, vp8, vp9) and Arabidopsis (aba 3) [Robertson, 1955; Koornneef, 1982]. These mutants all appear to reduce both ABA and carotenoid levels due to blocks in their common synthesis pathway [Robichaud et al., 1980; Neill et al., 1986; Rock and Zeevaart, 1991]. Vivipary may also be induced in wildtype maize kernels by application of fluridone, an inhibitor of carotenoid biosynthesis [Fong et al., 1983].

Although ABA appears critical for suppression of germination and the induction of maturation processes, little else is known about the regulation of these two discrete developmental pathways. What transduces the ABA signal into initiation
of maturation processes? What underlying developmental program initiates germination in *viviparous* mutants and is it regulated? The answer to the first question is, as yet, poorly understood although ABA-response mutants have provided some clues. The second question is not commonly posed since vivipary is tacitly assumed to reflect a default state yet clearly ABA must act to suppress genes underlying a germination pathway. Because of its developmental importance, this germination pathway must have some endogenous regulation of its own.

**cis- and trans- acting factors in ABA signal transduction**

ABA appears to act, in part, by regulating transcription. A number of Late embryogenesis abundant (Lea) genes are up-regulated by ABA in *in vitro* assays [reviewed by Kermode, 1990]. In addition, transcription from barley amylase genes is suppressed in the presence of ABA. A promoter element common to both types of genes, those turned on by ABA and those turned off, has been identified and shown to confer ABA-responsiveness in heterologous promoter function assays [reviewed by Ho and Hagen, 1993]. The ABA response element (ABRE) consists of a highly conserved G-box core CACGTG. G-box sequences have been found in the promoters of many plant genes and are known to respond to divergent environmental and hormonal signals [Williams et al. and citations therein, 1992]. Several different G-box binding activities have been identified in plant extracts and those characterized thus far belong to the bZip class of DNA binding proteins [Guiltinan et al., 1990; Mundy et al., 1990; Nelson et al., 1994]. A protein (EmBP) which specifically binds ABRE sequences has been isolated from wheat and like other G-box binding proteins, its binding is constitutive and cannot be directly related to ABA regulation of its target gene [Guiltinan et al., 1990].
ABA response mutants specifically affecting seed processes have been isolated from both maize (vp1) and Arabidopsis (abi 3). Maize vp1 mutants were first described by Robertson [1955]. Unlike the other vp mutants of maize which are deficient in carotenoids but competent in anthocyanin synthesis, vp1 synthesizes carotenoids but is impaired in kernel anthocyanin production. These observations place vp1 in a separate class. Subsequent work with cultured vp1 embryos has shown that, unlike the other viviparous maize mutants, their growth in culture is unimpaired by the presence of exogenous ABA [Robichaud et al., 1980]. Thus it appears that vp1 vivipary reflects a lesion in ABA response rather than production. This is corroborated by the finding of normal ABA levels in vp1 kernels and seedlings [Neill et al., 1986]. The maize Vp1 gene was cloned via transposon tagging [McCarty et al., 1989]. More recently the ABI3 gene of Arabidopsis has been isolated and shown to share amino acid sequence homology to the VP1 protein in several domains [Giraudat et al., 1992].

VP1 protein has been suggested to act as a transcriptional activator. Experiments using electroporated protoplasts show that wildtype VP1 protein can transactivate a promoter containing abscisic acid response elements (ABREs) in a manner synergistic with increasing exogenous ABA [McCarty et al., 1991]. While the VP1 protein does not contain any domains previously identified as transcription factor motifs, and it cannot be shown to interact with promoter sequences, McCarty [1991] has proposed that VP1 is a member of a new class of transcription factors which responds to ABA in a manner reminiscent of steroid hormone transcription factors.

The possibility exists however that VP1 itself is not a DNA binding protein per se and may exert its effect by enzymatic modification of transcription machinery.
Alternatively, VP1 may not bind DNA directly but may participate in the transcription complex via protein-protein interactions. Recent experiments indicate that ABRE sequences are necessary but may not be sufficient for ABA-induced transcription [Nelson et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994]. One interpretation of these results is that bZip proteins form a core transcription complex which functions to anchor additional proteins that actually participate in the signal transduction chain. This possibility is particularly intriguing in light of the fact that the ABRE sequence elements are separable by a small number of nucleotides from the VP1-responsive region of a promoter known to require both ABA and VP1 for transcriptional activation [Hattori et al., 1992]. If this is the case, VP1 may later be shown to interact with EmBP in some manner.

Another possible link in the ABA pathway is an ABA-inducible protein kinase homologue that has recently been isolated from wheat [Anderberg and Walker-Simmons, 1992]. Whether the product of this gene has bona fide kinase function and whether it acts in a structural or regulatory capacity is unknown.

Is maturation strictly controlled by ABA alone?

Despite the isolation of Vp1, the relative roles of ABA and changing embryo water relations play in initiating and maintaining maturation remain a source of confusion. Several suggestions exist in the literature that ABA acts in part to stimulate desiccation which in turn acts as a proximal regulator of maturation [Schopfer and Plachy, 1984; 1985; Finklestein and Crouch, 1986; Fischer et al., 1987; Morris et al. 1988; Kermode, 1990]. This view was originally supported by the observation that with embryos isolated from a variety of species, culture under conditions of osmotic stress could induce a number of maturation markers also
induced by treatment with ABA alone. Attempts to measure ABA levels under such culture conditions have yielded conflicting results, with some groups reporting no net ABA increase [Finklestein and Crouch, 1986; Morris et al. 1988; Hughes and Galau, 1991] and other groups reporting elevated ABA synthesis in response to osmotic stress [Bray and Beachy, 1985; Morris et al. 1988; Wilen et al., 1990] This latter observation is consistent with the role ABA plays in water stress of vegetative tissues: rising ABA levels in response to water stress play a crucial role in initiating stomatal closure [for a review see Walton, 1980; for more recent work see Harris and Outlaw, 1991 and citations therein]. Confusion regarding the role of ABA in regulating embryo water relationships has been complicated by the various experimental systems and developmental ages of embryos used. An additional confusion is the fact that some studies [Bray and Beachy, 1985; Finklestein and Crouch, 1986] used ELISA detection systems for ABA quantitation with an antibody subsequently to be shown to have poor affinity for the naturally occurring (+) enantiomer. This antibody appears to underestimate endogenous ABA levels by a factor of 200 fold when used with racemic internal controls [Raikhel et al., 1987].

Attempts to correlate the kinetics of maturation gene expression with ABA levels in vivo have yielded additional problems with analysis. In cotton, accumulation of some Late embryogenesis abundant (Lea) mRNAs occurs much later than the period of highest endogenous embryo ABA levels yet these genes depend on exogenous ABA for maximal expression in cultured embryos [Galau et al., 1986; Galau et al. 1987; Hughes and Galau, 1989]. Likewise, the accumulation of napin mRNA precedes peak ABA levels in developing rapeseed embryos although the method of hormone quantitation in that study is in doubt [Finklestein et al., 1985]. Although it could be that sensitivity to ABA is more important than absolute levels in
regulating embryo responses [Trewavas, 1981] it has been argued that sensitivity to ABA declines during embryo maturation and thus cannot account for kinetic discrepancies between ABA levels and expression of putative target genes [Galau et al., 1987]. However, the parameters by which sensitivity is assayed usually rely on either suppression of germination or measurement of steady state mRNA levels, both of which presumably result from interaction of a number of regulatory factors operating at various levels.

An additional factor that complicates the use of some Lea genes as markers for ABA regulation of seed-specific processes is their response to water stress in vegetative tissues. Unlike most seed storage proteins, some Lea genes also are up-regulated in dehydrating plant tissue, probably in response to increased ABA and are known also as the RAB genes [responsive to ABA, for a review see Skriver and Mundy, 1990]. Thus the regulation of Lea expression in cultured embryos may not be indicative of their normal regulation during seed development but of a generalized stress response. The fact that excised embryos can sometimes simultaneously express germination-associated genes and Lea genes has suggested to some that switching between developmental programs is not a binary decision [Kermode, 1990] but has suggested to others that cultured embryos may simply be behaving as stressed seedlings [Hughes and Galau, 1991; Jakobsen et al., 1994].

**What programs vivipary?**

Much of the work on embryo maturation has focused on the role of ABA to the exclusion of assessing the roles of other plant growth regulators. Although it has long been known that the gibberellins (GAs) are potent inducers of many germination-associated processes [for a review, see Jacobsen and Chandler, 1987],
little attention has been paid to the role of GAs in control of vivipary. Since Paleg's [1960] discovery that GA could substitute for diffusible embryo substances in mobilizing endosperm reserves, the role of GA in inducing various hydrolytic enzyme activities in aleurone tissue has become a model system for hormone action [for reviews see Jacobsen, 1987; Fincher, 1989; Ho and Hagen, 1993]. GA is known to induce the activities of cysteine proteases, amylases, lipases, phosphatases, and nucleases in barley and appears to function in a number cases by up-regulating transcription from these genes.

The role that GA plays in modulating germination per se is less clear than its role in endosperm mobilization. A number of GA-deficient dwarfs of different species germinate with approximately wildtype efficiency [reviewed by Reid, 1986]. However, several GA-deficient mutants of both Arabidopsis and tomato are impaired in this process [Koornneef and Van der Veen 1980; Koornneef et al., 1981]. In addition, dormancy in *Avena fatua* is correlated with lowered GA levels and can be broken only either by after-ripening or by treatment with GA [reviewed by Jones, 1973]. Moreover, phytochrome-mediated photocontrol of lettuce seed germination appears to operate by increasing endogenous GA levels, as does light-induction of germination in Arabidopsis [Derks and Karssen, 1993; Toyomasu et al., 1993]. Because of the absence of a clear GA requirement (as defined by mutants) for germination in many species, one might conclude these cases are more the exception rather than the rule in the control of germination. However, even those mutants with impaired germination have "leaky" alleles in which germination competence is intact but internode elongation is still significantly impaired. It is suggested that GA requirements for germination are much less than for vegetative processes or, alternatively, that the GA species active in germination differ from those important for
growth functions [Koornneef and van der Veen, 1980]. If either of these possibilities is true then only severe alleles of GA deficient mutants might be expected to have impaired germination capacity in addition to dwarfed growth habit, and these are likely to have been missed in mutant screens.

Although GA clearly has a role in seed germination processes its role in seed development is not well defined. Seeds and fruits are rich sources of a variety of gibberellins with a general positive correlation with seed growth and rising GA levels. However, manipulation of endogenous GA concentrations by either biochemical or genetic means has not completely elucidated a role for GA.[for review see Pharis and King, 1985; Swain et al. and citations therein, 1993, ]. Treatment of developing seeds of Pharbitis nil or Pisum sativum with GA biosynthesis inhibitors after early embryogeny has no effect on seed development [Zeevaart, 1966; Garcia-Martinez et al., 1987]. However, treatment at earlier time periods causes increased abortion. In parallel with these results, experiments using cultured Phaseolus coccineus embryos have indicated a clear requirement for suspensor-derived gibberellins in modulating early embryogenesis [Cionini et al., 1976; Yeung and Sussex, 1979]. One allele of a dwarf pea mutant with an early block in GA synthesis (lh1) has been shown to cause increased seed abortion but a number of other GA-deficient mutants in this and other species (including other dwarf alleles of this gene) cause very little alteration in seed set or size [Barendse et al., 1986; Santes et al., 1993; Swain et al., 1993]. One interpretation of these data is that, as for germination, GA is required for early seed development but its requirement is minute and unmasked only by severe biosynthetic impairment.

Interestingly, ABA is a strong antagonist of many GA regulated functions. Although GA levels have been implicated in preharvest sprouting of wheat, so too
have ABA levels and/or sensitivity [Walker-Simmons, 1987]. Likewise, treatment with ABA can suppress germination of photoblastic lettuce [Kahn, 1968; Sankhla and Sankhla, 1968]. In fact, Koorneef [1982] used a selection for revertants of non-germinating GA mutants to isolate a second set of Arabidopsis mutants which were subsequently shown to be deficient in ABA (aba). In maize, simultaneous incubation of germinating seeds with fluridone and GA3 enhances the GA stimulation of shoot elongation [Devlin et al., 1980]. The antagonism of GA action by ABA extends to the control of gene expression. GA-induced expression of a number of genes may be squelched by the addition of physiological concentrations of ABA [reviewed by Ho and Hagen, 1993]. The promotor regions of barley amylase contain ABRE sequences and it is thought that ABA suppression acts via these elements [Rogers and Rogers, 1992]. Although GA's role in regulating maize amylases is not clear, it is noteworthy that amylase activity is elevated in vp1 embryos [Wilson and Rhodes, 1973].

A model of seed germination and dormancy first proposed by Khan [1968; 1975] assigns primary, preventive, and permissive roles to gibberellins, abscisic acid, and cytokinins respectively. In this model, germination control results from a matrix of interactions of the three hormones and was first formulated to account for anomalous cases where germination occurred irrespective of inhibitors, and dormancy occurred even in the presence of high gibberellin levels. Although cytokinins have faded from view in germination literature, there is clear molecular support for the idea that some germination processes are controlled by a balance of GA and ABA. However, all such work has been done with mature seed, where the developmental decision is to germinate or not to germinate. In developing embryos, the apparent decision is whether to germinate or to mature. Clearly ABA plays a role
in maturation, but in viviparous embryos, does GA play a role in precocious germination?

This thesis concerns experiments aimed at uncovering more clues to the regulation of maturation processes in maize kernels. The experiments comprised two approaches: first, the isolation of a more extensive set of maturation-associated genes and the characterization of their regulation both in vivo and in vitro, and second, experiments designed to elucidate unexamined hormonal aspects of germination physiology both with cultured embryos and kernels developing in situ. By using maize mutants defective in ABA synthesis it is possible to experimentally uncouple the effects of lowered water potential from de novo ABA synthesis in cultured embryos and ask whether ABA or osmotic stress controls expression of maize maturation genes. To do so, a set of maturation-associated cDNAs was isolated and their expression under various culture conditions and in vivo was examined in both wildtype embryos and in viviparous mutants. Several of the cDNAs isolated represented novel clones from maize, and sequence and tissue-specific expression characteristics were also examined. In addition, a clone corresponding to a maize Lea polypeptide (Lea 3) not isolated in the initial screen was identified by a combination of genetic and molecular approaches which utilized a homologous wheat gene. This gene and its product are unique in that their expression is ABA-inducible but may also be directly controlled by water stress.

The second approach used genetic and chemical means both in vitro and in vivo, to ask whether gibberellins promote vivipary. The results of these experiments indicate a role for gibberellins in modulating precocious germination and indicate epistasis with Vp1. They suggest that gibberellins act prior to Vp1 in modulating the
decision to germinate or to mature, either by acting on ABA levels directly or via the signal transduction chain that lies between ABA and Vp1.
LITERATURE CITED


Anderberg RJ, Walker-Simmons MK (1992) Isolation of a wheat cDNA clone for an abscisic acid-inducible transcript with homology to protein kinases. Proc Natl Acad Sci USA 89: 10183-10187


Finklestein RR, Crouch ML (1986) Rapeseed embryo development in culture on high osmoticum is similar to that in seeds. Plant Physiol 81: 907-912


Hattori t, Vasil V, Rosenkrans L, Hannah LC, McCarty DR (1992) The Viviparous 1 gene and abscisic acid activate the Cl regulatory gene for anthocyanin biosynthesis during seed maturation in maize. Genes Devel 6: 609-618


Khan AA (1968) Inhibition of gibberellic acid-induced germination by abscisc acid and reversal by cytokinins. Science 125: 645-646


Robertson D (1955) The genetics of vivipary in maize. Genetics 40: 745-760


Schopfer P, Plachy C (1985) Control of seed germination by abscisic acid. III Effect on embryo growth potential (minimal turgor pressure) and growth coefficient (cell wall extensibility) in Brassica napus L. Plant Physiol 77: 155-160


Zeevaart JAD (1966) Reduction of the gibberellin content of Pharbitis seeds by CCC and the after effects in the progeny. Plant Physiol 41: 856-862

Chapter Two

Cloning and characterization of cDNAs of maturing maize embryos

Constance N. White and Carol J. Rivin
Department of Botany and Plant Pathology
Program in Genetics
Center for Gene Research and Biotechnology
Oregon State University, Corvallis, OR 97331-7304
ABSTRACT

A cDNA library prepared from maturing maize embryos was screened for messages that are highly expressed in late embryo development and which are absent in viviparous 1 embryos. Two novel clones in addition to two Globulin-encoding cDNAs and the maize Em gene were isolated. Message levels of each are reduced in viviparous mutant embryos. All are induced in wildtype embryos cultured with either exogenous ABA or under conditions of water stress. Expression is elevated in ABA-deficient embryos (viviparous 5) cultured in ABA but not under conditions of water stress, suggesting that ABA synthesis is required for message accumulation during declining water potentials. Expression of each message is primarily confined to seed tissues. One clone (pM44) is suggested to represent a maize homologue of the wheat E_c gene, while the second (pM69) may encode a novel seed storage protein of maize.

INTRODUCTION

In flowering plants, embryogeny is followed by a maturation process in which storage proteins are synthesized and desiccation tolerance is acquired. Mutants which block production or response to the hormone abscisic acid (ABA) block maturation in seeds from a variety of species. In addition, many maturation-associated proteins and mRNAs accumulate precociously when embryos are cultured with exogenous ABA [reviewed by Kermode, 1990].

Whether maturation is signalled by ABA in toto or may also be regulated by the declining water potentials typical of later embryo development has been a subject
of considerable debate [for reviews see Kermode, 1990; Bray, 1993]. It has been suggested that ABA is important in directing early maturation events but then mediates later maturation only through an indirect effect of restricting water uptake and stimulating desiccation [Schopfer and Plachy, 1984; 1985; Finkelstein and Crouch 1986; Hughes and Galau; 1987]. Several groups have reported up-regulation of maturation-associated markers by water stress in the absence of de novo ABA synthesis [Finkelstein and Crouch, 1986; Hughes and Galau, 1991]. Additionally, the accumulation kinetics of several late embryogenesis associated (Lea) messages in cotton suggested that, although these genes are ABA-responsive in culture, desiccation is a more proximal regulator of their expression [Galau et al., 1987]. Two reports have described genes that are directly induced by water stress alone [Guerrero et al., 1990; Yamaguchi-Shinozaki et al., 1992]. Recently, a drought-responsive promoter element (DRE) has been described which does not depend on ABA for function [Yamaguchi-Shinozaki and Shinozaki, 1994].

We have used maize mutants which are blocked in maturation to dissect the regulation of late embryo development [Robertson, 1955]. In maize most viviparous (vp) mutants are ABA-deficient by dint of blocks in the synthesis of carotenoid precursors (vp 2, vp5, vp 7, vp 8, vp 9) [Neill et al., 1986]. viviparous mutants germinate midway through kernel development (ca. 25-35 days after pollination-DAP) while still on the ear. Embryos of these mutants are devoid of most maturation-associated proteins when analysed by two-dimensional gel electrophoresis but will synthesize nearly a full complement of maturation-phase proteins when cultured with exogenous ABA [Rivin and Grudt, manuscript in preparation]. In contrast, one ABA-insensitive mutant (vp1) does not respond to ABA, either in vitro or in vivo [Robichaud et al., 1986]. However, both ABA-deficient (vp5) and ABA-
insensitive (vp1) mutant embryos synthesize slightly different subsets of maturation proteins in response to culture in high osmoticum. Based on these results, three independent regulatory pathways controlling the expression of maturation-phase genes have been proposed: genes requiring ABA directly, genes requiring water stress alone, and genes which specifically require the Vp1 gene product [Rivin and Grudt, manuscript in preparation].

Relatively few maturation-specific genes have been isolated from maize. We sought to expand the set and to examine their regulation to assay for the existence of multiple regulatory pathways suggested by two-dimensional polypeptide analysis. Because it had previously been shown that vp1 embryos lack all maturation proteins in vivo [Rivin and Grudt, manuscript in preparation], we chose to construct a cDNA library from maturing wildtype embryo RNA and then screen for messages absent in vp1 embryos of identical developmental age. Using the clones isolated with this differential screen, we then examined message levels in ABA-deficient and ABA-insensitive embryos cultured either with exogenous ABA or under conditions of water stress. In this paper we report the isolation of two novel maturation associated cDNAs from maize, as well as three previously described sequences and their expression characteristics both in cultured embryos and in vivo.
MATERIALS AND METHODS

Plant material

All plants were propagated either at the Corvallis, OR field nursery or in the greenhouse. Inbred W22 stock was originally obtained from J. Kermicle (University of Wisconsin). vpl, $vp1^{mc}$, and $vp5$ mutants were originally obtained from D. Robertson (Iowa State University). $vp1$ and $vp5$ plants are in primarily W22 backgrounds. $vp1$ plants were propagated as homozygotes from rescued viviparous kernels. $vp5$ kernels can be identified on segregating ears by their lack of carotenoids starting at approximately 15 days after pollination (DAP). $vp1^{mc}$ kernels are dormant but still suffer from the reduced levels of kernel anthocyanins common to all mutant $vp1$ alleles.

Embryo isolation and culture

For use in culture, ears were first surface sterilized in 2.5% sodium hypochlorite and embryos were removed under aseptic conditions. Samples were staged according to the number of leaf primordia [Abbe and Stein, 1954]. Embryos were cultured scutella side down in petri dishes on 3M Whatman Filters saturated with Murashige and Skoog growth medium (Sigma) supplemented with 100mg/L myo-inositol, 0.4 mg/L thiamine HCl, and 3% sucrose as a carbon source. For high
osmoticum cultures, sucrose concentration was raised to 20%. ABA (mixed enantiomers) was obtained from Sigma. Cultures were incubated for three days at 26°C in the dark.

RNA Extraction

RNA used for the construction of the embryo cDNA library was prepared from 30 DAP stage 4 W22 inbred embryos. RNA was extracted by lysis in 2% SDS, 100 mM NaCl, 50 mM Tris-HCL pH 7.4, 200 µg/ml proteinase K, 50 mM EDTA for 15 min at 25°C followed by multiple extractions with buffered phenol:chloroform:isoamyl (24:24:1). Following two ethanol/sodium acetate precipitations, concentration was assayed using OD_{260} and salt concentration adjusted for Oligo dT-cellulose chromatography. Two passes were made over Oligo dT-cellulose (New England Biolabs) and the subsequently enriched poly A+ RNA fraction was used for library construction.

For northern analysis, RNA was prepared following the method of Chomczynski and Sacchi [1987] with the following modifications. Embryos were ground in 5-10X volumes of lysis buffer using short bursts of a tissuemizer. The resulting extract was made 0.3 M Na-Acetate and extracted several times with phenol:chloroform:isoamyl alcohol (24:24:1) until no interface remained. After a single ethanol-sodium acetate precipitation, the resulting pellet was resuspended in water in a volume 1/3 the original lysis buffer volume and frozen (-20°C) for two hours to overnight. After this treatment, bulk starch became insoluble and could be eliminated by centrifugation at 7-10G for 10 minutes prior to a second ethanol
precipitation. The second pellet was resuspended in 1% SDS in ddH2O and the concentration was assayed spectrophotometrically and by gel electrophoresis in combination with ethidium bromide staining.

**Library construction and screening**

Poly A+ RNA prepared as described above was used to prime first strand cDNA synthesis using Moloney murine leukemia virus (MMLV) reverse transcriptase and Oligo dT-XhoI-linkered primer according to the manufacturer’s instructions (Zap cDNA synthesis kit, Stratagene, La Jolla, CA). Following conversion to hemi-methylated doubled stranded cDNA, EcoRI adapters were ligated and the mix was subjected to XhoI digestion to provide 5' and 3' overhangs ends for cloning. cDNA thus modified was then unidirectionally ligated into XhoI/EcoRI digested Lambda Zap II arms (Stratagene) and packaged using Gigapack packaging extracts (Stratagene).

Plaque lifts were made using nitrocellulose filters (Schleicher and Schull). Duplicate lifts were hybridized with first strand 32P-dATP labelled cDNA primed from poly A+ RNA using MMLV [Gasser et al., 1989]. Hybridization conditions were as for Southern analysis described below. Clones were converted to Bluescript plasmid according to the manufacturer’s instructions (Stratagene)
RNA and DNA analysis

RNA blots for northern analysis were prepared by electrophoresing total RNA on 2% formaldehyde, 1.2% agarose submarine gels using MOPS buffer. Gels were capillary-blotted onto either MSI Magnagraph (MSI, Burlington, MA) or Genatran (Plasco, Inc, Woburn, MA) membranes. Gel loading was determined by staining duplicate blots with methylene blue [Sambrook et al., 1989]. RNA slot blots were prepared by denaturing total RNA in formaldehyde-MOPS buffer [Sambrook et al., 1989] without dyes and capillary blotting (Steed Manufacturing, Palo Alto, CA). Southern blots were prepared by electrophoresing total genomic DNA prepared from W22 cobs [Cone, 1989] on 0.8% agarose gels using Tris-borate buffer. Blotting and membranes for Southern and slot blot analysis were as described for northerns. All blots were baked at 80°C for two hours prior to hybridization. All hybridizations were performed using hexamer-labelled DNA probes at a specific activity of 1-1.5 x 10^6 dpm/ml in seal-a-meal bags at 68°C overnight using a hybridization buffer consisting of 250 mM NaH₂PO₄, 7% SDS, 100 μg/ml denatured salmon sperm DNA [Church and Gilbert, 1984]. Generally, blots were prehybridized in this solution for 8-16 hours before the addition of probe. Standard stringent washing conditions were used.

Sequence analysis

Clones of interest were initially grouped into families using a combination of cross-hybridization assays on slot blots and by manual 3' end sequencing. Clones chosen for further analysis were sequenced both by manual Sequenase based methods (US Biochemicals, Pittsburgh, PA) and by Taq based automated methods.
RESULTS

Library Screening

To identify messages specific to maturing embryos, we constructed a cDNA library from poly A+ RNA isolated from stage 4 (30 DAP) wildtype embryos and then screened for cDNAs absent in stage 4 (28 DAP) *vpl* embryos. Of 10,000 primary recombinants screened, 206 plaques hybridized with cDNA probes from wildtype but not *vpl* embryos. 100 of these recombinants were rescreened with first strand cDNA probes prepared from wildtype embryos isolated at successive developmental stages (18, 21, 24, and 27 DAP). Forty seven recombinants whose message accumulation increased in maturing embryos were identified (data not shown). Sequence and cross-hybridization analyses indicated that these represented seven clone families. Five families were selected for further analysis. Sequence analysis revealed that of these, two clone sets corresponded to the Globulin 1 and Globulin 2 genes of maize [Belanger and Kriz, 1989, Wallace and Kriz, 1991]. The remaining families (pM44, pM52, and pM69) did not correspond to any previously described genes of maize at the time of initial sequencing.

Accumulation kinetics were examined by densitometry of slot-blotted RNA prepared from wildtype embryos at successive developmental stages (Figure II.1).
Three kinetic subgroups are apparent: pM69 transcript is expressed at high levels and peaks prior to other messages examined (24 DAP). Globulin 1 and pM52 transcripts accumulate in parallel, with maximum steady state levels achieved at 27 DAP. Finally, Globulin 2 and pM44 transcripts accumulate with slower kinetics. All five clone families detect uniquely sized RNA species that are low in abundance in immature embryos but whose expression is greatly increased during maturation. The sizes of transcripts detected by each clone were estimated based on their electrophoretic mobility relative to molecular weight markers (Table II.1). The sizes of the Globulin class transcripts agree generally with those previously reported [Belanger and Kriz, 1989, Wallace and Kriz, 1991].

Table II.1: Estimated sizes of maturation-associated transcripts

<table>
<thead>
<tr>
<th>Clone name</th>
<th>transcript (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Globulin 1</td>
<td>2200</td>
</tr>
<tr>
<td>Globulin 2</td>
<td>1412</td>
</tr>
<tr>
<td>pM44</td>
<td>530</td>
</tr>
<tr>
<td>pM52</td>
<td>707</td>
</tr>
<tr>
<td>pM69</td>
<td>2238</td>
</tr>
</tbody>
</table>

Northern blots of wildtype and viviparous embryo RNA showed that expression of these RNAs is dramatically reduced in mutant embryos (Figure II.2A). Slot blotted RNA hybridized against the same probes confirmed this result (Figure II.2B). Densitometric quantitation indicates that all messages other than pM69 were reduced by at least four-fold in vp5 mutants and at least four- to ten-fold in vp1
mutants (data not shown). pM69 expression is severely reduced in *vp1* mutant embryos (ten-fold) but reduced by only 25 percent in *vp5* embryos (data not shown). Strangely, we found that expression of two messages, Globulin 2 and pM52 is diminished in embryos homozygous for the dormant *vp1mc* allele (Figure 11.2B). This allele is unusual in that it impairs only kernel anthocyanin accumulation and not dormancy function; it has not been reported to affect expression of genes other than those controlling anthocyanin synthesis.
FIGURE II.1 Accumulation of 5 maturation-associated mRNAs in developing wild type embryos. Data represent mean densitometric determinations from three slot blot experiments normalized to levels of ubiquitin transcript ± sem. (A) levels of pM69 transcript (●). (B) levels of Globulin 1(●) and pM52 transcript (○). (C) levels of Globulin 2 (□) and pM44 transcript (■).
FIGURE II.2A. Expression of maturation messages in stage 4 (28-30 DAP) wildtype and *viviparous* embryos.

Each lane loaded with 7.5 μg of total RNA from either 30 DAP wildtype W22 inbred embryos (*Vp1*), 28 DAP *vp1* mutant embryos in a W22 background (*vp1*), and 29 DAP sibling embryos from *vp5* segregating ears (*Vp5* -wildtype embryos, *vp5* -mutant embryos). Blot hybridized with cDNA isolates as described in the text.
FIGURE II.2B. Expression of maturation messages in wildtype and *viviparous* mutants assayed by RNA slot blot.

10 μg total RNA prepared from maturing embryos capillary blotted and probed with cDNA isolates. Wildtype and mutant *vp1* and *vp5* materials as described in figure II.2A. *Vp1*<sup>mc</sup> and *vp1*<sup>mc</sup> RNA prepared from 36 DAP embryos from segregating ears. Two additional probes used were maize ubiquitin (uq) and maize Lea 3 (pMlg) clones. Both are normally expressed in *viviparous* mutants.
We assayed the effects on Globulin 2 expression conferred by different combinations of \( vp1 \) alleles. On northern blots, \( vp1^{mc} \) in combination with the wildtype allele allowed for accumulation of normal levels of Globulin 2 transcript. When homozygous or in combination with a standard non-dormant \( vp1 \) allele, Globulin 2 message levels were severely reduced (Figure II.3). These results were confirmed by densitometric quantitation (data not shown).

The reduced kernel anthocyanins in \( vp1^{mc} \) and other \( vp1 \) mutants result from the reduced expression of the regulatory gene C1 [McCarty et al., 1989a, Hattori et al., 1992]. This \( myb \) homologue activates structural genes along the anthocyanin biosynthetic pathway [Roth et al., 1991 and citations therein]. We tested the possibility that C1, or other anthocyanin regulators also activate expression of Globulin 2. Embryos carrying mutations in the anthocyanin regulatory genes C1, Pl, B, and R were screened for diminished Globulin 2 expression but levels appear to be normal in these embryos (Figure II.4).

Northern analysis of RNA prepared from a variety of tissues suggests that the cDNA isolates are expressed maximally only in seed tissue (Figure II.5). No transcripts corresponding to any isolate were detected in RNA prepared from leaf, cob, or seedling tissue. Results with tassle were variable, with trace amounts of message detected occasionally by clones pM44 and pM52.

When expression of these transcripts was assayed directly on northern blots of cultured wildtype and viviparous materials (Figure II.6), all of the isolates detected elevated message levels in wildtype embryos cultured with either 10 \( \mu \)M ABA or high osmoticum. Message levels of all but Globulin 2 were elevated in \( vp5 \) embryos cultured with ABA but not with osmoticum alone. No transcripts were detected in RNA prepared from \( vp1 \) embryos cultured under either condition.
FIGURE 11.3. Expression of Globulin 2 in embryos carrying the \( vp1^{mc} \) allele

Duplicate northern blots of 7.5 \( \mu \)g total RNA from 35 DAP embryos carrying the \( vp1^{mc} \) allele in combination with wildtype \( Vp1 (Vp1/vp1^{mc}) \), in a homozygous state (\( vp1^{mc}/vp1^{mc} \)), or with the standard mutant allele (\( vp1^{mc}/vpl \)) probed with clone pM44 and the Globulin 2 cDNA. Expression of pM44 message is normal in \( vp1^{mc} \) embryos.
FIGURE 11.4 Globulin 2 message levels in embryos with mutations in anthocyanin regulatory genes.

Northern blot of 7.5 μg total RNA from 30 DAP embryos per lane probed with Globulin 2 partial cDNA. Embryo genotype indicated by a (+) or (-) sign next to appropriate locus. Regulatory genes $B$ (+) or $b$ (-), $R$ (+) or $r$ (-), $Pl$ (+) or $pl$ (-), $Cl$ (+) or $cl$ (-). Most alleles of $R$ and $Cl$ affect kernel anthocyanins while those of $B$ and $Pl$ frequently control plant pigmentation.
FIGURE II.5 Maturation message levels in different plant tissues.

Northern blots of 7.5 μg total RNA prepared from different tissues and probed with cDNAs described in text. **em**-embryo, **lf**-mature leaf, **cb**-immature cob, **sr**-two week seedling root, **ss**-two-week seedling shoot, **ts**-maturing tassle. rRNA stained with methylene blue for loading comparisons.
FIGURE II.6 Expression of maturation messages in cultured immature embryos.

Northern blot of RNA from untreated 19 DAP wildtype and *viviparous* stage 2 embryos (19) and following three days of culture in Murishige and Skoog medium alone (MS) or supplemented with either 10 μM ABA (A) or 20% sucrose (O). Transcript levels were not elevated by culture in basal media alone (data not shown). rRNA stained with methylene blue for loading comparison.
Sequence characteristics of clones pM44, pM52, and pM69

Although initial database searches revealed no previously described maize genes homologous to the three non-Globulin isolates, clones pM44 and pM52 showed distinct homology to genes isolated from other monocot species. To date, searches of both Genbank and EMBL databases have not identified any genes strictly homologous with pM69. Its sequence characteristics will be discussed in a separate section below. During the course of these experiments, the sequence of pM52 was reported by another group [Williams and Tsang, 1991]; it shares extensive homology with the wheat $E_m$ gene [Williamson et al., 1985].

Clone pM44 shares distinct sequence homology with the metallothionein $E_c$ gene of wheat

Sequence analysis revealed strong homology between pM44 and transcripts encoded by the wheat $E_c$ (early cysteine) genes [Kawashima et al. 1992]. The sequence of pM44, the longest insert of its clone family, is shown in Figure II.7A. The size of this insert corresponds well with the size of its transcript. Including the 27 mer polyadenylated tail residing on this clone, the total cDNA sequence excluding adapters comprises 468 nucleotides. The size estimate of pM44 transcript (530 nt) suggests that pM44 represents a nearly full length cDNA. One long open reading frame which, if translated, would encode a polypeptide of 7.8 kD is present. The predicted polypeptide shares extensive homology with the wheat $E_c$ protein (Fig. II.7B).
Southern analysis of genomic DNA derived from W22 cobs showed a single band in samples digested with either EcoRI, BamHI, or HindIII (Figure II.8). In addition, we observed complete sequence identity between all pM44 isolates. While the possibility of multiple genes encoding pM44 message cannot be eliminated without quantitative reconstruction, these results suggest that pM44 is encoded by a single gene. The observation that wheat contains only one $E_c$ gene per genome supports this idea since copy number per genome of monocot homologues is often conserved [White, unpublished observation].
1 GAATTCGGCAGGAG GCGTCGATCGGGC ATG GGG TGC GAC GAC AAG TGC TGG MET Gly Cys Asp Asp Lys Cys Gly Cys

56 GCC GTG CCG TGC CCC GGC GGC AAA GAC TGG TGC AGC TCG GGG AGC GGC GGG Ala Val Pro Cys Pro Gly Gly Lys Asp Cys Arg Cys Thr Ser Gly Ser Gly Gly

110 CAG CGG GAG CAC ACG ACT TGG GGC TGC GGG GAG CAC TGC GAG TGC ACG TCG GGG AGC GGC GGG Gln Arg Glu His Thr Thr Cys Gly Cys Gly Glu His Cys Glu Cys Ser Pro Cys

163 ACG TGT GCC CCG GCC ACG ATG CCG TCC GCC CGC GAG AAC AGG AGG GCT AAC TGC Thr Cys Gly Arg Ala Thr MET Pro Ser Gly Arg Glu Asn Arg Ala Asn Cys

218 TCC TGC GGG GCG TCC TGC AAC TGC GCA TCC TGC GCC TCG GCC TGA TCGTGC GCC Ser Cys Gly Ala Thr MET Pro Ser Gly Arg Glu Asn Arg Ala Asn Cys

273 CTGC CCGCCTCGTACCGCGCGCTGCCTAGTGGAGGGAGTTGTCTAGTGAGGCTGGAGACGAAGC

336 AACTAGCACTACTTCTAAATAAAGGGGGTTGTCTCATTGGGCTAGGCTGGAGACGAAGC

399 TGATCGTGGATTATCGTGAGTAAGTTTGTATGATAATAAACACTACCACCTTTATGTC

FIGURE II.7A: Sequence of pM44 insert. 15 mer EcoRI adapter sequence in bold. Putative polyadenylation signals underlined. Predicted translation product of the longest open reading frame under nucleic acid sequence. A polyadenylated tail of 27 mer was found at position 457.
FIGURE II.7B  Alignment of maize putative pM44 translation product with polypeptide encoded by wheat Ec cDNA.
FIGURE II.8 Southern blot of W22 genomic DNA probed with pM44. Restriction enzymes *EcoRI* (R), *BamHI* (B), *HindIII* (H). 10 μg DNA per lane. Mobility of molecular weight standards shown at left.
Sequence characteristics of pM69

Sequence analysis of cDNA pM69 revealed that it is unusually rich in guanidine and adenine residues (Figure II.9). This isolate must contain only a partial cDNA sequence since the transcript it detects is significantly longer. Several screenings of both the original library and a second embryo cDNA library (kind gift of Alan Kriz) with clone pM69 allowed the isolation of a number of homologous clones. Of twenty additional isolates, none was of greater length than the original clone. The two libraries were constructed by reverse transcription from polyadenylated 3' message ends, thus screening of a library constructed from random priming may have be a more fruitful strategy than those undertaken. The failure to isolate a full length insert may be a result of the transcript's particular nuclease sensitivity (as detected on northerns) or a consequence of the peculiar sequence structure which carries a number of repeated tracts.

Southern analysis reveals a simple restriction pattern of hybridizing genomic fragments thus suggesting that pM69 is not encoded by a complex gene family (data not shown).
FIGURE II.9 Sequence of pM69. Bold face type shows possible stop codons. Putative polyadenylation signals are underlined. Polyadenylated tail found at 3' end (*).
DISCUSSION

Five cDNAs which are expressed in developing wildtype but not *viviparous* embryos were isolated in these experiments; two (pM44, pM69) represent novel clones not previously described. Expression of both of these is maximal only in seed and not in other tissues examined. Message accumulation is enhanced in both wildtype and ABA-deficient embryos cultured in the presence of ABA but not in embryos with a mutation in the *Vp1* gene. Additionally, message levels were elevated in wildtype embryos cultured in high osmoticum but not in *vp5* embryos. This suggests that osmoticum acts via stimulating *de novo* synthesis of ABA. Although we do not know whether ABA acts by stimulating transcription or by increasing message stability, we believe that the former is likely. *Vp1* upregulates expression of maturation-associated genes by stimulating transcription in response to increased ABA levels [McCarty et al., 1991]. The absence of transcripts in any *vp1* embryos cultured with high osmoticum suggests that their expression requires a functional *Vp1* gene product in addition to high levels of ABA.

While sequence homology in the absence of biochemical characterization is insufficient to identify the pM44 gene product as a true metallothionein, developmental and tissue specificity of its hybridizing message strongly suggests that this cDNA does encode the maize *Ec* homologue. Moreover, promoter regions of wheat *Ec* genes have been found to contain ABA response elements (ABREs) and their expression is ABA-inducible [Kawashima et al. 1992]. These results are consistent with the ABA induction of pM44 message in cultured embryos as well as with its impaired expression in *viviparous* mutants.

The wheat *Ec* protein is a bonafide plant metallothionein. Native wheat *Ec* is
found associated with Zn\(^{+2}\) [Lane et al., 1987]. While the native translation product of pM44 has not been characterized, its predicted sequence homology to the wheat protein suggests that they share a common function. The expression of pM44 RNA during maize maturation would suggest that \(E_c\) protein may either play a role in preparing seed tissues for desiccation or serve as a metal storage system in preparation for germination processes. Wheat \(E_c\) is suggested to act as part of a zinc homeostasis system in conjunction with nucleic acid synthesis during periods of cellular proliferation [Kawishima et al., 1992]. Although a similar function has been proposed for some mammalian metallothioneins, they may also be induced by physical or chemical stress [reviewed by Kagi and Schaffer, 1987]. The ABA-inducibility of pM44 and its accumulation after completion of embryogenesis indicate that \(E_c\) may be important in responding to the stress of desiccation. The failure of viviparous mutants to express pM44 indicates that if \(E_c\) is somehow involved in cellular proliferation, then this function is not obligate for germination to occur.

One other metallothionein-like gene has been isolated from maize [de Framond, 1991]. Unlike pM44, its expression is confined to root tissue. Maize and Wheat \(E_c\) coding elements diverge from this metallothionein, as well as other metallothioneins isolated from higher plants, in that they do not contain a cysteine poor internal spacer [Kawashima et al., 1992 and citations therein].

A second isolate, clone pM52, was identified as a maize homologue of wheat \(E_m\). Its sequence and expression characteristics were reported by another group during the course of these experiments [Williams and Tsang, 1991]. Their results are in general agreement with those reported here. However, they did not examine expression in \(vp1^{mc}\) embryos, and our observation of diminished expression in embryos homozygous for this allele represents a novel finding.
The second novel clone, pM69, hybridizes with a transcript of ca. 2200 nucleotides which is highly expressed in developing maize embryos and, like all other clones examined, is ABA-inducible. Unlike other mRNAs described here, the pM69 transcript appears to require ABA for expression in cultured embryos, yet it is relatively abundant in ABA-deficient mutants (vp5). We do not understand the nature of this discrepancy but suggest that pM69 expression in vivo may result from a maternal contribution of ABA which dissipates prior to initiation of expression of the other genes examined here. Embryo ABA levels peak at 21 DAP, a stage at which a portion of the ABA found in developing kernels has been reported to be maternally derived [Smith et al., 1987; White et al., manuscript in preparation].

While identity of its product awaits characterization of a full length cDNA and/or translation product, clone pM69 may encode a novel seed storage protein. Partial translation of the three possible frames of pM69 yields polypeptides rich in arginine in all cases (data not shown). Frames 1 and 3 yield peptides rich in glutamic acid and arginine. Frame 2 yields a peptide high in lysine and arginine. The peptide composition of all three frames is similar to that found for many seed storage proteins (invariably high in nitrogenous amino acids) [Higgins, 1984]. pM69 shares greater than 50 percent homology with cDNAs encoding storage proteins from a number of plant species but with no obvious pattern of sequence similarity [Figure II.10]. Likewise, structural analysis of the three possible peptides and comparison with structures derived from known seed storage proteins was of limited value in assigning identity to any of pM69's putative translation products (data not shown). It is possible that the cDNA does not contain any coding sequences and is an untranslated region of the pM69 transcript. The product encoded by pM69 may represent a novel storage protein of maize for a number of reasons. First, the
developmental timing of message accumulation is in agreement with that found for other known storage proteins. Second, the restriction of pM69 expression to seed tissues and its high level of expression is characteristic of other storage protein transcripts. Third, both the ABA inducibility of pM69 message and the amino acid composition of all three putative partial translation products is reminiscent of other storage proteins. Only characterization of full length pM69 message will provide definitive evidence for this possible identity.

The molecular phenotype of \textit{vp1}\textsuperscript{mc} embryos is intriguing. Levels of both Globulin 2 and E\textsubscript{m} message are reduced in these embryos. \textit{vp1}\textsuperscript{mc} is known to reduce anthocyanin levels by failing to activate transcription from the C1 regulatory gene [McCarty et al., 1989a, Hattori et al., 1992]. Here we show that Globulin 2 expression is not coordinately regulated with anthocyanin synthesis and thus represents a separate regulatory pathway that is impaired by the \textit{vp1}\textsuperscript{mc} allele. This allele has been characterized and is found to produce a protein truncated at its C-terminal end [McCarty et al., 1989b]. The apparent separation of \textit{vp1} regulation of dormancy and anthocyanin synthesis has suggested that wildtype VP1 protein may encompass two regulatory domains directed at dormancy functions and anthocyanin synthesis respectively [McCarty et al., 1991]. Our data add a third function of \textit{Vp1} that is separable from the regulation of anthocyanin synthesis but which is still impaired in \textit{vp1}\textsuperscript{mc} mutants. Examination of promoter regions from Globulin 2 and E\textsubscript{m}, as well as C1 may reveal sequence differences which explain the low expression of these genes in this mutant allele as compared to other genes which are not similarly impaired and may provide clues to the mechanism of \textit{Vp1} gene activation.

The initial aim of these experiments was to separate water stress and ABA as regulatory components of seed maturation processes. While the failure to isolate
markers that are induced by osmotic stress directly does not exclude a direct role for desiccation in regulating seed maturation, it may be significant that there are only two plant genes reported for which expression may be directly induced by desiccation [Guerrero et al., 1990, Yamaguchi-Shinozaki et al., 1992]. Our screen of mid-maturation embryos identified genes which depend solely on the presence of ABA and a functional Vp1 gene product for expression. We think it likely that if ABA-independent, desiccation-inducible gene-expression occurs in maturing embryos, it represents a small portion of maturation-phase developmental programming and is not fruitfully approached via a differential screen such as the one described here.
Figure II.10A  Alignment of clone pM69 (top sequence) with *Pisum sativum* cvcA gene for convicilin polypeptide (PEACVCA).
Figure II.10B  Alignment of clone pM69 (top sequence) with *G. hirsutum* (cotton) storage protein (beta-globulin = COTSPB)
Figure II.10C  Alignment of clone pM69 (top sequence) with Maize embryo globulin S allele (7S-like) cDNA (MZEGLB1SA)
Figure II.10D  Alignment of clone pM69 (top sequence) with Barley embryo globulin (BEG1) cDNA (BLYBEG1)
Figure II.10E  Alignment of clone pM69 (top sequence) with *Pisum sativum* legJ gene for minor legumin (PEALEGJ).
LITERATURE CITED


Cone KC (1989) Yet another rapid plant DNA prep. Maize Genet Coop Newslet 63: 68


Finklestein RR, Crouch ML (1986) Rapeseed embryo development in culture on high osmoticum is similar to that in seeds. Plant Physiol 81: 907-912


Guerrero FD, Jones JT, Mullet JE (1990) Turgor responsive gene transcription and RNA levels increase rapidly when pea shoots are wilted. Sequence and expression of three inducible genes. Plant Mol Biol 15: 11-26

Hattori T, Vasil V, Rosenkrans L, Hannah LC, McCarty DR (1992) The Viviparous 1 gene and abscisic acid activate the C1 regulatory gene for anthocyanin biosynthesis during seed maturation in maize. Genes Devel 6: 609-618


Robertson D (1955) The genetics of vivipary in maize. Genetics 40: 745-760


Schopfer P, Plachy C (1985) Control of seed germination by abscisic acid. III. Effect on embryo growth potential (minimal turgor pressure) and growth coefficient (cell wall extensibility) in Brassica napus L. Plant Physiol. 77: 155-160


Smith JD, Cobb BG, Magill CW, Hole DJ, Blakely CA (1987) Partitioning the sources of abscisic acid found in developing embryos. Maize Genetics Coop Newslet 61: 39


Williamson JD, Quatrano RS, Cuming AC (1985) Em polypeptide and its messenger RNA levels are modulated by ABA during embryogenesis in wheat. Eur J Biochem 152: 501-507


Chapter Three

Sequence and expression of the message encoding a Lea Group 3 protein of maize (Zea mays L.)

Constance N. White and Carol J. Rivin
Department of Botany and Plant Pathology
Program in Genetics
Center for Gene Research and Biotechnology
Oregon State University
Corvallis Oregon 97331-2902
ABSTRACT

A full length cDNA that encodes a putative maize (*Zea mays* L.) group 3 Lea protein (MLG3) was isolated using a probe from wheat. The predicted translation product is a 22.9 kD protein which shares distinct homology features with other members of this class of proteins, including having repeating tracts of an 11 amino acid sequence and a basic pI. Expression of MLG3 message occurs during maturation phase in developing seed. It can also be induced in cultured immature embryos by treatment with either ABA or osmotic stress. The pattern of MLG3 protein accumulation reflects the changes in messenger RNA abundance.

INTRODUCTION

Abscisic acid (ABA) plays dual regulatory roles in angiosperm tissues. In the developing seed ABA modulates many events associated with maturation and desiccation tolerance: exogenous ABA supplied to cultured embryos induces expression of both storage protein and Lea (Late embryogenesis abundant) genes. ABA also governs the response of vegetative tissue to drought stress: a transient rise in hormone levels is induced by water stress and the expression of some RAB (Responsive to ABA) genes is elevated. Some of these RAB genes are also Lea genes and are thought to play a role as desiccation protectants in both embryonic and adult tissues [reviewed by Skriver and Mundy, 1990].

One class of Lea proteins that is expressed both in seeds and in drought-stressed tissues is distinguished by containing a tandemly repeated 11 amino acid motif that is thought to form an amphiphilic alpha helix [Dure et al., 1989]. Genes for these Lea Group 3 proteins have been isolated from cotton [Dure et al., 1981; Baker et al, 1988], barley [Hong et al. 1988], rape [Harada et al., 1989], carrot
[Seffens et al., 1990], and wheat [Curry et al., 1991]. Other proteins containing related 11-mer repeats have been found in cotton [Baker et al., 1988] and the desiccation-tolerant plant *Craterostigma plantagineum* [Piatkowski et al., 1990]. Dure [1993] has compared the sequences of these proteins and proposed a model for the structure of the tandemly arrayed units and for the possible function of these proteins in desiccation protection. In cotton embryos, Lea 3 protein appears to accumulate in high concentration in various cell types [Roberts et al., 1993]. Strong evidence correlating the expression of Lea 3 with the resistance of wheat tissues to drought-stress has recently been published [Ried and Walker-Simmons, 1993].

A previous paper described a maize protein, MLG3 (Maize Lea Group 3), with antigenic similarity to wheat Lea 3 protein and characterized its accumulation in developing and cultured embryos [Thomann et al. 1992]. Unlike many ABA-inducible embryo proteins, MLG3 was found in *viviparous* mutants as well as in wildtype maize embryos. Like many Lea proteins, it was also found in water-stressed vegetative tissues. In this paper, we describe the isolation of a cDNA clone which encodes a maize Lea group 3 protein. Like the other proteins of this class, the predicted translation product has a basic pI and consists primarily of tandemly arrayed repeats of an 11 amino acid motif. M1g3 mRNA is expressed during kernel development, with maximum message levels occurring in late maturation phase, coincident with the onset of embryo desiccation tolerance. Traces of the message can be found in seedling leaves, but it is not detected in any adult tissues of well-watered plants. Precocious accumulation of M1g3 message, like MLG3 protein, can be induced in immature embryos cultured with ABA or under conditions of osmotic stress. Accumulation of MLG3 in cultured embryos appears to be governed by two discreet regulatory pathways.
MATERIALS AND METHODS

Clones for maize Lea Group 3 (Mlg3) were isolated from a Lambda Zap (Stratagene) maize embryo cDNA library (gift of Alan Kriz, University of Illinois) probed with the cDNA insert of pMA2005 encoding wheat Lea 3 (gift of M.K. Walker-Simmons, Washington State University). Positive plaques were confirmed via secondary screen and converted to Bluescript (Stratagene) plasmid via in vivo excision. Nested deletions were made of the longest insert (pMlg3) using Exo III (New England Biolabs) and these were sequenced from double stranded templates in both directions. Both manual sequencing using Sequenase (US Biochemical Corp.) and automated methods (Applied Biosystems) were performed. Sequence analysis was done with the Intelligenetics v. 5.4 software package.

Inbred W22 stock was originally obtained from J. Kermicle (University of Wisconsin). vp1, and vp5 mutants were originally obtained from D. Robertson (Iowa State University). Both are in primarily W22 backgrounds. Developing embryos were staged using the scheme of Abbe and Stein [1954]. Immature (19 days after pollination -DAP) embryos were cultured as previously described [Thomann et. al. 1992].

Genomic DNA was prepared from immature cobs using the method of Cone [1989]. Total RNA was prepared from all tissues examined using method of Chomczynski and Sacchi [1987]. Northern analysis was performed using low formaldehyde (2%), 1.2% agarose, MOPS gel electrophoresis. Hybridizations were done overnight at 68°C in 250 mM NaH2PO4, 7% SDS, 100 μg/ml salmon sperm DNA [Church and Gilbert,1984] using hexamer-labelled pMlg3 insert as probe and standard washing conditions.
Proteins were isolated using two methods: Using the method of Thomann et al. [1992] (Tris method), frozen embryos were ground in 50 mM Tris-HCl pH 6.8 followed by sonication and boiling; the resulting supernatant contains only heat-stable proteins [Thomann et al., 1992]. A second method (SDS method) was used for some of these experiments; it consisted of adding hot (95°C) SDS-PAGE gel loading buffer to ground frozen embryos, followed by vigorous vortexing and a second heat treatment at 95°C for two minutes, after which the material was centrifuged at 10 G for 10 minutes and the resulting supernatant was analysed. Protein gel electrophoresis and western analysis were performed as previously described [Thomann et al., 1992].

RESULTS AND DISCUSSION

Maize Lea 3 shows significant homology to Lea 3 from other monocots

In a previous paper [Thomann et. al., 1992], we described MLG3, an ABA-responsive embryo maturation protein that was antigenically related to the Lea group 3 protein from wheat. Using a cDNA of wheat Lea Group 3, we probed a maize embryo cDNA library and isolated a full-length (1.1 kb) cDNA insert (pMlg3) that showed significant cross hybridization with the wheat Lea 3 gene. Sequence analysis (Figure III.1) identified one long open reading frame of 666 bases. The predicted translation product contains a tandemly repeated eleven amino acid motif characteristic of this class of Lea proteins, having apolar residues (chiefly alanine and threonine) at positions 1, 2, 5, and 9, positively charged amino acids (chiefly lysine) at positions 6 and 8, and negative charges or amide residues at positions 3, 7 and 11 [Dure, 1993].
MLG3 is very similar to the group 3 Lea proteins found in barley and wheat in calculated molecular mass (22.9 kD), predicted pI (9.68) and general structure, [Hong et al., 1988; Curry et al., 1991]. The predicted mass is less than the 27-29 kD estimated by SDS-PAGE mobility [Thomann et al., 1991]. This discrepancy is found for other Lea 3 proteins [Ried and Walker-Simmons, 1993] and has been attributed to anomalous electrophoretic behavior due to the unique composition and charge characteristics of these proteins[Hong et. al., 1988]. The three monocot Lea 3 proteins are compared in Figure III.2. MLG3 has nine conserved repeat units (shown boxed), one degenerate repeat and a truncated repeat (both circled). The 11-mer arrays are interrupted twice; once by an 11 amino acid peptide having some homology to the repeat, and once by a 7 amino acid sequence that is unrelated to the repeat motif but homologous to an interruption found in the wheat and barley proteins. Dure [1993] has proposed that these interruptions may function as "hinges" to give the molecule flexibility. In addition, Lea 3 proteins from the three monocot species share strong homologies in both C-terminal and N-terminal peptide sequences that appear unrelated to the 11 amino acid repeat. Database searches did not reveal any known function for these peptide sequences in protein targeting, but their strong conservation suggests that they are of structural importance.
FIGURE III.1 Nucleotide sequence of pMlg3. The open reading frame is shown in upper case. The start of the open reading frame, the translational stop and the putative polyadenylation signal in the 3' untranslated region are boxed. A 50-mer poly-A tract (not shown) was found at the 3' end of this clone. The EcoRI linker site is underlined.
FIGURE III.2 Alignment of maize MLG3 with Lea 3 proteins from wheat and barley. Homologous 11-mers are shown boxed. Truncated and degenerate 11-mer motifs are circled. The sequences have been aligned to reflect both the homology of motif variants and the placement of an internal conserved sequence interrupting the 11-mer arrays. There is also considerable conservation of peptides flanking the repeats at both N and C ends.
Lea 3 appears to be derived from a single locus in maize

In Southern blots using W22 genomic DNA, pMlg3 detected a single band of approximately 4500 bp in EcoRI digested DNA and a single band of approximately 900 bp in HindIII digested DNA (Figure III.3). Low stringency Southern analysis did not reveal any additional hybridizing bands (data not shown). While the possibility of multiple genes encoding Lea 3 cannot be ruled out without quantitative genomic reconstructions, these results are consistent with our genetic evidence for a single locus [Thomann et al., 1992]. This is similar to wheat, in which there appears to be a single major locus for Lea 3 in each genome [Ried and Walker-Simmons, 1993]. Unlike these monocots, cotton Lea 3 appears to be a small multigene family [Baker et al., 1988].
**FIGURE III.3.** Southern blot of maize genomic DNA (W22 inbred) digested with either A) *EcoRI* or B) *HindIII* and probed with pMlg3.
Expression of the Lea 3 transcript in maize embryos and adult tissues

In a previous study of MLG3 protein [Thomann et al., 1992], levels were found to be low in early embryo development and dramatically rose during late maturation, coincident with the acquisition of embryo desiccation tolerance. High levels of the protein could be induced to accumulate precociously in wildtype embryos that were excised prior to maturity and cultured in the presence of either 10 μM ABA or in high osmoticum. Like some other Lea proteins, MLG3 also could be shown to accumulate during drought-stress in seedling shoots [Thomann et al., 1992], as well as in drought-stressed mature leaves [White, Sollinger, Rivin, unpublished].

To assess the contribution of message levels to the accumulation of MLG3 in developing embryos, RNA and protein were prepared from the split samples of developing embryos. The patterns of protein accumulation reflect the levels of Mlg3 message. The 29 kD form of MLG3 was first detected in mid stage 3 embryos (24 DAP) but does not become abundant until stage 4 (30 DAP) (Figure III.4). As measured on northern blots, pM1g3 detected a single transcript of approximately 1.1 kb in length in developing embryos (Figure III.5A). The transcript abundance increases greatly during late maturation (30 DAP, stage 4) and its level remains high after stage 4, suggesting that either message stability or steady state transcription plays a role in maintaining mRNA abundance throughout the remainder of maturation and seed desiccation. Together, these results suggest that message abundance is a major factor determining MLG3 accumulation in maturing embryos.

A smaller (25 kD) antigenically-related peptide was detected in stage 2 embryos (21DAP) which disappears as embryos mature (Figure III.4). This is probably not a precursor form of MLG3 but rather the product of a separate locus
because the 29 kD MLG3 was not converted to a lower molecular weight protein by either dephosphorylation or deglycosylation (data not shown). In addition, this protein is similarly sized in backgrounds carrying either the 27 or 29 kD allele of MLG3 (data not shown). If the size variation of mature MLG3 is due only to native structural differences (as our genetic analysis indicated), a precursor would be expected to size in parallel with the specific allele carried. If the 25 kD cross-reacting species is the product of a separate locus, as seems likely, it is sufficiently divergent at the nucleotide level to go undetected in low stringency Southern hybridizations.

To determine whether basal Mlg3 mRNA expression occurs in unstressed plant tissues, we tested for the presence of Mlg3 message in RNA from several tissues of well-watered adult plants and from seedling roots and shoots. As shown in Figure III.5B, a trace amount was found in two week old seedling shoot RNA, but no signal was detectable in RNA samples from mature leaf, tassle, and cob or from seedling root.
FIGURE III.4 Accumulation of MLG3 polypeptide during wildtype embryo development. Equal amounts of protein from embryos isolated at 21, 24, 27, 30, 40, 50 DAP and mature seed (MS) were separated by SDS-PAGE, blotted and reacted with anti-MLG3 serum.
FIGURE III.5 Northern analysis of maize tissues probed with pMl g3. A) RNA from embryos isolated at 21, 24, 27, 30, 40, and 50 days after pollination. Each lane contains 7.5 µg of total RNA. B) RNA from various maize tissues from well-watered W22 inbred plants: T= tassel; L= leaf; C= cob. S= 2 week seedling shoots; R= 2 week seedling roots; E= 30 DAP embryos. Each lane contains 7.5 µg of total RNA.
Expression of maize Lea 3 is regulated by two independent pathways

Drought-stressed plant tissues often respond to such stress by synthesizing ABA and expressing a battery of ABA-inducible stress-related genes [reviewed by Skriver and Mundy, 1990]. It was previously shown that MLG3 accumulated in wildtype embryos excised prior to maturity and cultured for 5 days in either 10 μM ABA or in high osmoticum [Thomann et al., 1992]. A viviparous mutant (vp5) unable to synthesize ABA accumulated MLG3 when cultured with ABA but not with high osmoticum. In addition, an ABA-insensitive mutant (vp1) accumulated the protein when cultured in high osmoticum. These results suggested that MLG3 protein accumulation requires ABA but not the Vp1 gene product. In order to assay the relative contribution of RNA levels to protein accumulation, RNA and protein were prepared from split culture samples. In this case however, embryos were cultured for only 3 days in order to minimize any effects of germination and proteins were extracted using the SDS method rather than grinding in Tris buffer as was previously done [Thomann et al., 1992]. As previously reported, wildtype embryos accumulate significant amounts of MLG3 under both conditions of high osmoticum and ABA. This rise is paralleled by the accumulation of transcript (Figure III.6). Also, as expected, MLG3 was detected in vp5 embryos cultured with ABA, albeit at significantly reduced levels, which were also paralleled by diminished transcript levels. MLG3 was found in vp1 embryos cultured under both conditions but again at reduced levels as compared to wildtype embryos; no Mlg3 transcript was detected in cultured vp1 embryos. We believe that this may be due either to the sensitivity limits of the northern technique or to decreased transcript stability in viviparous embryos; the latter is possible due to the increased hydrolytic enzyme activities reported for viviparous mutants [Wilson et al., 1973; White and Rivin, unpublished]
observations]. Taken together, these data suggest that MLG3 accumulation in cultured embryos is, at least in part, a reflection of increased Mlg3 message levels.

An unexpected result of this experiment was the accumulation of MLG3 in vp5 embryos cultured in high osmoticum (Figure III.6). In order to resolve this discrepancy, additional protein preparations from embryos cultured in different years and for different times (3 and 5 days) were assayed. In each instance, MLG3 was found (data not shown). In prior reported experiments, all cultured materials prepared by Tris extraction gave an additional and often preponderant MLG3 cross-reactive species that was approximately 6 kD smaller than the native protein [Thomann et al., 1992]. Figure III.7A shows the results of such an experiment using embryos cultured for 5 days on either basal media alone, or with the addition of 10 μM ABA or high osmoticum. In contrast, SDS extracts contained little of the smaller species, suggesting that endogenous proteases might be responsible for the differences. To test this hypothesis, unboiled Tris extracts from vp1 and vp5 embryos cultured in high osmoticum were assayed for their ability to proteolyse MLG3 from mature seed. Incubation of mature seed extract in Tris buffer alone at room temperature for 20 minutes indicated no endogenous protease activity, whereas incubation with either vp 5 or vp1 extracts under the same conditions resulted in reduction of the 29 kD MLG3 accompanied by the appearance of a 23 kD cross-reacting protein (Figure III.7B). Appearance of this smaller molecular weight species could not be attributed to MLG3 contributed by viviparous extracts because of both size and abundance considerations (data not shown). It is likely that the 23 kD protein represents a proteolytic product of MLG3 degradation, and that the absence of MLG3 in osmoticum-cultured vp5 embryos previously reported [Thomann et al., 1992] was probably due to complete proteolysis prior to boiling during sample preparation.
**FIGURE III.6** Accumulation of maize Lea 3 protein (top) and mRNA (bottom) in cultured embryos.

19 DAP embryos of wildtype, *vp5*, or *vp1* genotypes were cultured for three days in the dark in either 10 μM ABA (A) or high osmoticum (O). Protein and message levels in uncultured embryos of the same age (19).
FIGURE III.7 Endogenous protease activity in cultured embryos

A) Embryos cultured as in Fig. 6. Extracted with Tris buffer.

B) Conversion of native MLG3 to lower MW species by treatment with cultured viviparous embryo Tris extracts. lane 1 - mature seed extract, no treatment; lane 2 - mature seed extract incubated with Tris extraction buffer; lane 3 - mature seed extract incubated with vp1-osmoticum extract; lane 4 mature seed extract incubated with vp5-osmoticum extract. 10 μg protein per lane.
In light of the results described here the model of MLG3 regulation proposed by Thomann et al. [1992] must be revised. It is shown here that vp5 embryos accumulate the protein under conditions of water stress in the absence of endogenous ABA. Regulation of MLG3 expression appears to operate via at least two pathways, one requiring ABA directly and a second which can be induced by water-stress independent of *de novo* ABA synthesis. At least one of these pathways does not require a functional Vp1 gene product. Since this work was undertaken, several genes showing this combinatorial type of regulation in response to dessication have been reported [Guerrero et al., 1990; Yamaguchi-Shinozaki and Shinozaki, 1992; reviewed by Bray, 1993]. The promoter of one of these genes contains ABA (ABRE) and drought response (DRE) elements which bind distinctly different nuclear proteins when assayed by gel retardation and DNA footprinting analysis [Yamaguchi-Shinozaki and Shinozaki, 1994]. Whether MLG3 shares these characteristics of combinatorial control awaits isolation of the genomic clone and dissection of its promoter function.
LITERATURE CITED


Cone KC (1989) Yet another rapid plant DNA prep. Maize Genet Coop Newslet. 63: 68


Guerrero FD, Jones JT, Mullet JE (1990) Turgor responsive gene transcription and RNA levels increase rapidly when pea shoots are wilted. Sequence and expression of three inducible genes. Plant Mol Biol 15: 11-26


Chapter Four

Evidence for a role for gibberellins in the precocious germination of maize (Zea mays L.) embryos

Constance N. White and Carol J. Rivin
Department of Botany and Plant Pathology
Program in Genetics
Center for Gene Research and Biotechnology
Oregon State University
Corvallis Oregon 97331-2902
ABSTRACT

The effects of abscisic acid (ABA) in suppressing precocious germination and promoting maturation of immature embryos is well-established. Immature maize embryos germinate when excised and placed in hormone-free medium, but in the presence of ABA, germination is inhibited and maturation-phase products accumulate. Using this cultured embryo model, we examined whether the gibberellin (GA) class of growth regulators might also be a factor modulating the germination vs. maturation programs. Two gibberellin biosynthesis inhibitors, ancymidol and paclobutrazol, were tested for their effects on germination and anthocyanin pigment accumulation in maize (Zea mays L.) embryos that were isolated at successive developmental stages. Gibberellin inhibitors applied to maturing wildtype embryos suppressed precocious germination and enhanced anthocyanin accumulation in a developmentally specific manner. These behaviors mimicked the effect of ABA on cultured embryos, and were reversed by the addition of exogenous GA$_3$. We also investigated the effects of GA inhibitors on viviparous mutants, one deficient in ABA (vp5) and one insensitive to ABA (vp1). Our results suggest that immature embryos require GA for germination and that GA antagonizes ABA-regulated anthocyanin synthesis. We hypothesize that antagonism between ABA and GA levels, rather than the concentration of ABA alone, controls developmental choice between maturation and germination programs in culture and in vivo.
INTRODUCTION

Embryo development in flowering plants can generally be separated into two sequential programs: embryogenesis, characterized by rapid growth and morphogenesis, is followed by a period of maturation in which storage macromolecules are accumulated and desiccation tolerance is acquired. Development closes with dehydration and quiescence or dormancy. Experiments with many species have shown that maturation is not an obligate process; embryos excised from the seed environment prior to maturation and placed in culture are capable of germination and post-embryonic development [reviewed by Kermode, 1990].

Cultured plant embryos have been widely used to model physiological cues for maturation. Experiments using rape, cotton, soybean, wheat and maize have identified abscisic acid (ABA) as a positive regulator of seed maturation processes. Isolated embryos of these species either initiate or maintain maturation processes when cultured in the presence of exogenous ABA. Typically, germination is suppressed and a battery of maturation-associated genes is expressed [for reviews see Kermode, 1990; Skriver and Mundy, 1990].

Strong evidence for the role of ABA in suppressing precocious germination in vivo comes from the study of viviparous (vp) mutants in maize [Robertson, 1955; Robichaud et al. 1980; Neill et al. 1886]. Mutant embryos fail to complete maturation and instead germinate precociously while still on the ear. Most vp mutants appear to be ABA-deficient, while one (vp1) is unique in that it has normal ABA levels but compromised sensitivity. Work by McCarty and his collaborators [1989; 1991] has tentatively identified the Vp1 gene product as being a transcriptional regulator of ABA-responsive genes. Treatment of maize embryos early in development with the
herbicide fluridone also results in vivipary by blocking synthesis of ABA by the embryo [Fong et al., 1983].

The rise in the level of ABA during seed development is consistent with the conclusion that ABA plays a critical part in determining the developmental path of the immature embryo by inhibiting precocious germination and modulating the expression of maturation phase genes [Neill et al., 1987; reviewed in Quatrano, 1987]. However, the flux in ABA levels and the timing of ABA-induced maturation events are not always perfectly correlated. In cotton and maize, for example, induction of Late embryogenesis abundant (Lea) gene expression occurs much later than the time of highest endogenous ABA levels even though many of these genes are known to require ABA for expression in culture [Galau et al., 1987; Thomann et al., 1992; White and Rivin, unpublished observations]. In maize this paradox also extends to germination behavior of immature embryos. Embryos excised at stage 2 or 3 (when endogenous ABA levels are known to be high) germinate readily when cultured with basal media. Stage 4 embryos, which have diminished ABA levels, are nonetheless quiescent in culture [Rivin and Grudt, 1991].

It has been suggested that altered water relations, rather than ABA levels, suppress germination late in maturation phase, a time when events associated with maturation drying occur (Lea gene expression, acquisition of desiccation tolerance). Indeed, in cultured embryos, osmotic stress alone can induce some maturation-associated events, leading to several suggestions in the literature that water stress is a more proximal regulator of some "ABA-regulated" genes [Finkelstein and Crouch, 1986; Galau et al., 1987].

An unexplored factor in any of these studies is the possible involvement of the gibberellin (GA) class of growth regulators. Although GA is known to be a strong...
positive regulator of germination processes in the mature grain of several cereals and to be antagonized by ABA in the control of some of those processes [reviewed in Jacobsen and Chandler, 1987], a role for GA in altering seed maturation processes has been little examined. A few reports exist of added GA down-regulating maturation-associated gene expression in culture [Leah and Mundy, 1989; Bartels et al., 1991; Hughes and Galau, 1991]. Also, viviparous maize embryos exhibit increased amylase and RNAse activities [Wilson, et al., 1973; White and Rivin, unpublished observations] which are known to be influenced by GA in embryos of other cereals. The role which GA plays in the germination of mature maize kernels is ambiguous. GA stimulation of amylase production has been shown only in endosperms of cultivars known to have lowered endogenous GA levels [Harvey and Oakes, 1974; Rood and Larsen, 1988] although this stimulation is enhanced by pretreatment with fluridone, suggesting that ABA antagonism of amylase synthesis, as widely documented in barley, also exists in maize [Oishi and Bewley, 1990].

In the experiments reported here, we examined the effects of GA inhibitors on two aspects of maturation phase behavior in cultured wildtype maize (Zea mays L.) embryos, suppression of germination and accumulation of anthocyanin pigments. These visual markers of maturation are ABA-responsive. We also investigated the effects of these inhibitors on viviparous mutants, one deficient in ABA (vp5) and one insensitive to ABA (vp1). Our results indicate that immature embryos require GA for germination and that GA antagonizes ABA-regulated anthocyanin synthesis. We suggest that antagonism between ABA and GA, rather than the concentration of ABA alone, may control developmental choice between maturation and germination programs in culture and in vivo.
MATERIALS AND METHODS

Plant Material

The maize (Zea mays L.) viviparous mutants vp5, vp1, and vp1-McWhirter (vp1mc) were obtained from D. Robertson (Iowa State University). The inbred line W22 used in this research was originally obtained from J. Kermicle (University of Wisconsin). It carries the R-sc allele. Stock constructions carrying R-r and R-g alleles originated from the vp1 and vp1mc lines respectively.

All stocks were propagated in Corvallis, Oregon. Homozygous vp5 kernels were identified on segregating ears by their lack of carotenoid pigments. These mutant kernels showed 100% precocious germination by stage 4 of development. Kernels homozygous for the vp1 allele were obtained from homozygous mutant plants propagated from precociously germinating seeds or were identified on segregating ears by the absence of anthocyanin pigmented aleurone. Kernels homozygous for the vp1mc allele were unpigmented but dormant.

Embryo Culture

Plants were field-grown in 1992. Ears were harvested between 18 and 30 days after pollination (DAP) and surface sterilized in 2.5% sodium hypochlorite. Embryos were removed aseptically and staged morphologically by examining leaf primordia development according to the scheme of Abbe and Stein [1954]. Stages 2, 3, and 4 of embryo development typically corresponded to 18-22, 23-27, and 28-30 DAP respectively. In the experiments where viviparous mutants were used, care was taken to sample embryos prior to detectable vivipary (onset is normally 26 to 30 DAP for vp 5 mutants and 30 to 35 DAP for vp1 mutants). For culture in petri
dishes, 10-25 embryos were placed scutella down on filters saturated with a growth medium consisting of Murashige and Skoog medium (Sigma) supplemented with 3% sucrose, 100 mg/L myoinositol and 0.4 mg/L thiamine HCl plus growth regulators where noted. ABA (mixed enantiomers), ancymidol, and GA3 were purchased from Sigma. Paclobutrazol was obtained from ICI Americas via the kind gift of William Proebsting (Oregon State University). Cultures were incubated at 26° C in the dark. Germination was scored daily based on the criterion of radicle emergence from the coleorhiza. Unless otherwise noted, data were pooled from replicate experiments and values are given as mean ± sem.

Anthocyanin Determinations

Anthocyanins were extracted from embryos with acidified methanol and absorbance determined at 530 nm in the linear range [Mancinelli et al., 1988].

RESULTS

Maturing embryos require GA for germination in culture

Wildtype maize embryos germinate when they are removed from the seed and placed in a hormone-free culture medium. The rate of germination and the percentage of embryos that ultimately germinate are different for each developmental stage [Rivin and Grudt, 1991]. To determine whether the germination of immature embryos requires GA synthesis, we tested the effect of two GA synthesis inhibitors, paclobutrazol and ancymidol, on isolated embryos cultured at successive stages of development.
The embryos were dissected from sib-pollinated ears at times corresponding to stages 2, 3, and 4 (early, mid, and late maturation phase). Samples of staged embryos from several ears were placed in GM (hormone free media), in GM plus 10 or 100 µM ancymidol, in GM plus 10 or 100 µM paclobutrazol, and in GM plus 10 µM ABA. Germination was measured daily for eight days. The results (Figure IV.1) indicate that GA synthesis inhibitors can decrease both the rate of germination and the fraction of embryos that germinate, and that these effects are dependent on the developmental stage. Stage 2 embryos readily germinated in GM and were completely suppressed by the presence of ABA. Neither GA synthesis inhibitor had much effect on the germination of these embryos. Stage 3 embryos also germinated readily in GM and showed complete suppression with ABA. Germination of these embryos was also strongly suppressed by the presence of the GA synthesis inhibitors. Stage 4 embryos were nearly quiescent when cultured on GM alone. They were completely arrested by 10 µM concentrations of ABA and by either GA synthesis inhibitor.

In order to determine whether the effects of ancymidol and paclobutrazol were mediated by a decline in GA levels, we tested whether the addition of exogenous GA would restore germination to embryos cultured in the presence of these inhibitors. The addition of either 10 or 100 µM GA3 to stage 3 embryos cultured in 100 µM paclobutrazol restored germination levels to those of stage 3 embryos cultured on GM alone (Figure IV.2). This indicates that paclobutrazol suppresses the germination of these embryos by suppressing de novo GA biosynthesis. Similar results were obtained with ancymidol cultures (data not shown). Exogenous GA3 alone also decreases the lag time to germination in stage 3 embryos (Figure IV.3A). The
addition of exogenous GA3 also induces rapid and complete germination of stage 4 embryos (Figure IV.3B), which remain ungerminated in hormone-free medium.

Taken together, these results imply that gibberellins are required for the germination of immature embryos in culture and that there is a marked change in either GA sensitivity or GA content as embryos progress from early to late maturation phase. Additionally, germination kinetics are positively correlated with GA availability.
FIGURE IV.1. Germination of wildtype embryos isolated at successive developmental stages when cultured in the presence and absence of growth regulators and inhibitors.

(A, B, C) Stage 2 embryos; (D, E, F) Early stage 3 embryos; (G, H, I) Late stage 3 embryos, (J, K, L) Stage 4 embryos. Cultures were maintained for eight days on hormone-free growth medium, GM (Δ), or on GM supplemented with 10 μM ABA (▲), 10 μM ancymidol (○), 100 μM ancymidol (●), 10 μM paclobutrazol (□), 100 μM paclobutrazol (■). Where required for clarity, error bars are shown in only one direction.
FIGURE IV.2. Effects of exogenous GA3 on the germination of paclobutrazol-treated embryos.

Wildtype embryos at late stage 3 were isolated and cultured for eight days in GM +100 μM paclobutrazol and either 10 μM GA3 (■) or 100 μM GA3 (○).
FIGURE IV.3. Effects of exogenous GA$_3$ on the germination of wildtype embryos in culture.

Wildtype embryos at (A) Late stage 3, or (B) Stage 4 were cultured for eight days on GM alone (Δ) or on GM supplemented with 10 μM GA$_3$ (■) or 100 μM GA$_3$ (O).
Cultured *viviparous* embryos are relatively insensitive to GA synthesis inhibitors

*Viviparous* mutants germinate precociously within the developing seed environment. These mutants have defects in ABA synthesis or responsiveness, implicating ABA in the suppression of precocious germination. Therefore, it was of interest to ask whether *viviparous* mutant embryos also require de novo GA synthesis for germination in culture. Two types of *viviparous* mutants were tested: *vp5* embryos are ABA deficient; *vp1* embryos synthesize normal levels of ABA but are not responsive to the hormone.

When placed in culture, the *viviparous* mutant embryos displayed a different germination behavior than their wildtype sibs (Figure IV.4). The *vp5* embryos were isolated at stage 3 prior to detectable vivipary. These mutants germinated much more rapidly on hormone-free medium than wildtype stage 3 embryos. Supplementing the medium with 10 mM ABA suppressed germination, as previously reported [Robichaud et al., 1980; Rivin and Grudt, 1991]. In contrast to wildtype stage 3 embryos, *vp5* embryos cultured with paclobutrazol showed a considerable lag, but went on to germinate at a level comparable to embryos cultured on the basal medium (Figure IV.4A).

When *vp1* embryos were cultured under the same conditions, they showed no significant inhibition of germination by ABA or paclobutrazol (Figure IV.4B). However, shoot elongation rates were severely reduced in this and in the other *viviparous* mutants cultured with GA inhibitors, indicating that these embryos were sensitive to GA levels in this aspect of their growth (data not shown). We also tested the response of embryos homozygous for a different *vp1* allele, *vp1mc*. Like the standard *vp1* mutants, this mutant is compromised in kernel anthocyanin formation, but it is thought to have normal dormancy function [McCarty et al., 1991]. In the
culture assay with ABA and paclobutrazol, \( vp1^{mc} \) embryos displayed an intermediate sensitivity. They were not completely suppressed, but were substantially delayed in germination (Figure IV.5). In this and all experiments with \( vp1 \) and \( vp5 \) mutants, ancymidol cultures gave results highly similar to those with paclobutrazol (data not shown).

The data from \( vp1 \) and \( vp5 \) cultures combined with that obtained from wildtype embryos indicates that there is a dramatic transition in developmental programs accompanying the progression from stage 2 to stage 3. *Viviparous* embryos of either class appear to be "ready-to-go" after entrance to stage 3 and are relatively unaffected by inhibition of \textit{de novo} GA synthesis.
FIGURE IV.4 Germination of *viviparous* mutant embryos in culture.

(A) *vp5*, and (B) *vp1* embryos cultured for eight days in either GM (△), or GM supplemented with 10 μM ABA (▲) or 100 μM paclobutrazol (■)
FIGURE IV.5 Germination of cultured embryos carrying vp1 (viviparous) and vp1-mcwhirter (dormant) alleles.

Wildtype embryos (Δ), and embryos homozygous for either vp1 (O) or vp1° (●) were isolated at stage 4 and cultured for eight days in (A) GM; (B) 100 μM paclobutrazol (C) 10 μM ABA. Similar results were obtained from stage 3 vp1° embryos cultured under parallel conditions.

FOLLOWING PAGE
Fig. IV.5A
GM

Fig. IV.5B
PAC

Fig. IV.5C
ABA

% GERMINATION

DAYS IN CULTURE
Developmentally-regulated anthocyanin synthesis in cultured embryos is antagonized by GA

An apparent antagonism of embryo ABA and GA was also seen in the accumulation of anthocyanin pigments in cultured embryos. In vivo, anthocyanin synthesis in the embryo requires a functional Vp1 gene product in addition to an appropriate allele of the R locus. R encodes a transcriptional activator that functions in combination with the C1 gene product to activate structural genes along the anthocyanin biosynthetic pathway [Roth et al., 1991]. Many R alleles exist, but only a few condition anthocyanin synthesis in embryo tissue. A direct ABA requirement for kernel anthocyanins has also been shown [Smith and Cobb, 1989] that may exert itself via VP1 regulation of C1 expression [McCarty et al., 1989].

On the ear, embryos that carry the R-sc allele begin to accumulate significant anthocyanin pigments in early stage 4. Isolated stage 4 embryos cultured in GM alone show vigorous anthocyanin accumulation, and precocious pigmentation can be induced in younger embryos by culturing them in the presence of 10 μM ABA (Figure IV.6A). The addition of paclobutrazol or ancymidol similarly results in anthocyanin accumulation in embryos, an effect that is reversed by supplementing inhibitor-treated cultures with exogenous GA3 (Figures IV.6B, IV.7). We suspect that anthocyanin synthesis in cultured embryos relies on ABA whose action or synthesis is antagonized by increased GA levels. If fluridone is added to stage 2 cultures along with paclobutrazol, anthocyanin accumulation is diminished in comparison to treatment with paclobutrazol alone (data not shown).

Anthocyanins in maize accumulate in a developmentally and tissue specific manner, but they can also be synthesized in response to stress. In order to correlate the regulation of anthocyanin accumulation in vitro with developmentally programmed pigment accumulation in vivo, we tested whether the synthesis of
anthocyanins in cultured embryos was dependent on an appropriate $R$ allele. Embryos carrying either $R$-r, $R$-g (both purple aleurone, yellow embryo), or $R$-sc (purple aleurone, purple embryo) were cultured in the presence of exogenous ABA or GA synthesis inhibitors. The results (Figure IV.8) indicate that anthocyanin accumulation in cultured embryos requires the presence of the $R$ allele that conditions embryos anthocyanin accumulation in vivo. This suggests to us that the GA and ABA effects seen in vitro are being exerted via developmentally specific regulatory pathways and not as a result of a non-specific stress response.
FIGURE IV.6 Effects of developmental stage and growth regulators on anthocyanin accumulation.

(A) Anthocyanin accumulation in embryos isolated at successive developmental stages and cultured for eight days in GM or GM supplemented with 10 μM ABA. (B) Anthocyanin accumulation in stage 3 embryos cultured for eight days in GM alone or in GM supplemented with ABA, ancyimidol (ancy), paclobutrazol (pac) or 100 μM paclobutrazol with two concentrations of GA3. Anthocyanin extraction and measurement are described in Methods.
FIGURE IV.7 Anthocyanin accumulation in cultured embryos.

Stage 4 embryos were cultured for one day in (A) GM, or in GM supplemented with (B) 10 μM ABA, (C) 10 μM GA3, (D) 100 μM paclobutrazol, (E) 100 μM paclobutrazol and 10 μM GA3, (F) 100 μM paclobutrazol and 100 μM GA3.
FIGURE IV.8. Anthocyanin accumulation in cultured embryos requires the $R$-sc allele.

Stage 3 embryos carrying different $R$ alleles were cultured for 8 days on GM, or GM supplemented with 10 $\mu$M ABA, 100 $\mu$M ancymidol, or 100 $\mu$M paclobutrazol. Anthocyanin extraction and measurement are described in Methods.
DISCUSSION

The critical role played by ABA in regulating transit from embryogenesis to maturation is supported by experiments using cultured maize embryos and viviparous mutants. The observation of powerful effects by ABA on both precocious germination in culture and vivipary in vivo have led to the implicit idea that the potential to germinate is a default state throughout maize embryo development. In this scheme, failure to germinate reflects the continuous action of a germination suppressor, so that in early maize development inhibition may be attributed to the presence of ABA, while later in embryogeny, desiccation is thought to play an important role.

An alternate model of precocious germination and vivipary can also be entertained, under which positive cues for germination are available during embryo development. One such positive cue for germination could be signalled by the gibberellins. By testing the effects of GA synthesis inhibitors on cultured embryos, we found evidence for a GA-response in two visual markers of the developmental switch between germination and maturation. When de novo gibberellin synthesis was inhibited in wildtype embryos, precocious germination was suppressed and developmentally specific anthocyanin accumulation occurred. The behaviors mimicked the effect of ABA on cultured embryos, and they were reversed by the addition of exogenous GA, suggesting that an ABA/GA balance regulates the maturation and germination programs. The increasing sensitivity of wildtype embryos to GA inhibitors may be explained by either a decline in GA levels or sensitivity (or both) as embryos progress from early to late maturation. We speculate that a high GA/ABA ratio will switch embryos to the germination pathway while a high ABA/GA ratio will program maturation processes instead.
This notion is also consistent with the observation that diminished GA levels block germination only in embryos able to synthesize and sense ABA. Our results from cultured viviparous embryos indicated that GA synthesis was not required for their germination in vitro. It may be that vivipary is independent of GA. Alternatively, GA-mediated commitment to germination may have occurred in these mutants prior to excision. The dramatic decrease in the lag time to germination of these embryos in basal media alone suggests that the latter possibility may be correct. This interpretation is also supported by preliminary experiments in which in vivo treatment of vp5 segregating ears with ancymidol resulted in a nearly complete suppression of vivipary (data not shown). Regardless of the possible role for GA in vivipary, the differing response of wildtype and viviparous mutant embryos to the presence of GA synthesis inhibitors implies that germination suppression by these inhibitors relies on the presence of endogenous ABA.

The intermediate response of the embryos homozygous for the vp1\textsuperscript{mc} allele was unexpected. The phenotype of this allele has suggested to some that the Vp1 gene product is discretely bifunctional, with separable regulatory domains governing anthocyanin synthesis and dormancy [McCarty et al., 1991]. The results presented in this report, coupled with our (unpublished) observation that vp1\textsuperscript{mc} embryos exhibit reduced expression of several maturation associated genes, suggests that this allele may be generally impaired in maturation functions. Some features of this impairment can be masked by maintenance in the seed environment, perhaps due to the presence of maternal ABA.

In normal situations, embryos follow either specific maturation or germination programs. However, in genetically or experimentally perturbed conditions, there may be simultaneous expression of aspects of both pathways, perhaps as a result of
perturbed ABA/GA ratios. For example, Hughes and Galau [1991] found that excised cotton embryos simultaneously express a post abscission program (characterized by Lea gene expression) and a germination program (characterized by the accumulation of germination-associated mRNAs). Treatment of these cultured embryos with either ABA or GA could shift the magnitude of either program's expression in a manner consistent with our model. Likewise, Kriz et. al. [1990] found that several ABA-deficient viviparous mutants express the ABA regulated Glb 1 message, even while preparing for vivipary. Perhaps the maternal ABA contribution, which may be up to 50% of seed ABA [Smith et al., 1987] is sufficient to induce some maturation processes, but does not achieve sufficient levels to present the proper ABA/GA ratio for dormancy. The obverse could account for the germination of embryos removed from the maternal environment. If embryo programming is exquisitely sensitive to hormone balance, then removal from a maternal ABA source may be sufficient for developmental reprogramming.

Our results suggest that the idea of hormone balance previously used to model germination processes in mature seed may have application to the regulation of seed development. Gibberellins are known to accumulate in immature seeds of a variety of species, including cereals, but their role in regulating seed development is not well understood [reviewed in Jacobsen and Chandler, 1987; Quatrano, 1987]. In several dicot species, the suspensor has been shown to be a rich source of gibberellins. In Phaseolus coccineus, gibberellins synthesized in suspensor tissue are required for the growth of young embryos maintained in culture [Cionnini et al. 1976; Yeung and Sussex1979]. No similar study has been made with monocots. It is plausible that gibberellins stimulate early grain growth that, if left unchecked, will continue on to germination. For development to proceed towards a dry, quiescent seed, a negative
effector (presumably ABA) must act. In studies examining the response of GA-regulated germination-associated genes, ABA has often been found to act as a negative regulator of their expression [Koehler and Ho, 1990 and citations therein]. We obtained parallel results with maize embryos cultured in the presence of both of these hormones. ABA-treated embryos will not germinate even in the presence of exogenous GA3 [White and Rivin, unpublished observations].

Finally, our model could account for the lack of correspondence of embryo ABA levels with ABA-induced gene expression. It is possible that the balance of GA and ABA levels, rather than the absolute concentration of ABA itself may be responsible for induction of late maturation events. With this interpretation, relatively low ABA in the absence of GA may exert a more powerful induction than higher ABA levels in combination with GA. Such a model obviates the need to entertain alternate regulatory pathways, such as those that have been proposed for water stress or local concentration effects. In this view, late-maturation expressed genes (such as the Lea messages) may constitute a class of "Gib-off" (or more correctly "Gib-down") genes, as described for the BAS1 gene of barley [Lea and Mundy, 1989]; indeed there is evidence that this may be the case [Hughes and Galan, 1991]. Although ABA levels have been measured throughout maize kernel development, there are no parallel studies of the GA content of maize seeds [Murofushi et al., 1991]. A direct test of this model would be the measurement of GA and ABA levels throughout embryo development as well as direct examination of GA and ABA antagonism on the expression of maturation associated events other than anthocyanin synthesis.
LITERATURE CITED


Finkelstein RR, Crouch ML (1986) Rapeseed embryo development in culture on high osmoticum is similar to that in seeds. Plant Physiol 81: 907-912


Robertson DS (1955) The genetics of vivipary in maize. Genetics 40: 745-760


Chapter Five

Effects of altered gibberellin metabolism on viviparous maize kernels developing on the ear: correlations of endogenous gibberellin levels with precocious germination in vitro and in vivo

Constance N. White¹, William R. Proebsting², Peter Hedden³, and Carol J. Rivin¹

1. Department of Botany and Plant Pathology, Center for Gene Research and Biotechnology, Oregon State University, Corvallis, OR 97331-2902

2. Department of Horticulture, Oregon State University, Corvallis, OR 97331-7304

3. Department of Agricultural Sciences, University of Bristol, Agricultural and Food Research Council Institute of Arable Crops Research, Long Ashton Research Station, Long Ashton, Bristol BS18 9AF United Kingdom
ABSTRACT

The hormone abscisic acid (ABA) plays an important role in suppressing precocious germination of developing seeds. *Viviparous* (vp) mutants of maize do not mature on the ear but rather germinate midway through kernel development. Most vp mutants exhibit reduced ABA levels but one (vp1) suffers from insensitivity to the hormone. Gibberellins stimulate germination processes in a variety of plant species and are essential for germination of maturation stage maize embryos *in vitro*. Using viviparous maize kernels we examined whether gibberellins (GAs) are required for precocious germination on the ear. Vivipary that results from diminished ABA levels could be suppressed by either chemical or genetic reduction of GA levels in immature kernels. In contrast, reduction of endogenous GAs did not suppress vivipary of the ABA-insensitive mutant vp1. Temporal analysis of GA accumulation in developing kernels reveals the accumulation of two bioactive species (GA1 and GA3) during a developmental window just prior to peak ABA levels. We hypothesize that these species stimulate a developmental program leading to vivipary in the absence of sufficient levels of ABA and that reduction of GA levels reestablishes an ABA/GA ratio appropriate for suppression of germination and induction of maturation in ABA-deficient kernels. The failure to suppress vivipary via reduction of GA levels in the ABA-insensitive mutant vp1 suggests that the wildtype gene product functions downstream of the sites of GA and ABA action in regulation of maturation versus germination.
INTRODUCTION

Developing plant embryos have the capacity to germinate or to mature. After early embryogenesis is complete, seeds begin to mature with the synthesis of storage molecules and the acquisition of desiccation tolerance. Maturation is not a obligate process; embryos cultured prior to maturation can form normal seedlings [reviewed by Kermode, 1990]. Induction of maturation appears to rely on the hormone abscisic acid (ABA). Germination in vitro may be suppressed and many maturation-associated processes can be induced with exogenous ABA [reviewed by Skriver and Mundy, 1990]. In addition, mutants of Arabidopsis, tomato, and maize which are deficient in either synthesis or response to ABA suffer from impaired maturation [Robertson, 1955; Koornneef and van der Veen, 1980; Koornneef et al., 1981; Neill et al., 1986]. In maize these viviparous (vp) mutant precociously germinate while still on the ear at ca. 25-35 days after pollination (DAP). Like wildtype embryos, in vitro germination of ABA-deficient embryos (vp2, vp5, vp7, vp8, vp9) is suppressed by exogenous ABA, while one insensitive mutant (vpl) germinates efficiently even when cultured with this hormone [Robichaud et al., 1980]. Finally, treatment of maize embryos early in development with the herbicide fluridone also results in vivipary, by blocking synthesis of ABA in the embryo [Fong et al., 1983].

The importance of ABA in promoting maturation has focussed attention on its regulation of seed development and the genes underlying this developmental program. Much of this work implicitly considers germination to be a default developmental program that is suppressed by an appropriately timed ABA signal. While such a model is attractive in its simplicity, we have found that the germinability of excised maize embryos varies in a stage-specific manner that cannot be directly correlated with endogenous ABA levels. Undesiccated stage 2 and 3 embryos (18-22 and 23-27 DAP
respectively) germinate efficiently in culture while Stage 4 embryos (28-30 DAP) will not germinate unless treated with acid pH or the fungal toxin fusicoccin, although endogenous ABA levels are quite low at this developmental stage [Rivin and Grudt, 1991]. Similar results have been reported in wheat [Radley, 1976]. We wondered if a positive factor was needed for germination of immature maize embryos.

Because of the well established role for gibberellins (GAs) in mediating many germination processes, we chose to examine its role in our cultured embryo system. In another paper [White and Rivin, manuscript in preparation] we showed that GA$_3$ could induce germination of refractory stage 4 embryos in culture. Moreover, inhibitors of gibberellin biosynthesis could substitute for ABA in suppressing the germination of stage 3 wildtype embryos. The sensitivity of embryos to GA inhibitor treatment increased with developmental age, with stage 2 embryos being relatively insensitive to inhibitors and stage 3 and 4 embryos increasingly arrested under these culture conditions. In contrast, viviparous mutant embryos (vp5 and vp1) germinate efficiently even when GA synthesis is blocked.

On the basis of these effects, coupled with the fact that ABA is known to antagonize a number of germination processes in mature seed [reviewed by Jacobsen and Chandler, 1987], we proposed that developmental choice between germination and maturation may be mediated not solely by ABA alone but rather results from an interplay of this hormone with GA in immature embryos. In this scheme, cultured stage 2 embryos do not require de novo GA synthesis for germination either because endogenous GA levels are sufficient or because GAs have already activated a germination program that is unmasked by excising and culturing embryos away from the seed and maternal environment. This model made some testable predictions regarding the temporal pattern of GA and ABA: if ABA acts as a repressor of GA-mediated
germination processes then GA should accumulate prior to or coincident with peak ABA levels. In addition, if vivipary is induced by accumulating GAs, reduction of GA levels should suppress precocious germination. We sought to test this hypothesis by 1) testing whether decreased GA levels affect germination of viviparous kernels developing on the ear and 2) examining accumulation of both GA and ABA in developing wildtype embryos. Results presented here show that reduction of endogenous GA levels can suppress precocious germination of ABA-deficient kernels developing on the ear. In addition, of the number of GA species detected in developing embryos, species with greatest bioactivity are present at higher levels in stage 2 kernels, prior to peak ABA levels, and decline as embryos transit from stage 2 to stages 3 and 4 of development.

**MATERIALS AND METHODS**

**Plant material**

The maize *viviparous* mutants *vp1* and *vp5* were obtained from D. Robertson (Iowa State University) and have been propagated in primarily W22 backgrounds. Inbred W22 was originally obtained from J. Kermicle (University of Wisconsin). The GA-deficient dwarf mutant *d1* was originally obtained from the Maize Genetics Cooperative. All stocks were propagated in OSU greenhouse facilities and at the Botany and Plant Pathology Department field farm (Corvallis, OR). *vp5* must be maintained as a heterozygote since carotenoid-deficiency is seedling-lethal; homozygous *vp5* kernels were identified on segregating ears by their lack of carotenoid pigments. The *vp1* mutation affects only seed-specific processes and results in precocious germination and impaired kernel anthocyanin synthesis. Kernels homozygous for the *vp1* allele were obtained from homozygous mutant
plants propagated from precociously germinating seeds. The experiments reported here were undertaken in 1992 and 1993 summer plots.

**Kernel treatment with hormone biosynthesis inhibitors**

Paclobutrazol and ancymidol were obtained in a commercial formulations with surfactant ("Bonzi", ICI Americas Inc. and "A-rest", Dow Elanco respectively). Dilutions to 100 µM were made with water according to manufacturer's instructions. Gibberellic acid (GA₃) was obtained from Sigma Chemical Co. and diluted to 100 µM according to manufacturer's instructions. Fluridone was obtained from Elanco and diluted to 150 µg/ml in 1% acetone [Fong et al., 1983].

Ears were treated in the following manner [Fong et al., 1983]: following hand pollination, ears were covered and allowed to develop for various time periods. Kernels on one half of each ear were then exposed by peeling back the husk and removing the silks and then treated with saturating sprays of growth regulator solutions; husks were then replaced and the ears re-covered with water proof bags.

Ears were harvested at 55 - 60 DAP, dried, and individual kernels shelled and scored. Kernels to be scored were taken from the center of the treatment area. Generally this meant taking kernels from the middle 3 or 4 rows in strips of approximately 3 inches (30 to 50 kernels), except in the case of segregating vp5 ears where the scant number of mutant kernels necessitated sampling from a wider zone of the treatment area. Scoring criteria for germination were based on plumule extension past the scutellar border and on swelling or necrosis of the embryo or overlaying pericarp. Embryos having a single one of these characteristics were scored positive for germination even if plumule extension was not excessive. Data were pooled
from a minimum of three ears per treatment and values are given as mean percentage germinating ± sem.

**Hormone extraction and quantitation**

Five gram samples of flash frozen kernels were homogenized in 80% methanol and stirred overnight at 40°C. GAs were extracted and analysed according to previously published methods [Proebsting et al., 1992]. To determine recoveries and elution profiles, tritiated species of GAs and ABA were added to tissue prior to homogenization. Reversed phase HPLC, using a gradient of 30 to 100% methanol in acetic acid, allowed separation of GA species and ABA. Combined HPLC fractions were converted to methyl ester trimethylsilyl ethers and analysed by gas chromatography-mass spectroscopy. ABA cofractionated with bulk polar GA species but was resolved upon HPLC (ABA elutes just prior to GA20 in the separation system described above).

**RESULTS**

**Inhibition of GA synthesis suppresses germination in ABA-deficient but not ABA-nonresponsive viviparous kernels**

We tested the idea that GA/ABA balance, rather than absolute levels of ABA, regulates precocious germination by reducing GA levels in ABA-deficient kernels and assaying for reduction of vivipary. Both genetic and chemical means were used to manipulate hormone levels. The maize *vp5* mutant is deficient in ABA due to a block in carotenoid synthesis [Neill et al., 1986]. These mutants fail to undergo maturation and instead precociously germinate while still on the ear. In addition, a chemically-
induced phenocopy of \(vp5\) was produced by treatment of 11 DAP wildtype kernels with the herbicide fluridone, an inhibitor of ABA and carotenoid biosynthesis [Fong et al., 1983]. Reduction of endogenous GA levels was accomplished either with the use of biosynthesis inhibitor sprays (paclobutrazol and ancymidol) or with the use of a GA-deficient dwarf mutant, \(d1\). Ancymidol and paclobutrazol retard oxidation of ent-kaurene to ent-kaurenioic acid [Rademacher et al., 1984], thus both block GA synthesis at a step in the pathway close to the \(d5\) lesion (Figure V.1)). The \(d1\) mutation impairs conversion of GA\(_{20}\) to GA\(_1\), a much later step in the pathway. GA and ABA share a common precursor in mevalonic acid (MVA).

**Figure V.1:** Hypothetical GA biosynthetic pathway for maize (from Phinney and Spray, 1985)

![Hypothetical GA biosynthetic pathway for maize](image)

We assayed the effects of treating either fluridone-treated or \(vp5\) kernels with paclobutrazol or ancymydol between 9 and 20 DAP. As expected, treatment of
wildtype kernels with fluridone alone induced a substantial amount of vivipary. A second treatment at 11 DAP with either 100 µM ancymidol or paclobutrazol (Figure V.2) completely suppressed vivipary. Treatment at subsequent time points was less effective with either inhibitor, although paclobutrazol uniformly reduced vivipary to ca. 30% at later treatments while ancymidol gradually diminished in effectiveness. Treatment of fluridone-treated kernels with ancymidol at 20 DAP (stage 2) had no significant effect. This observation is in concordance with our previous results that paclobutrazol is more effective in suppressing germination of embryos cultured at any developmental time point [White and Rivin, manuscript in preparation].

Vivipary of vp5 kernels treated with either ancymidol or paclobutrazol at 9 DAP was severely suppressed (Figure V.3). As with fluridone-treated kernels, effectiveness of ancymidol decreases with increasing kernel age at time of treatment. Treatment with either inhibitor at 13 DAP or points beyond no longer inhibited vivipary.

Our presumption was that both of the GA-inhibitors used were exerting their effect by reducing kernel GA levels. To test this hypothesis, we asked whether treatment with GA3 could restore germination to GA-inhibitor-treated kernels. vp5 embryos treated at 9 DAP with either 100 µM GA3 plus 100 µM ancymidol or 100 µM GA3 plus 100 µM ancymidol showed substantially greater germination than ears treated with GA-inhibitor alone (Figure V.4). This strongly suggests that ancymidol and paclobutrazol both suppress vivipary via a reduction in kernel GA synthesis.

GA-inhibitors were also tested on another viviparous maize mutant, vp1. Unlike other maize viviparous mutants, this mutant has normal ABA levels but appears unable to respond to the hormone either in vitro or in vivo [Robichaud et al., 1980; Neill et al., 1986]. The Vp1 gene has been isolated and is proposed to act as a
transcriptional activator responsive to ABA [McCarty et al., 1989; 1991]. In contrast to the results with ABA-deficient viviparous kernels, vp1 germination was not suppressed by treatment with either ancymidol or paclobutrazol at times between 6 and 26 DAP (data not shown). Plumule length was uniformly reduced on kernels sprayed with either inhibitor, indicating that the treatments did alter GA levels, however pericarp swelling coupled with either plumule greening or embryo necrosis occurred on all treated ears, indicating that vivipary had occurred.
FIGURE V.2. Suppression of fluridone-induced vivipary by treatment with GA biosynthesis inhibitors.

A) Fluridone-treated W22 inbred wildtype kernels were treated at the times indicated with 100 μM ancymidol and scored at maturity for germination (▲).
B) Fluridone-treated W22 inbred wildtype kernels were treated at the times indicated with 100 μM paclobutrazol and scored at maturity for germination (■).

All fluridone treatments were performed at 11 DAP. Vivipary of control ears treated with fluridone only given for each treatment set (○).
FIGURE V.3 Suppression of vp5-induced vivipary by treatment with GA biosynthesis inhibitors.

A) vp5 kernels were treated at the times indicated with 100 μM ancymidol and scored at maturity for germination (▲).
B) vp5 kernels were treated at the times indicated with 100 μM paclobutrazol and scored at maturity for germination (■).

Vivipary of mutant kernels from the untreated side of ears from each treatment set is indicated. (○).
FIGURE V.4 Restoration of vivipary to vp5 inhibitor-treated kernels with GA3 treatment.

vp5 kernels were treated at 9 DAP with either 100 µM ancymidol (ANC) or 100 µM paclobutrazol (PAC) alone or in combination with a second treatment of 100 µM GA3 (GA).
In addition to the chemical manipulations of hormone levels, we sought to reduce endogenous kernel gibberellins by genetic means. The *d1* mutation blocks conversion of GA$_{20}$ to GA$_{1}$ and results in a dwarfed plant when carried in a homozygous state. We tested whether the *d1* mutation could suppress vivipary when combined in double mutants with the ABA-insensitive mutant *vp1* or the ABA-deficient mutant *vp5*.

To construct *vp1-d1* double mutants, *vp1* homozygous plants were crossed with *d1* homozygotes and resulting double heterozygotes were self-pollinated. Wild type kernels from these ears were planted and the resulting plants were scored for dwarfism. To assay for suppression of vivipary by the *d1* mutation, dwarf plants were self-pollinated and the resulting kernels were examined for precocious germination. To ensure against misscores in the event of phenotype suppression, reciprocal crosses were made between these *vp1* dwarf plants and standard *vp1* homozygote stocks.

Segregation ratios of wildtype to dwarf plants resulting from *vp1 d1* stock constructions conformed to the 3:1 expectation for an F$_2$ population (Table V.1, $X^2$ = .0526, df = 1). While independent segregation of *d1* from *vp1* could not be statistically verified owing to the small sample size, it is apparent that kernel vivipary was not suppressed in dwarf plants. Similarly, segregation ratios of *viviparous* versus wildtype kernels on normal plants (2/3 of which would be expected to be *d1 / +*) were not altered from the 3:1 ratio expected (data not shown). The appearance of two *vp1 / vp1* plants was probably a result of miscoring kernels for dormancy prior to planting since none of the original kernel stock was dried to completion prior to propagation. These results show no epistasis between the *d1* and *vp1* mutations. Vivipary resulting from the *vp1* mutation occurs in kernels of *d1* homozygotes as
readily as in those of plants having normal GA₁ levels. However, as with the results using chemical inhibitors, plumule elongation was suppressed on vp1 kernels developing on dwarf mother plants relative to that of kernels developing on non-dwarf mother plants (data not shown).

Table V.1: vivipary of vp1/d1 mutants

<table>
<thead>
<tr>
<th>plant height</th>
<th>+/+</th>
<th>+/vp1</th>
<th>vp1/vp1</th>
<th>?</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>wildtype</td>
<td>1</td>
<td>20</td>
<td>1</td>
<td>22</td>
<td>44</td>
</tr>
<tr>
<td>dwarf</td>
<td>7</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>total</td>
<td>8</td>
<td>25</td>
<td>2</td>
<td>22</td>
<td>57</td>
</tr>
</tbody>
</table>

To obtain vp5 segregants in d1 plants, vp5 heterozygotes were crossed with d1 homozygotes and the resulting F1 kernels were propagated and self-pollinated. Ears resulting from these self-crosses were scored for vivipary. Dormant seeds from segregating ears were planted and the plants scored for dwarfism. We succeeded in obtaining one ear from the self-pollination of a bonafide vp5/+ , d1/d1 plant. vp5/vp5 segregants from this ear resembled wildtype sibling kernels in every way except for the absence of carotenoids (Figure V.5). Self-pollinated ears from sibling plants of the genotype vp5/+ , d1/+ all showed normal levels of vivipary (data not shown), indicating to us that suppressed maternal GA synthesis may be important in obtaining this result.
**FIGURE V.5** *vp5* segregants from *d1* ear. Left are wildtype kernels, right are *vp5* mutants.
Reduction of GA levels in ABA-deficient viviparous kernels results in desiccation-tolerant, germinable seed

Remarkably, even after extensive drying these kernels germinated, albeit at very delayed rates, to produce albino seedlings thus verifying presence of the \(vp5\) mutation (Figure V.6A). It was not obvious that suppression of germination in these or any other kernels by reduction of GAs would necessarily confer desiccation-tolerance. We tested for germination of inhibitor-treated \(vp5\) kernels which had been dried and stored for ten months at room temperature. Like the double \(d1\ \ vp5\) mutant kernels, they also germinated but at a very delayed rate compared to their wildtype inhibitor-treated sibling kernels (Figure V.6B and 6C).
FIGURE V.6A Germinating $vp5$ $d1$ double mutant kernels.

Kernels were dried and stored for ten months. Following surface sterilization, kernels were placed on water-moistened filter papers in magenta vessels and incubated at 26°C in the dark for two weeks. Picture at left are wild type sibling kernels from same ear germinated in parallel with mutant kernels (right).
FIGURE V.6B Germinating ancyimidol-treated \textit{vp5} kernels.

Kernels from \textit{vp5} segregating ears were treated with 100 $\mu$M ancyimidol at 11 DAP. After dry storage for ten months, kernels were germinated as described in Figure V.6A. Pictured at top are wildtype kernels taken from same treatment area and germinated in parallel with mutant kernels (bottom).
FIGURE V.6C Germinating paclobutrazol-treated *vp5* kernels.

Kernels from *vp5* segregating ears were treated with 100 μM paclobutrazol at 11 DAP. After dry storage for ten months, kernels were germinated as described in Figure V.6A. Pictured at top are wildtype kernels taken from same treatment area and germinated in parallel with mutant kernels (bottom).
Endogenous gibberellins in developing maize kernels

From our prior work with cultured embryos, we surmised that endogenous GA levels may be high in stage 2 (18-22 DAP) embryos when de novo synthesis is not required for germination in vitro but that those levels appear to decline in stage 3 and stage 4 embryos (23-27 and 28-30 DAP respectively) when germination in culture can be blocked by application of GA biosynthesis inhibitors [White and Rivin, manuscript in preparation]. In addition, the timing of inhibitor sprays described above suggests that a sufficient level of gibberellins is synthesized after 13 DAP to allow vivipary to occur even with additional GA synthesis blocked. We examined gibberellin and ABA levels in developing wildtype kernels to test the hypothesis that kernel GA levels are high in embryos between 13 and 22 DAP. Several different GA species were detected in developing W22 kernels (Figure V.7, Table V.2). Overall, three GA species show precipitous declines in as embryos age from stage 2 to stage 3. GA₁ is detectable only in 15 or 18 DAP embryos (late stage 1, early stage 2 respectively) and decreases abruptly in 21 DAP embryos (late stage 2). GA₃ and GA₈ decline continuously as embryos age from 15 (stage 1) to 27 DAP (early stage 4). Two additional species (GA₁₉ and GA₂₉) are at peak levels in 21 DAP kernels, a time point at which peak ABA levels are also achieved.
FIGURE V.7 GA and ABA levels in developing wildtype W22 embryos.
TABLE V.2 GC-MS quantitation of gibberellins and abscisic acid in developing W22 kernels.

Hormone species and levels (ng/g fresh weight) identified in flash-frozen kernels at successive developmental time points (dap = days after pollination). Averages from two determinations plus standard deviation (sd) given. Where only one determination was made, standard deviation is represented as (-). The same data are pictured in Figure V.7.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>15dap</th>
<th>15 sd</th>
<th>18dap</th>
<th>18 sd</th>
<th>21dap</th>
<th>21 sd</th>
<th>24dap</th>
<th>24 sd</th>
<th>27dap</th>
<th>27</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA1</td>
<td>0.02</td>
<td>-</td>
<td>0.065</td>
<td>0.09</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>GA3</td>
<td>0.75</td>
<td>-</td>
<td>0.60</td>
<td>-</td>
<td>0.35</td>
<td>-</td>
<td>0.31</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GA8</td>
<td>0.53</td>
<td>-</td>
<td>0.36</td>
<td>0.14</td>
<td>0.41</td>
<td>0.04</td>
<td>0.175</td>
<td>0.35</td>
<td>0.9</td>
<td>-</td>
</tr>
<tr>
<td>GA19</td>
<td>4.99</td>
<td>-</td>
<td>7.0</td>
<td>0.22</td>
<td>10.3</td>
<td>0.45</td>
<td>7.1</td>
<td>0.37</td>
<td>9.6</td>
<td>-</td>
</tr>
<tr>
<td>GA20</td>
<td>5.36</td>
<td>-</td>
<td>5.61</td>
<td>0.86</td>
<td>6.53</td>
<td>0.10</td>
<td>4.35</td>
<td>0.21</td>
<td>11.4</td>
<td>-</td>
</tr>
<tr>
<td>GA29</td>
<td>0.7</td>
<td>-</td>
<td>1.34</td>
<td>0.30</td>
<td>1.63</td>
<td>0.13</td>
<td>1.09</td>
<td>0.04</td>
<td>1.21</td>
<td>-</td>
</tr>
<tr>
<td>GA44</td>
<td>3.50</td>
<td>-</td>
<td>5.26</td>
<td>1.96</td>
<td>7.9</td>
<td>1.31</td>
<td>8.14</td>
<td>3.44</td>
<td>6.6</td>
<td>-</td>
</tr>
<tr>
<td>ABA</td>
<td>30.1</td>
<td>-</td>
<td>31.1</td>
<td>8.63</td>
<td>93.6</td>
<td>20.7</td>
<td>21.4</td>
<td>3.40</td>
<td>20</td>
<td>-</td>
</tr>
</tbody>
</table>
DISCUSSION

Previously we suggested that relative levels of ABA and GA, rather than the concentration of ABA alone, may control the developmental choice between maturation and germination in developing maize embryos. In this paper, we sought to extend results suggesting a role for GAs in mediating in vitro germination to vivipary occurring in vivo. We predicted that bioactive gibberellin species should accumulate at higher levels in stage 2 and younger kernels, prior to or coincident with peak ABA levels, and that disruption of GA synthesis in vivo prior to this time might suppress vivipary in ABA-deficient viviparous kernels. The results presented in this paper satisfy these predictions and, additionally, suggest a model of epistasis between ABA, GA, and the Vp1 gene.

Our results show that inhibition of GA biosynthesis can suppress vivipary in ABA-deficient but not in ABA-insensitive kernels. Both chemical and genetic reduction of GA levels inhibited germination of vp5 kernels, while neither method of altering GA levels affected vivipary of vpl mutants. Additionally, GA inhibitors suppress fluridone-induced vivipary of wildtype kernels.

Suppression of vp5 vivipary by both chemical and genetic reduction of GAs was accompanied by acquisition of desiccation tolerance and the capacity to germinate after prolonged storage. This is a novel and unexpected result. It was anticipated that, while germination might be suppressed by reduction of gibberellins, this suppression would not necessarily induce maturation. We do not understand the molecular basis of this result but it suggests that in addition to germination, maturation processes which confer desiccation tolerance also are regulated by relative levels of ABA and GAs.
The suppression of vivipary of vp5 kernels reported here contrasts with our result in using GA-inhibitors in culture: in those experiments, stage 3 vp5 embryos germinated even in the presence of ancymidol or paclobutrazol [White and Rivin, manuscript in preparation]. We note, however, that inhibitor treatment of vp5 kernels reported here effectively suppressed vivipary only when applied prior to 13 DAP while the embryos used in in vitro germination assays were 18 DAP or older. These results suggest that gibberellin levels sufficient for germination are synthesized by 13 DAP.

Gibberellins in developing wildtype kernels were identified and quantified. Of the species identified, only GA1 and GA3 possess significant activity in biological assays [Reeve and Crozier, 1973]. Their peak accumulation at 15-18 DAP is consistent with our hypothesis that bioactive kernel gibberellins are high in stage 2 embryos. Moreover, their temporal accumulation profiles is consistent with the timing of GA-inhibitor effectiveness. In addition, suppression of vivipary in d1 vp5 double mutant kernels suggests that GA1 may indeed play a role in vivipary. Moreover, restoration of vivipary to inhibitor-treated vp5 kernels by GA3 treatment suggests that this species can stimulate precocious germination. Additional crosses of vp5 with d1, as well as with other GA-deficient mutants with defined biosynthetic blocks will establish which GA species are vital for precocious germination.

There is one other report quantifying and identifying kernel GAs of maize [Murofushi et al, 1991]. 7 DAP and 9 DAP kernels from two different field years were examined using both gas chromatography-mass spectrophotometry and immunoassays. As with the material analysed in this report, GA1, GA8, GA19, GA20, GA29, and GA44 were detected. However, that group also reported species not detected here (GA4, GA9, GA17, GA34, GA53). While it is difficult to
make direct quantitative comparisons between the data of both groups because of
assay differences and differing genetic backgrounds, they report generally reduced
levels of species which we have identified and quantitated. Of the species found in
common, only GA1 is detected in higher quantities in 7 or 9 DAP seed than in the
kernels assayed in this report. All other species are substantially reduced in 7 or 9
DAP seed.

Our results suggest that the idea of hormone balance previously used to model
germination processes in mature seed may have relevance to the regulation of seed
development and further suggest that GA and ABA interact upstream from the Vp1
gene in regulating precocious germination. In kernels with a functional Vp1 gene
product but reduced levels of ABA, reduction of endogenous GA levels suppresses
vivipary. However, in the absence of a functional Vp1 gene, vivipary occurs
regardless of relative ABA and GA levels. This suggests to us a model of epistasis in
which the ratio of ABA to GA is a key determinant in signalling maturation versus
germination but that the Vp1 gene is absolutely required for maturation to occur.

Although the role of Vp1 in upregulating maturation-associated genes is well
known, its corollary function is to suppress germination processes. While the
relationship of vivipary to germination of mature seeds has not been established, it
seems likely that the developmental program underlying precocious germination may
involve gibberellin-induced gene expression. Genetic regulators of germination have
not been described but it seems probable that at least some of these would govern cell
cycle. Another group has recently described homologues of mammalian proto-
oncogenes which are coordinately regulated with cellular division in germinating
mature maize kernels [Georgieva et al., 1994]. We think it possible that cell cycle
regulators such as these may play a key role in sensing and integrating hormonal
signals in viviparous as well as mature germinating seed and that these may be likely targets of Vp1 and ABA in suppressing vivipary.


Robertson DS (1955) The genetics of vivipary in maize. Genetics 40: 745-760


Smith JD, Cobb BG, Magill CW, Hole DJ, Blakely CA (1987) Partitioning the sources of abscisic acid found in developing embryos. Maize Genetics Coop. Newslet. 61: 39

Swain SM, Reid JB, Ross JJ (1993) Seed development in Pisum:: the lh-i allele reduces gibberellin levels in developing seeds, and increases seed abortion. Planta 191: 482-488
Chapter Six

Physiological and molecular aspects of maize embryo maturation revisited:
A model for the ontogeny and regulation of seed maturation

Constance N. White
Department of Botany and Plant Pathology
Program in Genetics
Center for Gene Research and Biotechnology
Oregon State University, Corvallis, OR
The experiments described here used two approaches to dissect the regulation of maize embryo maturation. Experiments described in chapters two and three sought to isolate additional genes expressed in maturing embryos and examine regulation of their expression. Using a differential screen to search for mRNAs absent in developing $vp1$ embryos, five cDNAs were isolated. Three corresponded to previously described genes of maize (Globulin 1, Globulin 2, and $E_m$). One, pM44, appears to represent the maize homologue of a metallothionein gene isolated from wheat ($Ec$). The second novel cDNA, pM69, is not full length, so identity cannot be assigned. Its unusual GA-rich sequence and extremely high and rapid accumulation in embryos suggests that it may represent a novel seed storage protein. All five cDNAs appear to absolutely require a functional $Vp1$ gene and the hormone abscisic acid (ABA) for expression of their corresponding transcripts both $in$ $vivo$ and $in$ $vitro$. In addition, expression of two messages (Globulin 2 and $E_m$) was found to be reduced in embryos carrying the dormant anthocyanin-less $vp1$ allele, $vp1^{mc}$.

The initial impetus for undertaking the differential screen was to assess the relative roles of ABA and water stress in controlling maturation. However none of the markers isolated in the screen shows regulation independent of ABA and $Vp1$, so extension of two-dimensional protein gel analysis that suggested alternate pathways controlling maturation-associated genes was not possible. It is possible that the original screen design precluded isolation of alternately regulated clones. One cDNA isolated in a different manner (pM1g) appears to be independently regulated by water stress apart from ABA and should have been present in high abundance in our library but was not identified in the screen. This is no longer a surprise since the product of this gene is known to accumulate in developing $vp1$ (as well as $vp5$) mutant embryos [Thomann et al., 1992, White, unpublished observation]. The results of the screen
reflect both the importance of *a priori* assumptions in designing differential screens as well as the inherent difficulty in using such a strategy to collect novel and interesting clones. Screens of the type used here tend to identify only high abundance messages so the isolation of three previously described cDNAs is not unexpected.

Similar work by other groups has met with similar results and success. Many high-abundance maturation genes have been isolated from a variety of systems but it is difficult to correlate *in vitro* effects with true developmental programming. The kinetics of message accumulation as well as their expression characteristics in mutant backgrounds vary between the clones described here so it can be inferred that some aspects of their regulation are discretely different. However their behavior in cultured embryos is nearly identical. Moreover, since steady state mRNA levels result from both transcriptional and post-transcriptional controls which cannot be assayed *in vivo*, it is not known at what level differential expression characteristics are regulated. The regulation of pMLG is of particular interest. Does the promoter of this gene contain DRE-related sequences which can respond to osmotic stress independent of ABA synthesis or does accumulation of both message and protein in *viviparous* mutants result from some other form of regulation? In addition, the high endogenous expression of pM69 message coupled with its relative abundance in ABA-deficient embryos warrants examination for increased ABA-responsiveness in its promoter elements. And finally, why is expression of Globulin 2 and Em diminished in *vp1*<sup>mc</sup> mutants? Do they share promoter elements in common with the C1 gene and, if so, how are those elements different from other ABA-inducible genes whose expression is not impaired by the *vp1*<sup>mc</sup> allele? Extension of the analysis presented here will require isolation of genomic sequences and dissection of their respective promoter functions. This may be accomplished via transient assays in maize tissue or by
construction of transgenic plants. Unfortunately maize does not yet offer a convenient transformation system so the latter approach would necessarily be undertaken in a heterologous system such as tobacco.

The second set of experiments reported here suggest that gibberellins (GAs) play an important role in modulating precocious germination. By systematically assaying germinability of embryos isolated at various developmental stages, it was shown that germination of excised stage 2 embryos is independent of GA biosynthesis, but that as embryos develop through stages 3 and 4, their germination capacity becomes increasingly dependent on de novo GA biosynthesis. Based on these results, we hypothesized that bioactive GA species which can stimulate precocious germination are present at high levels in stage 2 embryos but then decline with advancing embryo age. Measurement and identification of GAs in developing kernels support this model. In addition, inhibition of GA biosynthesis prior to stage 2 suppressed vivipary in ABA-deficient kernels developing in situ, while similar treatments did not prevent precocious germination of vpl kernels. These results suggest that gibberellins and ABA mediate antagonistic programs in developing seed. The high levels of ABA relative to gibberellins found in wildtype kernels suggest that in the normal situation, the balance is strongly tilted to ABA-mediated maturation. Shifting the ratios of these two hormones may shift developmental programming from maturation to germination, but germination suppression absolutely requires the presence of a functional Vp1 gene product.

This suggests a model of epistasis in which ABA and GA mediate opposite and antagonistic processes upstream of the site of Vp1 action (Figure VI.1). The mechanism of such a pathway is unclear. Whether ABA works by reducing GA levels directly or by reducing expression of GA inducible genes or vice versa is
unknown. Sparse evidence exists to favor one model over the other. Because the
two hormone classes share early biosynthetic steps in common, their respective levels
could conceivably be coupled: such coupling could take many forms. Recently GA
has been shown to be capable of suppressing ABA synthesis. This report is
consistent with our own observation of diminished carotenoids in GA treated kernels
suggests that GA could act by diminishing ABA levels [Toyomasu et al., 1994,
White, unpublished observations]. However, in order for this to be true,
suppression of vp 5 vivipary by GA-reduction implies that residual ABA biosynthetic
activity is present and unmasked by absence of the other hormone. In addition,
although Vp 1 expression is not up-regulated by ABA [Allan Kriz, personal
communication], the effect of gibberellins on the expression of this gene has not been
examined; it is certainly possible that gibberellins could down-regulate Vp1
expression or activity.

FIGURE VI.1 Schematic of ABA, Vp1, and GA function in modulating seed
development
These results suggest that future studies of seed maturation should consider not only the effects of ABA but of gibberellins as well. The failure to correlate maturation gene expression with temporal accumulation of ABA resulted in suggestions that water-stress played a more important role in regulation of these genes [reviewed by Kermode, 1990]. Our observation that three features of maize embryo development: vivipary, desiccation-tolerance, and anthocyanin synthesis are regulated by hormone balance rather than absolute ABA concentrations suggests an alternate model to those invoking water-stress. A direct test of this model would be to examine the effects of altered ABA/GA balance on expression of maturation associated genes. Although this has not been done in our lab, evidence from others suggests that at least some maturation genes are down-regulated by increased GA levels in cultured embryos, suggesting that our observation of gibberellin effects on visual markers of maturation may be paralleled by changes in programmed gene expression [Leah and Mundy, 1989; Hughes and Galau, 1991; Bartels et al., 1991].

Although considerable attention has been paid to genes and programs turned "on" by ABA, little effort has been directed at identifying processes which are turned "off" during early seed maturation. Clearly, ABA and Vp1 suppress germination processes of developing kernels. What are the genes underlying this germination program and how are they regulated? For example, amylases from wheat, barley and rice are suppressible by ABA and are known to possess ABRE promoter elements. Does ABA suppression require a Vp1 homologue in these plant species? Surprisingly, neither amylases nor any other genes known to be induced by GA have been isolated from maize. A cursory attempt was made to find maize sequences homologous to wheat and barley amylases using low stringency Southern and northern analysis but no cross hybridization was detected [White, unpublished].
A more direct and useful approach would be to isolate cDNAs specific to pre-germination viviparous mutants. Because we found ABA to peak at 21 DAP, such clones would be expected to be expressed at, or shortly, prior to that time point. A subtraction library could be made using 21 DAP vpl embryo RNA as (+) material and wild type embryos as (-) RNA. Such a library would be expected to contain clones turned "off" by ABA and Vpl. An additional advantage using this method of construction is that subtraction libraries generally offer a greater possibility of isolating rare messages and hence, possibly regulators rather than just target genes. Once such clones are isolated, they may be assayed for induction by GA and suppression by ABA and Vp1 using the cultured embryo system established in our laboratory.

Underlying the assumption that ABA down-regulates genes expressed during transit from embryogeny to maturation is the question of why such genes would be turned on at all. Of what use is a program which, when uncovered, allows for germination without heed of environmental conditions? While the relationship of vivipary to germination of mature seeds has not been established, homologues of mammalian proto-oncogenes are coordinately regulated with cellular division in germinating mature maize kernels [Georgieva et al., 1994]. We think it possible that cell cycle regulators such as these may play a key role first in embryogeny and later, during initial germination. Although Vp1's role in upregulating maturation-associated genes is well known, its corollary function is to suppress germination processes. We suggest that proto-oncogenes may be likely targets of this activity and moreover may also be regulated by gibberellins. In a simple model Vp1 and ABA may suppress vivipary by upregulating expression of homologue of the p53 tumor suppressor, which then in turn suppresses activity of GA-inducible positive cell cycle
regulators such as c-myc, c-jun, and c-fos. Consistent with this model is the report of high levels of a p53 homologue in quiescent seed which rapidly disappears during germination [Georgieva et al., 1994].

Seed maturation may be profitably viewed as an adaptive pause in development. In non-vascular plants and many lower vascular species, embryos develop into mature sporophyte with no intervening period of arrest [Salisbury and Parke, 1970]. This system of reproduction demands continuous moisture and the acquisition of desiccation tolerance represents an adaptation enabling the plant kingdom to survive dry conditions and greatly expand potential habitat. Some tropical plants (eg. mangroves) which do not suffer seasonal interruption of their reproductive cycle are naturally viviparous. This is sensible if moist and warm conditions prevail and the objective is to maximize progeny number and minimize generation time. With this in mind, it is tempting to speculate that viviparous mutants in maize and other species uncover a primitive developmental program still extant in the plant genome. Such a system may be conserved for some adaptive advantage, such as coupling of GA levels with seed growth, but may also account for the high levels of GAs encountered in seeds of many plant species and their apparent lack of function. If these represent vestigial hormonal remnants of an antiquated developmental program then it may be, in maize viviparous mutants at least, that ontogeny recapitulates phylogeny. Viviparous mutants may unmask a primitive developmental program modulated by GA which has been retained to regulate prematuration embryo development and growth but which is then checked by superimposition of ABA and the Vp 1 gene product to enable survival in dry conditions.


BIBLIOGRAPHY


Anderberg RJ, Walker-Simmons MK (1992) Isolation of a wheat cDNA clone for an abscisic acid-inducible transcript with homology to protein kinases. Proc Natl Acad Sci USA 89: 10183-10187


Cone KC (1989) Yet another rapid plant DNA prep. Maize Genet Coop Newslet. 63: 68


Finkelman RR, Crouch ML (1986) Rapeseed embryo development in culture on high osmoticum is similar to that in seeds. Plant Physiol 81: 907-912


Guerrero FD, Jones JT, Mullet JE (1990) Turgor responsive gene transcription and RNA levels increase rapidly when pea shoots are wilted. Sequence and expression of three inducible genes. *Plant Mol Biol* 15: 11-26


Kawashima I, Dennedy TD, Chino M, Lane BG (1992) Wheat Ec metallothionein genes. Like mammalian Zn\textsuperscript{2+} metallothionein genes, wheat Zn\textsuperscript{2+} metallothionein genes are conspicuously expressed during embryogenesis. Eur J Biochem 209: 971-976


Khan AA (1968) Inhibition of gibberellic acid-induced germination by abscisc acid and reversal by cytokinins. Science 125: 645-646


Robertson DS (1955) The genetics of vivipary in maize. Genetics 40: 745-760


Schopfer P, Plachy C (1985) Control of seed germination by abscisic acid. III Effect on embryo growth potential (minimal turgor pressure) and growth coefficient (cell wall extensibility) in Brassica napus L. Plant Physiol 77: 155-160


White CN, Proebsting WR, Hedden P, Rivin CJ (1994) Effects of altered gibberellin metabolism on viviparous maize kernels developing on the ear:
correlations of endogenous gibberellin levels with precocious germination in vitro and in vivo. manuscript in preparation.


Williamson JD, Quatrano RS, Cuming AC (1985) E
m polypeptide and its messenger RNA levels are modulated by ABA during embryogenesis in wheat. Eur J Biochem 152: 501-507


Yamaguchi-Shinozaki K, Kozumi M, Urao S, Shinozaki K (1992) Molecular coning and characterization of 9 cDNAs for genes that are responsive to desiccation in Arabidopsis thaliana:: Sequence analysis of one cDNA clone that encodes a putative transmembrane channel protein. Plant Cell Physiol 33: 217-224


Zeevaart JAD (1966) Reduction of the gibberellin content of Pharbitis seeds by CCC and the after effects in the progeny. Plant Physiol 41: 856-862