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Accumulation of Group 3 Late Embryogenesis Abundant Proteins in *Zea mays* Embryos[1]

Roles of Abscisic Acid and the *Viviparous*-1 Gene Product

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ABSTRACT

Several different types of proteins that are modulated by abscisic acid (ABA) accumulate in developing embryos of maize (*Zea mays* L.). Some of these proteins are specific to the developing seed, such as the storage globulin, GLB1, whereas others are involved in general responses to water deficit. Here we describe a maize protein family of this second type, a Group 3 late embryogenesis abundant (MLG3). Like other proteins of this class, MLG3 polypeptides are ABA-responsive. They are found in maturing seeds and in dehydrating plant tissues. Antigenically related proteins are found in other cereals. To distinguish the regulation of developmentally programmed ABA responses from those that are environmentally induced, we compared the ontological pattern and accumulation requirements of MLG3 polypeptides with those we previously described for GLB1. GLB1 accumulation begins early in the maturation phase and specifically requires high levels of ABA and the participation of the *Viviparous*-1 (*Vp*1) gene product. *Vp*1 is required for other ABA-modulated events in maize seed development as well. In experiments using *vp*1 mutants and mutants deficient in ABA synthesis (*vp*5 mutation), we show that MLG3 accumulation also is dependent upon ABA, but it shows striking differences from GLB1. MLG3 accumulates much later in embryogenesis, coincident with the onset of dehydration. In contrast to GLB1, MLG3 proteins can be induced by *de novo* ABA synthesis in response to culturing in high osmoticum. Unlike GLB1, MLG3 has no specific requirement for the *Vp*1 gene product.

Several types of proteins that accumulate in maturing embryos have been shown to be responsive to ABA. In rapeseed, maize, soybean, and wheat, storage proteins accumulate precociously in immature embryos exposed to exogenous ABA (reviewed in ref. 28). Exogenous ABA also results in the accumulation of a set of unusual, highly hydrophilic proteins that normally accumulate late in embryogenesis. First identified among LEA[2] messenger RNAs in cotton (8, 11), three conserved families of LEA proteins have been found to have cognates in a wide range of monocot and dicot embryos (reviewed in ref. 9). In maize, one LEA protein of 23 to 25 kD has been described, known variously as DHN or RAB-17 (5, 30).

Unlike the storage proteins, the LEA proteins are not limited to the developing seed. Various members of this group have been shown to accumulate in plant tissues under conditions of water deficit, salt and osmotic stress, and cold; application of ABA to plants will also induce the proteins in the absence of environmental stress (5, 17, 21, 29, reviewed in 28). It has been proposed that LEA proteins have a general role in desiccation tolerance (2, 9).

The mechanism of ABA modulation of embryo-specific and LEA proteins is unknown, and it is still unclear whether the hormone directly or indirectly regulates these proteins in different plants. Maize seedlings with genetic defects in ABA synthesis do not accumulate DHN under drought-stress conditions as wild-type seedlings do (4, 21). Other evidence of ABA regulation comes from the study of gene promoter regions. An ABA-responsive consensus sequence has been found in the 5′ upstream region of both storage protein and nonseed specific genes (14, 18, 29). In developing embryos, a programmed accumulation of ABA occurs prior to seed desiccation (3, 20). Here the hormone is thought to play a dual role, suppressing precocious germination of the embryo and stimulating the expression of products associated with maturation phase (reviewed in ref. 22).

The distinction between ABA effects that are developmentally regulated and those that are stress-induced is under investigation at the molecular level (29). In maize, the product of the *Vp*1 gene is a candidate for a regulator of seed-specific ABA response. Homozygous *vp*1 mutant embryos have wild-type levels of ABA (19), but they lack normal ABA effects. These mutants precociously germinate on the ear beginning in late stage 3 and they do not accumulate globulin storage protein or globulin mRNA (13, 25). Unlike wild-type embryos, *vp*1 embryos placed in tissue culture with ABA germi-

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[2] Abbreviations: LEA, late embryogenesis abundant; DAP, days after pollination; vp, viviparous; DHN, dehydrin; VP1, *vp*1 gene product.

nate and they do not show precocious accumulation of globulin (24, 26). The mutant seeds also show a variety of enzyme deficiencies in the aleurone layer that are not known to be ABA-modulated (7). For some of these products, the relevent mRNAs are missing (15).

In contrast, homozygous *vp*1 plants appear to be normal for these enzymes and in their ABA responses (15, 20). The sequence of the *Vp*1 gene shows no homology with known regulatory proteins, but in a series of domain-switching experiments, it was shown to function as a transcriptional activator of the ABA-inducible wheat Em promoter (14, 16).

To uncover the regulatory programs that control developmental *versus* environmentally induced roles of ABA, we have been comparing the accumulation of seed-specific and nonspecific maturation proteins in wild-type maize embryos and in mutants that are deficient either in ABA synthesis (*vp*5 mutant) or ABA response in seeds (*vp*1 mutant). By culturing these embryos in either high osmoticum or high levels of ABA, we can block precocious germination and examine the individual roles of ABA, germination inhibition, and the *Vp*1 gene product in the regulation of the two classes of proteins. In a previous study of the maize embryo storage protein GLB1, we have shown that both ABA and the *Vp*1 gene product are required to initiate synthesis and accumulation, and that inhibition of germination is required for the continued accumulation of this protein (24).

In this report, we describe a family of ABA-reponsive polypeptides, called MLG3, that abruptly increase from low to high abundance late in maize embryogenesis. Antigenically, MLG3 is a member of the Group 3 LEAs. Like the LEA proteins that have been described, MLG3 polypeptides also accumulate in plant tissues undergoing water deficit. Accumulation of MLG3 polypeptides in maturing embryos is dependent both on ABA and on the limitation of water uptake, as is GLB1 accumulation. However, unlike GLB1, MLG3 accumulation has no specific requirement for the *Vp*1 gene product.

MATERIALS AND METHODS

Plant Material

Maize (*Zea mays* L.) varieties and vp mutants were obtained from several sources. Ky21 was obtained from E. Coe, Gaspe Flint from J. Beckett, and B37 from M.G. Neuffer (all of the University of Missouri, Columbia); W22 from J. Kermicle (University of Wisconsin, Madison); the vp mutants *vp*1 and *vp*5 (27) from D. Robertson (Iowa State University, Ames IA). The genetic background for both *vp*1 and *vp*5 is chiefly W22. All lines were propagated in Corvallis, OR.

Homozygous *vp*5 kernels can be identified on a segregating ear because they do not produce cartenoid pigments (19). The mutant kernels appear white, whereas their normal sibling kernels are yellow in color (25). Mutant kernels showed 100% precocious germination by stage 4 of development. Homozygous *vp*1 kernels can be identified on a segregating ear because they fail to make anthocyanins in the aleurone layer, unlike their wild-type and heterozygous siblings (7, 25). Because aleurone anthocyanin is not visible in early development, stage 1 and stage 2 *vp*1 mutant embryos were isolated from homozygous *vp*1 plants propagated from precociously germinating seeds. Vivipary was more variable in *vp*1 kernels.

Seed of barley (*Hordeum vulgare* cv Himalaya) was obtained from Andrew Kleinhofs at Washington State University, Pullman, WA. Seed of wheat (*Triticum aestivum* cv OR8313) was obtained from Warren Kronstad, Oregon State University, Corvallis, OR.

Embryo Culture

Ears were harvested 15 to 25 d after controlled pollinations and surface sterilized with 2.5% bleach. The embryos were dissected free from maternal and endosperm tissue under sterile conditions and staged according to the number of leaf primordia, using the scheme of Abbe and Stein (1). For growth in tissue culture, up to 30 embryos were placed in Petri plates, scutellum down, on a filter saturated with a minimal-nutrients medium supplemented with 3% (w/v) sucrose, 100 mg/L *myo*-inositol, and 0.4 mg/L thiamine-HCl (24). For some experiments, ABA (Sigma, mixed enantiomers) was added to 10^{-5} M. For experiments with high osmoticum, the sucrose concentration was raised to 20%. Both of these additions effectively blocked precocious germination of cultured embryos 35 DAP and younger. Embryos cultured in lower levels of ABA or osmoticum germinated within a few days (24). Embryos were incubated in the dark at 26°C, and the media were changed daily. Embryo samples were frozen in liquid nitrogen and stored at −80°C. Each experiment was repeated with embryos from different pollination dates.

Protein Isolation

Embryos were powdered in liquid N_2 and further ground in a buffer containing 0.05 M Tris-HCl (pH 6.8) and 1 mM PMSF. This slurry was heated to 65°C for 5 min, vortexed, and heated at 95°C for 2 min, vortexed again, and then centrifuged at 35,000*g* for 15 min to remove cellular debris. The supernatant was recovered and the centrifugation repeated for 10 min. When it was necessary to concentrate the protein sample, 4 volumes of ice-cold acetone with 10 mM 2-mercaptoethanol was added to precipitate proteins. After centrifugation, the resulting pellet was dried and resuspended in SDS-PAGE sample buffer. The protein content was determined by Bradford microassay (Bio-Rad) using BSA standards.

SDS-PAGE Gels and Western Blots

Proteins were analyzed by SDS-PAGE using 15% acrylamide. Gels were stained with Coomassie brilliant blue. For western blots, duplicate gels were run, an unstained gel was electroblotted to Immobilon-P membrane (Millipore), and MLG3 polypeptides were detected using polyclonal antibodies. The anti-MLG3 serum was obtained from rabbits that had been injected with 29 kD MLG3 purified from mature W22 embryos by three passes through SDS-PAGE. DHN polypeptides were detected using polyclonal antibodies raised in rabbits to a conserved synthetic peptide from the carboxyl terminus of barley DHN. This serum was kindly provided by Timothy Close (University of California, Riverside). Mono-

specific Em antiserum was provided by Ralph Quatrano (University of North Carolina, Chapel Hill). Polyclonal serum to a fusion protein product of a wheat Group 3 LEA cDNA clone (6, 23) was obtained from Jeff Reid and M.K. Walker-Simmons (ARS-USDA, Pullman, WA). Antibody binding was observed using the Vectastain ABC horseradish peroxidase kit (Vector Laboratories, Inc.). The manufacturer's directions were followed except for the detection step, when the ECL chemiluminescent reagent (Amersham) was used to obtain greater sensitivity.

RESULTS

The MLG3 Family of Polypeptides Accumulates in Late Maturation Phase

As maize embryos move from embryogenesis through maturation phase, they accumulate a new set of polypeptides. The first phase of maturation is characterized by the accumulation of high mol wt storage globulins that are detectable by early stage 3 of development, about 20 DAP (12, 24). In late stage 4 (about 30 DAP) a new set of low mol wt proteins become visible in the polypeptide profile. Figure 1 compares the low mol wt proteins of maize embryos between prematuration stage 1 (15 DAP) and kernel maturity (stage 6, 60 DAP), showing the accumulation of new polypeptides in late maturation phase.

To study the identity and regulation of this class of late maturation phase proteins, antiserum was raised to a late-appearing abundant protein of 29 kD from W22. The serum was tested on western blots with mature embryo proteins of several maize varieties (Fig. 2, A and B), detecting a small family of polypeptides named MLG3.

The size of MLG3 polypeptides varies among maize strains. In W22, Gaspe Flint, and the *vp1* heterozygote stock, the major band is 29 kD; in Ky21, B37, and the *vp5* heterozygote stock, the major band detected is 27 kD. The source of this variation was studied by examining the western band patterns of F1 hybrids and F2 individuals. Figure 2C shows the MLG3 pattern of Ky21 × W22, 12 F2 individuals from that hybrid, and the reciprocal F1. Both F1s show a codominant pattern and the F2s segregate for parental or F1 patterns in the ratios expected for a single gene or a complex of closely linked genes. We expect, therefore, that the 27 and 29 kD bands represent allelic forms of MLG3 from a single locus. A weaker, cross-reacting band of 31 kD is seen in all lines except Ky21. In the hybrids and F2 individuals shown in Figure 2C, this segregates as the product of another, unlinked locus.

A faint band of 24 kD is seen in all lines having the major 29-kD band, whereas those lines having the major 27-kD MLG3 each have a weak band of 21 kD. The levels of these smaller bands vary considerably among individual seeds of the same genotype, so we cannot interpret their apparent segregation in F2 individuals with confidence.

The accumulation of MLG3 polypeptides in embryogenesis, germination, and seedling growth was followed for two distinct varieties of maize, W22, a midwestern dent line, and Gaspe Flint, a northern flint strain. As shown in Figure 3A,

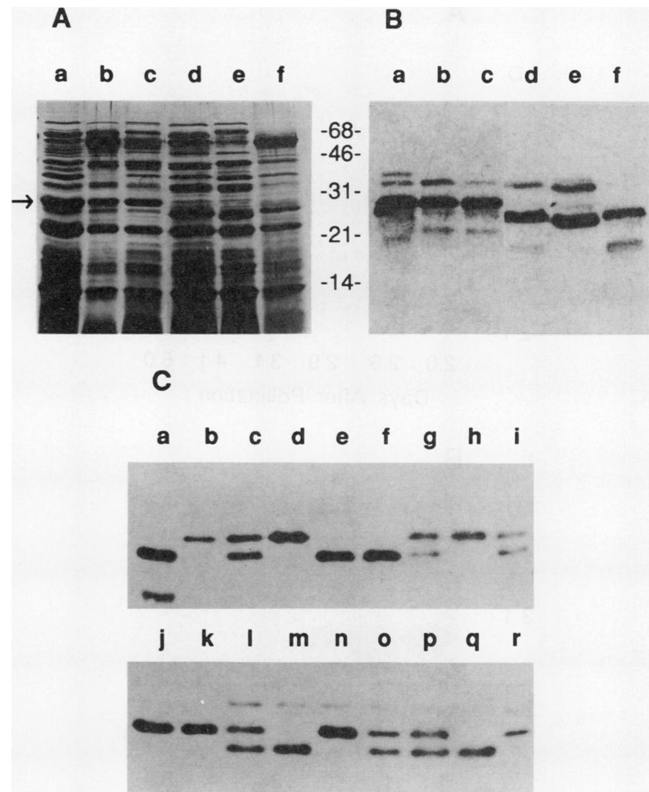

Figure 2. Allelic variants of MLG3 polypeptides. A, Coomassie-stained gel of mature embryo proteins from lanes a, Gaspe Flint; b, W22; c, *vp1*/+; d, *vp5*/+; e, B37; f, Ky21. Arrow shows band excised for antibody production. B, Western blot of duplicate gel using anti-MLG3 serum. C, Western blot of MLG3 proteins in mature embryos of lanes a, q, Ky21; b, r, W22; c, Ky21 × W22; d-o, F2 individuals from Ky21 × W22 self-pollination; p, W22 × Ky21.

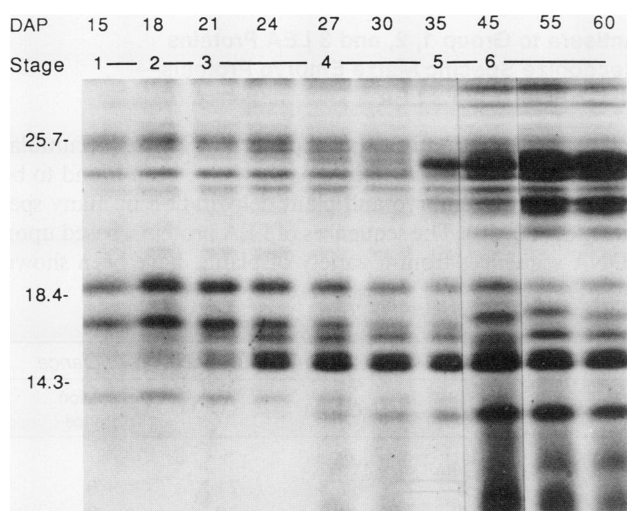

Figure 1. Low mol wt proteins of Gaspe Flint embryos between stages 1 and 6 of development. Equal amounts of protein from each stage were run on SDS-PAGE and the gel was stained with Coomassie brilliant blue, demonstrating the accumulation of several low mol wt polypeptides.

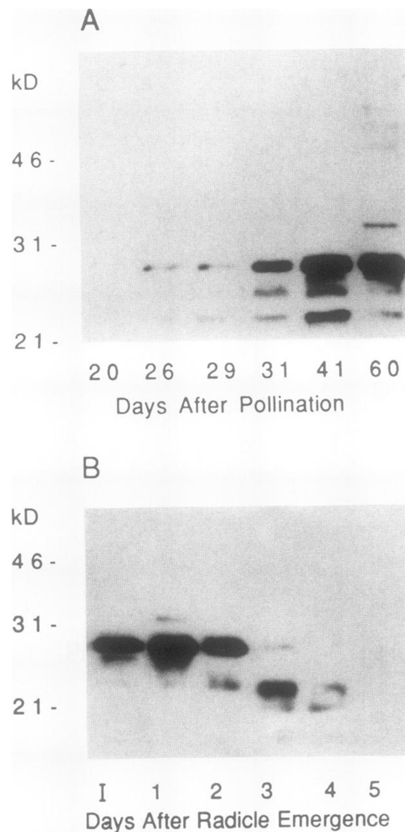

Figure 3. Accumulation and turnover of MLG3 polypeptides during maturation and germination of W22 embryos. Equal amounts of protein from embryos at different stages were separated by SDS-PAGE, blotted, and reacted with anti-MLG3 serum. A, Protein samples of embryos throughout maturation phase, 20 to 60 DAP. An abrupt increase in the level of the 29- and 27-kD proteins is seen between 29 and 31 DAP. B, Protein samples of embryos during imbibition and germination. Proteins were isolated from embryos of mature seeds after 24 h of imbibition (I), and at 1-d intervals following radicle emergence.

the abundant 29-kD MLG3 polypeptide can be detected at low levels in W22 embryos in early maturation phase (20 DAP). Amounts 20-fold less can be detected in prematuration stage embryos (not shown). The MLG3 polypeptides accumulate slowly during early maturation phase and then increase sharply at approximately 30 DAP. This time corresponds to the onset of dehydration and the acquisition of desiccation tolerance in maize embryos (Tables I and II). The abrupt increase in MLG3 abundance was seen in both genetic backgrounds and in ears with different pollination dates. Upon germination (Fig. 3B), MLG3 polypeptides declined in abundance and were undetectable in the germinating seedling within 5 d of radicle emergence. The low mol wt band seems to accumulate as the 29 kD MLG3 disappears, suggesting that it may be a breakdown product. The temporal patterns of accumulation and disappearance are the same for W22 and Gaspe Flint (data not shown).

Table I. *W22 Embryo Growth and Desiccation Tolerance*

DAP	Stage	Fresh Wt	H_2O	Desiccation Tolerance
		mg	%	%
15	1	0.3	73	0
18	2	2.7	74	0
21	3	4.7	72	0
24	3–4	11.2	72	0
28	4	15.1	65	90
31	5	19.1	62	100
35	5–6	26.7	56	100
46	6	22.4	51	100

MLG3 Polypeptides Are Associated with Dehydration in Plant Tissues

The late embryogenesis accumulation profile of MLG3 polypeptides suggested that MLG3 polypeptides might be among the class of proteins that accumulate upon water deficit both in seeds and plant tissues (5, 17, reviewed in 28). To test this possibility, W22 and Gaspe Flint seeds were germinated in well-watered vermiculite for 5 d to allow the maturation-phase accumulation of MLG3 to turn over, and then half of the seedlings were transplanted to dry vermiculite and the remainder were returned to the fully hydrated condition. After 4 d, the seedlings were harvested and the proteins of the two sets of roots and shoots were compared. The western blot in Figure 4 shows the profile of MLG3 proteins in these samples. Dehydrated seedlings, particularly the roots, accumulated the 29 and 24 kD polypeptides that were observed in dehydrating embryos, as well as lower mol wt bands. None of these proteins were detected in well-watered roots and little 29-kD protein was observed in well-watered shoots. As was observed by Close *et al.* for other LEA proteins (5), MLG3 polypeptides are not precipitated by 10 min of boiling.

Antisera to Group 1, 2, and 3 LEA Proteins Recognize Specific Maize Embryo Proteins—MLG3 Is a Group 3 LEA

The regulation and temporal pattern of MLG3 accumulation follows that of LEA proteins that have been found to be associated with embryo and plant dehydration in many species (4, 5, 23, 30). The sequences of LEA proteins, based upon cDNA sequences from a variety of plants, have been shown

Table II. *Gaspe Flint Embryo Growth and Desiccation Tolerance*

DAP	Stage	Fresh Wt	H_2O	Desiccation Tolerance
		mg	%	%
21	3	7.3	71	0
24	4	14.8	72	0
27	4	23.6	59	100
30	4–5	26.8	54	100
40	5	49.7	47	100
50	6	56.7	43	100

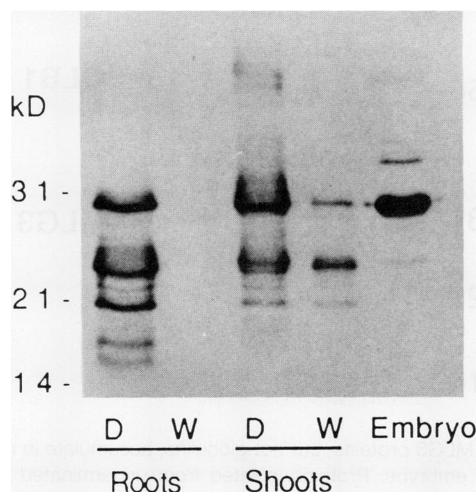

Figure 4. Western blot of root and shoot proteins from dehydrated and well-watered seedlings reacted with anti-MLG3 serum. Proteins were isolated from the roots and shoots of 8-d-old Gaspe Flint seedlings that had been grown for 4 d in either dry (D) or well-watered (W) vermiculite. Equal amounts of these proteins were separated on SDS-PAGE, along with an equal amount of protein from mature dry embryos. The gel was blotted and reacted with anti-MLG3 serum.

to fall into three groups (9). We tested for the ability of antiserum for each class of LEA protein to recognize maize embryo proteins on western blots, and compared these patterns with that created by anti-MLG3 serum.

Group 1 identity was tested using a monospecific antibody to the Em protein of wheat. This serum reacted with a single maize embryo protein of less than 14 kD, the same size as in wheat embryos (Ralph Quatrano, personal communication). Class 2 LEA homology was tested using an antiserum raised to a barley DHN synthetic peptide. In maize embryos, DHN is found as 23- to 25-kD phosphorylated polypeptides (21), similar to the sizes of MLG3 proteins. To test whether MLG3 and DHN are distinct families, embryo and endosperm proteins from mature maize and barley seeds were run on duplicate SDS-PAGE, blotted, and reacted with either antiserum (data not shown). MLG3 antiserum did not detect barley DHN, although it strongly reacted with another barley seed protein of 27 kD. DHN antiserum did not react with the 29- to 27-kD MLG3 bands. Both antisera detected a 24-kD maize polypeptide, but this appears to be coincidental. Anti-DHN recognized a 24-kD polypeptide in all lines tested, not the 24- to 21-kD polymorphism observed with anti-MLG3.

Group 3 LEA antibody derived from a fusion protein using the wheat cDNA (6, 23), strongly recognized a 29-kD maize embryo protein, and reacted more weakly with a 24-kD protein. Proof that these were the same proteins detected by anti-MLG3 was obtained by showing that the wheat antibody detects the same 29- to 27-kD polymorphisms as anti-MLG3 in a western blot of F2s from Ky21 × W22 (Fig. 5). Abundant proteins of 29 and 28 kD were detected in the wheat seed sample, along with fainter cross-reacting bands.

MLG3 Accumulation in Embryos Requires ABA and Dehydration, but not the *Vp1* Gene Product

ABA and dehydration are important developmental factors in seed development, and both may be involved in modulating embryo proteins (3, 10). The involvement of ABA and dehydration in the accumulation of MLG3 proteins was investigated by comparing the proteins of wild-type embryos with those of vp mutants that are deficient in either ABA synthesis (*vp5* homozygotes) or ABA response (*vp1* homozygotes). The mutants and wild types were compared for the proteins that accumulate during development on the ear, and those that can be induced in embryo culture by exogenous ABA or a high osmoticum (20% sucrose).

Stage 2 (18 DAP) and stage 3 (23 DAP) embryos from *vp5* segregating ears, from *vp1* homozygous ears and from wild-type (W22) ears were isolated under sterile conditions. Samples of wild-type and mutant embryos were frozen directly after dissection. Stage 2 embryo samples of each phenotype were also cultured for 5 d in a growth medium supplemented with either 10^{-5} M ABA or high osmoticum. Both of these culture treatments block precocious germination of wild-type and *vp5* homozygous embryos, but *vp1* mutant embryos germinate in ABA. Embryos of all genotypes germinate within a few days when cultured in growth medium alone (24). At least 40 embryos of each genotype and treatment sample were used to prepare proteins for western blot analysis.

The results of these experiments show that precocious accumulation of the maize Group 3 LEA proteins detected by anti-MLG3 can be induced in wild-type embryos by either exogenous ABA or high osmoticum (Fig. 6). At 18 and 23 DAP, freshly isolated embryos from *vp5* segregating ears have negligible amounts of MLG3 polypeptides. Precocious accumulation of the 27- and 21-kD proteins was observed when the 18 DAP wild-type embryos were placed in culture with either ABA or high osmoticum. These proteins are characteristic of mature wild-type embryos of this stock. The ABA

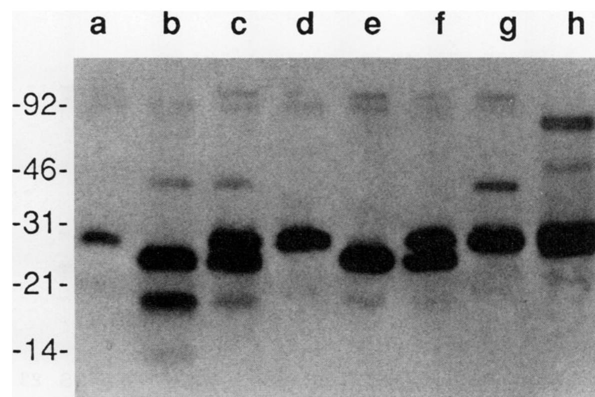

Figure 5. Western blot of embryo proteins of maize and wheat reacted with Group 3 LEA antiserum. Embryo proteins from mature maize and wheat seed were separated by SDS-PAGE, electroblotted, and immunodetected using antiserum to a wheat LEA Group 3 fusion protein (see "Materials and Methods"). a, W22; b, Ky21; c, Ky21 × W22; d-g, F2 individuals of Ky21 × W22; h, wheat.

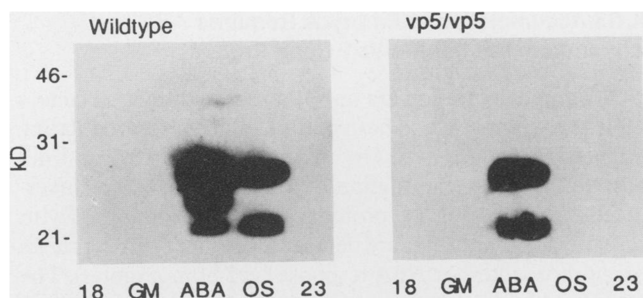

Figure 6. Induction of MLG3 accumulation by ABA and high osmoticum. Wild-type and *vp5* homozygous embryos were excised from segregating ears at 18 and 23 DAP. Proteins were isolated from freshly dissected embryos (18, 23), and from 18 DAP embryos that were cultured for 5 d in a hormone-free medium (GM), in GM + 10 μM ABA (ABA), or in GM + 20% sucrose (OS). Equal amounts of protein from each sample were run on SDS-PAGE and blotted. The proteins were detected using anti-MLG3 serum.

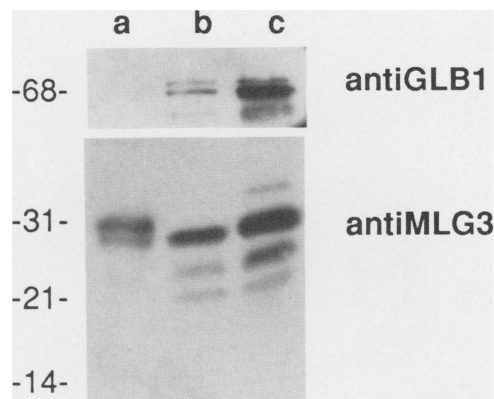

Figure 8. MLG3 proteins, but not globulins, accumulate in ungerminated *vp1* embryos. Proteins isolated from ungerminated 35 DAP embryos of *vp1* were compared on western blots with protein of 35 DAP and mature embryos of W22. Duplicate blots were reacted with anti-GLB1 and anti-MGL3 sera. Top panel shows accumulation of globulins, bottom panel shows accumulation of MGL3 proteins.

treatment also induces an intermediate-sized band that is not seen in the mature embryos. Embryos cultured in unsupplemented growth medium germinated and failed to accumulate any of these proteins.

The contrasting behavior of the cultured *vp5* homozygous embryos demonstrate that ABA, rather than dehydration, is specifically required for the accumulation of MLG3 polypeptides in isolated embryos. Stage 2 mutant embryos cultured with ABA accumulated 27- and 21-kD MLG3. However, they accumulated no MLG3 proteins when cultured with high osmoticum, a nonlethal treatment that blocks precocious germination. Unlike their wild-type siblings, *vp5* mutant embryos are unable to synthesize ABA in response to water deficit (20). These results suggest that ABA, provided exogenously or synthesized *de novo* in response to high osmoticum, is a specific requirement for MLG3 accumulation.

Other ABA-regulated activities of the maize seed have been shown to require the participation of VP1, including suppression of precocious germination on the ear and the accumulation of the globulin storage proteins (13, 15, 24). The role of VP1 in the accumulation of MLG3 proteins was tested in a similar experiment. As shown in Figure 7, the stage 2 (18 DAP) wild-type embryos that were frozen after dissection or cultured in growth medium gave no detectable signal. When cultured with ABA, the 29-, 27-, and 24-kD proteins typical of W22 accumulated to levels comparable to those observed in the freshly dissected 60 DAP embryos. The 27-kD band was lacking from those embryos cultured in high osmoticum. The behavior of cultured 18 DAP *vp1* homozygous embryos was different from that of wild-type or *vp5* embryos. Culturing the 18 DAP *vp1* homozygous embryos in exogenous ABA resulted in precocious germination, and no MLG3 accumulation occurred. Supplementing the medium with high osmoticum blocked the germination of *vp1* mutants, and in this treatment the 29- and 24-kD polypeptides accumulated.

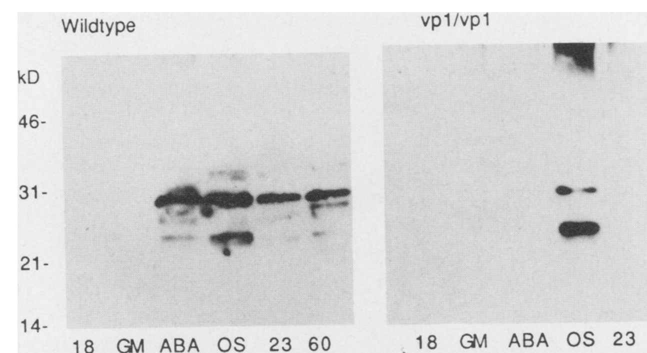

Figure 7. Role of *vp1* in the induction of MLG3 accumulation. Embryos were isolated from W22 ears at 18 and 23 DAP, and from *vp1* homozygous ears at 18 and 23 DAP. Proteins were isolated from freshly dissected embryos (18, 23, 60), and from 18 DAP embryos that were cultured for 5 d in a hormone-free medium (GM), in GM + 10 μM ABA (ABA), or in GM + 20% sucrose (OS). Equal amounts of protein from each sample were run on SDS-PAGE and blotted. The proteins were detected using anti-MLG3 serum.

This observation that nongerminating *vp1* embryos can accumulate MLG3 polypeptides was confirmed by examining *vp1* embryos that remain ungerminated on the ear. The timing of *vp1* precocious germination is variable and late season pollinations frequently show more delayed germination. Figure 8 compares proteins of mature and 35 DAP wild-type embryos with those of ungerminated 35 DAP (stage 5) *vp1* embryos. The sibling *vp1* embryos on these ears went on to precociously germinate. MLG3 polypeptides accumulate in 35 DAP *vp1* embryos in amounts comparable to wild-type, although the 31-kD polypeptide is more prominent. However, GLB1 is undetectable in the mutant. These data suggest that suppression of precocious germination or dehydration is a prerequisite for MLG3 accumulation, whereas VP1 is not directly required to accumulate these polypeptides.

DISCUSSION

The proteins of the maize embryo change markedly over the course of seed development (27). To study proteins that

accumulate late in embryo development, antibodies were raised to a 29-kD protein that is prominent in mature maize embryos of W22. The antiserum detects a Group 3 LEA protein family that we have named MLG3 (Maize LEA Group 3). The size of the major MLG3 proteins varies slightly between different inbred lines, and segregation studies show that this is due to alleles of a single gene or a closely linked gene complex. At least some of the other cross-reacting proteins are products of unlinked genes of this family. The MLG3 proteins have the characterstics of the LEA proteins reported in other species. They increase in embryogenesis coincident with the advent of dehydration and the acquisition of desiccation tolerance and they remain soluble in water at high temperature. The proteins decline gradually in abundance during germination, but high levels accumulate when seedlings are grown in conditions of water deficit.

We investigated the possibility that MLG3 is regulated by ABA, because several lines of evidence point to a pivotal role for ABA in modulating the expression of some of the LEA proteins in plant tissues undergoing water stress: (a) there is a strong correspondence between the level of ABA and the level of mRNA; (b) blocking ABA synthesis genetically or with inhibitors prevents accumulation during water stress; and (c) exogenous application of ABA stimulates accumulation in the absence of water stress (4, 5, 17, 21).

We found that ABA supplied exogenously to immature maize embryos does stimulate the accumulation of MLG3, but this observation does not prove that the hormone regulates the appearance of these proteins during seed development. Indeed, cotton embryos exposed to exogenous ABA induce expression of the LEA mRNAs, but *in vivo*, only a subset of these genes appear to be ABA-regulated (11). The actual cue may be changing water relations, which can be altered by ABA. As regulatory cues, ABA and restricted water uptake are difficult to clearly separate, because ABA may change water relations and drought-stress may lead to an increase in ABA levels (31).

Evidence for a specific ABA requirement for MLG3 accumulation in embryos was obtained by comparing the responses of wild-type and *vp5* embryos to exogenous ABA or high osmoticum. ABA was effective in inducing these proteins in *vp5* mutant embryos, but high osmoticum was not. Wild-type maize embryos cultured with high osmoticum show a small increase in ABA level (20). Because the *vp5* embryos are blocked in ABA synthesis, we interpret our results to mean that exogenous ABA, or ABA synthesized *de novo* in response to water limitation, can stimulate MLG3 accumulation, but that water stress in the absence of ABA is not a sufficient inducer. We did not detect MLG3 proteins in young vp mutant embryos developing on the ear, suggesting that ABA is directly or indirectly involved in the developmental accumulation of these polypeptides. However, because the germination of *vp5* embryos occurs in early stage 4, we could not compare wild-type and mutant embryos at times when MLG3 was very abundant.

The accumulation patterns of MLG3 polypeptides are in interesting contrast to another abundant ABA-regulated embryo protein, GLB1. This storage protein begins to accumulate much earlier in embryo development (approximately 20 DAP) and peaks in abundance 30 to 35 DAP (12, 13, 24). The initiation of GLB1 closely follows the sharp increase in maize embryo ABA that occurs about 16 to 18 DAP (20), consistent with the idea of ABA modulation. In contrast, MLG3 increases only as the embryo begins to dehydrate, suggesting that a different or an additional physiological cue is required. The two classes of polypeptides also show divergent responses to high osmoticum in cultured wild-type embryos. GLB1 does not accumulate in these embryos (24), whereas MLG3 does. The distinction may be due to the level of *de novo* ABA required to stimulate accumulation or to cell specificity in hormone synthesis and response.

The most interesting difference between GLB1 and the MLG3 polypeptides is that the *Vp1* gene product is a specific requirement for GLB1, but not the others. No protein or mRNA for GLB1 can be found in *vp1* mutant embryos *in vivo* (13). Homozygous *vp1* embryos that remain ungerminated on the ear or are blocked from germination in culture by a high osmoticum do not accumulate GLB1, but they do accumulate MLG3. This result was unexpected because VP1 participates in other ABA-mediated events in seed maturation (7, 15, 24, 25). Also, recent investigations into VP1 function provide evidence that this protein positively regulates the expression of Group 1 LEA in maize (16). It may be that ABA alone is sufficient to modulate accumulation of Group 3 LEAs, whereas other proteins require ABA and VP1 to act in concert. Or another activating factor may be important in late maturation, when the level of *vp1* expression declines (16).

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LITERATURE CITED

1. **Abbe EC, Stein OL** (1954) The growth of the shoot apex in maize: embryogeny. Am J Bot **41**: 285–293
2. **Baker JC, Steele C, Dure L III** (1988) Sequence and characterization of 6 Lea proteins and their genes from cotton. Plant Mol Biol **11**: 277–291
3. **Bewley DJ, Black M** (1985) Seeds: Physiology of Development and Germination. Plenum Press, New York
4. **Chandler PM, Walker-Simmons M, King RW, Crouch M, Close TJ** (1988) Expression of ABA-inducible genes in water-stressed cereal seedlings. J Cell Biochem **12C** (Suppl): 143
5. **Close TJ, Kortt AA, Chandler PM** (1989) A cDNA-based comparison of dehydration-induced proteins (dehydrins) in barley and corn. Plant Mol Biol **13**: 95–108
6. **Curry J, Morris CF, Walker-Simmons MK** (1991) Sequence analysis of a cDNA encoding a group 3 LEA mRNA inducible by ABA or dehydration stress in wheat. Plant Mol Biol **16**: 1073–1076
7. **Dooner HK** (1985) *Viviparous*-1 mutation in maize conditions pleiotropic enzyme deficiencies in the aleurone. Plant Physiol **77**: 486–488
8. **Dure L III** (1985) Embryogenesis and gene expression during seed formation. *In* BS Miflin, ed, Oxford Surveys of Plant Molecular and Cell Biology, Vol 2. Oxford University Press, Oxford, UK, pp 179–197
9. **Dure L III, Crouch M, Harada J, Ho T-HD, Mundy J, Thomas T, Sung ZR** (1989) Common amino acid sequence domains among the LEA proteins of higher plants. Plant Mol Biol **12**: 475–486

10. **Finkelstein RR, Crouch ML** (1986) Rapeseed embryo development in culture on high osmoticum is similar to that in seeds. Plant Physiol **81:** 907–912
11. **Galau GA, Bijaisoradat N, Hughes DW** (1987) Accumulation kinetics of cotton late embryogenesis-abundant (Lea) mRNAs and storage protein mRNAs: coordinate regulation during embryogenesis and the role of abscisic acid. Dev Biol **123:** 198–212
12. **Kriz AR** (1989) Characterization of embryo globulins encoded by the maize *Glb* genes. Biochem Genet **27:** 239–251
13. **Kriz AR, Wallace MS, Paiva R** (1990) Globulin gene expression in embryos of maize viviparous mutants. Plant Physiol **92:** 538–542
14. **Marcotte WR Jr, Russell SH, Quatrano RS** (1989) Abscisic acid-responsive sequences for the Em gene of wheat. Plant Cell **1:** 969–976
15. **McCarty DR, Carson CB, Stinard PS, Robertson DS** (1989) Molecular analysis of *viviparous*-1: an abscisic acid insensitive mutant of maize. Plant Cell **1:** 523–532
16. **McCarty DR, Hattori T, Carson CB, Vasil V, Lazar M, Vasil IK** (1991) The *Viviparous*-1 developmental gene of maize encodes a novel transcriptional activator. Cell **66:** 895–905
17. **Mundy J, Chua NH** (1988) Abscisic acid and water stress induce the expression of a novel rice gene. EMBO J **7:** 2279–2286
18. **Mundy J, Yamaguchi-Shinozaki K, Chua NH** (1990) Nuclear proteins bind conserved elements in the abscisic acid-responsive promoter of a rice *rab* gene. Proc Natl Acad Sci USA **87:** 406–410
19. **Neill SJ, Horgan R, Parry AD** (1986) The carotenoid and abscisic acid content of viviparous kernels and seedlings of *Zea mays* L. Planta **169:** 87–96
20. **Neill SJ, Horgan R, Rees AF** (1987) Seed development and vivipary in *Zea mays* L. Planta **171:** 358–364
21. **Pla M, Goday A, Vilardell J, Gomez J, Pages M** (1989) Differential regulation of the ABA-induced 23–25 kD proteins in embryos and vegetative tissues of the viviparous mutants of maize. Plant Mol Biol **13:** 385–394
22. **Quatrano RS** (1987) The roles of hormones during seed development. *In* P Davies, ed, Plant Hormones and their Roles in Plant Growth and Development. Nijhoff, Boston, pp 494–514
23. **Reid JL, Walker-Simmons MK** (1991) Expression of ABA-responsive mRNAs and proteins in dehydrated wheat seedlings capable of regrowth (abstract No. 519). Plant Physiol **96:** 5–81
24. **Rivin CJ, Grudt T** (1991) Abscisic acid and the developmental regulation of embryo storage proteins in maize. Plant Physiol **95:** 358–365
25. **Robertson DS** (1955) The genetics of vivipary in maize. Genetics **40:** 745–760
26. **Robichaud CS, Wong J, Sussex IM** (1980) Control of *in vitro* growth of viviparous embryo mutants of maize by abscisic acid. Dev Genet **1:** 325–330
27. **Sanchez-Martinez D, Puigdomenech P, Pages M** (1986) Regulation of gene expression in developing *Zea mays* embryos. Protein synthesis during embryogenesis and early germination of maize. Plant Physiol **82:** 543–549
28. **Skriver K, Mundy J** (1990) Gene expression in response to abscisic acid and osmotic stress. Plant Cell **2:** 503–512
29. **Thomas T, Vivekananda J, Bogue MA** (1992) ABA regulation of gene expression in embryos and mature plants. *In* WJ Davies, ed, Topics in Environmental Physiology. BIOS, London, pp 125–135
30. **Vilardell J, Goday A, Freire MA, Torrent M, Martinez C, Torne JM, Pages M** (1990) Gene sequence, developmental expression and protein phosphorylation of RAB-17 in maize. Plant Mol Biol **14:** 423–432
31. **Zeevart JA, Creelman RA** (1988) Metabolism and physiology of abscisic acid. Annu Rev Plant Physiol Plant Mol Biol **39:** 439–473