The Plant Cell, Vol. 1, 523-532, May 1989

Molecular Analysis of *viviparous-1*: An Abscisic Acid-Insensitive Mutant of Maize

Donald R. McCarty[a, 1] Christian B. Carson,[a] Philip S. Stinard,[b] and Donald S. Robertson[b]

[a] Vegetable Crops Department, University of Florida, Gainesville, Florida 32611
[b] Department of Genetics, Iowa State University, Ames, Iowa 50010

The *viviparous-1 (vp1)* gene in maize controls multiple developmental responses associated with the maturation phase of seed formation. Most notably, mutant embryos have reduced sensitivity to the hormone abscisic acid, resulting in precocious germination, and blocked anthocyanin synthesis in aleurone and embryo tissues. The *Vp1* locus was cloned by transposon tagging, using the *Robertson's Mutator* element present in the *vp1-mum1* mutant allele. Detection of DNA rearrangements in several spontaneous and transposable element-induced mutant *vp1* alleles, including a partial deletion of the locus, confirmed the identity of the clone. The *Vp1* gene encodes a 2500-nucleotide mRNA that is expressed specifically in embryo and endosperm tissues of the developing seed. This transcript is absent in seed tissues of *vp1* mutant stocks. Expression of *C1*, a regulatory gene for the anthocyanin pathway, is selectively blocked at the mRNA level in *vp1* mutant seed tissues, indicating the *Vp1* may control the anthocyanin pathway by regulating *C1*. We suggest that the *Vp1* gene product functions to potentiate multiple signal transduction pathways in specific seed tissues.

INTRODUCTION

The maturation phase of seed formation in higher plants is a complex developmental process marked by the arrest of embryo growth and the onset of desiccation tolerance in embryo tissues. In maize and cereal grains, cells of the aleurone, the outermost layer of the seed endosperm, also undergo a maturation process and remain viable in the dry seed. In pigmented maize stocks, these events coincide with the accumulation of anthocyanin pigments in aleurone and scutellum tissue. Mutations at the *viviparous-1* (*vp1*) locus in maize block expression of many maturation-related events in embryo and aleurone tissues. In mutant seed, the embryo fails to enter developmental arrest, causing precocious germination of the seed on the mother plant (vivipary), and anthocyanin synthesis in the aleurone is inhibited (Robertson, 1955). In addition, *vp1* mutants have multiple enzyme deficiencies in the aleurone, indicating that diverse metabolic pathways unrelated to the anthocyanin pathway are also affected (Dooner, 1985). However, *vp1* action is apparently limited to embryo and aleurone tissues. Plants grown from mutant embryos appear normal (Dooner, 1985), and the composition and texture of the subaleurone region of the endosperm is normal prior to precocious germination (Wilson, Rhodes, and Dickenson, 1973). Tissue-autonomous expression of the *vp1* phenotype is observed in embryo and aleurone of genetically nonconcordant kernels formed from crosses of the *vp1* mutant with a T-B3La translocation stock (Robertson, 1955; Dooner, 1985). Certain *vp1* alleles, notably *vp1-Mc* (Robertson, 1965), condition near normal embryo dormancy, but block anthocyanin expression, thus at least partially separating control of developmental arrest from regulation of the anthocyanin pathway. These properties suggest a developmentally specific regulatory function for the *Vp1* product.

The *vp1* gene is one of nine known loci that control vivipary in maize. Most of the other eight mutants have been shown to affect levels of the phytohormone abscisic acid (ABA) in the developing seed (Neill, Horgan, and Parry, 1986; Neill, Horgan, and Rees, 1987) and to alter carotenoid synthesis (Robertson, 1955). Significantly, mutants of this class do not affect anthocyanin synthesis in the aleurone (Robertson, 1955). These mutants, which are probable lesions in a common ABA/carotenoid biosynthetic pathway, implicate ABA in the arrest of maize embryo development (Neill, Horgan, and Parry, 1986). In contrast, the *vp1* mutation does not alter ABA levels in the embryo (Neill, Horgan, and Rees, 1987); rather, mutant embryos exhibit a reduced sensitivity to the hormone (Robichaud, Wong, and Sussex, 1980; Robichaud and Sussex, 1986), suggesting that the *vp1* may alter ABA receptor activity or signal transduction. However, the effect of *vp1* on the ABA response does readily account for the block in the anthocyanin pathway.

Anthocyanin synthesis in the maize seed requires expression of at least seven unlinked genes in addition to *Vp1* (Coe and Neuffer, 1977). Of these, *A1, A2, Bz1, Bz2,*

[1] To whom correspondence should be addressed.

and *C2* are structural genes for enzymes in the anthocyanin pathway, whereas *C1* and *R1* are regulatory genes specific to the pathway. Enzymes in this pathway are typically expressed at low levels early in development and become induced to high levels during seed maturation (Dooner, 1983a, 1985). At least three enzymes in the pathway, UDP glucose:flavonoid glucosyltranferase, the *Bz1* gene product (Dooner and Nelson, 1979; Dooner, 1985); flavanone synthase, the *C2* product (Dooner, 1983a, 1983b); and phenylalanine ammonia lyase (Dooner, 1983a, 1985) fail to accumulate in *vp1* mutant aleurone tissue. A second seed-specific regulatory gene for the anthocyanin pathway is encoded by the *C1* locus (Cone, Burr, and Burr, 1986; Paz-Ares et al., 1987). *C1* expression is required for transcription of at least the *A1* and *Bz1* genes (Cone, Burr, and Burr, 1986). Partial amino acid sequence similarity of the *C1* protein to the DNA-binding domain of the *myb* oncogene product suggests that *C1* protein is a nuclear regulatory factor (Paz-Ares et al., 1987).

In this report, we describe cloning of the *vp1* locus by transposon tagging with a *Robertson's Mutator* element and some features of *vp1* structure and expression. We describe DNA rearrangements, including a partial deletion of *vp1* sequences, that occur in *vp1* mutant alleles that genetically define the locus. In addition, we show that *Vp1* is expressed specifically in embryo and endosperm tissues of the plant and that the *Vp1* product is required for expression of *C1* mRNA in seed tissues.

RESULTS

A *Robertson's Mutator*-Induced *vp1* Allele

The *Robertson's Mutator*-induced *vp1-mum1* allele of *vp1* was identified on a segregating ear among self-pollinated progeny of a cross involving an active *Robertson's Mutator* line (Stinard, 1986). Figure 1A shows an ear segregating for *vp1-mum1*. The homozygous mutant kernels exhibit rampant embryo development and lack purple anthocyanin pigment in the aleurone cell layer of the endosperm. Somatic mutability, visible as single cell-sized pigmented revertant aleurone sectors (Figure 1B), is low to absent in this stock. The small sector size, indicating reversion very late in endosperm development, is characteristic of many *Mutator*-induced mutations (Robertson, 1980). The well-defined somatic sectors also indicate that *Vp1* expression is cell autonomous in aleurone tissue.

Identification and Cloning of a *vp1-mum1*-Linked *Mutator* Element

Our strategy for cloning the *vp1-mum1* allele was to (1) reduce the background copy number of *Mutator* elements

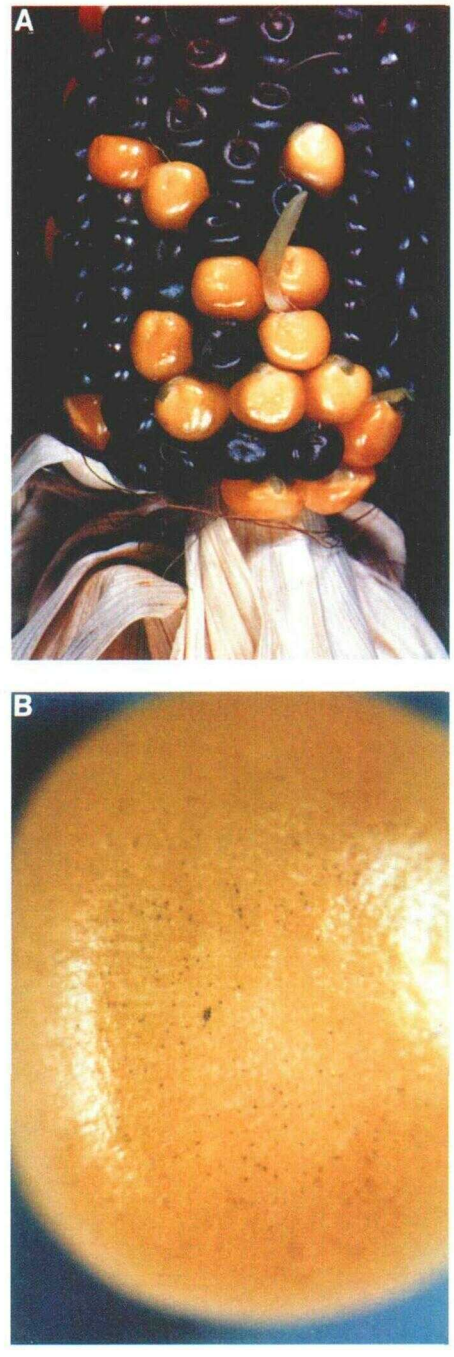

Figure 1. A *Robertson's Mutator*-Induced Mutation at *vp1*.

(A) A self-pollinated ear from a *vp1-mum1* heterozygote segregating in a 3:1 Mendelian ratio. Mutant kernels produce rampant embryos and lack purple anthocyanin pigment in the aleurone layer of the endosperm.

(B) A magnification of a mutant kernel showing somatic mutability of *vp1-mum1*. Occasional transposition of the *Robertson's Mutator* transposable element away from the *vp1* locus late in endosperm development produces single-cell sized pigmented aleurone sectors.

by outcrossing the *vp1-mum1* stock to a line that has no detectable *Mu* elements, (2) identify a *Mu* element linked to *vp1* through DNA blot hybridization analysis of segregating populations, and (3) select clones from a λ phage library constructed from DNA size-selected for the linked *Mu* containing fragment.

Figure 2 shows a segregation analysis of homozygous mutant and wild-type descendents of a plant heterozygous for *vp1-mum1*. The internal ~750-bp AvaI/TaqI fragment of the cloned *Mu1* element (Barker et al., 1984) used as probe will detect *Mu1*- and *Mu2*-like elements (Taylor, Chandler, and Walbot, 1986) but not *Mu3* (Chen et al., 1987) or other elements in the *Mutator* family that lack homology to *Mu1* internal to the 213-bp to 215-bp inverted terminal repeats. The apparent number of *Mu1* hybridizing elements present in these plants was sufficiently low after a single outcross to allow tentative identification of a *vp1*-linked element. The seven plants homozygous for *vp1-mum1* exhibited a 6.2-kb BamHI fragment that was not present in homozygous wild-type plants. In total, 45 plants (25 carried *vp1-mum1*, 20 were homozygous wild-type) representing four populations were analyzed. In all cases, the 6.2-kb BamHI fragment was associated with the *vp1-mum1* allele.

The 6.2-kb BamHI fragment was cloned from a library constructed in EMBL 3 from size-selected BamHI-digested *vp1-mum1* genomic DNA, using the internal *Mu1* fragment as a hybridization probe. This clone (subcloned into pUC19 plasmid) is designated pVPM1B (Figure 3A). Restriction mapping and blot hybridization data revealed a 1.4-kb *Mu1*-like sequence in this clone. That this was the same *Mu* element identified by our original segregation analysis was confirmed by re-analysis of one of the populations using a probe derived from DNA flanking the insertion. As expected, plants that carried *vp1-mum1* exhibited a 6.2-kb BamHI restriction fragment, whereas in wild-type plants a 4.8-kb fragment was detected (data not shown). Moreover, a restriction fragment length polymorphism detected by pVPM1B maps to the position expected for *vp1* on the long arm of chromosome 3 (B. Burr, personal communication). Finally, two independent forms of evidence described in detail below establish that pVPM1B is from *vp1* locus: (1) Flanking probes detect DNA rearrangements in independent *vp1* mutant alleles and (2) sequences from pVPM1B detect a poly(A) RNA transcript in developing kernels that is absent in *vp1* mutants.

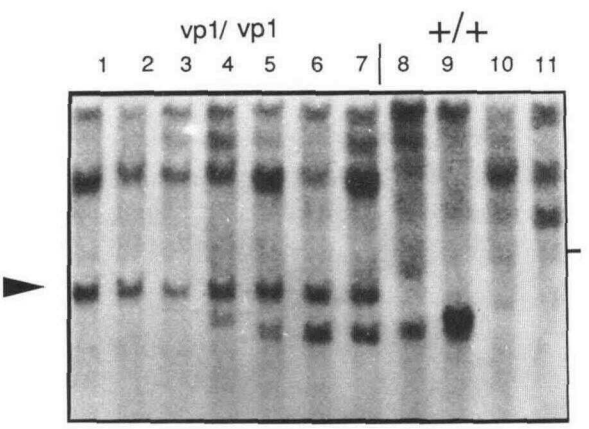

Figure 2. Identification of *vp1-mum1*-Linked *Robertson's Mutator* Transposable Element.

To generate a segregating population, purple kernels were taken from a single segregating ear and grown, and the plants were self-pollinated. A single mutant kernel was planted from each of seven ears that segregated *vp1-mum1*, and single normal seeds were planted from each of four fully pigmented ears. DNA from each plant was digested with BamHI, resolved by agarose gel electrophoresis, blotted, and probed with the 750-bp internal AvaI/TaqI fragment of *Mu1* (Barker et al., 1984) as described in Methods. A 6.2-kb fragment detected in DNA digests from homozygous *vp1-mum1* plants (vp1/vp1) and absent in homozygous normal plants (+/+) is indicated by the arrow.

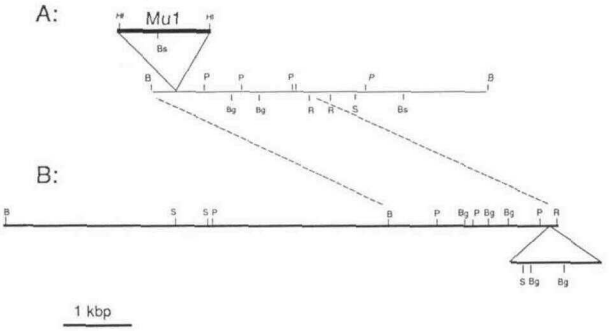

Figure 3. Restriction Maps of pVPM1B and pVPM2R.

(A) pVPM1B was isolated as described in the text.
(B) A partial restriction map of pVPM2R showing the region of overlap with pVPM1B and the position of the 1.5-kb insertion. (Only the HinfI sites within the *Mu1* element are shown.)
B=BamHI; Bg=BglII; BS=BstEII; Hf=HinfI; P=PstI; R=EcoRI; S=SstI.

Analysis of *vp1* Mutant Alleles

To confirm the identity of pVPM1B, DNA probes derived from sequences flanking the *Mu1* insertion were used to analyze independent *vp1* mutations for DNA rearrangements. Total DNA was extracted from wild-type and homozygous mutant plants recovered from each stock and subjected to DNA blot hybridization analysis (Figure 4). In every case, we detect differences between the mutant and wild-type alleles, and the wild-type alleles uniformly resemble those found in the W22 inbred line. However, we can rule out pre-existing restriction site polymorphisms only in instances where the wild-type progenitor of the mutant allele is known. Two of the mutants,

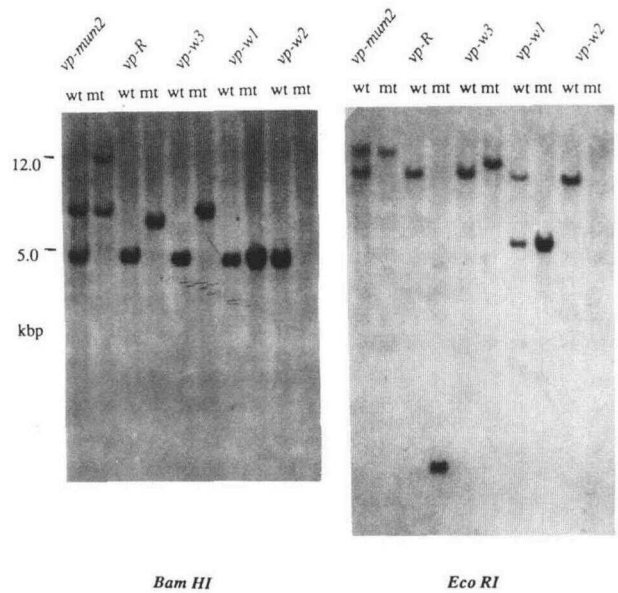

Figure 4. DNA Blot Hybridization Analysis of *vp1* Mutant Stocks.

Total DNA was isolated from mutant (mt) and wild-type (wt) seedlings of each segregating stock, digested with BamHI or EcoRI, resolved by agarose gel electrophoresis, blotted to nylon, and probed as described in Methods. The probe was prepared by digestion of the 2.1-kb BamHI/PstI fragment of pVPM1B with HinfI to remove most of the *Mu1* sequence (except approximately 40 bp of the inverted terminal repeat), and the small flanking fragments were purified by agarose gel electrophoresis and labeled with ^{32}P as described in Methods. The normal segregants from the *vp1-mum2* and *vp1-w1* stocks were heterozygous individuals. The 12-kb BamHI fragment detected *vp1-mum2* mutant lane is a incomplete digestion product resulting from apparent DNA modification in this individual. Other *vp1-mum2* plants exhibited only the smaller 6.5-kb fragment.

vp1-w1 and *vp1-w2,* arose spontaneously in homogeneous W22 inbred lines and are known to be derivatives of the wild-type W22 allele. Similarly, the parental stock that gave rise to *vp1-w3* was found to be homozygous for the W22 wild-type allele using pVPM1B probes (D.R. McCarty, unpublished data). The rearrangements detected in these stocks, particularly the deletion apparent in the *vp1-w2* allele, establish that pVPM1B is from the *vp1* locus.

The *vp1-w2* deletion is notable in that the breakpoints are located within the cloned regions. Figure 5 shows blots of mutant and wild-type DNA restriction digests probed with subregions of pVPM1B. Probes derived from 1.2 region defined by the 0.7-kb and 0.5-kb PstI fragments (Figure 4 and Figure 5, probe B) do not hybridize to *vp1-w2* DNA, whereas probes to the left (probe A) and right (probe C) of this region detect a common 9.0-kb BamHI fragment in the mutant. The size of the mutant restriction fragment indicates that approximately 1.3 kb of sequence (including the internal BamHI site) is deleted.

In addition to *vp1-mum1,* at least four mutants, *vp1-mum2, vp-mum3* (not shown), *vp1-w1,* and *vp1-w3,* bear evidence of insertional rearrangements. Of these, only *vp1-mum2* and *vp1-mum3,* both of which arose in *Robertson's Mutator* lines, have mutable phenotypes. The *vp1-mum2* mutant is of particular interest because of its distinctive phenotype. Mutant kernals exhibit a highly variable pattern of somatic sectoring in the aleurone, with many reversion events that occur early in development. This pattern is atypical of most *Robertson's Mutator*-induced mutations, which characteristically revert late in development (Robertson, 1980), producing small somatic sectors of normal tissue (e.g., *vp1-mum1;* Figure 1B).

To more clearly define the rearrangement in *vp1-mum2* allele, the 15-kb EcoRI genomic fragment (designated pVPM2R) was cloned from the mutant. Restriction mapping and hybridization analysis of this clone revealed a 1.5-kb insertion element in the mutant DNA (Figure 3B). That this insertion did not pre-exist in the wild-type progenitor was confirmed by DNA blot analysis of the parental *Mutator* stocks, which were possible contributors of *vp1* alleles. Two wild-type alleles were identified: the W22 inbred form and a second allele that differed detectably from the mutant only in lacking the 1.5-kb insertion (data not shown). This second allele is the presumed progenitor of *vp1-mum2.* Although the size of *vp1-mum2* insertion is typical of the *Mutator* family of transposable elements (Bennetzen, 1984;

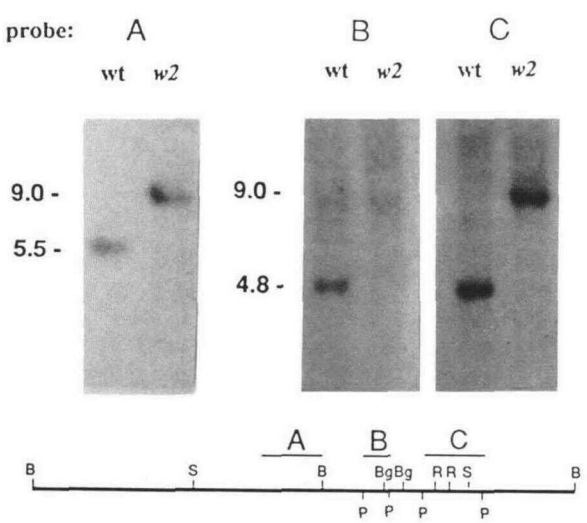

Figure 5. DNA Blot Hybridization Analysis of the *vp1-w2* Deletion Mutant.

DNA was extracted from wild-type (wt) and mutant (*w2*) segregants of the *vp1-w2* stock, digested with the indicated restriction enzyme, and analyzed as in Figure 4. The probes hybridized to each pair of lanes are indicated in the diagram.
(A) A 1.6-kb HincII/BamHI fragment of pVPM2R.
(B) The 0.5-kb PstI fragment of pVPM1B.
(C) The 1.1-kb PstI fragment of pVPM1B.

Chen et al., 1987; Taylor and Walbot, 1987), we detect no hybridization to a probe that includes the entire *Mu1* sequence (D.R. McCarty, unpublished data). Therefore, the *Robertson's Mutator* phenomenon may include transposable elements that have little or no sequence similarity to the inverted terminal repeat sequences of *Mu1* and other previously characterized *Mu* elements (Chen et al., 1987; Taylor and Walbot, 1987). On the other hand, we cannot rule out the possibility that the *mum2* insert belongs to an unrelated class of transposable element that was fortuitously active in this stock. Analysis of genomic DNA of a third *Robertson's Mutator*-derived mutant, *vp1-mum3,* also revealed a 1.5-kb insertion (see Figure 6). It is not yet known whether this element shares sequence similarity with the *Robertson's Mutator* family.

The *vp1-w3* and *vp1-w1* mutants were also derived from stocks known to carry active transposable elements. The *vp1-w3* allele that arose in an *Ac/Ds* stock shows clear evidence of a 2-kb insertion (Figure 4). However, the mutant is phenotypically stable and does not respond to *Ac* (see Methods). Therefore, the insertion is not a genetically defined *Ds* element. Similarly, the data for the *vp1-w1* mutant are consistent with either a large insertion (>4.7 kb) containing several relevant restriction sites or a large deletion of DNA (>10.0 kb) adjacent to the pVPM1B region.

A physical map of the *vp1* locus derived from these data is shown in Figure 6. The locus as defined by the available mutants occupies approximately 5 kb of genomic DNA. However, the transcriptional unit as delimited by RNA blot analysis using various subclones of pVPM1B and pVPM2R (see Figure 6) extends to 1 kb to 2 kb beyond the *vp1-mum2* insertion.

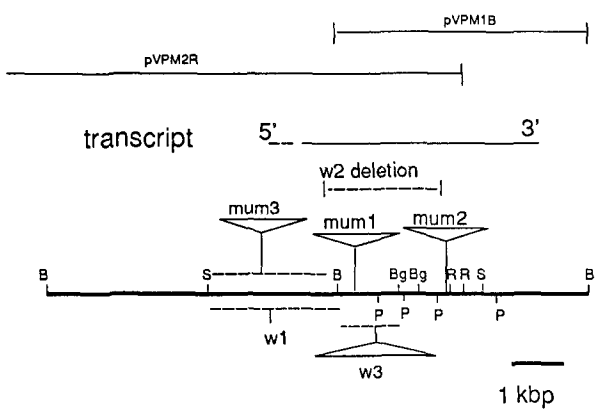

Figure 6. A Physical Map of the *viviparous*-1 Locus.

The diagram summarizes the rearrangements detected in the various *vp1* mutants relative to the W22 wild-type allele. The positions of the lesions in the *vp1-w1, vp1-w3,* and *vp1-mum3* alleles were determined by blot hybridization analysis of genomic DNA to within the limits indicated by the dashed lines. The W22 allele was the apparent progenitor of all the mutants shown except *vp1-mum2.* The approximate limits of the transcriptional unit were mapped by RNA blot analysis of wild-type embryo RNA.

Vp1 Expression in Mutant and Wild-Type Developing Kernels

Expression of *Vp1* sequences in developing seeds was assessed by RNA blot hybridization. Figure 7 shows a blot hybridization analysis of poly(A) RNA isolated from mutant and wild-type maize seeds 22 days after pollination, shortly after the onset of anthocyanin pigment synthesis in the aleurone of wild-type. *Vp1* probes detect a 2500-nucleotide (nt) transcript in wild-type seeds that is absent in the three mutants examined. We conclude that this mRNA encodes the *Vp1* gene product. In addition to this transcript, probes derived from sequences immediately flanking the *Mu1* insertion site in *vp1-mum1* detect two minor RNAs of 900 nt and 1350 nt, respectively, in both wild-type and mutant poly(A) RNA preparations. However, their presence in the *vp1-w2* mutant in which the probe sequence is deleted from the genome suggests that the smaller transcripts may originate from a related sequence elsewhere in the genome. Consistent with this suggestion, at reduced hybridization stringency, additional bands are detected on DNA blots with *vp1* probes (C.B. Carson and D.R. McCarty, unpublished data). Other *Vp1*-specific probes derived from pVPM1B and pVPM2R detect only the 2500-nt transcript in wild-type (not shown).

Interestingly, altered transcription of the *vp1* locus is detected for at least two of the mutant alleles. The relationship between the minor 1350-nt RNA and the relatively abundant transcript detected in the *vp1-mum1* mutant is unclear. The mutant transcript may be a truncation of the normal 2500-nt *Vp1* mRNA. A *Mu1* insertion into the first intron of *Adh1* is reported to cause premature termination of transcription (Vayda and Freeling, 1986). In addition, the 1.7-kb PstI/BamHI fragment from the right end of pVPM1B detects a low abundance 2300-nt transcript in poly(A) RNA from *vp1-R* mutant embryos that is not present in wild-type (Figure 8, top panel; C.B. Carson and D.R. McCarty, unpublished data). This transcript is not detected by other *Vp1* probes (Figure 7). These allele-specific transcripts apparently have little or no functional significance because all of the mutants analyzed are phenotypically similar.

Because the *vp1* mutant phenotype affects only the embryo and endosperm tissues of the seed and does not involve other plant tissues, we would expect *Vp1* to be expressed primarily, if not exclusively, in seed tissues. RNA blot hybridization data for poly(A) RNA from developing seed and plant tissues are in good agreement with this prediction. Figure 8 (top panel) shows an RNA blot probed with the 1.7-kb PstI/BamHI fragment from the right end of pVPM1B. This probe detects only the 2500-nt transcript in wild-type. The *Vp1* transcript is detected in embryo and endosperm tissues (the latter at low but detectable levels) as early as 16 days post-pollination.

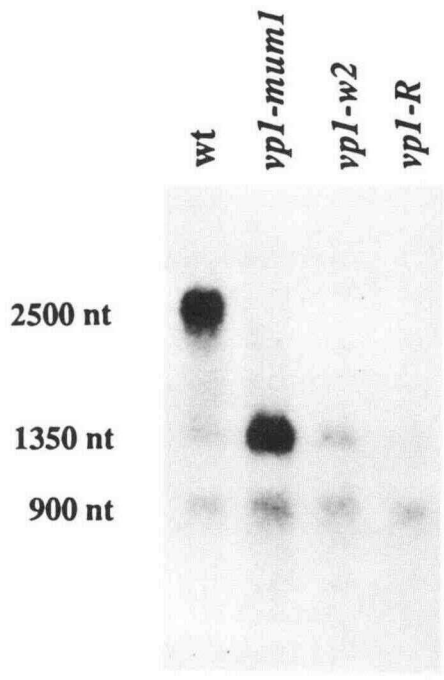

Figure 7. RNA Blot Analysis of Developing Wild-Type and *vp1* Mutant Seeds.

Polyadenylated RNA was isolated from normal and mutant kernels 22 days after pollination, resolved by formaldehyde agarose gel electrophoresis, blotted, and probed as described in Methods. The probe consisted of the ~750-bp BamHI/PstI fragment of pVPM2R, which corresponds to the region immediately flanking the *Mu1* insertion in pVPM1B.

However, the *Vp1* mRNA is not detected in poly(A) RNA from root and shoot tissues of the developing plant. Similar results are obtained with other *Vp1* probes, including the *vp1-mum1* flanking probe (data not shown), which detects the two minor transcripts in whole seed poly(A) RNA (Figure 7). Therefore, no detectable *Vp1* homologous transcripts are expressed in seedling tissues.

Within the endosperm, the *vp1* mutant phenotype is limited to the aleurone layer. While the present data do not resolve *Vp1* expression at the cellular level, the relative low abundance of *Vp1* message we detect in the endosperm is consistent with the small contribution of the single cell aleurone layer to the total endosperm mass, implying that expression is perhaps limited to aleurone cells.

Vp1 Regulation of Gene Expression in Seed Development

The pleiotropic phenotype of *vp1* suggests that the *Vp1* product has a regulatory function in seed development. To assess the effect of *vp1* on gene expression in seed tissues, we examined steady-state levels of mRNAs (Figure 8) of two genes (*C1* and *Adh1*) that are implicated in *vp1*-regulated processes (Dooner, 1985). The *C1* locus is a seed-specific regulatory gene of the anthocyanin pathway (Cone, Burr, and Burr, 1986; Paz-Ares et al., 1986, 1987), and *Adh1* encodes the major isozyme of alcohol dehydrogenase expressed in seed tissues (Freeling and Bennett, 1985). In addition, as a control for mRNA integrity and sample load, we probed identical blots with a clone of the *Sus1* gene (McCarty, Shaw, and Hannah, 1986), which encodes an isozyme of sucrose synthase that is expressed in both the embryo and endosperm (Chourey, 1981). Sucrose synthase activity is not known to be affected by *vp1* (Dooner, 1985).

C1 probes detect two major mRNAs (1200 nt and 1500 nt, respectively) in developing seeds of most genotypes

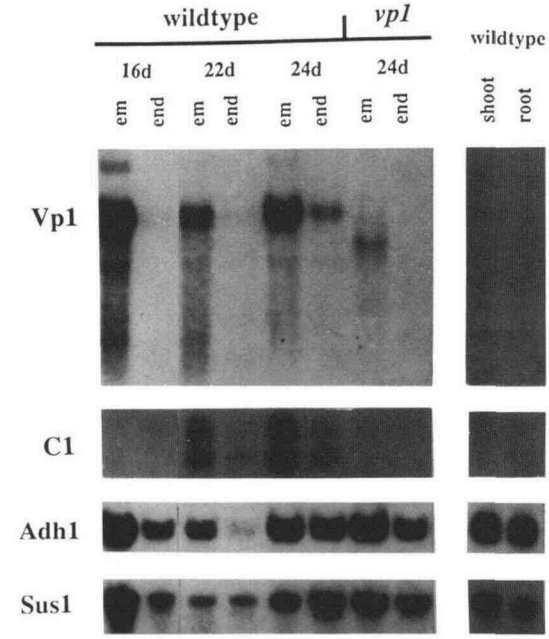

Figure 8. Developmental Expression of *Vp1* and *Vp1*-Regulated Genes.

Polyadenylated RNA was isolated from embryo (em) and endosperm (end) tissues dissected from developing kernels and root and shoot tissues of 5-day-old germinating seedlings. The mutant allele shown is *vp1-R*. RNA blots were prepared as described in Methods. Identical blots were hybridized as indicated with the following probes: *Vp1*, the right end 1.7-kb BamHI/PstI fragment of pVPM1B; *C1*, a 1.0-kb EcoRI genomic fragment of the *C1* locus (Cone, Burr, and Burr, 1986); *Adh1:* a 610-bp genomic fragment containing the first exon of *Adh1* (Dennis et al., 1984); *Sus1,* the 6.0-kb genomic insert of p21.2 (McCarty, Shaw, and Hannah, 1986). *Vp1* and *C1* blots were exposed to x-ray film for 48 hr to 72 hr, whereas the *Adh1* and *Sus1* blots were exposed for 5 hr to 6 hr.

(Cone, Burr, and Burr, 1986; Paz-Ares et al. 1986). The timing of *C1* expression coincides roughly with activation of the anthocyanin pathway in normal tissues. At 16 days post-pollination, prior to the onset of pigment accumulation, the *C1* mRNAs are not detected in either the embryo or the endosperm, whereas by 22 days, when anthocyanins begin to appear in the aleurone, relatively high *C1* message levels are present in the embryo and to a lesser extent in the endosperm. Temporally, *Vp1* expression significantly precedes induction of *C1*. It should be pointed out that the level of *Vp1* and *C1* transcript present in 22-day wild-type endosperm is under-represented in this experiment because anomalously low levels of *Adh1* and *Sus1* mRNA are also detected. This difference is attributable, therefore, to inefficient extraction of poly(A) RNA in this sample, rather than a developmental fluctuation in expression.

Selective regulation of *C1* expression by *Vp1* is evident at the mRNA level. We detect no *C1* mRNA in *vp1* mutant seed tissues. It is perhaps surprising that *C1* mRNA is more abundant in the normally unpigmented embryo than in the endosperm. However, as was suggested for *Vp1*, the low level of *C1* message detected in the endosperm may reflect aleurone-specific expression. Moreover, the relative abundance of *C1* expression in the embryo is consistent with the capacity of a large fraction of the embryo tissue mass to become pigmented when appropriate alleles of the *R1* locus (e.g., *R-sc*) are present (Coe and Neuffer, 1977). Notably, in an *R-sc* background, the *vp1* mutant blocks pigmentation in both embryo and aleurone tissues (D.R. McCarty, unpublished data).

Although the absence of *C1* product (Dooner and Nelson, 1979) alone is sufficient to account for the block of anthocyanin expression associated with *vp1*, Dooner (1985) reported that *vp1* mutant aleurone is also deficient in several unrelated enzyme activities, including alcohol dehydrogenase. However, we find that, in contrast to *C1*, expression of the *Adh1* gene is not significantly altered in mutant tissues relative to wild-type. Therefore, we find no evidence for selective regulation of *Adh1* by *Vp1* at the mRNA level. One possibility is that *Adh1* expression is affected at the translational or post-translational level. Alternatively, we cannot rule out an aleurone-specific effect of *Vp1* on *Adh1* message level because the present data average the aleurone with the bulk endosperm. However, the latter situation would imply that *Adh1* is regulated differentially by *Vp1* in embryo and aleurone tissues.

DISCUSSION

The *vp1* mutant produces pleiotropic effects on maturation-related gene expression in two histologically and genetically distinct seed tissues, the diploid embryo and the aleruone cell layer of the triploid endosperm (Robertson, 1955; Dooner, 1985). Physiological studies indicate that the viviparous conditions of *vp1* mutant embryos is due to a reduced sensitivity to ABA (Robichaud and Sussex, 1986). Thus, the cloning of the *vp1* locus by transposon tagging has allowed the first molecular analysis of a plant hormone response mutant.

However, it must be emphasized that the connection between the ABA response and the block in anthocyanin induction remains unclear, as viviparous mutants that block ABA synthesis (e.g., *vp5*) do not affect anthocyanin expression (Robertson, 1955). Moreover, while the majority of *vp1* mutant alleles affect both vivipary and pigment synthesis, certain *vp1* alleles (e.g., *vp1-Mc:* Robertson, 1965) have a dormant/anthocyaninless phenotype, suggesting that control of vivipary and pigment synthesis may be genetically separable. Nonetheless, our results confirm that control of vivipary and anthocyanin synthesis are functions of a single gene at the molecular level. The six *vp1* mutant alleles analyzed exhibit discrete rearrangements that define a compact genomic region (~5 kb) with no evidence of unusual genic complexity. The pleiotropic effects of these mutants apparently correlate with disruption of a single transcriptional unit that encodes a 2500-nt mature transcript. It remains to be determined whether the *vp1-Mc* phenotype truly reflects a loss of one function in a bifunctional gene or simply a difference in the threshold level of *Vp1* product required for embryo dormancy and pigment synthesis. The molecular basis of the *vp1-Mc* mutation is under investigation.

Analysis of gene expression in *vp1* mutant and wild-type seeds indicates that *Vp1* is required for transcription, or mRNA stability, of specific genes. However, transcriptional regulation may not account for all effects of the *vp1* mutation. Although *Adh1* is apparently not expressed in mutant aleurone at the level of enzyme activity (Dooner, 1985), we detect no effect on *Adh1* mRNA levels in whole endosperm or embryos. Whether this reflects post-transcriptional control of *Adh1* expression or aleurone-specific gene regulation remains to be determined.

The selective block of *C1* expression in the *vp1* mutant is of particular interest because the *C1* gene product is itself believed to be a transcription regulatory factor responsible for activation of the anthocyanin pathway in seed tissues (Paz-Ares et al., 1987). Our results and the fact that the *c1* phenotype is a subset of the broader *vp1* mutant phenotype support the suggestion that *Vp1* and *C1* are part of a regulatory hierarchy controlling seed development. That *Vp1* and *C1* have very similar, if not identical, tissue specificity further underscores a regulatory link between these genes. However, *Vp1* and *C1* are not coordinately expressed at the mRNA level in these tissues. *Vp1* mRNA is clearly abundant at 16 days post-pollination, before the *C1* transcripts are detected, suggesting that other factors in addition to *Vp1* regulate *C1* expression in the seed. Therefore, the *Vp1* product may only potentiate *C1* expression. Alternatively, *Vp1* expression and subse-

quent interaction with *C1* may be subject to translational or post-translational control. In any case, it is interesting that the requirement for *Vp1* is logically sufficient to account for the tissue specificity of *C1* expression. In other words, *Vp1* may specify the place, rather than the timing, of *C1* expression. The timing of *C1* induction may be controlled by other developmental signals, such as light (Chen and Coe, 1977).

The interaction of *Vp1* with *C1* may offer some insight into the broader role of *Vp1* in seed development. A feature common to both vivipary and the block in pigment synthesis in the *vp1* mutant seed is an inability of specific seed tissues to respond normally to developmental signals (i.e., light and ABA). This suggests that the *Vp1* may potentiate multiple signal transduction pathways in the tissues where it is expressed. One possibility is that *Vp1* may regulate genes encoding key components (e.g., an ABA receptor gene and *C1*) of the regulatory mechanisms that mediate the ABA and light responses. This is consistent with the notion of a regulatory hierarchy in which *Vp1* interacts with a set of subordinate regulatory genes. Alternatively, the *Vp1* gene could encode a tissue-specific component of a signal transduction mechanism that is common to ABA and light-mediated responses. In either case, a key implication is that *Vp1* may confer tissue specificity to ABA and light-regulated gene expression associated with seed maturation. However, this suggestion ultimately raises the issue of what factors in turn regulate *Vp1* expression. The good correlation between the tissue specificity of *Vp1* mRNA expression and that of the mutant phenotype suggests that transcriptional control likely plays a role in regulating *Vp1* expression.

METHODS

Genetic Stocks

The wild-type maize stocks used in this study were a color-converted W22 inbred carrying all factors required for anthocyanin pigment in the aleurone and a nonpigmented W64Ax182E hybrid. The *vp1* mutant stocks used are described below.

The *vp1-mum1*, *vp1-mum2*, and *vp1-mum3* alleles (Stinard, 1986; P.S. Stinard, unpublished data) arose independently in *Robertson's Mutator* transposable element stocks (Robertson, 1978) maintained at Ames, IA. These alleles differ markedly in the developmental timing and frequency of somatic reversion. The *vp1-mum1* and *vp1-mum3* alleles revert very late in development, producing single cell-sized sectors in the aleurone, whereas *vp1-mum2* produces frequent large sectors corresponding to early events.

The *vp1-R* allele is the standard mutant allele described by Robertson (1955). The progenitor stock is unknown.

The *vp1-w3* allele arose as a stable mutant in an active *Activator/Dissociation* stock (O. Nelson, personal communication). We have confirmed that this stock contains *Ac* by activation of the *Ds* element at *sh2-m*. However, there is no evidence of *vp1* mutability.

The *vp1-w1* and *vp1-w2* alleles arose spontaneously in homogeneous W22 inbred sublines. Both progenitor stocks carried the *R-st* allele at the *R1* locus (J. L. Kermicle, personal communication), which contains an uncharacterized transposable element (Ashman, 1960). Neither allele shows somatic mutability.

All plants were grown in the field or greenhouses at Gainesville, FL. Because the viviparous seed cannot be stored, the mutant stocks were routinely maintained by self-pollination of heterozygous plants. Homozygous mutant plants were rescued from segregating ears and grown in the greenhouse.

Materials

Restriction enzymes and DNA modifying enzymes (Bethesda Research Laboratories) were used according to manufacturer's instructions.

DNA and RNA Isolation and Blot Hybridization

Total cellular DNA was isolated from leaf tissue as described by Dellaporta, Wood, and Hicks (1984), digested with restriction enzymes, fractionated by agarose gel electrophoresis (Maniatis, Fritsch, and Sambrook, 1983), blotted to nylon membrane (Amersham), and hybridized for 24 hr (Southern, 1975; Church and Gilbert, 1984) with probe DNA ^{32}P-labeled (10^8 dpm 10^9 dpm/μg) by nick-translation (BRL Kit; according to manufacturer's instructions) or random primer extension (Boehringer Mannheim Biochemicals; according to manufacturer's instructions). Membranes were washed at 65°C for 1.5 hr to 2 hr according to Church and Gilbert (1984) and exposed to x-ray film with intensifier screens at −70°C for 24 hr to 72 hr.

Total RNA was extracted from maize tissues as previously described (McCarty, 1986). The polyadenylated RNA-enriched fraction obtained by oligo(dT)-cellulose chromatography (Maniatis, Fritsch, and Sambrook, 1983) was denatured in 50% formamide and fractionated (5 μg of RNA/lane) by electrophoresis in agarose containing formaldehyde (Thomas, 1980; Maniatis, Fritsch, and Sambrook, 1983), blotted to nylon membrane, and hybridized to DNA probes as described above.

Genomic Cloning

To obtain pVPM1B, total DNA isolated from a homozygous *vp1-mum1* plant was digested with excess BamHI enzyme. DNA fragments in the ~6 kb range were purified by preparative agarose gel electrophoresis and electroelution into dialysis tubing, and ligated to BamHI-cut EMBL 3 arms (Glover, 1985) at a high insert to vector ratio to favor inclusion of multiple inserts. Approximately 7×10^4 primary plaques were lifted on nylon membrane and screened by plaque hybridization (Church and Gilbert, 1984), using the internal 750 AvaI/TaqI fragment of the 1.4-kb *Mu1* transposable element (Barker et al., 1984) as a probe. A single very strongly hybridizing plaque was identified and purified. This phage contained a 6.2-kb BamHI fragment with very strong

homology to the internal *Mu* probe. This fragment, subcloned into pUC19, is designated pVPM1B.

To obtain pVPM2R, ~15-kb EcoRI genomic DNA fragments (from a *vp1-mum2* homozygote) were size-selected as above and ligated into EcoRI-cut EMBL 4 arms. Approximately 2×10^5 primary plaques were screened using the ~1.0-kb BglII/EcoRI fragment of pVPM1B as probe. Nine identical phage clones were isolated and purified. The insert from one of these was subcloned into pUC19.

ACKNOWLEDGMENTS

We are indebted to S. Caprice Simonds for technical assistance, L. C. Hannah for support and helpful discussions. We thank Karen Cone and Udo Wienand for providing molecular probes for *C1*; Hugo Dooner for providing spontaneous mutant *vp1* stocks; Judith Strommer for providing a *Mu1* clone; and Ben and Frances Burr for providing RFLP mapping data for pVPM1B. We acknowledge support from the National Science Foundation grants DCB-87-18374 (D. R. M.) and DCB-86-08188 (D. S. R.). Florida Agriculture Experiment Station Journal Series No. 9902.

Received February 9, 1989; revised March 17, 1989.

REFERENCES

Ashman, R.B. (1960). Stippled aleurone in maize. Genetics **45,** 19–34.

Barker, R.F., Thompson, D.V., Talbot, D.R., Swanson, J., and Bennetzen, J. (1984). Nucleotide sequence of the maize transposable element *Mu1*. Nucl. Acids Res. **12,** 5955–5967.

Bennetzen, J. (1984). Transposable element *Mu1* is found in multiple copies in *Robertson's Mutator* maize lines. J. Mol. Appl. Genet. **2,** 519–524.

Chen, C.-H., Oishi, K.K., Kleockener-Gruissem, B., and Freeling, M. (1987). Organ specific expression of maize *Adh1* is altered after a *Mu1* transposon insertion. Genetics **116,** 469–477.

Chen, S.M., and Coe, E.H., Jr. (1977). Control of anthocyanin synthesis by the *C* locus in maize. Biochem. Genet. **15,** 333–346.

Chourey, P.S. (1981). Genetic control of sucrose synthetase in maize endosperm. Mol. Gen. Genet. **184,** 372–376.

Church, G.M., and Gilbert, W. (1984). Genomic sequencing. Proc. Natl. Acad. Sci. USA **81,** 1991–1995.

Coe, E.H., and Neuffer, M.G. (1977). The genetics of corn. In Corn and Corn Improvement, G.F. Sprague, ed, (Madison, WI: American Society of Agronomy), pp. 111–213.

Cone, K.C., Burr, F.A., and Burr, B. (1986). Molecular analysis of the maize anthocyanin regulatory locus *c1*. Proc. Natl. Acad. Sci. USA **83,** 9631–9635.

Dellaporta, S.L., Wood, J., and Hicks, J.B. (1983). A plant minipreparation. Plant Mol. Biol. Rep. **1,** 19–23.

Dennis, E., Gerlach, W., Proyor, A., Bennetzen, J., Inglis, A., Llewellyn, D., Sachs, M., Ferl, R., and Peacock, W. (1984). Molecular analysis of the alcohol dehydrogenase gene of maize. Nucl. Acids Res. **12,** 3983–3990.

Dooner, H.K. (1983a). Gene-enzyme relationships in anthocyanin biosynthesis in maize. In Maize for Biological Research, W.F. Sheridan, ed (Charlottesville, VA: Plant Molecular Biology Association), p. 123.

Dooner, H.K. (1983b). Coordinate genetic regulation of flavonoid biosynthetic enzymes in maize. Mol. Gen. Genet., **189,** 136–141.

Dooner, H.K. (1985). *Viviparous-1* mutation in maize conditions pleiotropic enzyme deficiencies in the aleurone. Plant Physiol **77,** 486–488.

Dooner, H.K., and Nelson, O.E. (1979). Interaction among *C1, R1,* and *Vp1* in the control of the *Bz* glucosyltransferase during endosperm development. Genetics, **91,** 309–315.

Freeling, M., and Bennett, D.C. (1985). Maize *Adh1*. Annu. Rev. Genet. **19,** 297–323.

Glover, D.M., ed (1985). DNA Cloning, Volume I: A Practical Approach. (Oxford: IRL Press).

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1983). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

McCarty, D.R. (1986). A simple method for extraction of RNA from maize tissues. Maize Gen. Coop. Newsl. **60,** 61.

McCarty, D.R., Shaw, J., and Hannah, L.C. (1986). Cloning, mapping, and expression of the *Constitutive sucrose synthase* locus in maize. Proc. Natl. Acad. Sci. USA **83,** 9099–9103.

Neill, S.J., Horgan, R., and Parry, A.D. (1986). The carotenoid and abscisic acid content of viviparous kernels and seedlings of *Zea mays* L. Planta **169,** 87–96.

Neill, S.J., Horgan, R., and Rees, A.F. (1987). Seed development and vivipary in *Zea mays* L. Planta **171,** 358–364.

Paz-Ares, J., Wienand, U., Peterson, P. A., and Saedler, H. (1986). Molecular cloning of the *c1* locus of *Zea mays:* A locus regulating the anthocyanin pathway in maize. *EMBO J.* **5,** 829–833.

Paz-Ares, J., Ghosal, D., Wienand, U., Peterson, P.A., and Saedler, H. (1987). The regulatory locus *c1* of *Zea mays* encodes a protein with homology to *myb* proto-oncogene products and with structural similarities to transcriptional activators. EMBO J. **6,** 3553–3558.

Robertson, D.S. (1955). The genetics of vivipary in maize. Genetics **40,** 745–760.

Robertson, D.S. (1965). A dormant allele of *vp1*. Maize Gen. Coop. News1. **39,** 104.

Robertson, D.S. (1978). Characterization of a mutator system in maize. Mutation Res. **51,** 21–28.

Robertson, D.S. (1980). The timing of *Mu* activity in maize. Genetics **94,** 969–978.

Robichaud, C.S., and Sussex, I.M. (1986). The response of *viviparous-1* and wildtype embryos of *Zea mays* to culture in the presence of abscisic acid. J. Plant Physiol **126,** 235–242.

Robichaud, C.S., Wong, J., and Sussex, I.M. (1980). Control of *in vitro* growth of viviparous embryo mutants of maize by abscisic acid. Dev. Genet. **1,** 325–330.

Southern, E. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol. Biol. **98,** 503–517.

Stinard, P. (1986). A putative *Mu* induced viviparous mutant. Maize Gen. Coop. Newsl. **60,** 7.

Taylor, L.P., and Walbot, V. (1987). Isolation and characterization of a 1.7 kb transposable element from a mutator line of maize. Genetics **117,** 297–307.

Taylor, L.P., Chandler, V., and Walbot, V. (1986). Insertion of 1.4 kb and 1.7 kb *Mu1* elements into the *bronze-1* gene of *Zea mays* L. Maydica. **31,** 31–45.

Thomas, P.S. (1980). Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose paper. Proc. Natl. Acad. Sci. USA **77,** 5201–5205.

Vayda, M.E., and Freeling, M. (1986). Insertion of the *Mu1* transposable element into the first intron of maize *Adh1* interferes with transcript elongation, but does not disrupt chromatin structure. Plant Mol. Biol. **6,** 441–454.

Wilson, G.F., Rhodes, A.M., and Dickinson, D.B., (1973). Some physiological effects of viviparous genes *vp1* and *vp5* on developing maize kernels. Plant Physiol. **52,** 350–356.