The Maize *Viviparous8* Locus, Encoding a Putative ALTERED MERISTEM PROGRAM1-Like Peptidase, Regulates Abscisic Acid Accumulation and Coordinates Embryo and Endosperm Development[1][W]

Masaharu Suzuki*, Susan Latshaw, Yutaka Sato, A. Mark Settles, Karen E. Koch, L. Curtis Hannah, Mikiko Kojima, Hitoshi Sakakibara, and Donald R. McCarty

Plant Molecular and Cellular Biology Program, Horticultural Sciences Department, University of Florida, Gainesville, Florida 32611 (M.S., S.L., A.M.S., K.E.K., L.C.H., D.R.M.); Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, Aichi 464–8601, Japan (Y.S.); and RIKEN Plant Science Center, Yokohama, Kanagawa 230–0045, Japan (M.K., H.S.)

We describe a mutant of *Zea mays* isolated from a W22 inbred transposon population, *widow's peak mutant1* (*wpk1*), with an altered pattern of anthocyanin synthesis and aleurone cell differentiation in endosperm. In addition, a failure of the developing mutant embryo to form leaf initials is associated with decreased expression of a subset of meristem regulatory genes that includes *Abphyl1* and *Td1*. We show that the *viviparous8* (*vp8*) mutant has a similar pleiotropic phenotype in the W22 inbred background in contrast to the viviparous embryo phenotype exhibited in the standard genetic background, and we confirmed that *wpk1* is allelic to *vp8*. Further genetic analysis revealed that the standard *vp8* stock contains an unlinked, partially dominant suppressor of the *vp8* mutation that is not present in W22. Consistent with the early-onset viviparous phenotype of *vp8*, expression of several embryonic regulators, including *LEC1/B3* domain transcription factors, was reduced in the mutant embryo. Moreover, reduced abscisic acid (ABA) content of *vp8/wpk1* embryos was correlated with altered regulation of ABA biosynthesis, as well as ABA catabolic pathways. The ABA biosynthetic gene *Vp14* was down-regulated in the nonsuppressed background, whereas the *ZmABA8′oxA1a* ABA 8′-hydroxylase gene was strongly up-regulated in both genetic backgrounds. Molecular analysis revealed that *Vp8* encodes a putative peptidase closely related to *Arabidopsis thaliana* ALTERED MERISTEM PROGRAM1. Because the *Vp8* regulates meristem development as well as seed maturation processes, including ABA accumulation, we propose that VP8 is required for synthesis of an unidentified signal that integrates meristem and embryo formation in seeds.

In flowering plants, seed development begins with double fertilization generating a diploid zygote that undergoes embryogenesis and a triploid central cell that develops as endosperm. As organogenesis nears completion, the embryo and endosperm enter a maturation phase characterized by developmental arrest and acquisition of dormancy.

Genetic studies in Arabidopsis (*Arabidopsis thaliana*) and maize (*Zea mays*) have identified two classes of transcription factors that are essential for seed maturation and dormancy processes. The first class of genes, exemplified by Arabidopsis *LEC1* and *L1L*, encode HAP3-related transcription factors (Lotan et al., 1998; Kwong et al., 2003). The second class of genes, which includes Arabidopsis *LEC2*, *FUS3*, and *ABI3*, as well as maize *Viviparous1* (*Vp1*), encodes B3 domain transcription factors (McCarty et al., 1991; Giraudat et al., 1992; Luerssen et al., 1998; Stone et al., 2001). Loss of function of these genes causes precocious germination of developing seeds (McCarty et al., 1989; Meinke, 1992, 1994; West et al., 1994). Although these genes have overlapping roles in regulation of downstream gene expression mediated by common cis-elements (Suzuki et al., 1997; Monke et al., 2004; Braybrook et al., 2006), differences in time of expression and spatial localization confer differential functions during seed development (Parcy et al., 1997; Nambara et al., 2000; Raz et al., 2001; Brocard-Gifford et al., 2003; Baumbusch et al., 2004; Santos Mendoza et al., 2005; To et al., 2006). Upon germination, expression of the embryonic regulators in seedlings is strictly repressed by the closely related VAL B3 factors (Suzuki et al., 2007).

Control of maturation by the B3 transcription factor network is further determined by interactions with hormone signaling pathways. For example, the ABI3/VP1 transcription factor has a unique capacity to interact with abscisic acid (ABA) signaling conferred by physical interaction with ABI5 (Hobo et al., 1999; Nakamura et al., 2001). This functionality enables integration of the *LEC1/B3* network controlling em-

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* Corresponding author; e-mail masaharu@ufl.edu.

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bryogenesis with ABA signaling during the late phase of embryo maturation. *FUS3*, in turn, has been implicated in developmental regulation of ABA and GA biosynthesis in the seed (Nambara et al., 2000; Curaba et al., 2004; Gazzarrini et al., 2004) through regulation of key genes involved in hormone biosynthesis as well as turnover (for review, see Olszewski et al., 2002; Nambara and Marion-Poll, 2005). Finally, repression of embryonic development by the VAL B3 factors is promoted by an interaction with GA signaling (Suzuki et al., 2007).

Endosperm differentiation and maturation proceed in parallel with embryo development (for review, see Olsen, 2001). The mature endosperm of grass seeds consists of three principal cell types, starchy endosperm cells, aleurone cells, and basal endosperm transfer cells. Maize mutants that affect aleurone differentiation include *Defective kernel1* (*Dek1*), *Supernumerary aleurone layers1* (*Sal1*), and *Crinkly4* (*Cr4*; Becraft et al., 1996, 2002; Lid et al., 2002; Shen et al., 2003). Whereas *Dek1* and *Sal1* genes are required during early endosperm development, *Cr4* is required for aleurone differentiation late in endosperm formation (Becraft and Asuncion-Crabb, 2000). In addition, maize *Vp1*, an ortholog of Arabidopsis *ABI3*, is required for embryo maturation (McCarty et al., 1991) as well as activation of the anthocyanin biosynthesis pathway in aleurone cells (Hattori et al., 1992; Carson et al. 1997), indicating that the embryonic B3 genes also function in aleurone differentiation.

Here we describe genetic and molecular analysis of the maize *vp8* mutant in embryo and endosperm development. We show that novel *widow's peak1* (*wpk1*) mutations that alter the pattern of aleurone differentiation in the adgerminal region of the endosperm are allelic to *vp8*. Genetic analyses reveal that the *vp8* phenotype is strongly conditioned by genetic background in maize due to action of an unlinked semidominant suppressor locus. Our results suggest that the pleiotropic effects of the *vp8* mutation are mediated through regulation of specific meristem and embryonic regulatory genes and by regulation of genes controlling ABA biosynthesis and turnover in the developing seed. Finally, we cloned the *Vp8* gene and show that it encodes a putative membrane peptidase closely related to Arabidopsis ALTERED MERISTEM PROGRAM1 (AMP1).

RESULTS

Isolation of *wpk1* Mutants

To search for new mutations that affect differentiation of aleurone in maize, we screened the UniformMu inbred transposon-tagging population (McCarty et al., 2005). We identified the *wpk1* (*wpk1-umu1*) mutation that causes a distinctive pattern of pigmentation in the aleurone (Fig. 1A). A deficiency of anthocyanin accumulation in aleurone of *wpk1* mutant seeds was most pronounced along the interface of the endosperm and embryo (Fig. 1, A and B) extending around the silk attachment site at the top of the kernel. In addition, the subaleurone endosperm of mature *wpk1* kernels had a floury texture, resulting in less vitreous endosperm compared to wild-type kernels (Supplemental Fig. S1). Embryo development in *wpk1* seeds was also severely affected. Compared to wild-type embryos, developing mutant embryos had irregular morphology, smaller size (Fig. 1C), and a slightly translucent appearance (Fig. 1D). Mutant embryos of dry seed were necrotic and nonviable (Supplemental Fig. S1). Although the dome structure of the shoot apical meristem (SAM) could be discerned in developing mutant embryos, leaf initials were frequently absent (Fig. 1, E and F). The cells in meristem as well as nonmeristem regions of the *wpk1* embryo were strikingly enlarged. The cell enlargement phenotype was already apparent in the *wpk1* developing embryo at 12 d after pollination (DAP; Supplemental Fig. S2). Consistent with the defect in the SAM structure of the embryo, the *wpk1* mutants rarely formed a coleoptile and invariably failed to form leaves when developing embryos were rescued and grown on sterile culture medium (Fig. 1, G and H). Instead, the cultured mutant embryos formed prolific adventitious roots, suggesting that primary root meristem function was affected as well. We further noted that *wpk1* mutant kernels were prone to abort early in development in growing seasons with high temperatures (Supplemental Table S1; i.e. compare the spring and fall seasons). In addition to visible seed abortion, we also detected significantly lower frequencies of *wpk1* mutant seeds on self-pollinated heterozygous ears (Supplemental Table S1; e.g. 06S-e; $P = 5 \times 10^{-4}$). This result suggested that transmission of either male and/or female gametophytes carrying the *wpk1* mutation was also affected.

Aleurone Development in the *wpk1* Mutant

Taking advantage of the genetic background of the UniformMu population, we examined the pattern of aleurone differentiation marked by anthocyanin accumulation in the *wpk1* mutant seeds. In wild-type kernels, pigmentation of aleurone was typically visible by 14 DAP in our spring field conditions, initiating in a ring of aleurone cells (Fig. 2A). In the *wpk1* mutant, the onset of anthocyanin accumulation was delayed by up to several days and typically failed to occur at all in aleurone cells surrounding the embryo on the adgerminal face of the kernel. Within several days following the onset of pigment accumulation, the crown and adgerminal regions of wild-type kernels were completely pigmented, whereas the lower portion of the abgerminal aleurone remained colorless (Fig. 2B). In contrast to wild type, pigmentation of the abgerminal aleurone of *wpk1* mutant seed was frequently enhanced relative to wild type, forming irregular patches of pigmented aleurone (Fig. 2C). This result suggested that *Wpk1* regulation of anthocyanin accumulation is region specific.

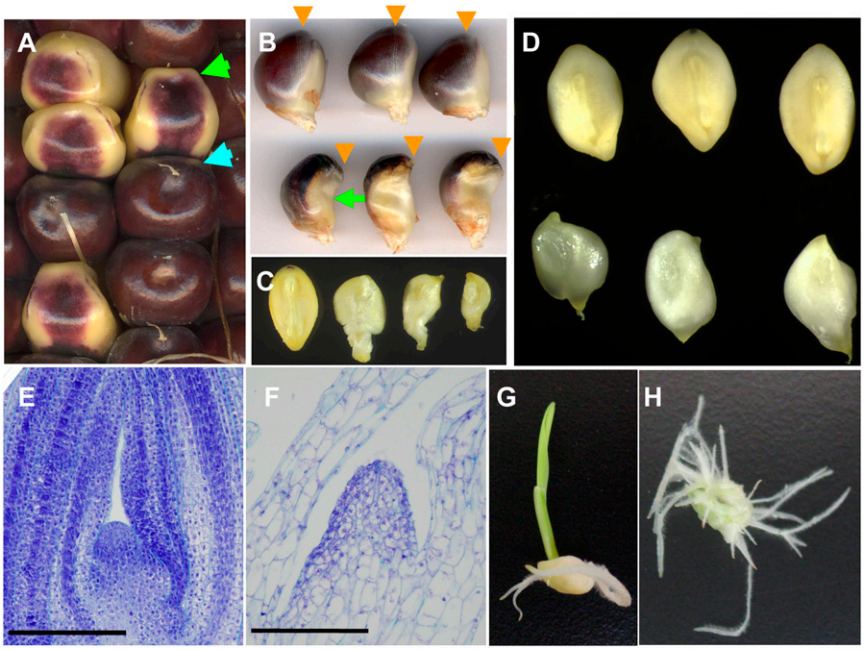

Figure 1. Phenotypes of the *wpk1* mutant seeds and seedling. A, Ear segregating *wpk1-umu1*. Wild-type and *wpk1* mutant seeds are indicated by blue and green arrows, respectively. B, Lateral views of mature seeds of wild type (top) and *wpk1* (bottom) are shown. The positions of silk attachment are indicated by orange arrows. The green arrow highlights deficiency of anthocyanin accumulation in the germinal region of *wpk1*. C, A wild-type embryo (left) and three *wpk1* embryos are shown. The embryos were excised from the seeds shown in B. D, Three developing embryos for wild type (top) and *wpk1* (bottom) are shown. The embryos were excised from the seeds at 20 DAP. E and F, Embryonic SAMs of wild-type (E) and *wpk1* (F) embryos at 16 DAP. Scale bars = 200 μm. The multiple embryos were sectioned and examined through histological analysis to confirm the altered development of SAM of the *wpk1* mutant embryos. G and H, Seedlings from rescued wild-type control and *wpk1* mutant embryos.

Maize *Cr4*, which encodes a plasma membrane receptor kinase, is a positive regulator of abgerminal aleurone differentiation in endosperm (Becraft et al., 1996). To test whether *Cr4* and *Wpk1* genetically interact in aleurone differentiation, we constructed *cr4 wpk1* double-mutant seeds to examine pigmentation and patterning of the aleurone (Supplemental Fig. S3). The double-mutant seeds exhibited anthocyanin deficiencies in both adgerminal and abgerminal regions. The aleurone phenotype was consistent with an additive superposition of the *cr4* and *wpk1* single-mutant patterns of anthocyanin accumulation in the aleurone. This result implies that these two genes most likely function independently in aleurone development. The opposite polarities of the *vp8* and *cr4* phenotypes with respect to the adgerminal/abgerminal axis of the endosperm suggest that development of adgerminal and abgerminal domains may be separately regulated (Becraft and Asuncion-Crabb, 2000).

In addition to the distinctive pattern of anthocyanin pigmentation, the size and morphology of aleurone cells was strongly affected in *wpk1* mutant seeds. The *wpk1* mutant aleurone cells in the abgerminal, as well as adgerminal, region were markedly elongated in the anticlinal direction, but not in the periclinal plane (Fig. 2, D–G). Measurement of cell lengths of 15 cells shown in Figure 2E indicated an average length 1.6-fold greater than comparable aleurone cells in wild-type kernels. This result indicated that the *Wpk1* gene suppresses anticlinal cell expansion of aleurone cells of the endosperm. Whereas cell elongation was most pronounced in the anthocyanin-deficient cells proximal to the embryo, slight elongation of aleurone cells was detected throughout the aleurone of *wpk1* seeds, suggesting that cell size and anthocyanin accumulation patterns may be independently regulated by the *Wpk1* gene.

Expression of Meristem-Related Genes in *wpk1* Developing Embryo

Because the *wpk1* mutation significantly altered organization of the embryonic SAM, we determined whether expression of genes that are known to regulate SAM formation in grasses was affected during mid-to-late embryo development (12, 14, and 16 DAP) when wild-type W22 embryos continue to produce new leaf primordia (Supplemental Fig. S2). Interestingly, the *wpk1* mutant affected expression of a subset, but not all, of SAM markers (Fig. 3). Expression of *Abnormal phyllotaxy1* (*Abphyl1*; Asakura et al., 2003; Giulini et al., 2004) and *Thick tassel dwarf1* (*Td1*; Bommert et al., 2005) genes was significantly reduced in the *wpk1* developing embryo, whereas, on a total RNA basis, *Fasciated ear2* (*Fea2*), *Knotted1* (*Kn1*), *Rough sheath2* (*Rs2*), and maize *PLASTOCHRON1-like* (*ZmPLA1*) genes (Hake et al., 1989; Timmermans et al., 1999; Tsiantis et al., 1999; Taguchi-Shiobara et al., 2001; Miyoshi et al., 2004) were expressed at comparable levels in wild-type and *wpk1* embryos. In a replicate experiment using embryo samples harvested in a separate growing season (Supplemental Fig. S4), *Abphyl1*, *Td1*, *Fea2*, and *ZmPLA1* genes showed similar patterns of expression, whereas expression of *Kn1* and *Rs2* genes was slightly reduced, suggesting an environmental effect on the gene expression. In any case, these results suggest that, in spite of having a strongly pleiotropic phenotype, the *wpk1* mutation causes limited alterations in gene expression during meristem development.

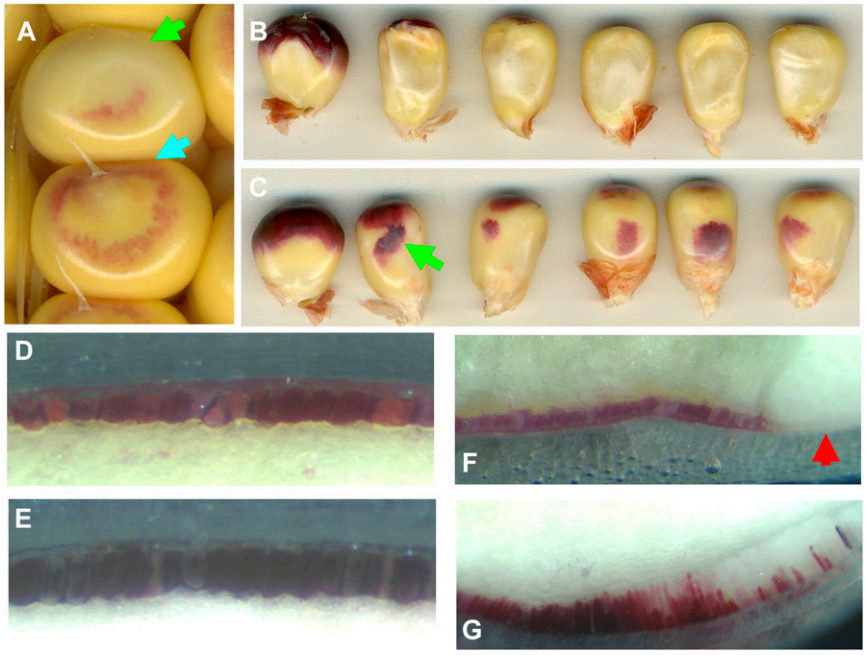

Figure 2. Aleurone development in *wpk1* seeds. A, Developing wild-type (blue arrow) and *wpk1* mutant (green arrow) seeds at 14 DAP are shown. B, Adgerminal views of developing wild-type embryo (left) and five *wpk1* seeds (right) at 26 DAP. C, Abgerminal views of 26-DAP seeds shown in B. Patches of precocious anthocyanin accumulation are indicated by a light green arrow. D to G, The aleurone layer in abgerminal (D and E) and germinal (F and G) regions of wild-type (D and F) and *wpk1* (E and G) kernels are shown. A red arrow indicates the position of the embryo in wild-type seed.

The *wpk1* Mutant Is Allelic to *vp8*

In a parallel study of the *vp8* mutant (Robertson, 1955), we introgressed the reference *vp8-R* allele into the W22 inbred. In the original genetic background, *vp8-R* has a viviparous seed phenotype with a heterochronic effect on vegetative development (Evans and Poethig, 1997). We noticed that, in the W22 background, *vp8-R* conditioned a defective embryo rather than a viviparous phenotype and that pigmentation of the endosperm resembled the pattern observed in *wpk1* kernels (Fig. 4, A and B). Genetic complementation tests performed by crossing mutant heterozygotes confirmed allelism of *vp8-R* and *wpk1-umu1* (hereafter *vp8-umu1*) based on noncomplementation of the seed phenotype. Because the genetic background of the *vp8-R* stock distributed by the Maize Genetics Cooperation Stock Center is not documented, we use the designations *vp8-R (SC)* and *vp8-R (W22)* to distinguish the genotypes conferring viviparous and defective embryo phenotypes, respectively.

To understand the basis for the altered expression of *vp8-R* in the SC and W22 backgrounds, we performed a characterization of embryo and aleurone development in *vp8-R (SC)* and *vp8-R (W22)* seeds and compared their phenotypes to *vp8-umu1*. In contrast to the *vp8-R* and *vp8-umu1* mutations in the W22 background, *vp8-R (SC)* mutant embryos were viable if rescued prior to desiccation as previously reported (Evans and Poethig, 1997; Supplemental Fig. S3). The mutant was capable of forming a well-organized SAM and leaf initials with comparable size of the cells in the developing embryo (Fig. 4, C and D). The aleurone layer of *vp8-R (SC)* kernels did not show marked differences from wild type (Fig. 4, E and F). Whereas the absence of *C1* and *R1* alleles required for anthocyanin biosynthesis prevented evaluation of pigmentation patterns in the SC background, mutant aleurone cells were elongated only slightly compared to wild type (1.15-fold longer in the anticlinal direction compared to the wild-type control). Furthermore, unlike *vp8-umu1*, we did not detect increased early seed abortion or less

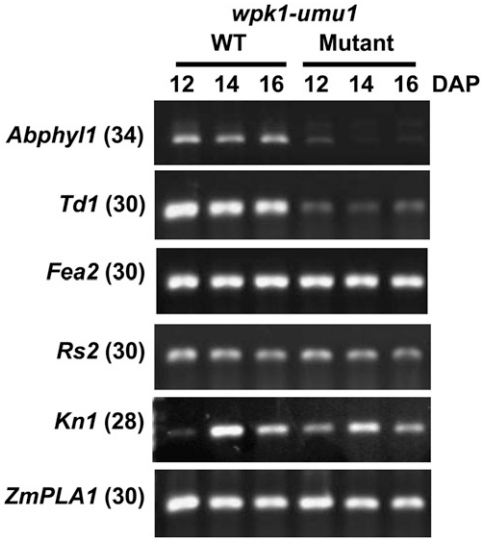

Figure 3. Expression of meristem-related genes in *wpk1* embryos. RT-PCR analysis of meristem-related genes in developing wild-type and *wpk1* mutant embryos at 12, 14, and 16 DAP. The numbers in parentheses indicate the numbers of cycles in the RT-PCR reactions. The cycle numbers were optimized to quantitatively examine expression for each gene.

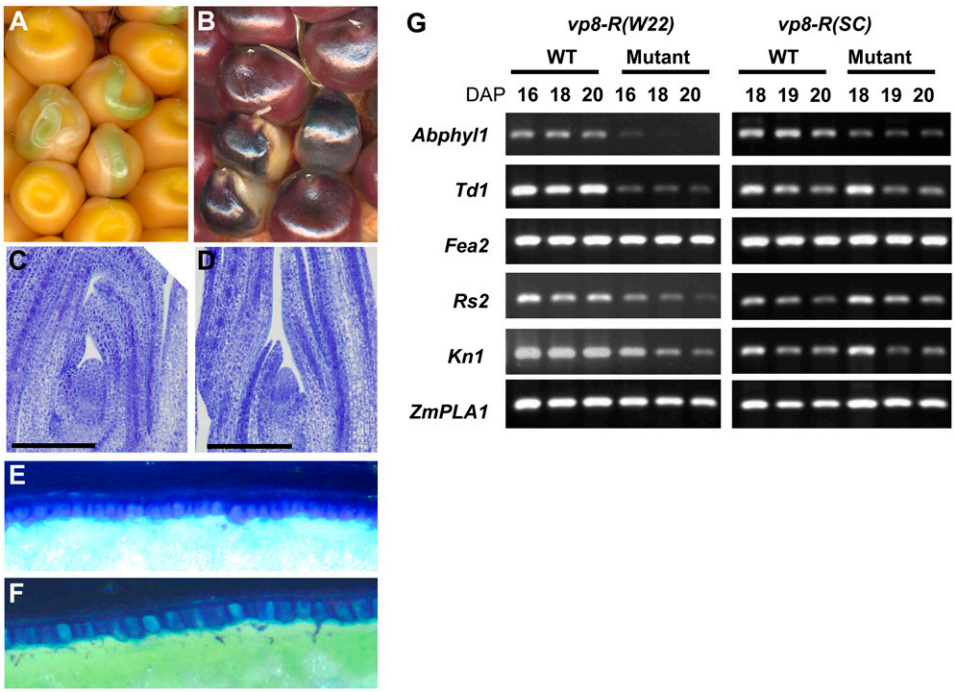

Figure 4. Phenotypic analysis of *vp8* seeds. A and B, Mature ears segregating *vp8-R* in SC (A) and W22 inbred (B) genetic backgrounds. C and D, Embryonic SAMs in developing wild-type and *vp8-R (SC)* embryos at 16 DAP. Scale bars = 200 μm. E and F, Abgerminal aleurone of wild-type and *vp8-R* mutant endosperms. G, RT-PCR analysis of meristem-related genes in *vp8-R (SC)* and *vp8-R (W22)* embryos. The RT-PCR condition for each gene was identical to that in Figure 3. The plants were grown simultaneously in a greenhouse.

than expected frequencies of viviparous seeds on the self-pollinated ears (Supplemental Table S2).

To compare meristem function in the SC and W22 backgrounds, expression profiles of meristem marker genes in developing *vp8-R (SC)* and *vp8-R (W22)* embryos were determined by reverse transcription (RT)-PCR (Fig. 4G). In the SC background, all of the meristem markers, except *Abphyl1*, showed comparable expression profiles in wild-type and mutant embryos. In marked contrast to *vp8-R (W22)* and *vp8-umu1* embryos, *vp8-R (SC)* showed no evidence of altered expression of *Td1*. Whereas *Abphyl1* expression was reduced in both the W22 and SC backgrounds, the effect was more pronounced in the W22 background. The comparatively subtle changes in gene expression of meristem-related genes in the SC background were consistent with the observation that the *vp8-R (SC)* mutant embryo was able to develop a functional SAM. Similar to *vp8-umu1*, *Fea2* and *ZmPLA1* genes were unaffected by *vp8-R* in both the W22 and SC backgrounds. Expression of *Rs2* and *Kn1* genes was slightly decreased in *vp8-R (W22)* embryos compared to wild type. These subtle differences are most likely due to environmental effects on embryo development because we detected similar differences in the *vp8-umu1* between two seasons (Fig. 3; Supplemental Fig. S4). Overall, these results indicated that genetic background differences between W22 and SC had a much more profound effect on the *vp8* phenotype than allele differences.

The SC Genetic Background Contains a Partially Dominant Suppressor of *vp8*

To identify genetic factors that interact with the *vp8* mutation in W22 and SC genetic backgrounds, we analyzed the seed and seedling phenotypes of F1 plants generated by reciprocal crosses between *vp8-R (SC)* and *vp8-umu1*. All of the heteroallelic *vp8* mutant seed from crosses made in either direction developed embryos comparable in size to wild type (Table I; Supplemental Fig. S5). The characteristic *wpk1* phenotypes, including severe defective embryo and patterned anthocyanin deficiency, were not observed in the F1 seed. These results indicated that the SC background is able to suppress the *wpk1* defective embryo sufficiently to produce a well-developed embryo that is in some cases viviparous. In spring (warm) as well as fall (cool) field environments, viviparous embryos were frequently observed in the F1 seeds derived from crosses between *vp8-R (SC)* females and *vp8-umu1* male parents, whereas F1 seed from the reciprocal cross predominantly had an intermediate phenotype with near full-size embryos that were weakly viviparous. The qualitative difference in the phenotypes of reciprocal F1 hybrid seed suggested that either maternal factors or gametophytic transmission affected *vp8* function in the developing seed. In this respect, it is noteworthy that, in some of the crosses involving *vp8-R (W22)* made in either direction, mutant seed was recovered at a lower than expected frequency consistent with *Vp8* having a function in both male and female gametophytes (Table I).

To determine whether embryos with strong and intermediate viviparous phenotypes were viable and capable of growing into seedlings, we rescued these mutant embryos and placed them in sterile culture. Unlike *vp8-umu1*, the viviparous *vp8-R (SC)* mutants and heteroallelic F1 mutant embryos developed shoots at >90% frequency (Supplemental Fig. S6).

Table I. *F1 seed phenotypes from heteroallelic crosses between vp8-R (SC) and vp8-umu1*

06F and 06S represent the 2006 fall and 2006 spring seasons, respectively. Mutant embryos were classified as viviparous, intermediate, and wpk (aborted) based on the extent of shoot and scutellum development, as indicated in Supplemental Figure S3. χ^2 tests and *P* values were calculated for a model that assumed that normal and all mutant classes (viviparous + intermediate + wpk) segregate 3:1.

Female	Male	Normal	Mutant			*P* Value
			Viviparous	Intermediate	wpk	
06F +/*vp8-R (SC)*	06F +/*vp8-umu1* a	130	32	0	0	0.12
06F +/*vp8-R (SC)*	06F +/*vp8-umu1* c	65	11	0	0	0.03
06F +/*vp8-R (SC)*	06F +/*vp8-umu1*	127	29	0	0	0.06
06F +/*vp8-umu1*	06F +/*vp8-R (SC)*	153	0	29	0	4.7×10^{-3}
06F +/*vp8-umu1*	06F +/*vp8-R (SC)*	123	0	29	0	0.09
06F +/*vp8-umu1*	06F +/*vp8-R (SC)*	139	1	22	0	1.5×10^{-3}
06S +/*vp8-R (SC)*	06S +/*vp8-umu1*	92	33	1	0	0.61
06S +/*vp8-R (SC)*	06S +/*vp8-umu1*	83	5	0	0	2.9×10^{-5}
06S +/*vp8-R (SC)*	06S +/*vp8-umu1*	68	17	0	0	0.29
06S +/*vp8-umu1*	06S +/*vp8-R (SC)*	99	3	3	0	5.0×10^{-6}
06S +/*vp8-umu1*	06S +/*vp8-R (SC)*	87	3	15	0	0.06
06S +/*vp8-umu1*	06S +/*vp8-R (SC)*	150	1	10	0	1.0×10^{-7}

The results of reciprocal crosses indicated that one or more dominant genetic factors in the SC background partially suppress the severe developmental defects caused by the *vp8* mutation in W22. To estimate the number of genetic loci involved in suppression of *vp8* in the SC background, we analyzed the phenotypes of F2 seeds generated from the heteroallelic F1 seeds as well as of F2 seeds from backcrosses of *vp8-R (SC)* with W22 (Table II). The mutant F2 seeds were grouped in three phenotype classes: strongly viviparous, intermediate, and wpk-like, respectively. Within the mutant class, the viviparous plus intermediate and wpk-like embryos occurred in a ratio that was consistent with segregation of a single, unlinked partially dominant suppressor. The SC and W22 inbreds are evidently homozygous for dominant and recessive alleles of the suppressor, respectively.

Altered Regulation of ABA Synthesis and Turnover in the *vp8* Mutant

The *vp8-R (SC)* genotype has been previously reported to have moderately reduced levels of ABA in the developing embryo (Neill et al., 1986), suggesting that ABA deficiency may contribute to the viviparous phenotype. We observed that viviparous *vp8-R (SC)* mutant embryos could be distinguished from wild-type embryos as early as 16 DAP consistent with the timing of ABA biosynthesis in maize embryos (Tan et al., 1997). However, the pleiotropic phenotypes of *vp8-R (SC)* exhibited during vegetative development are not readily attributed to ABA deficiency (Evans and Poethig, 1997).

To examine the potential role of hormone biosynthesis in the complex phenotype of *vp8* mutants, we analyzed levels of three key plant hormones, ABA, auxin, and cytokinin, in developing embryo and endosperm tissues of wild-type and mutant seeds. Consistent with the previous report by Neill et al. (1986), the amount of ABA was significantly reduced in *vp8-R (SC)* developing embryo at 16 DAP (Fig. 5A), whereas auxin and cytokinin levels did not show clear differences between mutant and wild type (Supplemental Table S3). Moreover, consistent with the enhanced *vp8* phenotype in the W22 background, accumulation of ABA in *vp8-umu1* embryos was dramatically lower compared to the *vp8-R (SC)* and wild-type genotypes at 14 and 16 DAP (Fig. 5A; Supplemental Table S3). Whereas ABA levels measured on a fresh-weight basis in the SC and W22 backgrounds correlated with the

Table II. *F2 seed phenotypes from self-crosses of F1 backcrossed seeds and heteroallelic seeds*

Fall season crosses (2005 [05F] and 2006 [06F]) were analyzed. The *P* values were determined from χ^2 tests of a model assuming segregation of an incompletely dominant suppressor (i.e. the class of viviparous and intermediate phenotypes combined, and the wpk class segregate 3:1). The *vp8* alleles segregating in F2 of heteroallelic hybrids were not distinguished.

Female	Male	Normal	Mutant			*P* Value
			Viviparous	Intermediate	wpk	
05F +/+ (W22)	05F +/*vp8-R (SC)*	316	41	25	26	0.47
05F +/+ (W22)	05F +/*vp8-R (SC)*	326	40	45	18	0.08
05F +/+ (W22)	05F +/*vp8-R (SC)*	340	42	0	16	0.65
06F +/*vp8-R (SC)*	06F +/*vp8-umu1*	350	17	28	15	1
06F +/*vp8-R (SC)*	06F +/*vp8-umu1*	330	38	29	14	0.11
06F +/*vp8-R (SC)*	06F +/*vp8-umu1*	232	35	19	18	1

severity of the mutant phenotype, we cannot rule out the possibility that this difference is due to indirect effect caused by the profound developmental defects of *vp8 (W22)* embryos. We did not detect significant differences in hormone levels in endosperms of wild-type and *vp8* seed (Supplemental Table S3), indicating that *Vp8* specifically affects ABA accumulation in the embryo.

To gain insight into how ABA accumulation is regulated by *Vp8*, we analyzed expression of key genes, *Vp14* and *ZmABA8′oxA1a*, that are implicated in control of ABA biosynthesis and degradation, respectively. The *Vp14* gene encodes the major 9-cis-carotenoid dioxygenase expressed during maize embryo development (Tan et al., 1997), and *ZmABA8′oxA1a* is an ortholog of the ABA 8′-hydroxylases (Kushiro et al., 2004; Saito et al., 2004; Millar et al., 2006; Okamoto et al., 2006; Yang and Choi, 2006; Yang and Zeevaart, 2006; Saika et al., 2007) that catalyzes the first step in catabolism of ABA. As shown in Figure 5B, *Vp14* expression was significantly lower in developing *vp8-umu1 (W22)* embryos, whereas expression of *Vp14* in *vp8-R (SC)* embryos was similar to wild type. In contrast, *ZmABA8′oxA1a* expression was markedly elevated in *vp8* mutant embryos in both the SC and W22 genetic backgrounds. These results indicate that the severe ABA deficiency evident in the W22 background correlates with simultaneous down-regulation of ABA biosynthesis and up-regulation of ABA catabolism pathways, whereas the moderate ABA deficiency conditioned by *vp8* in the SC background is primarily due to elevated ABA catabolism. A key implication is that the dominant suppressor in the SC background restores regulation of ABA biosynthesis, but not repression of ABA catabolism. These results are consistent with the independent evidence that both ABA biosynthesis and turnover contribute to regulation of seed development and germination (for review, see Nambara and Marion-Poll, 2005).

Expression of Embryonic Regulators in the *vp8* Mutant

Although the reduced ABA accumulation in *vp8* mutant embryos is consistent with the viviparous phenotype, ABA deficiency alone seems unlikely to account for the pleiotropic phenotypes of *vp8* in embryo, aleurone, and vegetative organs. To better understand the complex embryo phenotype, we analyzed expression of the LEC1-related factors and B3 domain transcription factors that regulate embryogenesis and maturation. In Arabidopsis, loss-of-function mutations at four loci, *lec1*, *lec2*, *fus3*, and *abi3*, prevent embryo maturation and induce a potential for viviparous seed development. The *ABI3* gene and its maize ortholog, *Vp1*, are required for ABA-regulated gene expression late in seed development, whereas *lec1*, *lec2*, and *fus3* genes affect earlier stages of seed development. Because aspects of the *vp8* phenotype are manifest at early stages of seed development, we considered the possibility that *Vp8* may also interact with the early-acting regulators of embryogenesis in maize.

To develop RT-PCR assays for expression of the maize embryo pathway, we searched available genome and EST databases to identify maize orthologs of the Arabidopsis HAP3 and B3 transcription factor genes. We identified three homologs of the Arabidopsis *LEC1* and *L1L* HAP3-related genes (*ZmLEC1*, *ZmL1La*, and *ZmL1Lb*, respectively) based on the similarity of the HAP3 domains (M. Suzuki, unpublished data). A *LEC1* ortholog identical to *ZmL1La* has been described previously (Zhang et al., 2002). Based on alignments of B3 domain sequences, we identified a single maize gene with roughly equal similarity to the Arabidopsis *FUS3* and *LEC2* genes, which we designated *ZmFUS3*.

As shown in Figure 6, expression of *ZmLEC1*, *ZmL1La*, *ZmL1Lb*, *ZmFUS3*, and *Vp1* was reduced in *vp8* mutant embryos in both W22 and SC back-

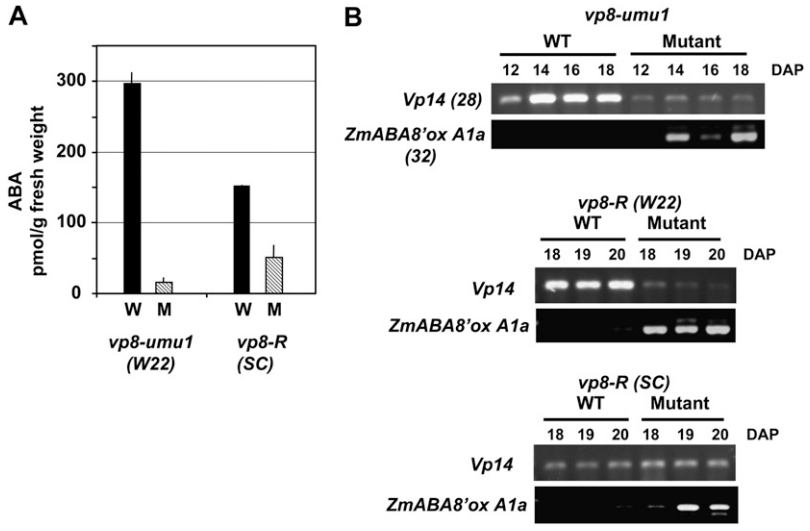

Figure 5. ABA content and expression of *Vp14* and *ZmABA8′oxA1a* in *vp8* embryos. A, ABA content of developing wild-type (W) and *vp8* mutant embryos (M) at 16 DAP. Error bars indicate the mean deviation of replicate experiments. B, RT-PCR analysis of *Vp14* and *ZmABA8′ox A1a* expression in *vp8* embryos in the W22 and SC genetic backgrounds. Total RNA was prepared from embryos of field-grown *vp8-umu1* developing seeds and greenhouse-grown *vp8-R (W22)* and *vp8-R (SC)* seeds.

grounds. Whereas all five genes showed quantitatively lower expression in *vp8* embryos late in development (18 and 20 DAP), *ZmL1Lb* expression, which was not detected in *vp8* embryos after 18 DAP, showed the most striking qualitative difference between wild type and *vp8* common to both backgrounds. Interestingly, the *ZmL1La* and *ZmL1Lb* genes were differentially expressed in the wild-type SC and W22 backgrounds. *ZmL1La* expression was relatively high compared to *ZmL1Lb* throughout development in SC embryos, whereas *ZmL1Lb* and *ZmL1La* were expressed at similar levels in W22 embryos. Hence, among the embryogenesis regulators tested, only *ZmL1Lb* and *ZmL1La* exhibited expression differences that correlated with partial suppression of the *vp8* phenotype. In any case, because *vp8* embryos in the suppressed background are fully formed, the qualitative differences in expression of the embryo pathway genes are likely to be caused by specific action of the *Vp8* gene, but not by nonspecific effects due to gross morphological defects in embryo formation. This interpretation is further supported by the observation that expression of *ZmL1Lb*, in contrast to the other embryonic regulators, was clearly reduced in the *vp8-R (SC)* mutant at 18 DAP under greenhouse conditions. Although the viviparous phenotype of greenhouse-grown *vp8-R (SC)* embryos is barely discerned at 18 DAP, embryo genotypes were confirmed by subsequent RT-PCR analysis for the presence of detectable *Vp8* mRNA (see Fig. 7B).

Cloning of the *Vp8* Gene by Transposon Tagging Performed in Silico

Our screen of the UniformMu population (McCarty et al., 2005) yielded three additional independent *vp8* alleles that were confirmed by genetic complementation tests (designated *vp8-umu2*, *vp8-umu3*, and *vp8-umu4*, respectively). No consistent differences in the embryo and endosperm phenotypes could be discerned among a total of five *vp8* alleles observed in the *W22* background. Skewed F2 segregation ratios from self-pollinated heterozygotes were detected for multiple alleles (Supplemental Table S2). Moreover, RT-PCR analysis of meristem genes, ABA biosynthesis pathway genes, and *LEC1/B3* embryo markers showed similar patterns as described for *vp8-umu1* and *vp8-R* alleles (Supplemental Fig. S7).

To clone the *vp8* locus, we performed MuTAIL-PCR (Settles et al., 2004), high-throughput sequencing, and bioinformatics cluster analysis (McCarty et al., 2005; Supplemental Information S1) to screen for allelic Mu insertions in the *vp8-umu1*, *vp8-umu2*, and *vp8-umu3* mutants. Bioinformatics analysis detected overlapping MuTAIL sequences from the *vp8-umu1* and *vp8-umu2* lines that were derived from closely spaced insertions in a maize gene. Using gene-specific PCR, we confirmed the presence of a third nearby Mu insertion in the *vp8-umu3* allele. Analysis of the flanking genomic sequences by BLASTX (Altschul et al., 1997) detected similarity to glutamate carboxypeptidases from various organisms, including homologs of the Arabidopsis AMP1 (Helliwell et al., 2001).

We performed RT-PCR to analyze expression of *Vp8* in wild-type and *vp8* mutant embryos (Fig. 7B). A *Vp8* transcript was detected in the W22 inbred but not in *vp8-umu1* and *vp8-umu2* mutants, indicating that the transposon insertions disrupted transcription or mRNA stability. Although the molecular lesion in the *vp8-R* allele was not determined, the reference allele was also null for mRNA expression based on RT-PCR. In the developing seed of wild type, expression of the *Vp8* gene was markedly higher in the embryo than in endosperm on a total RNA basis (Fig. 7C). *Vp8* expression was detected as early as in 6-DAP developing seed, as well as in various vegetative tissues at lower levels (Fig. 7D), consistent with *AMP1* gene expression in Arabidopsis (Helliwell et al., 2001; Vidaurre et al., 2007; Schmid et al., 2005).

Structure of the VP8 Protein

To determine the complete sequence of the VP8 protein, we isolated and sequenced a full-length cDNA of *Vp8* mRNA by RT-PCR using RNA prepared from W22 developing embryos. We designed primers based on maize genome survey sequences that contained the predicted 5′ and 3′ untranslated regions of the gene. The cDNA sequence predicted a protein of 714 amino acids that aligns with two membrane-localized glutamate carboxypeptidases from human

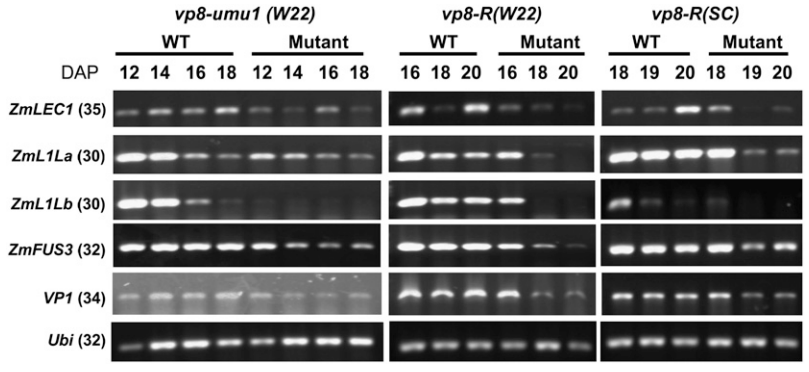

Figure 6. Expression of *LEC1/B3* embryonic genes in developing maize embryos. RT-PCR analysis of *LEC1/B3* genes in *vp8* developing embryos. Numbers in parentheses indicate the numbers of cycles in the RT-PCR reactions. The cycle numbers were optimized to quantitatively examine expression for each gene. The *Ubiquitin* (*Ubi*) gene was used as a control marker.

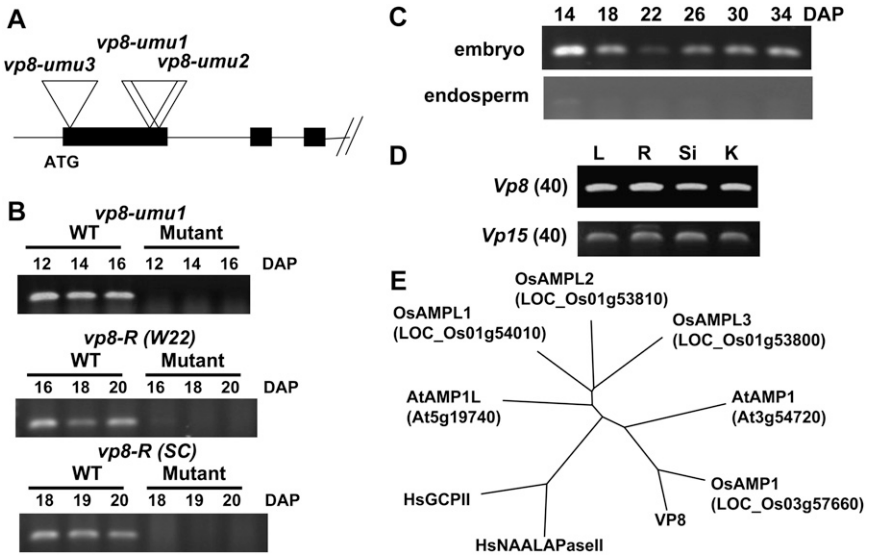

Figure 7. Structure and expression of the *Vp8* gene. A, Three independent Mu-tagged alleles of *vp8* mutant from the UniformMu population are shown. B, RT-PCR analysis of *Vp8* expression in developing embryos of *vp8* mutants. These results verified the genotype of embryos that were used for our RT-PCR analyses (Figs. 3–6) including embryo of *vp8-R (SC)* at 18 DAP. The 28 PCR cycles were run in the RT-PCR reactions for the *Vp8* gene. C, RT-PCT analysis of *Vp8* gene expression in embryo and endosperm of developing maize seeds. The 28 PCR cycles were run in the RT-PCR reactions for the *Vp8* gene. D, RT-PCR analysis of expression of *Vp8* gene in various plant tissues. The *Vp15* gene (Suzuki et al., 2006) was used for control. The 40 cycles of PCR were run in the RT-PCR reactions, as indicated in the parentheses for both genes, to detect amplified products. L, Leaf from 10-d-old seedling; R, primary root from germinating seeds; Si, unpollinated silk; K, whole kernel (6 DAP). E, Unrooted ClustalW tree of AMP1/VP8-related proteins from maize (VP8), Arabidopsis (AtAMP1, AtAMP1L), rice (OsAMP1, OsAMPL1-3), and human glutamate carboxypeptidases (HsGCPII, HsNAALAPaseII).

(Israeli et al., 1993; Pangalos et al., 1999). Comparison of amino acid sequences of the human glutamate carboxypeptidases and several plant homologs suggested that plants have two subfamilies of the peptidase-like proteins (Fig. 7D). In the tree, maize VP8, Arabidopsis AMP1, and rice (*Oryza sativa*) Os03g57660 form a distinct subfamily. In Arabidopsis, *AMP1* has been shown to regulate shoot meristem development (Chaudhury et al., 1993; Conway and Poethig, 1997; Helliwell et al., 2001). To search for potential *AMP/Vp8*-related genes in maize, we analyzed all publicly available maize sequences, including a near-complete draft sequence of the whole maize genome (including 15,750 phase I bacterial artificial chromosome sequence assemblies; www.maizesequence.org). No other candidates for related maize paralogs were detected.

DISCUSSION

Our results show that maize *Vp8* encodes a putative membrane-localized peptidase that is closely related to Arabidopsis AMP1. Loss of *Vp8* function causes either lethality or precocious germination of the developing embryo, depending on the genetic background. The *vp8* mutant is highly pleiotropic in the W22 inbred, indicating that the gene is essential for a wide range of developmental processes in maize. In the developing embryo, the *Vp8* gene is required for expression of *LEC1/B3* embryonic regulators, as well as for genes that regulate ABA synthesis and turnover. *Vp8* effects on SAM organization are associated with reduced expression of a specific subset of meristem-related genes. Finally, we have identified a partially dominant suppressor that genetically interacts with v*p8* in regulation of plant development in maize.

Although it remains to be determined whether maize *Vp8* is capable of complementing the Arabidopsis *amp1* mutant, the similarities in the pleiotropic phenotypes of the *amp1* and *vp8-R (SC)* mutants, as well as in the protein structures of AMP1 and VP8, suggest that these genes are likely orthologs. Whereas the precise mechanisms of SAM development and leaf differentiation are thought to be distinct in Arabidopsis and maize (Scanlon, 2000; Tsiantis and Hay, 2003; Champagne and Sinha, 2004), *amp1* and *vp8-R (SC)* mutants, respectively, cause acceleration of leaf formation in both species (Chaudhury et al., 1993; Evans and Poethig, 1997). *AMP1* suppression of lateral root formation (Vidaurre et al., 2007) is also consistent with *Vp8* function (Fig. 1). In addition, the *amp1* mutant gametophytes are less capable of producing seeds than wild type (Chaudhury et al., 1993), as observed in the *vp8* mutant. Although *amp1* does not consistently cause precocious germination, a possibly related phenotype, ectopic leaf initiation, has been described in the developing seeds (Conway and Poethig, 1997). Evans and Poethig (1997) noted observation of vivip-

arous seeds from an *amp1* allele. Furthermore, inspection of microscopy images of *amp1* mutant seed (Mordhorst et al., 1998) indicates the presence of enlarged aleurone cells, consistent with the *vp8 (W22)* phenotype in maize. Structurally, VP8 is most similar to LOC_Os03g57660, the apparent rice candidate ortholog of Arabidopsis AMP1. Whereas our searches of maize sequence databases did not detect any additional members of the AMP1/VP8/LOC_Os03g57660 group in the maize genome, we detected evidence of other maize AMP1-like peptidases belonging to the AMP1-like family composed of At5g19740 (AMP1L) and three rice AMP1L proteins.

Although *amp1* and *vp8* have analogous phenotypes, the mutants differ in their reported effects on hormone synthesis. In Arabidopsis, *amp1* mutant seedlings are reported to have elevated cytokinin levels compared to wild type (Chaudhury et al., 1993; Saibo et al., 2007), whereas we did not detect significant differences in cytokinin levels in wild-type and *vp8* mutant embryos of maize. This discrepancy may well be due to the different tissues analyzed in the two species (seedling versus embryo). Cytokinin biosynthesis is evidently tightly regulated by spatial and temporal signals. In Arabidopsis and cereals, cytokinin turnover, as well as cytokinin responses, has been shown to be highly localized in plant tissues at various developmental stages (D'Agostino et al., 2000; Werner et al., 2003; Higuchi et al., 2004; Miyawaki et al., 2004; Takei et al., 2004; Hutchison et al., 2006; Riefler et al., 2006). For instance, in maize, expression of *Abphyl1*, which encodes a negative regulator of cytokinin signaling, is restricted to a subdomain of the embryonic SAM (Giulini et al., 2004). A cytokinin-activating enzyme encoded by rice *LOG* is expressed in a similar pattern in the vegetative SAM (Kurakawa et al., 2007). Whereas we show a predominant effect of *vp8* on ABA synthesis and turnover in maize embryos, the hormone levels, including ABA content, have not been determined in the *amp1* developing seeds. To precisely compare function of *AMP1* and *Vp8* in hormone accumulation, a more comprehensive and extensive analysis of hormone quantification at the equivalent stages of development, as well as at the cellular level, will be required.

Among the embryonic regulatory genes we analyzed, *ZmL1Lb* shows the earliest detectable difference in the expression in *vp8* mutant embryos prior to discernible vivipary, thus suggesting that *ZmL1Lb* may be a primary target of unidentified factors derived from *Vp8* function. In Arabidopsis, *LEC1* has been proposed to be an upstream activator for *FUS3* and *ABI3* B3 domain genes (Kagaya et al., 2005). Therefore, decreased expression of *ZmL1La* and *ZmL1Lb* could account for down-regulation of *ZmFUS3* and *Vp1*. Interestingly, *lec1/fus3/lec2* and *amp1/vp8* class mutants, respectively, cause heterochronic shifts in development, although apparently in opposite directions. In contrast to *lec1*, *lec2*, and *fus3* mutants, *amp1* does not cause ectopic development of trichomes on the cotyledons (Chaudhury et al., 1993; Keith et al., 1994; Meinke et al., 1994; West et al., 1994). Similarly, in maize, *vp8* delays the expression of adult vegetative traits in leaves extending juvenile development (Evans and Poethig, 1997). Hence, there is not a simple relationship between heterochronic vegetative phenotypes and reduced expression of the *LEC1/B3* genes in *vp8* embryos. Similar pleiotropy is evident in regulation of *Abphyl1* expression. Although loss of *Abphyl1* function causes fasciation of the SAM in developing embryos as well as in plants (Jackson and Hake, 1999), *vp8-R (SC)* mutant plants do not show evidence of fasciation. We cannot completely rule out the possibility that the early decrease in *ZmL1Lb* and *Abphyl1* expression may be caused indirectly by subtle morphological changes in the mutant embryo.

The down-regulation of *LEC1/B3* genes in the *vp8* may account for regulation of ABA accumulation as well as affect maturation-related gene expression. *FUS3* has been shown to regulate ABA accumulation in developing seeds of Arabidopsis (Nambara et al., 2000; Gazzarrini et al., 2004). Hence, the up-regulation of the *ZmABA8′oxA1a* gene may be caused by reduced *ZmFUS3* expression. Interestingly, the 5′ regions of maize and Arabidopsis ABA 8′-hydroxylase genes have multiple Sph/RY motifs (data not shown), which have been shown to mediate *FUS3* binding (Reidt et al., 2000). The inference that ABA level is controlled indirectly through regulation of seed-specific factors is consistent with the fact that *vp8* mutant plants lack phenotypes associated with ABA deficiency. The observation that *vp8 (SC)* embryos respond to ABA with normal sensitivity (Robichaud et al., 1980) suggests that seed-specific ABA deficiency accounts for the viviparous phenotype. Moreover, the floury endosperm and translucent embryo phenotypes of *vp8*, which are frequently associated with lower protein content in cereal seeds (for review, see Lopes and Larkins, 1993), are consistent with the broader role of *LEC1/B3* regulators in gene expression for seed storage proteins and lipid accumulation (Keith et al., 1994; Meinke et al., 1994; Parcy et al., 1994; West et al., 1994; Nambara et al., 1995).

Whereas *vp8* mutation alters development and gene expression in meristem as well as nonmeristem tissues of the embryo, it is not yet clear whether the dual effects are mediated by the same or distinct independent mechanisms. One possibility is that *AMP1/Vp8* establishes a regulatory field that is interpreted locally to produce diverse responses in different tissues. In the meristem of Arabidopsis, *AMP1* function is implicated in cytokinin signaling (Chaudhury et al., 1993; Helliwell et al., 2001). We have shown that *Vp8* is required for normal expression of the *Abphyl1* cytokinin response regulator during maize embryo development. This result suggests that *Vp8* may at least indirectly regulate cytokinin signaling at a cellular level in the SAM of maize embryos. In another context, Vidaurre et al. (2007) have recently shown that the *MP/ARF5* transcription factor interacts locally with *AMP1* function in SAM development. Thus, a series of

domain-specific transcription factors may interact with a ubiquitous *AMP1/Vp8* function to precisely regulate meristem development. Likewise, *LEC1/B3* genes, which are primarily expressed in nonmeristem tissues of the embryo, regulate diverse downstream targets, including seed storage protein genes under the apparent influence of *AMP1/Vp8* function.

The isolation of *vp8* alleles in the nonsuppressed W22 inbred background proved crucial to uncovering the unexpectedly broad and essential role of *Vp8* in embryo and endosperm development. One of the notable findings is that *Vp8* is likely required for normal cell division and expansion processes in the developing embryo as well as in the aleurone. Although Arabidopsis *AMP1* is expressed throughout the tissues, expression of this gene is relatively higher in rapidly dividing tissues such as in shoot and root meristems (Schmid et al., 2005), implying involvement of *AMP1/Vp8* gene function in cell division. Moreover, the nonsuppressed background effect enabled us to identify a semidominant suppressor of *vp8* that rescues a discrete subset of phenotypes observed in the developing seed. The molecular basis for suppression is not known. Southern-blot analysis and searches of maize sequence databases failed to detect any closely related locus that correlated with the suppressed phenotype, suggesting that the suppressor is unlikely a partially redundant duplicate gene in the maize genome (data not shown). The evidence that the suppressor restores a subset of *Vp8* functions (e.g. activation of *Vp14* but not down-regulation of *ZmABA8′oxA1a*) suggests that the suppressor functions in the same pathway, but is not functionally redundant with *Vp8*. In addition, the differential effect on expression of *Vp14* and *ZmABA8′oxA1a* by the suppressor suggests that activation of ABA synthesis and repression of ABA catabolism pathways are mediated by distinct *Vp8*-dependent mechanisms.

Our finding of a suppressor, together with the highly pleiotropic nature of the *vp8* phenotype, suggests that a search for other interacting genes may be fruitful. Other genes that interact with *Vp8* function in cereal seed development have so far not been identified. Whereas the maize *terminal ear1* (*te1*), rice *pla1*, and *pla2* mutants have accelerated leaf formation similar to that seen in *vp8-R (SC)* plants during vegetative development, no differences in seed development have been described in these mutants (Itoh et al., 1998; Veit et al., 1998; Miyoshi et al., 2004; Kawakatsu et al., 2006). Consistent with those findings, *ZmPLA1* expression is unchanged in *vp8* embryos. The recent significant finding that the *MP/ARF5* auxin response factor genetically interacts with *AMP1* (Vidaurre et al., 2007) suggests the possibility that the *Vp8* and/or the suppressor might also interact with auxin signaling in developing seeds of maize.

The discovery that the *Vp8* gene encodes a putative peptidase, together with the irregular pattern of aleurone pigmentation, is consistent with diffusion of a nonautonomous signal derived from *Vp8* activity. Interestingly, *Vp8* is expressed at significantly lower levels in endosperm than in embryo, suggesting the possibility that the abnormal endosperm development might be caused by embryo-derived diffusible signals. Several classes of plant peptides have been identified in signaling (for review, see Boller, 2005) and the VP8 may be involved in processing of one or more of these peptides. Vidaurre et al. (2007) has shown that AMP1-GFP fusion protein is localized to endomembranes in Arabidopsis, suggesting that the AMP1/VP8 peptidases may process peptides in intracellular compartments. The incompletely dominant nature of the suppressor found in the SC background indicates that dosage-sensitive factors influence *Vp8* function in plant development.

MATERIALS AND METHODS

Plant Material

The *vp8-R* stock was obtained from the Maize Genetics Cooperation Stock Center. The *vp8-R (W22)* material used in this study was established by backcrossing the original *vp8-R* with the W22 inbred line five times. For the *vp8-umu1*, *vp8-umu2*, *vp8-umu3*, and *vp8-umu4* alleles, the complementation tests were performed by generating at least five independent crosses between *vp8-R* and each of the *vp8-umu* heterozygous mutants.

Tissue Culture with Rescued Embryos

The *vp8-umu1* and *vp8-R (SC)* heterozygous plants were self-pollinated and the resulting developing seeds were used for embryo rescue experiments. The heteroallelic F1 seeds were generated by crosses of *vp8-umu1 (W22)* and *vp8-R (SC)*. Embryos excised from 20- or 22-DAP seeds were placed on culture medium, as previously described (Suzuki et al., 2006).

MuTAIL Library Construction and Sequence Assembly

The MuTAIL library construction and processing and assembly of the sequences were described previously (Settles et al., 2004; McCarty et al., 2005). A full description of these processes specifically with the *vp8* mutants is available in the supplemental data.

RT-PCR Analysis

Total RNA was prepared from maize (*Zea mays*) embryos, DNaseI treated, and purified using the RNeasy kit (Qiagen). Total RNA from maize endosperm was extracted as previously described (McCarty, 1986). The RNA was further purified and DNaseI treated with the RNeasy kit. RT-PCR reactions were performed with 100 ng of total RNA in a total volume of 10 μL using the One-Step RT-PCR kit (Qiagen). The primers used for RT-PCR are listed in Supplemental Table S4.

Quantification of Hormones

Quantification of hormones was performed as described (Nakagawa et al., 2005; Naito et al., 2007) using a liquid chromatography-mass chromatography system (UPLC/Quattro Ultima Pt; Waters) with an ODS column (AQUITY-UPLC BEH-C_{18}, 1.7 μm, 2.1 $\times$ 50 mm; Waters).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number EU401893.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Hand sections of a dry mature kernel of W22 and *wpk1-umu1*.

Supplemental Figure S2. Thin sections of developing embryos at 12 DAP.

Supplemental Figure S3. Aleurone phenotype of *cr4 vp8* double-mutant seed.

Supplemental Figure S4. RT-PCR analysis of meristem-related genes and embryo-expressed genes in *wpk1-umu1* (*vp8-umu1*) developing embryos.

Supplemental Figure S5. Seedlings that were heteroallelic between *vp8-umu1* and *vp8-R (SC)*.

Supplemental Figure S6. F1 seeds between *vp8-umu1* and *vp8-R (SC)* heteroallelic crosses.

Supplemental Figure S7. RT-PCR analysis of meristem-related genes and embryo-expressed genes in *vp8-umu2* developing embryos.

Supplemental Table S1. Segregation of *wpk1-umu1* seeds.

Supplemental Table S2. Segregation of *vp8* seeds.

Supplemental Table S3. Hormone content in *vp8* mutant seeds.

Supplemental Table S4. RT-PCR primers used in this study.

Supplemental Information S1. Full descriptions of MuTAIL construction and sequence assembly.

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