# Variegated Phenotype and Developmental Methylation Changes of a Maize Allele Originating From Epimutation

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ABSTRACT

Two instances of genetic transmission of spontaneous epimutation of the maize *P-rr* gene were identified. Transmission gave rise to two similar, moderately stable alleles, designated *P-pr-1* and *P-pr-2*, that exhibited Mendelian behavior. Both isolates of *P-pr* conditioned a variable and variegated phenotype, unlike the uniform pigmentation conditioned by *P-rr*. Extensive genomic analysis failed to reveal insertions, deletions or restriction site polymorphisms between the new allele and its progenitor. However, methylation of the *P* gene was increased in *P-pr* relative to *P-rr*, and was greatly reduced (though not lost) in a revertant to uniform pigmentation. Variability in pigmentation conditioned by *P-pr* correlated with variability in transcript levels of the *P* gene, and both correlated inversely with variability in its methylation. Part of the variability in methylation could be accounted for by a developmental decrease in methylation in all tissues of plants carrying *P-pr*. We hypothesize that the variegated phenotype results from a general epigenetic pathway which causes a progressive decrease in methylation and increase in expression potential of the *P* gene as a function of cell divisions in each meristem of the plant. This renders all tissues chimeric for a functional gene; chimerism is visualized as variegation only in pericarp due to the tissue specificity of *P* gene expression. Therefore, this allele that originates from epimutation may exemplify an epigenetic mechanism for variegation in maize.

ALTHOUGH the principles of genetics account very well for the heritability of nucleotide sequences, few general rules apply to the changes in the expression of these sequences during development. Pathways that regulate developmental changes in gene activity can be termed epigenetic, following the initial use of this term by WADDINGTON (1939, 1957). Developmental control of gene expression is often mediated by gene products in specific cell types, usually via protein-DNA or protein-RNA interactions. This requires prior presence of the regulatory gene product in the specific tissue, and not elsewhere. Non-uniform distributions of this type can arise, for example, by asymmetric cell division leading to unequal segregation of material between daughter cells (reviewed in HORVITZ and HERSKOWITZ 1992). Alternately, chromosomes may directly generate asymmetric distribution of a regulatory gene product using information other than nucleotide sequence (BRINK 1964; MCCLINTOCK 1967a). As one possibility, chromatin structure can change in a predetermined way through development (BRINK 1964; McClintock 1967a); this may, in molecular terms, expose a critical *cis*-acting site only in a defined tissue or at a given developmental stage. Position-effect variegation in Drosophila (reviewed in SHAFFER *et al.* 1993) may be related to such a mechanism (BRINK 1964).

In higher eukaryotes, cytosine methylation provides an alternate mechanism by which an epigenetic pathway can regulate gene expression during development (HOLLIDAY 1987). Methylation patterns, comprised of the distribution of modified cytosines in CpG (and CpNpG in plants; GRUENBAUM *et al.* 1981) motifs in a given domain, can be inherited through mitosis by the action of a maintenance methylase (BESTOR and INGRAM 1983). However, they have the potential to be erased or subjected to change over a wide range during development. Epigenetic pathways can cause changes in the pattern at a given stage or in a given tissue by selective demethylation or remethylation (HOLLIDAY 1987; CEDAR and RAZIN 1990). Such a methylation change can affect gene expression, either by altering the binding affinity of the affected DNA for a protein factor (*e.g.*, WATT and MOLLOY 1988), or by hindering factor binding by interaction with other proteins specific for methylated DNA (MEEHAN *et al* 1989; BOYES and BIRD 1992). In addition, DNA methylation changes are often associated with changes in chromatin organization, and the two may act in concert (SELKER 1990a; RIGGS and PFEIFER 1992).

In agreement with such a regulatory role, precise control of methylation has been shown to be essential for development. A 70% reduction in the number of methylated CpG sites in the mouse embryo caused by targeted disruption of the murine methyltransferase gene was embryo-lethal (LI *et al.* 1992). As pointed out elsewhere (BIRD 1992), this result is all the more striking since other genes considered important in development have not shown such drastic phenotypes upon disruption. In another approach, perturbation of methylation patterns

in the developing sea urchin embryo by microinjection of 5-methyl-dCTP caused embryo arrest (CHEN *et al.* 1993). While such experiments clearly demonstrate the importance of methylation in development, they do not address its function, or the mechanisms that control methylation changes. These questions remain open (BESTOR 1993), despite extensive studies on developmental changes in methylation for individual genes (KAFRI *et al.* 1993), regions of genes (*e.g.*, MUELLER and WOLD 1989) and bulk DNA (MONK *et al.* 1987) in mammals.

A promising approach to such questions is provided by molecular studies of certain non-Mendelian phenomena associated with DNA methylation, such as genomic imprinting and *X* chromosome inactivation in mammals (reviewed in LYON 1993). Related phenomena are well known in plants as well, especially in maize. For instance the mottled phenotype of the *R* gene in endosperm was shown to result from genomic imprinting of the *R* gene (KERMICLE 1970). Paramutation of the *R* (BRINK 1964, 1973) and *B* (COE 1966) genes, which causes heritable changes in the pigmenting potential of these genes due to interaction with paramutagenic alleles such as *R-st* or *B'*, provides another example. Paramutation has been hypothesized to be mediated by chromatin changes during development (BRINK 1964), and may be associated with methylation (M. ALLEMAN and J. L. KERMICLE, unpublished, in JORGENSEN 1993). Gene activity changes mediated by epigenetic programs have been demonstrated for maize transposons (MCCLINTOCK 1967a), and methylation changes have been shown to correlate with transposon activity for *Ac*, *Spm* and *Mu* (BENNETZEN 1985; FEDOROFF 1989; FEDOROFF *et al.* 1989; MARTIENSSEN *et al.* 1990). MCCLINTOCK showed that the controlling element *Spm* could cause variegated expression of a responding gene carrying a defective element by a mechanism unrelated to transposition, termed "presetting" (MCCLINTOCK 1964, 1965, 1967a,b). Variegation caused by presetting required an active *Spm* initially, but did not require its continued presence during development, and was occasionally heritable (MCCLINTOCK 1965, 1967b), suggesting an epigenetic change at the responding locus mediated by *Spm*. MCCLINTOCK hypothesized that presetting was a general phenomenon, accounting for some instances of variegation in plants that would be otherwise attributed to transposition (MCCLINTOCK 1967a). She further predicted that if floral sectors displaying such variegation on a background of uniform pigmentation included reproductive structures, the genetic change responsible could be transmitted (MCCLINTOCK 1967a). We describe here a new allele of the maize *P* gene isolated in just this manner. Pigmentation conditioned by this allele can resemble patterns caused by presetting of gene expression described by MCCLINTOCK (1967b), even though different genes are involved. Furthermore, the phenotype and the developmental changes in methylation of this allele illustrate general epigenetic pathways that may control gene expression in maize.

## MATERIALS AND METHODS

**Maize stocks:** The A188 sublines displaying increased frequencies of somatic mutations that were used in this study have been described (DAS and MESSING 1993). The *P-rr-4026* allele in the W22 inbred background (ATHMA and PETERSON 1991), and the inbred line 4Co63 were obtained from THOMAS PETERSON of Cold Spring Harbor Laboratories. Other inbred lines were derived from stocks of RONALD PHILLIPS at the University of Minnesota.

**Genetic analysis:** Inbred A188 plants, and $F_2$ progeny of (A188 × A619) and (A188 × W64A) were employed in crosses to *P-rr-4026* to study somatic instability of this gene. These $F_2$ progeny had been previously shown to display somatic rearrangement of the duplicated 27-kD zein locus (DAS *et al.* 1990a). Both A188 and A619 carry *P-ww* alleles, whereas W64A carries *P-wr*. Therefore, from the $F_2$ population involving W64A, *P-ww/P-ww* plants were selected by determining the browning of cut silks, which is a reliable indicator of the *P* allele present (COE *et al.* 1988). Five plants from this $F_2$ population, 13 inbred A188 plants and 4 plants from the A619 $F_2$ population were crossed to *P-rr/P-rr* plants. From the resulting 22 *P-rr/P-ww* families, approximately 50 ears per family were scored for somatic mutations of *P-rr*, visualized as sectors on the uniform red phenotype. We recovered from these one multikernel sector covering 11 kernels of the light crown and colorless cob phenotype (DAS and MESSING 1993) that is expected to be non-transmissible (GREENBLATT 1985), and one half-kernel sector (not shown). Some of these plants were self-pollinated to generate families segregating for *P-rr* and *P-ww*. From 75 such segregating families, ~3,000 ears were similarly scored. Even though only 50% of these latter plants would have the desired *P-rr/P-ww* genotype required for detection of somatic mutations of *P-rr*, self-pollinated progeny were used to render putative genetic factor(s) responsible for somatic mutation homozygous. One sector (not shown) covering five kernels of the dark crown type (GREENBLATT 1985), one sector covering 13 kernels with dark crowns and light orange sides (not shown), two covering single kernels (one shown in Figure 1), two covering the germinal halves of single kernels (one in Figure 1), and one covering the embryo third of a kernel (not shown) were recovered from this group. In addition to these potentially transmissible instances, pericarp sectors were seen at an approximate frequency of 1 per 50 ears in both populations (DAS and MESSING 1993).

Since both plantings were under isolation conditions, all ears should have received only *P-ww* or *P-rr* pollen. Therefore the sectored kernels could carry the mutant allele transmitted via the female gamete at a probability of 50% or less (ANDERSON and BRINK 1952), and either *P-ww* or *P-rr* from pollen. Because the phenotype of the mutant allele in the next generation plant could be masked by a dominant *P-rr* allele from pollen, two generations of progeny of the sectored kernels were tested for phenotype. Plants grown from sectored kernels were scored for phenotype by browning of cut silks. Positives, *i.e.*, those carrying a functional *P* gene, were either self-pollinated or backcrossed to *P-ww/P-ww* plants to remove a potentially masking *P-rr* allele by segregation. Pericarp and cob phenotypes were determined on mature ears to confirm the readings based on silk color. A second generation of progeny from the above crosses from red kerneled ears was then tested similarly. In all cases except for the two isolates of *P-pr*, no new phenotypes were seen in either generation. *P-ww*-like alleles resulting from

loss-of-function mutations may have been transmitted, but are beyond the scope of this report.

The first generation plant grown from the kernel carrying *P-pr-1* was self pollinated, and the first generation plant carrying *P-pr-2* was both self-pollinated and crossed to *P-ww*. The resulting second generation progeny of both isolates were screened for *P* on the basis of phenotype in tassel glume edges, subsequently confirmed by ear phenotype. Based on the segregation ratios (see Figure 2), both first generation plants appeared to have inherited the new allele via the female gamete, and *P-ww* from pollen. Many of the second (and later) generation plants were genotyped by molecular analysis, and were tested for concordance of phenotype. These are listed in Table 2, and no exceptions were noted. Plants with genotypes determined thus were self-pollinated or crossed to homozygous *P-ww* plants which were either siblings or from the inbred line A619, and to *P-wr* plants from the inbred lines W64A, W22, Mo17 and BSSS53 to generate material for further analysis. Third, fourth and fifth generation progeny that did not arise from crosses to *P-wr* were obtained and tested similarly. In all cases, segregation ratios were determined by initial phenotype readings using silk and or tassel glume pigmentation, and ear phenotypes were used for confirmation. No lack of concordance between the two assays was noted. Progeny of crosses to *P-wr* could be screened for pericarp phenotype only, since *P-wr* conditions pigmentation in cob and tassel glumes. Note that only the first generation of matings to *P-wr* have been tested.

**Molecular analysis:** DNA from various tissues of mature plants, and from endosperms and embryos of immature and mature kernels was isolated as described (Das *et al.* 1990b). Pericarp DNA from mature kernels was isolated by soaking kernels in water for an hour and peeling pericarps, which were ground in liquid nitrogen and processed as above. For immature pericarps, the tip halves of ears were cut off from the rest of the ear 21 days post-pollination. The bottom half of the ear was allowed to remain on the plant to determine phenotype on mature kernels, and pericarps were peeled from immature kernels and quick-frozen in liquid nitrogen. The tissue was later ground in liquid nitrogen, and total nucleic acids extracted as described (Das *et al.* 1990b). Poly(A) RNA was isolated from this using magnetic separation. The supernate after poly(A) isolation was precipitated with isopropanol to obtain pericarp DNA. Poly(A) RNA from the equivalent of 10–20 kernels was electrophoresed on formaldehyde-agarose gels, and blotted to Nytran membranes. These blots were hybridized and washed as below. Restriction digests were performed as described by the manufacturers, and electrophoresis was on 0.8–1.2% agarose gels. Southern blots onto Nytran membranes were prepared by standard protocols, and probed with the appropriate fragments isolated by the Gene-Clean process. These were labeled with [$^{32}$P]dCTP by random priming. Blots were washed three times in 1 × SSC, O.1% sodium dodecyl sulfate (SDS) at room temperature, and two stringent washes were performed at 65° in 0.1 or 0.2 × SSC, 1% SDS for 20 min each. Blots were stripped by pouring a boiling solution of 0.1 × SSC, 0.1% SDS on wet blots. Autoradiograms from slot blots and Northern blots for RNA, and Southern blots for methylation were quantitated by laser densitometry.

A summary of Southern blot analysis of *P-pr-1* and *P-pr-2* is given below. Leaf DNA from 3 sibling progeny from generation 2 of genotype *P-pr-1/P-ww* was compared to *P-rr* and *P-ww* leaf DNA using the following 34 restriction enzymes in single digests: *Alu*I, *Ava*I, *Ban*I, *Bgl*II, *Bss*HI, *Cla*I, *Dde*I, *Dra*I, *Eco*RI, *Eco*RII, *Hae*II, *Hae*III, *Hin*dIII, *Hpa*I, *Hpa*II, *Kpn*I, *Mbo*I, *Mlu*I, *Msp*I, *Mva*I, *Nae*I, *Nar*I, *Nco*I, *Nde*I, *Pst*I, *Pvu*II, *Sal*I, *Sma*I, *Spe*I, *Ssp*I, *Sty*I, *Xba*I and *Xho*I. The resulting blots were probed sequentially with probes 15, 8B and 19. These results showed that *P-pr* did not differ from *P-rr* in restriction sites. Increased methylation in *P-pr* was detected with a number of tests, which included the following. Leaf DNA from two *P-pr-1* progeny of generation 5, and two *P-pr-2* progeny of generation 2 were digested with *Hpa*II, *Msp*I, and *Hha*I and hybridized sequentially to all the probes shown in Figure 5 to generate the methylation map for these enzymes (Figure 5). *Pst*I digests of leaf or endosperm DNA from generations 2, 3, 4 and 5 (10–20 samples each) of *P-pr-1* were also probed with all the probes in Figure 5. DNA from the 219 progeny listed in Table 2 was digested with *Sal*I and *Bgl*II or *Hin*dIII, and probed with probe 15. The latter enzyme determined whether *P-pr* was present in the particular plant, and whether the appropriate bands were seen; *Sal*I determined methylation. Increased methylation in *P-pr* could be detected with the methylation-sensitive enzymes *Alu*I, *Eco* RII, *Hae*II, *Hha*I, *Hpa*II, *Msp*I, *Nar*I, *Pst*I, *Pvu*II and *Sal*I using DNAs from three *P-pr-1/P-ww* plants. Many of the enzymes in the group mentioned earlier that showed no differences betweeen the two alleles are potentially sensitive to cytosine methylation in maize. The fact that they showed no difference between *P-pr* and *P-rr* may result from either from location of sites in regions whose methylation is unchanged in *P-pr*, such as CpG islands, or from a lack of the appropriate methylation motif flanking their recognition site.

Since inferences regarding methylation are strongly dependent on the absence of partial digests, several types of controls were performed to rule out partial digestion. For determination of the enzymes whose sites were methylated in *P-pr* and for mapping of these sites (Figure 5), blots were sequentially hybridized to a number of probes, always including 8B. Although this probe also detects a linked second locus, it is an excellent indicator of partial digests, since its binding region is generally flanked by sites unmethylated in either allele (open circles, Figure 5). Hybridization to probe 16 A, B or C was a particularly useful control for four-cutters such as *Hpa*II and *Msp*I, since one side of their binding region is flanked by well spaced sites rather than a cluster of sites. In addition, many samples were digested with several enzymes, and the degree of methylation was usually consistent between enzymes (*e.g.*, Figure 9). For routine analysis with *Pst*I and *Sal*I, which were the enzymes of choice for screening large sample numbers, we also used the observation that certain sites were never methylated (Figure 5); incomplete digestion at these sites, as revealed by the diagnostic bands, was a good indicator of partial digests. For example, the presence of bands between the 4.5-kb doublet and the 10-kb doublet with *Sal*I was diagnostic of partial digestion at sites 5 and 7, and their absence (*e.g.*, Figures 6 and 8) was indicative of complete digestion.

## RESULTS

**Origin of the new allele:** The *P* gene encodes a *myb*-like transcription factor (Lechelt *et al.* 1989, Grotewold *et al.* 1991a), consistent with its genetic characterization as a regulatory locus controlling the expression of red phlobaphene pigment in various tissues of the maize plant (Styles and Ceska 1977; Coe *et al.* 1988). These tissues include kernel pericarp (an extension of the ovary wall similar to a seed coat), the lemma, palea and glumes of the female flower (cob pigmentation), and similar organs of the male flower. *P* alleles (Table 1) are usually named based on pigmentation in these two tissues, *e.g.*, *P-wr* corresponds to white (colorless) pericarp and red cob. Because pericarp

**TABLE 1**

**Alleles of *P***

| Allele | Pericarp and cob phenotype | Origin | Structure | Reference |
|---|---|---|---|---|
| *P-vv* | Variegated in both | Unknown, from "calico" variegated corn | *Ac* in intron 2 of *P* gene | Emerson (1914) |
| *P-ovov* | Orange variegrated in both | From *P-vv*, by intragenic transposition of *Ac* | *Ac* in intron 2 of *P* gene, in opposite orientation from *P-vv* | Peterson (1990) |
| *P-rr* | Uniform red in both | Revertant by *Ac* loss from *P-vv* | No *Ac* present, see Figure 2 | Emerson (1914) |
| *P-rr-4026* | Uniform red in both | Revertant by *Ac* loss from *P-ovov* | No *Ac* present, see Figure 2 | Peterson (1990) |
| *P-pr-1* | Variegated pericarp, red cob | Somatic epimutation of *P-rr-4026* | Similar to *P-rr*, but methylated | This report |
| *P-pr-2* | Variegated pericarp, red cob | Somatic epimutation of *P-rr-4026* | Similar to *P-rr*, but methylated | This report |
| *P-wr* | Colorless pericarp, red cob | Unknown, present in many inbreds | Unknown | |
| *P-ww* | Colorless in both | Unknown, present in many inbreds | Unknown | |

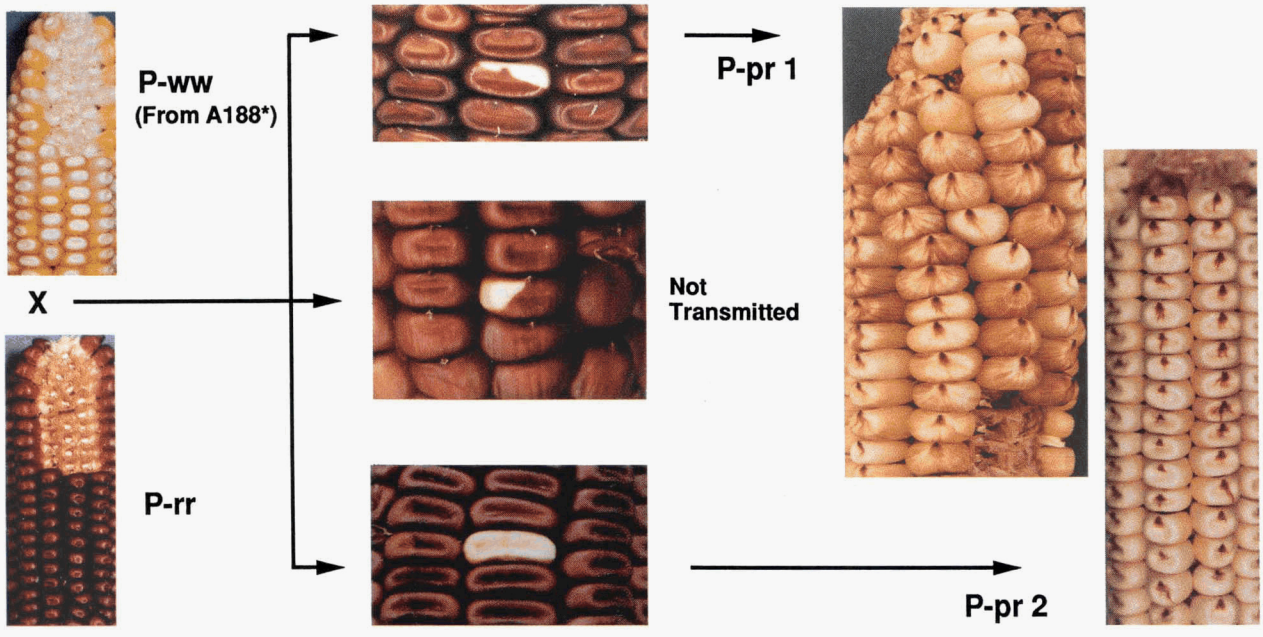


Figure 1.—Origin of the new allele. Phenotypes of *P-rr* and *P-ww* (alleles described in Table 1) are shown on the left. All kernels on ears of *P-rr/P-ww* plants are red because *P-rr* is dominant, and because pericarp is maternal tissue. Somatic mutations of *P-rr* can therefore be detected as sectors (middle panels). The potential for transmission of the mutations (see text) is indicated on the figure. The plant grown from the nearly colorless kernel gave the ear on the far right (*P-pr-2*), and a kernel very similar in phenotype to the one in the upper panel gave the other ear on the right (*P-pr-1*). The ear on the far right (*P-pr-2*) shows a consistent phenotype on all kernels, while the other (*P-pr-1*) shows a more variable pattern. However, both gave rise to similarly variable pigmentation in succeeding generations, as illustrated in Figure 8.

is maternal tissue, all kernels on the ear of a plant of genotype *P-rr/P-ww* are uniformly red regardless of the pollen parent (*P-rr* is dominant, *P-ww* is recessive, phenotypes in Figure 1, left). In kernels of such a plant, somatic mutations of *P-rr* can be detected as sectors (Figure 1, middle).

Because germline in plants is continuous with somatic tissue, somatic mutations can be transmitted through meiosis. In particular, pericarp and the female gamete share a cell lineage (Emerson 1914; Randolph 1926; Greenblatt 1985). Therefore, when pericarp sectors cover the embryo half or more of a kernel (*e.g.*, Figure 1, middle, upper and lower), the causal mutation is transmitted to the next generation represented by the embryo of the underlying kernel at frequencies close to a theoretical maximum of 50% (Anderson and Brink 1952). Mutations causing smaller sectors (*e.g.*, Figure 1, middle, center) are poorly transmitted, probably because later events in development are less likely to affect a common ancestor. These conclusions based on somatic mutations of the *P-rr* gene induced by *Ac* were employed here to screen spontaneous somatic mutations of *P-rr* in the absence of *Ac*. Mutations of this type were rare. We recovered only eight sectors covering the embryo face or more from $\sim 1 \times 10^6$ kernels (materials and methods), although we used a genetic background that increases the frequency of somatic mutations of other genes (Das and Messing 1993). Among plants grown from the sectored kernels, two gave ears with a new phenotype (Figure 1, right). The other six gave either red or white kernels for two generations (materials and methods), and will not be considered further. Var-

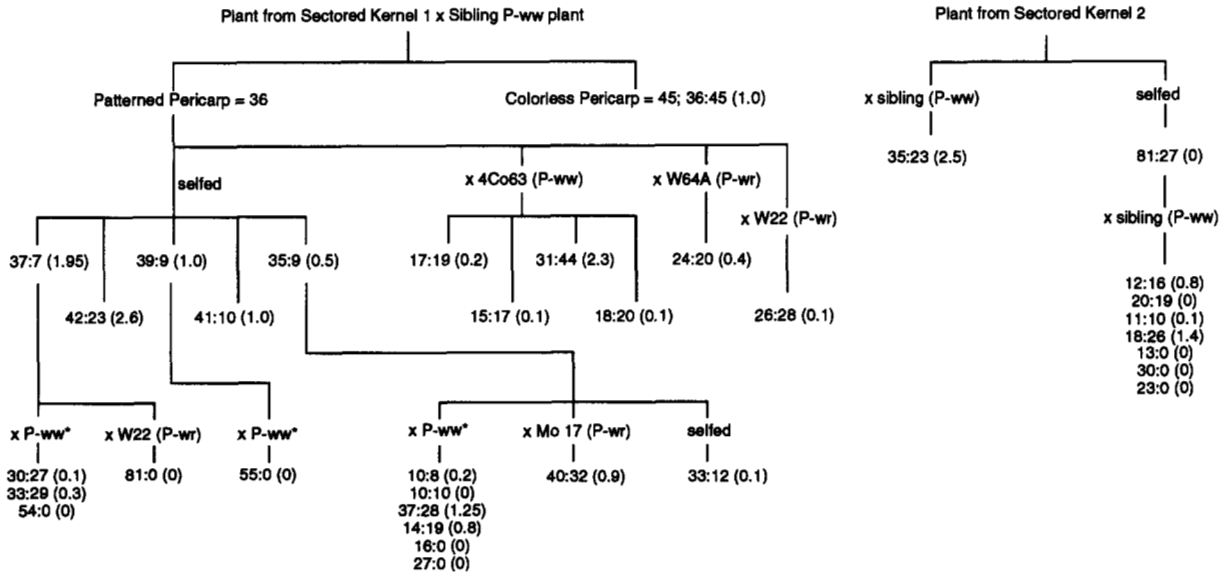


FIGURE 2.—Genetic analysis of *P-pr*. The lineage of the material used for the genetic analysis is shown. Generation 1 is the plant from the sectored kernel, giving the two ears shown in Figure 1. Segregation data are shown for three succeeding generations. Each entry gives the observed number of ears with pericarp pigmentation: ears without pericarp pigmentation for single families. The expected segregation ratios for the experiments represented, based on the genotype of the parent determined by molecular analysis, should be 1:1, 3:1 or 1:0, assuming dominance of *P-pr* to *P-ww*. The values in brackets give uncorrected chi-square values for deviation from these. None of these values were significantly different from expectations at a confidence level of 90%. Crosses to *P-ww* alleles marked by asterisks were to sibling *P-ww/P-ww* plants from each family.

iegation on these two ears must have resulted from transmission of mutations which caused sectors on the progenitor kernels because a similar phenotype was not seen in ~5,000 other plants screened over the course of these mutagenesis experiments (MATERIALS AND METHODS). Furthermore, this phenotype of variegated pericarp and red cob has not been previously described either for a spontaneous or transposon-induced derivative of *P-rr*.

We have studied six generations of progeny of the first isolate and four generations of the second (Figure 1, right, upper and lower, respectively) to determine the genetic basis of the variegated pericarp phenotype. Segregation data for four and two generations, respectively, are summarized in Figure 2; two additional generations have given similar results for both (not shown). The analysis (MATERIAL AND METHODS) showed that phenotype segregated as an allele of *P-ww* with normal penetrance, but variable expressivity, as described next. Unlinked loci were not required for its transmission, although we cannot rule out the influence of modifier genes upon pigmentation. The two isolates of this new allele were designated *P-pr-1* and *P-pr-2*, for patterned pericarp and red cob. The suffix "v" for "variegated" was avoided because variegation was not always detectable (below).

**Details of the *P-pr* phenotype:** Phenotype proved to be very informative regarding the mechanism of variegation in *P-pr*, due to the extensive literature on *P* allele phenotypes and pericarp development in maize (*e.g.*, EMERSON 1914; RANDOLPH 1926, 1936; MCCLINTOCK 1967b; GREENBLATT 1985; ATHMA *et al.* 1992; MORENO *et al.* 1992). Pericarp originates from a basal ring of cells of the ovary wall, which divide mostly orthogonal to the ring to give longitudinal files of elongated cells (MCCLINTOCK 1967b). Therefore, late clonal events at the *P* locus give rise to stripes on kernels. Two types of stripes are commonly seen. Stripes on the abgerminal side of kernels widen toward the silk attachment point (SAP) and cover it; these usually originate closer to the SAP than to the base. Stripes that originate in basal regions become narrower toward the SAP and disappear before reaching it (EMERSON 1914; GREENBLATT 1985). The origin of the two types of stripes is explained by a standard model, that the crown region of the pericarp originates from the tunica or L1 cell layer of the meristem, while the sides originate from the corpus or the L2 cell layer (RANDOLPH 1926; GREENBLATT 1985). With *P-vv*, red stripes are caused by switching of the *P* gene from a nonfunctional state to a functional one by *Ac* excision; switching in L1 and L2 cells, respectively, lead to the first or the second types of stripes. When this occurs in one of the two cell layers early in development, multikernel sectors with only dark crown or light crown phenotypes can result (RANDOLPH 1926; GREENBLATT 1985; ATHMA and PETERSON 1991).

Pericarp variegation with *P-pr* consists of similar stripes (examples of phenotypes in Figures 1, 8, and 10b), indicating that it should also undergo such a switch from a non-functional to a functional state. However, with *P-pr*, striping is highly variable (Figures 1, 8 and 10b); this can be explained by variation in the timing of the switch during pericarp development. In cases of heavy variegation, many broad pigmented cell files are interspersed with few narrow colorless cell files on

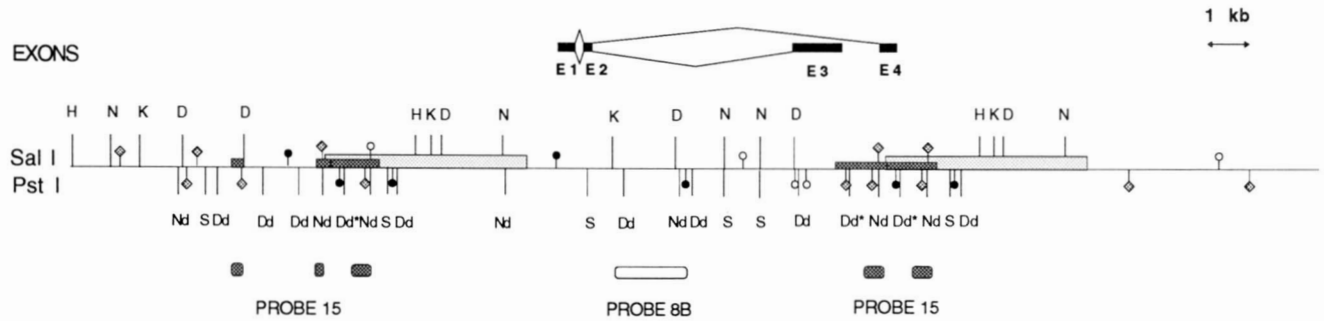


FIGURE 3.—Restriction site identity between *P-pr* and *P-rr*. Restriction sites that are shown to be conserved between *P-pr* and *P-rr* by the data in Figure 4 are represented by vertical lines; D, *Dra*I; Dd, *Dde*I; H, *Hin*dIII; K, *Kpn*I; *N*, *Nco*I; Nd, *Nde*I; S, *Sty*I. The *Dde*I sites marked with asterisks are potentially sensitive to maize DNA methylation (CTGAG) and are likely methylated based on the data (Figure 4). In addition, *Sal*I and *Pst*I sites are also shown, since they are also present at identical locations in the two alleles, although some sites are methylated (see legend to Figure 5 for the methylation status of these sites). These site assignments are based on published sequence and maps (LECHELT *et al.* 1989, ATHMA and PETERSON 1991; ATHMA *et al.* 1992), unpublished restriction data (from THOMAS PETERSON), unpublished sequence data for a 5-kb *Eco*RI fragment (Figure 5) at the 5′ end (from JYCHIAN CHEN and STEPHEN DELLAPORTA), and our analysis of genomic DNA and of clones provided by T. PETERSON. Note that additional restriction analysis described in MATERIALS AND METHODS further confirms the conclusion that no major rearrangements, insertions or deletions are associated with the generation of *P-pr* from *P-rr*. Some structural features of the *P* locus are also represented. A 4.6-kb direct repeat is shown by the dotted boxes, and a smaller repeat of 1.2 kb is represented by the hatched boxes. At the 5′ end, one complete and one truncated copy of the 1.2-kb repeat are present, and the latter is further disrupted by an insertion. Therefore probe 15, which is derived from the 3′ end of this repeat, can potentially detect five distinct regions of the locus, as shown. Probe 8B detects, in addition to the region shown, another linked locus (O. P. DAS and J. MESSING, unpublished data). The origin of the two major alternately spliced mRNAs from the locus is shown (exons 1 + 2 + 3 or 1 + 2 + 4); exons 3 and 4 originate from the 1.2-kb repeat. Two other minor mRNAs, presumably from incomplete splicing of the large intron (GROTEWOLD *et al.* 1991a), are also found (see Figure 10a).

the sides as well as the crown (*e.g.*, Figure 8, left, panels 5 and 6). Here, switching may occur relatively early, *i.e.*, after fewer cell divisions starting from an arbitrary precursor. Lighter patterns consist of pigmentation in fewer, narrower and more widely spaced lateral cell files, and/or shorter stripes on the crown that originate closer to the tip until only the silk attachment point is colored (examples in Figures 1, 8 and 10b). Here, switching may occur after more cell divisions have elapsed. Since patterns are often consistent over kernels consitituting large sectors or even the entire ear (*e.g.*, Figure 1, lower right) switching may occur after similar numbers of cell divisions have elapsed during development of individual pericarps. Therefore, it is likely that the number of cell divisions that elapse before switching is predetermined, probably by a developmental program (MCCLINTOCK 1967a). Patterns can also vary between multikernel sectors (*e.g.*, Figure 1, right upper), which can either be clonal with sharp boundaries (Figure 8, left, panel 5), or non-clonal with diffuse boundaries (*e.g.*, Figure 1, right upper). Therefore the developmental program may be subject to somatic change.

Based on *P-pr* phenotypes, cells in both L1 and L2 lineages appear to be able to switch independently (not shown). If switching occurs earlier in L1 than L2, well defined large pigmented crowns should result, with few faint lateral stripes (Figure 1, right upper); if it occurs earlier in L2, many broad lateral stripes with only tip pigmentation would result (Figure 8, left panel 6, middle panel 4). A large difference in timing between L1 and L2 would give either the dark crown or the light crown phenotype, similar to the sectors seen with *P-vv*. Phenotypes representing these extremes have also been observed with *P-pr* (not shown). Dark crown patterns were more frequent than the light crown type, both in the pure form or mixed with the other type of striping (not shown).

Despite these similarities, the *P-pr* phenotype differed in two important respects from *P-vv*. First, *P-vv* conditions variegation in both pericarp and cob, and colorless cob sectors underlie pericarp sectors of the light crown type (EMERSON 1914; GREENBLATT 1985). In contrast, with *P-pr*, we could not detect variegation in cobs, and cob pigmentation did not correlate with pericarp pigmentation (not shown). Rare cob sectors had indistinct boundaries, and did not underlie the frequently observed pericarp sectors (not shown). Second, variegation patterns are generally consistent among individuals carrying a given *P-vv* allele, permitting classification of *P-vv* alleles into medium, light and heavy variegation types (*e.g.*, MORENO *et al.* 1992). However, pericarp pigmentation given by *P-pr* was variable, and clonal and non-clonal sectors differing in the extent of variegation were common (above). Therefore, we suspected that variegation in *P-pr* was not caused by transposition, as shown below.

**Molecular characterization of *P-pr*:** Figures 3 and 5 show restriction maps of the *P-rr* gene (based on LECHELT *et al.* 1989; ATHMA *et al.* 1992; J. CHEN and S. DELLAPORTA, unpublished data; T. PETERSON, unpublished data). The transcription unit is closely flanked on both sides by a direct repeat (striped boxes); this itself con-

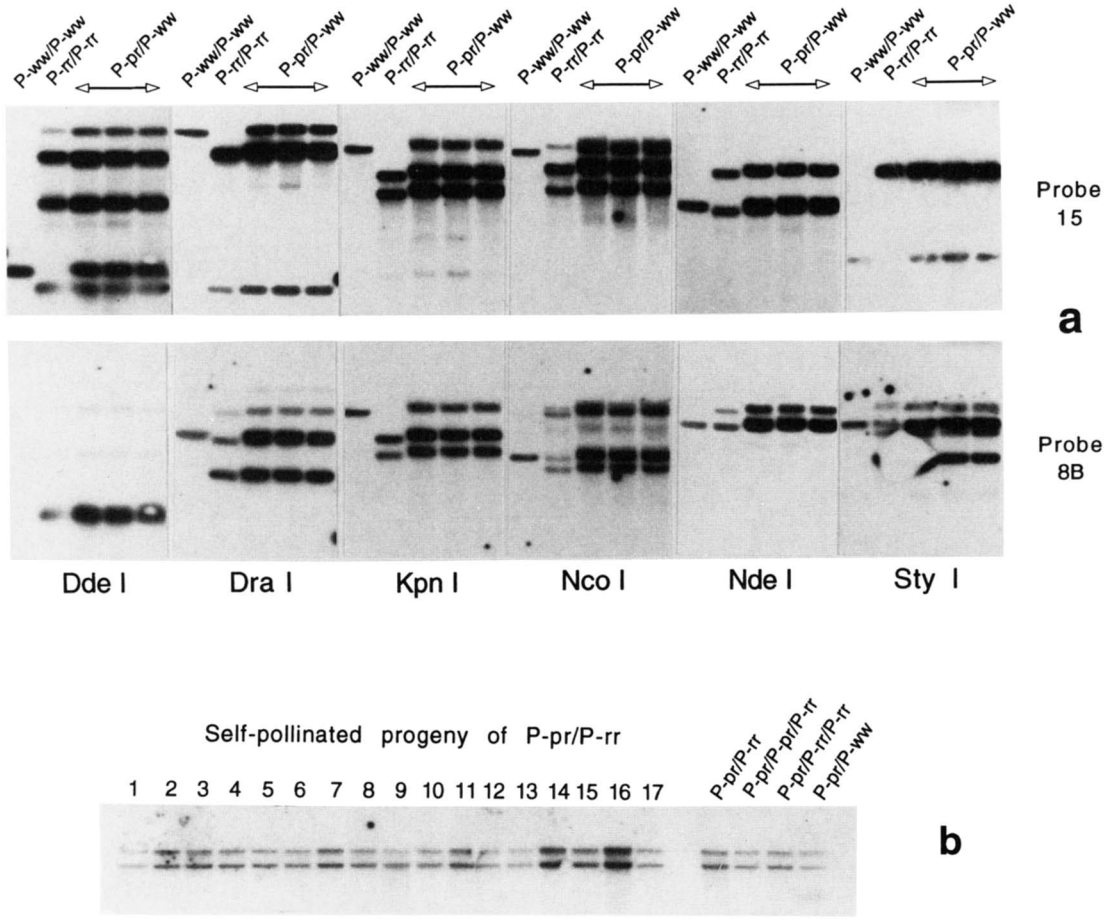


FIGURE 4.—Restriction Site Identity between *P-pr* and *P-rr*. DNAs of the indicated genotypes were digested with the restriction enzymes shown, and blots were sequentially hybridized to probes 15 and 8B. The results are represented in (a). Note that molecular weights are not comparable between panels; the sizes of the bands can be determined from Figure 3. In all cases, the three lanes containing *P-pr/P-ww* DNA shows only the bands seen in the lanes containing *P-rr* and *P-ww* DNA. The slightly higher mobility of the *P-pr* samples with *Kpn*I and *Nco*I is due to greater amounts of DNA loaded. Panel b shows another type of evidence for restriction site identity between *P-pr* and *P-rr*, using *Hin*dIII digests in all lanes, and probe 15. Of the four right lanes, the middle two represent endosperm DNA from reciprocal crosses of *P-pr* and *P-rr*; the left lane represents pericarp DNA of the genotype *P-pr/P-rr* and the right lane represents leaf DNA from *P-pr/P-ww* (the lower band in this lane is from the *P-ww* allele). These show no differences, and in addition, DNA from mature endosperms of kernels from a self-pollinated *P-pr/P-rr* plant also show no differences between lanes, confirming the identity of the two alleles.

tains 3 complete copies of a smaller 1.2-kb repeat (hatched boxes), and a fourth truncated copy interrupted by an insertion at the 5′ end. Alternate splicing gives two major (1.4 and 2 kb) and two minor (6.5 and 7 kb) mRNAs from the locus, and exon 3 is necessary for function (GROTEWOLD *et al.* 1991a; mRNAs visualized in Figure 10a). We have identified several potential CpG islands at the locus using criteria defined previously (BIRD 1992; GARDINER-GARDEN and FROMMER 1987) (described in the legend to Figure 5). In vertebrates, housekeeping genes have CpG islands at the 5′ end, whereas tissue-specific genes may have islands at either or both ends (GARDINER-GARDEN and FROMMER 1987). The tissue-specificity of the *P* phenotype (STYLES and CESKA 1977) is consistent with the presence of two islands in the gene, and the association of the 3′ island with an exon (GARDINER-GARDEN and FROMMER 1987).

**TABLE 2**

**Genetic analysis of methylation**

| Allele | Generation | Individuals carrying *P-pr* | Individuals with methylation |
|---|---|---|---|
| *P-pr-1* | 2 | 17 (1) [a] | 17 (1) |
| *P-pr-1* | 3 | 55 (3) | 55 (3) |
| *P-pr-1* | 4 | 74 (14) | 74 (14) |
| *P-pr-1* | 5 | 54 (3) | 54 (3) |
| *P-pr-2* | 2 | 13 (2) | 13 (2) |
| *P-pr-2* | 4 | 6 (1) | 6 (1) |
| Total | | 219 | 219 |

[a] Number of families is shown in parentheses.

Consistent with the interpretation that variegation in *P-pr* does not involve transposition, we failed to detect any insertions, deletions or restriction site polymorphisms in *P-pr* compared to its progenitor, *P-rr*, by extensive Southern blot analysis. Data using six restriction

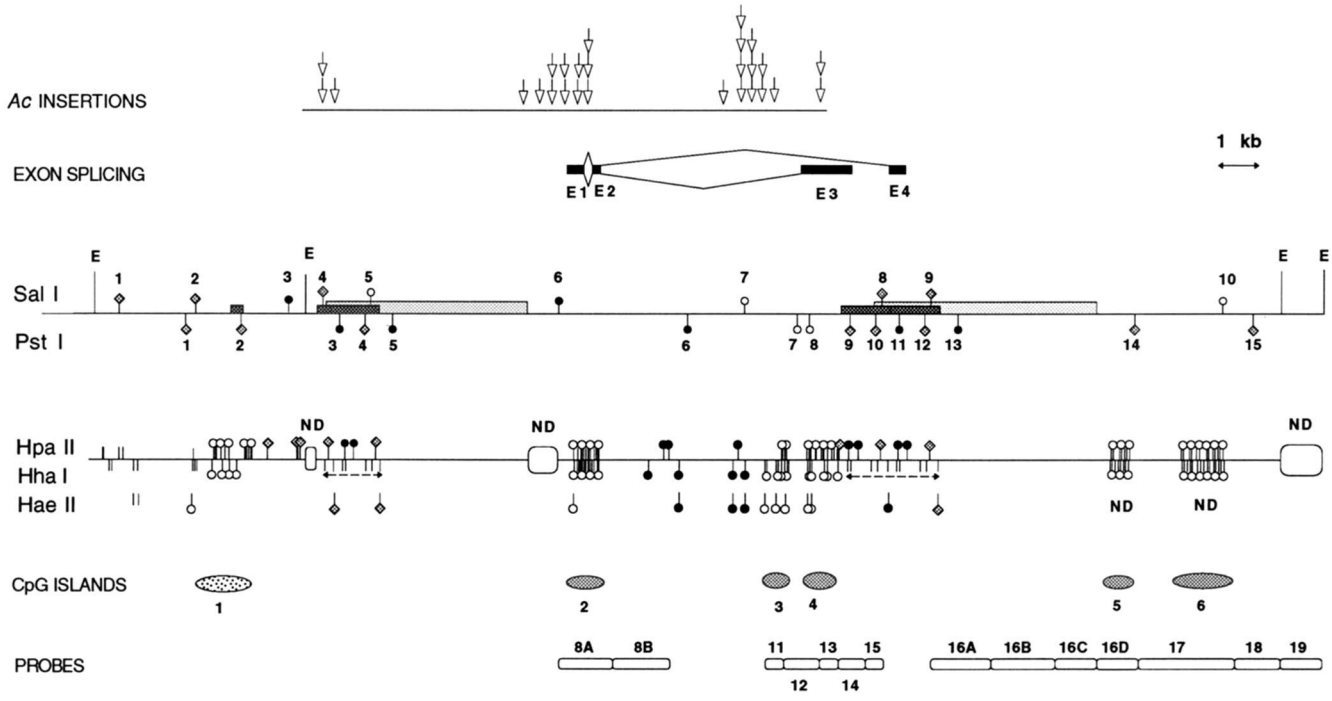


FIGURE 5.—Methylation maps of the *P* locus in *P-pr* and *P-rr*. In addition to the methylation data summarized in the middle two maps, other features of the *P* locus are also represented. The upper panel represents the location of ~250 *Ac* insertions that disrupt gene function, mapped by MORENO *et al.* (1992), and ATHMA *et al.* (1992). The number of arrows approximates the number of insertions. Note the insertions ~5 kb 5′ to exon 1, near the 5′ pair of repeats. Insertions show a marked bias, especially in the large central intron. The two middle maps show restriction sites at the locus, based on published sequence and maps, and unpublished restriction and sequence data as described in the legend to Figure 3. Methylation of these sites was determined by restriction analysis and probing with the fragments shown at the bottom (MATERIALS AND METHODS). The numbering of these probes follows the original description of the locus (LECHELT *et al.* 1989). Keys to the methylated sites are as follows: open circles represent sites unmethylated in both *P-rr* and *P-pr*; filled circles represent sites methylated in both alleles; stippled diamonds represent sites unmethylated in *P-rr*, but methylated in *P-pr*. The latter sites vary in methylation among progeny carrying *P-pr*, and any site which is even partially methylated in *P-pr* is represented thus. For example, *Pst* site 15 is partially methylated only in plants showing very high levels of methylation. In the two regions shown by the dashed lines, methylation of individual *Hha*I sites could not be determined. All could be methylated in *P-pr*, but some were methylated in *P-rr*. The boxes labeled ND represent regions for which *Hpa*II and *Hha*I data is not available. CpG islands 1, 2, 3 and 4 were identified by analysis of sequence data for the transcribed region (ATHMA *et al.* 1992) and for the 5-kb *Eco*RI fragment (J. CHEN and S. DELLAPORTA, unpublished). The criteria used were increased ratios of CpG/GpC, increased GC content, and clustering of *Hpa*II and *Hha*I sites (GARDINER-GARDEN and FROMMER 1987). Island 1 is borderline, and is therefore lightly stippled. Islands 5 and 6 were identified only by mapping *Hpa*II and *Hin*PI (isoschizomer of *Hha*I) sites in cloned DNA since sequence data was not available. The approximate number and distribution of sites in these cloned fragments was determined by end-labeling digested DNA with Klenow fragment. Islands 3/4 and 5/6 may represent single larger islands disrupted by insertions. Each site in these islands is represented by a vertical line; note that circles do not represent the number of sites. Since *Hpa*II and *Hha*I sites in all CpG islands are highly clustered, the methylation status of individual sites could not be determined. However, at least some of the sites in all six islands were unmethylated in both alleles.

enzymes and two probes are shown in Figure 4, and the sites mapped using only these digests cover most of the locus (Figure 3). Note particularly the evidence in Figure 4b, which shows both *P-rr* and *P-pr* DNA in the same lanes. In all, we have failed to find a size difference between the two alleles using 34 restriction enzymes (MATERIALS AND METHODS) in single digests with sequential hybridization to probes 15, 8B and 19. We have also not observed any new or different bands in DNA from the 219 plants represented in Table 2 with *Sal*I and at least one methylation-insensitive restriction enzymes, using probe 15.

However, analysis of leaf DNA with the methylation-sensitive restriction enzymes *Alu*I, *Eco*RII, *Hae*II, *Hha*I, *Hpa*II, *Msp*I, *Nar*I, *Pst*I, *Pvu*II and *Sal*I, in combination with certain probes, showed higher molecular weight bands in addition to parental bands, *i.e.*, those in *P-rr*. The sensitivity of these enzymes to cytosine methylation in the CpG (*Hae*II, *Hha*I, *Hpa*II, *Nar*I, *Sal*I) and CpXpG (*Alu*I, *Eco* RII, *Hpa*II, *Msp*I, *Pst*I, *Pvu*II) contexts suggested methylation of their sites in *P-pr*. Data for *Sal*I is shown in Figure 6, and the assignment of higher molecular weight bands based on methylation of sites are described in its legend. Similar data without band assignments are shown in Figure 7 for *Hae*II, *Hpa*II and *Msp*I, and with band assignments for *Pst*I in Figures 9 and 11.

We have mapped the methylation status of *Sal*I, *Pst*I, *Hae*II, *Hpa*II and *Hha*I sites in *P-rr* and *P-pr*, based on restriction data similar to that in Figures 6, 7, 8, 9 and

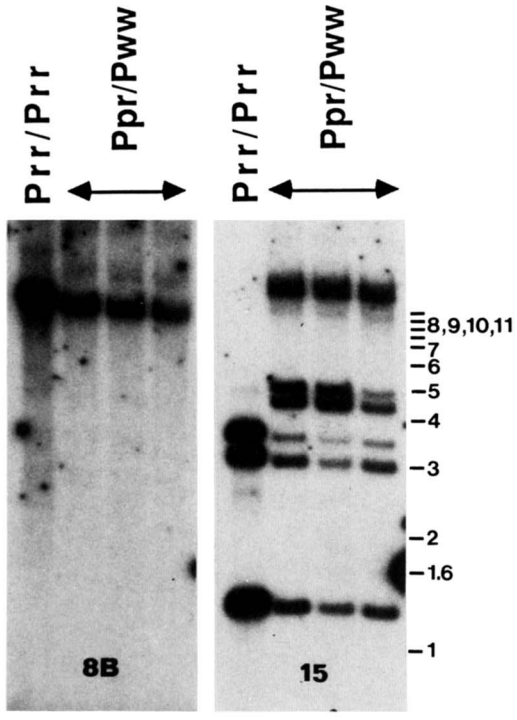


FIGURE 6.—Methylation of *SalI* sites in *P-pr* and *P-rr*. Leaf DNA from three sibling plants of genotype *P-pr/Pww* from the second generation (defined in Figure 2) of *P-pr-1* was compared to leaf DNA from plants homozygous for *P-rr* and *P-ww*. *SalI* digests were probed sequentially with probes 15 and 8B. Probe 8B shows no differences in the lanes. This indicates that sites 5 and 7 are unmethylated in *P-pr*, that site 6 remains methylated as in *P-rr*, and that partial digestion is absent. The pattern with probe 15 is more complex. *P-rr* gives a doublet of 1.2 kb from digestion at 4 + 5, and 8 + 9, and bands of 3 and 3.4 kb from sites 2 + 4, and 7 + 8, respectively. With *P-pr*, these bands are visible, in addition to higher molecular weight bands. The origin of the latter can be explained by methylation as follows. The two bands between 4 and 5 kb arise from methylation of sites 8 and 4, leading to fragments of 3 + 1.2 = 4.2 kb, and 3.4 + 1.2 = 4.6 kb. Two higher molecular weight bands (poorly resolved here) arise from methylation of sites 8 and 9, giving a fragment of ~12 kb from digestion at sites 7 and 10, and a similar sized fragment from methylation of 1, 2 and 4, and digestion at sites 5 and an unmapped site to the left. Site 3 remains methylated in *P-pr* and *P-rr*. The seven bands in *P-pr* are defined as indicating "basal" methylation (as in *P-rr*, 1.2-, 3- and 3.4-kb bands), "partial" methylation (4.2- and 4.6-kb bands), and "full" methylation (two bands of ~12 kb). None of these bands originate from the *P-ww* allele (data not shown).

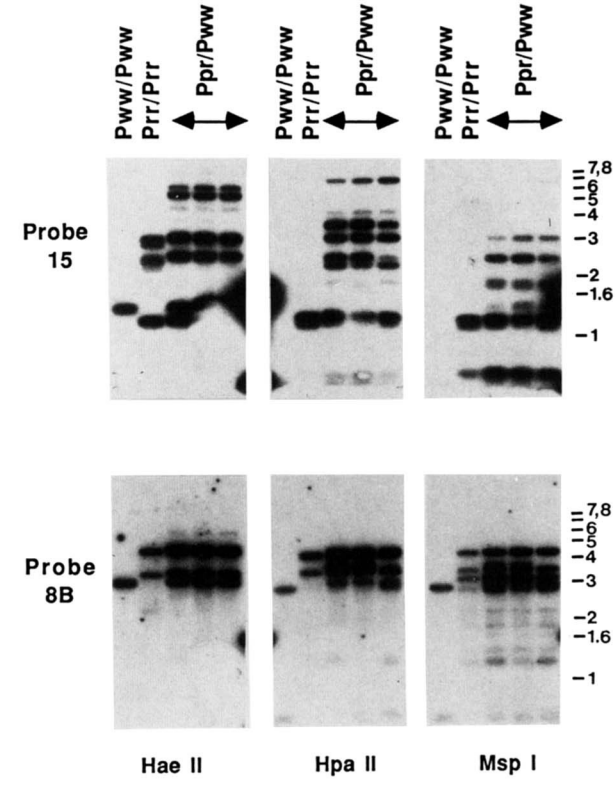


FIGURE 7.—Methylation of *Hae*II, *Hpa*II, *Msp*I sites in *P-pr*. The same DNA samples represented in Figures 4 and 6 were digested with *Hae*II, *Hpa*II and *Msp*I, and the resulting blot was sequentially hybridized to with probes 15 and 8B. With 8B, all three enzymes give a similar 4.5-kb band, because of methylation of all sites in the region between CpG islands 2 and 3. The weakly hybridizing 3.5-kb band(s) is from a linked unmapped locus (data not shown), and the lower molecular weight bands with *Msp*I arise from the frequent leakiness of CpNpG methylation. With probe 15, a series of higher molecular weight bands are seen with *P-pr*. These bands can also be accounted for by methylation (band assignments not shown). When all sites between islands 4 and 6 are methylated, a ~6-kb band should be seen, as detectable with *Hpa*II (and faintly with *Msp*I). Individuals with high methylation levels show high levels of this band, and a ~7-kb band from methylation of all sites between islands 1 and 2 (not shown).

11 (experiments summarized in MATERIALS AND METHODS). This is shown in the methylation maps in Figure 5. Open circles represent sites unmmethylated in either allele, filled circles represent sites methylated in both alleles, and stippled diamonds represent sites methylated specifically in *P-pr*. Many sites were unchanged in methylation in the new allele. For instance, all six CpG islands remained largely unmethylated (open circles) in *P-pr* as in *P-rr*, and *SalI* sites 5 and 7, and *PstI* sites 7 and 8 were also unmethylated in both. The highly methylated region between islands 2 and 3 remained methylated in *P-pr* as in *P-rr* (filled circles); this was also true of *SalI* sites 3 and 6, and *PstI* sites 3, 5 and 6. The major increase in methylation in *P-pr* was within the pairs of 1.2-kb repeats, and in regions flanking these in both directions. Owing to the striking absence of assayable sites over most of the large repeat (dotted boxes), we could not determine methylation in these two regions. The 5′ methylated region is ~5 kb distal to exon 1, but *Ac* insertions between *Sal* sites 4 and 5 can inactivate the gene (Figure 5) (based on MORENO *et al.* 1992), suggesting the presence of an expression signal here. This region, or the methylated region at the 3′ end may contain a *cis*-acting sequence necessary for expression in pericarp that may be sensitive to methylation (RAZIN and CEDAR 1991). Based on enzyme specificities, both CpG and CpNpG sites were modified. However, methylation

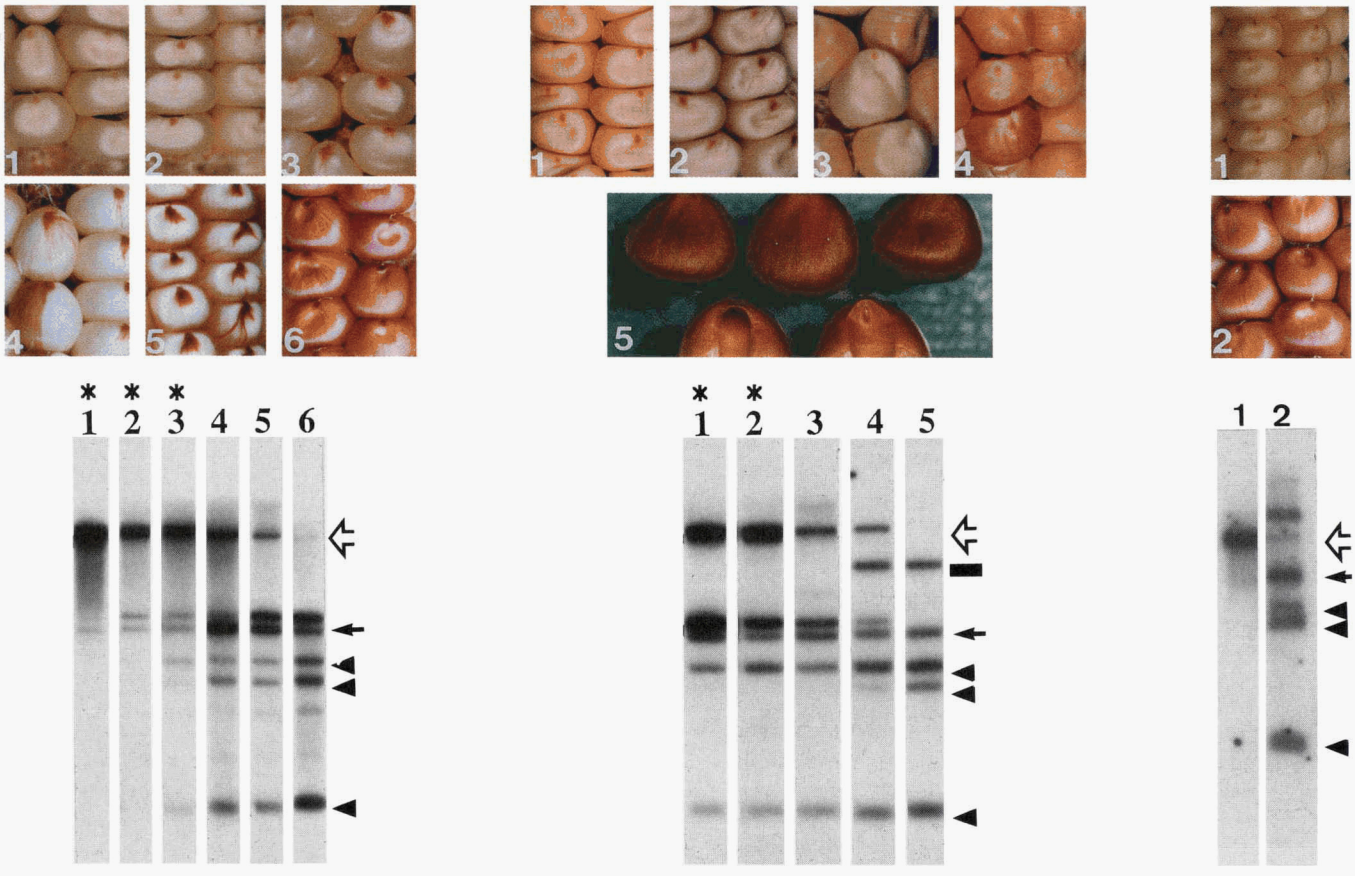


FIGURE 8.—Correlations between methylation and phenotype. Color photographs show the phenotype, and Southern analysis shows methylation. Results with leaf DNA are represented in the left and middle panels, and pericarp DNA from mature kernels is represented on the right. The first set of 6 samples represents 6 progeny of generation 3 (defined in Figure 2) of *P-pr-1*, obtained by self-pollination of a *P-pr/P-ww* plant (left, 1–6). The next set represents 5 progeny of generation 2 of *P-pr-2*, the first three from self-pollination of the original *P-pr-2/P-ww* plant, and the other two from outcrossing to *P-ww* (middle, 1–5). DNA analysis of mature pericarps of two sibling plants of the family in the left panels (genotypes *P-pr/P-pr* and *P-pr/P-ww*, respectively), and their corresponding phenotypes are shown on the two samples on the right. All blots were hybridized to probe 15, and reprobing with 8B controlled for partial digestion (not shown). DNAs in the left and middle panels were digested with *Sal*I. In the two right panels digestion was with *Sal*I + *Bam*HI to distinguish a *P-ww* band in lane 2 (highest molecular weight band). Triangles represent the "parental" *P-rr* bands, the filled arrow shows the "partial" methylation bands, and the open arrow indicates the "full" methylation bands (band molecular weights and origins are described in Figure 6). The band marked by a bar is from a *P-ww* allele in these two plants. Note the inverse correlation between the intensities of higher molecular weight bands and phenotype. Also note that homozygotes of *P-pr* (asterisks) show more methylation and less pigmentation than sibling heterozygotes; this difference has been seen in all families tested (not shown).

at CpNpG sites appeared to be more leaky (a small fraction of each site unprotected from digestion) in both *P-pr* and *P-rr*, as shown by *Msp*I (Figure 7) and *Pst*I digests (not shown).

Methylation varied greatly among plants carrying *P-pr* (examples in Figures 8 and 10). Some were modified at all possible sites (stippled diamonds in Figure 5), whereas those with lower levels of methylation were modified at sites located increasingly closer to the central highly methylated region between CpG islands 2 and 3/4. This is illustrated below for *Sal*I and *Pst*I sites. Thus, *Sal*I sites 4 and 8 were nearly always methylated, whereas plants with heavy methylation were additionally modified at sites 1, 2, and 9 (Figure 8). Methylation of *Sal*I site 2 was never observed without methylation of site 1 as well, implying that they were always methylated together as a unit. Similarly, low methylation led to masking of only *Pst*I sites 4, 9 and 10 (Figure 9), intermediate levels additionally masked sites 2 and 12 (Figure 11), and heavy methylation caused masking of sites 1, 14 and 15 (not shown). Similar results were also obtained with *Hpa*II, *Msp*I, *Hha*I and *Hae*II (not shown).

Furthermore, in individual plants, sites that were distant from the gene were not methylated unless more proximal sites were methylated. For example, *Sal*I sites 1 or 2 were not methylated unless site 4 was, and site 9 was not methylated unless 8 was. This is illustrated by the data in Figures 6 and 8. Only two higher molecular weight doublets are seen; if sites 1 or 2 were methylated without methylation of site 4, or if site 9 was methylated without methylation of site 8, intermediate bands reduced by 1.2 kb from the upper doublet should be seen.

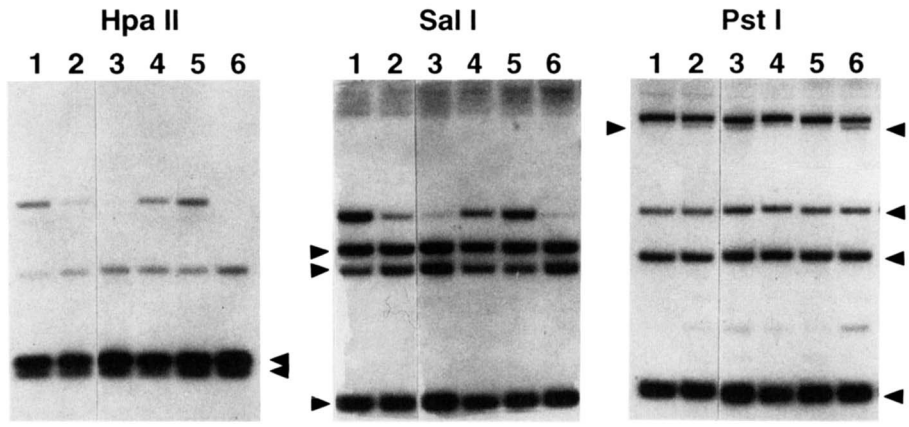


FIGURE 9.—Methylation in a revertant. Progeny of the plant represented in Figure 8, middle, lane and panel 5, gave red kernels for two more generations, indicating that this plant carried a revertant allele. Six sibling progeny carrying this allele from the third generation, obtained by crossing *P-pr-2(rev)/P-ww* to a *P-ww* tester, are represented. Leaf DNA was digested with the indicated enzymes, and hybridized to probe 15. Note that band sizes do not correspond between panels. Basal methylation (as in *P-rr*) is indicated by arrows. The low methylation seen with *Sal*I in lanes 2, 3 and 6 is confirmed by *Hpa*II digests, and also by *Pst*I, as described below. The basal *Pst*I bands (*i.e.*, same as in *P-rr*, triangles) originate, in increasing molecular weight, from: restriction at sites 1 + 2, and 10 + 12 to give the lowest molecular weight 1.2–1.3-kb doublet (site 11 is methylated); restriction at sites 2 and 4 to give a 2.8-kb band (site 3 is methylated); restriction at sites 12 and 14 to give a ~5-kb band (site 13 is methylated); restriction at sites 4 and 7 to give band of ~10 kb (sites 5 and 6 are methylated). Methylation of site 4 in *P-pr* converts the 10-kb band to a higher molecular weight band (seen in all lanes) by digestion at sites 2 and 7. Site 4 is the most sensitive indicator of methylation in *P-pr*, and in all our experiments, has been seen to be detectably unmethylated only in the lightly methylated samples in lanes 2, 3 and 6. The faint bands (molecular weight 1.7 kb) in lanes 2, 3 and 6 between the 1.2- and 3-kb basal bands correspond to methylation of site 9, and digestion at sites 8 and 10. Additional methylation of site 10 in lanes 1, 4 and 5 gives a band that comigrates with the 2.8-kb basal band. Thus, it is clear that low methylation detected by *Sal*I and *Hpa*II in lanes 2, 3 and 6 is also reflected by *Pst*I.

This was true of *Sal*I digests for all the plants listed in Table 2. Band assignments showed that this was also true of *Hpa*II, *Hae*II and *Hha*I in cases where these enzymes were used (not shown), and usually applicable to *Pst*I and *Msp*I, although the latter enzymes are affected by the slight leakiness of CpNpG methylation, and exceptions to this trend were occasionally noted (*e.g.*, Figure 11). The order of methylation of sites was also a valuable aid in ruling out partial digests. The presence of inappropriate bands representing violations to the trend was indicative of partial digests, and their absence was indicative of complete digestion.

Thus, to a first approximation, methylation appeared to have "spread" outward to different extents in individual plants from a central region, which is approximately demarcated by CpG islands 2 and 3 and comprises much of the transcription unit (Figure 5; GROTEWOLD *et al.* 1991a). The alternate possibility that methylation has spread into the *P* gene from neighboring regions is less likely in view of the above data. The spread could not have caused complete methylation in the flanking regions since at least some sites in all the CpG islands remained unmethylated. We have not yet determined how far the increased methylation can extend in either direction. The decrease in methylation that occurs during development (described below) appears to cause a reduction or shrinking of the region covered by the spread, *i.e.*, a progressive reduction in methylation from distal to proximal sites (not shown). This is illustrated by the *Sal* I digests in Figures 6 and 8 and especially by the quantitations in Figure 12. In almost all cases examined (Table 2), the intensities of bands corresponding to basal, partial and full methylation (defined in Figure 6) generally decrease or increase in any sample, *i.e.*, high levels of the highest and lowest molecular weight bands were not seen in combination with low levels of the intermediate molecular weight bands. Therefore, sites 4 or 8 were not significantly demethylated unless sites 1 and 2, and 9, respectively, were demethylated to a greater extent.

**Genetic analysis of methylation:** The above arguments suggest that methylation changes in the absence of sequence changes are associated with the generation of *P-pr* from *P-rr*, as expected for epimutation (HOLLIDAY 1987). The Mendelian inheritance of *P-pr* was used to confirm this conclusion. First, all of the tested 219 progeny (listed in Table 2) carrying *P-pr-1* or *P-pr-2* showed some methylation. *i.e.*, gave higher molecular weight bands with *Sal*I, including even the phenotypic revertant discussed below. Therefore, methylation was fully penetrant over at least five generations (Figure 2). Furthermore, control of methylation was tightly linked to the *P* locus because no recombinants, *i.e.*, progeny without methylation, were found in 219 progeny (map distance <0.46). Thus, methylation was likely controlled by a *cis*-acting modification of the locus, as expected for an epimutation. *Cis*-acting control also argues against a mechanism involving a controlling element located elsewhere, such as in presetting (*e.g.*, MCCLINTOCK 1967b), or a homologous

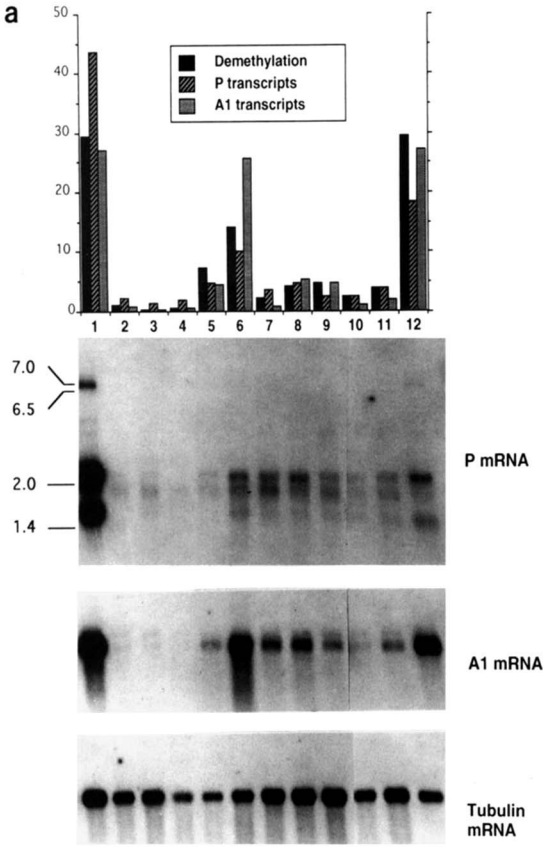


FIGURE 10.—Methylation, transcript levels and phenotype. Panel a represents molecular analysis of immature pericarps isolated from halves of ears, and panel b shows phenotypes on kernels from the other half of the ear at maturity. The genotypes and lineages (defined in Figure 2) represented are: lane 1, *P-rr/P-rr*; lanes 2, 3 and 4, sibling *P-pr-1/P-pr-1*, 5th generation; lanes 5, 6 and 7, *P-pr-1/P-ww* from independent families, 4th generation; lanes 8 and 9, *P-pr-1/P-ww* from sibling families, 3rd generation; lanes 10 and 11, sibling *P-pr-1/P-ww*, 5th generation; lane 12, *P-rr/Pww*. The autoradiograms show a Northern blot of poly(A) RNA hybridized sequentially with: a 840-bp *Pvu*II-*Sac*I fragment of the 1.8-kb *P* cDNA that includes 330 bp of exon 1, exon 2 and 420 bp of exon 3; a 1.8-kb cDNA from a maize α-tubulin gene (VILLEMUR *et al.* 1992); a 1.4-kb cDNA from the maize *A1* gene (SCHWARTZ-SOMMER *et al.* 1987). The expected mRNAs of molecular weights 1.4, 2, 6.5 and 7 kb from the *P* locus (LECHELT *et al.* 1989) can be visualized. All are reduced in *P-pr*, as are the levels of *A1* mRNA. Tubulin provides a control for loading. The faint bands of slightly higher molecular weight from the *A1* mRNA are from incomplete removal of the tubulin probe. For the bar chart, *A1* transcripts were quantitated relative to tubulin from slot blots loaded with similar amounts of poly(A) RNA, and probed sequentially with the *A1* and tubulin probes. *P* transcripts (only 2- and 1.4-kb bands) were quantitated from Northern blots, to eliminate the signal from the faint bands between 1.4 and 2 kb visible in some lanes, and normalized to tubulin data from the same blot. Methylation in immature pericarp DNA was quantitated by *Sal*I digests and hybridization to probe 15. The ratio of partial + basal methylation (defined in Figure 6) to total signal is represented. All units are arbitrary. Note the excellent correlation between methylation, *P* and *A1* mRNA levels among sibling plants (2, 3, 4, and 10, 11). Correlations are less precise for 8 and 9, which are from sibling families (*i.e.*, sibling parents), and even less for 5, 6 and 7, which are related by sibling grandparents (defined in Figure 2). The high level of *A1* mRNA in lane 6 is discussed in the text. *P-rr* seems to give dosage-dependent transcript levels, but this is not reflected either in *A1* transcript levels or pigmentation. The levels of all quantitative characteristics for the *P-pr* samples in panel a, especially *A1* transcript levels, correlate well with pigmentation in panel b. As in Figure 8, homozygotes show lower pigmentation, (and transcript levels), and more methylation.

gene located elsewhere, as in co-suppression (reviewed in JORGENSEN 1993; MATZKE and MATZKE 1993).

One of the 166 progeny of the initial *P-pr-2/P-ww* plant gave an ear with red kernels (Figure 8, middle, panel 5), and showed the lowest level of methylation with *Sal*I (Figure 8, middle, lane 5, only partial modification of site 4) among all the samples tested. Two more generations of its progeny also gave fully red kernels, indicating that it was a stable revertant. Other instances of reversion have been observed, and preliminary data suggest that reversion frequency may depend on the genetic background, and on pigmentation in the preceding generation. While the molecular analysis in the preceding section had shown that insertions or deletions were unlikely in the formation of *P-pr*, stable reversion argues against a point mutation, and further supports epimutation. Furthermore, the revertant was unchanged in band size from *P-pr* (Figure 9), arguing against reversion by excision of a transposable element.

**Methylation levels in the revertant allele and *P-rr*:** DNA analysis of sibling progeny from the third generation of the revertant is shown in Figure 9. *Sal*I digests showed variable methylation of site 4 only, and methylation levels in the samples represented in lanes 1 and 5 were comparable to the grandparent in Figure 8. The other plants showed even lower methylation of *Sal*I site 4. *Hpa*II and *Pst*I digests confirmed that methylation was low, but not absent in these samples. Therefore, even low levels of methylation may be transmitted stably. Similarly incomplete loss of methylation has been seen in reversion of epimutation in *Ascobulus immersus* (RHOUNIN *et al.* 1992), and activation of silent copies of the maize transposon *Ac* ( BRETTELL and DENNIS 1991). Despite the slight variation in methylation between these plants, all ears showed full pericarp and cob pigmentation, for reasons discussed below. Therefore, pigmentation phenotype cannot be used to select for reversion to the *P-rr*-like state of basal methylation; selection may, however, be possible if methylation itself is used as a phenotype.

In this context, it is useful to review the methylation in the parental *P-rr* allele and the reliability of these

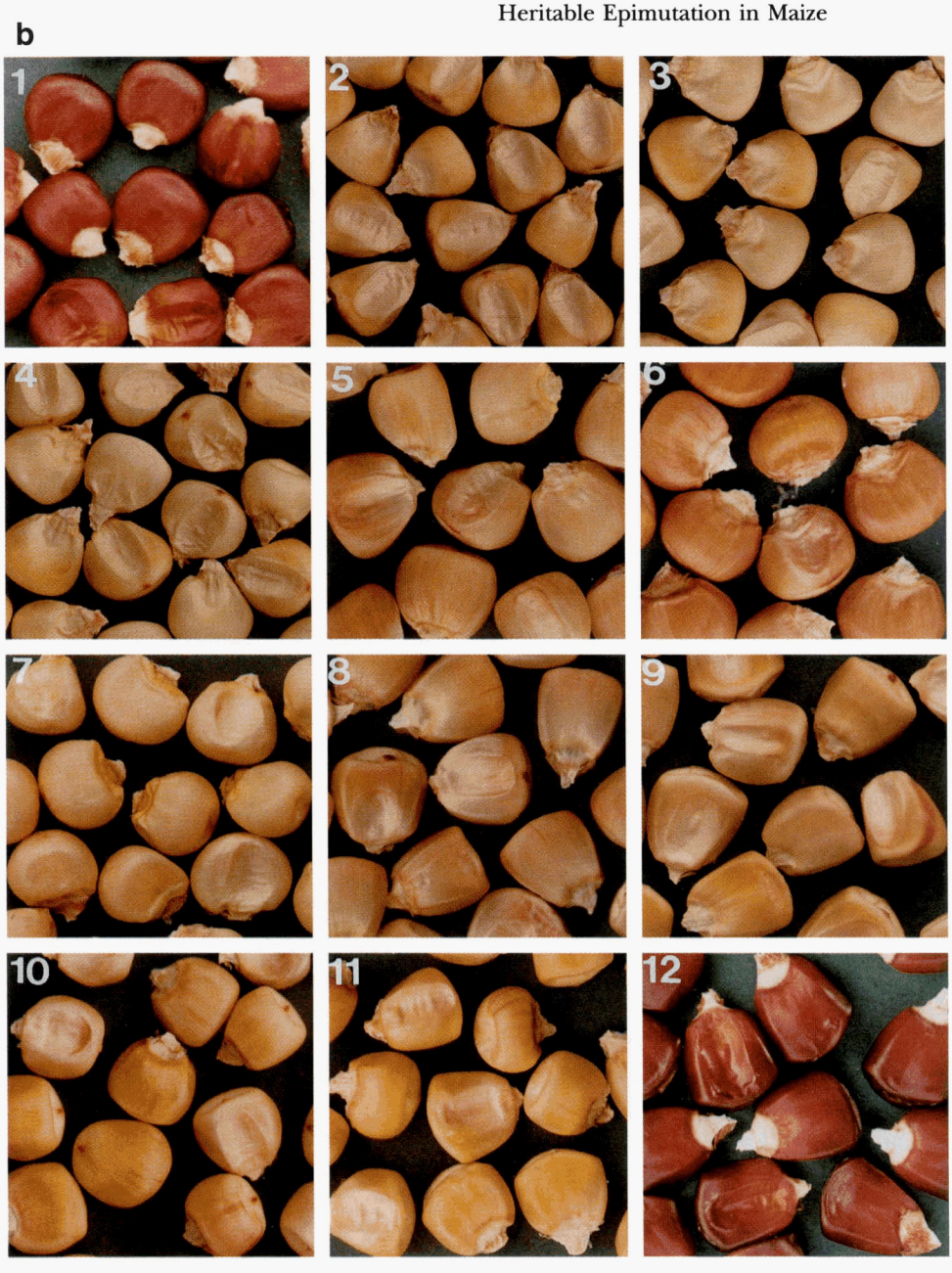


FIGURE 10.—*Continued*

measurements of low methylation levels in *P-pr*. The low levels of methylation in the samples in Figure 9 is consistently detected with all three enzymes, ensuring that they indicate a valid result. In contrast, although occasional digests of *P-rr* DNA can give higher molecular weight bands (*e.g.*, the faint band at 4.4 kb in Figure 6), they have never proved consistent among different enzymes and upon repetition, indicating that they arise from low levels of partial digestion. The map in Figure 5 represents the methylation of various enzyme sites in *P-rr*. No deviations from this map were observed for *P-rr* in different tissues (pericarp, seedling, juvenile leaf, mature leaf at DAP 30, mature endosperm and mature embryo), in different individuals carrying *P-rr* (numbering 35 in one experiment, using *Sal*I digests and probe 15), between genetic backgrounds (inbred W22 or the hybrid backgrounds described in MATERIALS AND METHODS) or between the *P-rr-4026* allele used in this study and the *P-rr-4B2* allele obtained by reversion of *P-vv* (GROTEWOLD and PETERSON 1991b).

**Correlations between methylation, phenotype and transcript levels in pericarp:** Several types of such correlations with *P-pr* are described below. First, methylation in pericarp DNA of mature kernels correlated inversely with pigmentation in three cases (two are shown in Figure 8, right, the third was intermediate in both pigmentation and methylation). Furthermore, pericarp DNA from a lightly variegated sector on a moderately variegated ear showed more methylation in the less pigmented sector (not shown). Thus, pericarp pigmenta-

tion correlated inversely with methylation, assayed after the tissue had undergone maturation and desiccation. Only qualitative correlations were possible here because the red pigment is difficult to extract quantitatively (STYLES and CESKA 1977).

Second, similar correlations were observed in immature pericarp at a stage when the gene is expressed (Figure 10). In these examples, because transcript levels in pericarp were also measured from the same nucleic acid preparation used to determine methylation, quantitative comparisons were possible. Transcript levels of the *P* gene were considerably reduced in *P-pr* (Figure 10a). This would be expected if methylation inhibits transcription, as for other genes whose expression correlates with methylation (RAZIN and CEDAR 1991). To quantify correlations to transcript levels, methylation in pericarp DNA was quantitated from Southern blots of *SalI* digests (representative blots in Figures 6 and 8) as follows. Bands in common with *P-rr* were defined (see legend to Figure 6) as basal methylation (triangles, Figure 8); partial methylation was defined as the first set of higher molecular weight bands (filled arrow, Figure 8); full methylation was defined as the highest molecular weight bands (open arrow, Figure 8). The ratio of the intensities of bands corresponding to basal + partial methylation to the total signal was used as a quantitative index. This is a measure of demethylation since the fully methylated state would give a value of 0, while *P-rr* or the revertant (Figure 9) would give a value of 1. Comparison of this index to transcript levels of the *P* gene (bar chart, Figure 10a, compare filled bars to striped bars) showed that transcripts were reduced in proportion to demethylation. Proportionality depended on the genetic relationship of the compared plants. Siblings showed the best correlation (samples 2, 3 and 4; 10 and 11), followed in order by progeny of sibling parents (samples 8 and 9), and progeny of sibling grandparents (samples 5, 6 and 7). Transcript levels also correlated qualitatively with pigmentation (Figure 10b), as expected.

In addition, we could also show that reduced levels of *P* mRNA were directly involved in reduced pigmentation by assaying for transcripts of the *A1* gene (Figure 10a, stippled bars). This gene encodes dihydroflavonol reductase, a key enzyme in the synthesis of flavan-4-ol (a precursor to the red phlobaphene pigment; STYLES and CESKA 1977). In the genetic background used (*r-r/r-r*; *b/b* or *b/B-w*), its expression in pericarp depends only on *P* (STYLES and CESKA 1977; COE *et al.* 1988; GROTEWOLD *et al.* 1991a,b). Quantitative comparisons showed that transcript levels of *A1* correlated well with transcript levels of *P* (Figure 10a, bar chart, compare stippled bars to striped bars). Again, the quality of correlations was best for siblings, next best for progeny of sibling parents and poorest for progeny of sibling grandparents. In the latter group, one sample showed almost wild-type *A1* transcript levels, but did not show correspondingly high *P* mRNA levels or methylation (Figure 10a, sample 6). Although this was the most pigmented ear in the group, its pigmentation was much less than wild-type (Figure 10b, panel 6). One explanation is that low levels of the P protein may stimulate higher levels of *A1* transcription in this plant than in others, giving higher average transcript levels even though not all pericarp cells express the gene.

From the analysis of phenotype discussed earlier, we could conclude that a switch from a non-expressing state to an expressing state occurred during pericarp development. The above correlations suggest that a methylation decrease may be associated with this switch. A likely possibility is that cell clones carrying a demethylated *P* gene express the gene while those that carry a methylated gene do not. Thus, methylation and lack of expression potential of the *P* gene may decrease during pericarp development, and when a threshold level of methylation associated with gene inactivation is passed, the gene may be activated. When this threshold is passed earlier in development, more and larger clonal sectors of pigmented cells would be present, and when it is passed late in development, fewer and smaller clones would be present. This accounts for the correlations observed. In the revertant, the threshold may be exceeded very early in development, accounting for their solid color, and lack of correlation with methylation.

**Developmental changes in methylation:** This type of a progressive change in methylation and expression potential with development suggests control by an epigenetic pathway (WADDINGTON 1957; HOLLIDAY 1987). The data presented next suggest that the pathway may be general in that it operates in all meristems of the plant, and not just the pericarp. One observation that suggested such a general pathway was that methylation in leaf DNA correlated inversely with pigmentation. The methylation index defined earlier, when measured for leaf DNA, was a good indicator of pericarp pigmentation (examples in Figure 8). These correlations were applicable to most of the progeny listed in Table 2. Again, correlations were best for siblings, and less so for less related individuals.

The correlation of phenotype with methylation in pericarp DNA was understandable since the gene is expressed in pericarp; however, the correlation with methylation in leaf DNA was surprising since *P* transcripts are undetectable in leaves, suggesting that the gene is not expressed in this tissue (T. PETERSON, personal communication). Apart from pericarp and cob, the *P* locus controls pigmentation in the palea, lemma and glumes of the tassel, husks, silks and cob pith, but has no phenotype in leaves (STYLES and CESKA 1977). Moreover, for the 10 plants carrying *P-pr* represented in Figure 10a, methylation in ear leaf DNA was slightly less than methylation in pericarp DNA (not shown), even though pericarp develops following pollination, after the ear leaf is fully

mature. One explanation for our results was that the epigenetic pathway operates in all tissues, regardless of whether the *P* gene is expressed in those tissues.

This was tested by analyzing methylation in various plant parts and sections. In one type of analysis, similar sections of three successive leaves from three mature plants was analyzed. The restriction enzyme used for this analysis, *Pst*I, was a sensitive indicator of differences in methylation since the affected sites are very close to each other, revealing minor methylation differences within a small region. The affected sites can be seen in Figure 5 (sites 9, 10, 12, and 1, 2 4), and the data in Figure 11. It is clear that subtle differences are present between methylation of these sites in the three plants, as indicated by the relative intensities of the two bands shown by triangles, and the two bands shown by arrows. However, the pattern of methylated sites is quite similar between the three leaves of each plant, since each pair of bands shows similar relative intensities in each of the three sets of three lanes. Therefore, methylation may change similarly during growth of three successive lateral meristems of a plant.

In another approach, methylation in different tissues was examined, using the basal, partial and full states of methylation (represented by dotted, striped and filled bars, respectively, Figure 12) based on *Sal*I digests defined previously (legend to Figure 6). Methylation decreased slightly with development in endosperms and embryos of kernels obtained from self-pollination of a plant homozygous for *P-pr* (Figure 12, increase in partial methylation marked by the striped bar). Analysis of sections of a mature leaf of the genotype *P-pr/P-ww* was also consistent with a slight methylation decrease with cell divisions, since edges and tips of a leaf were slightly more methylated than regions located closer to the meristem (Figure 12, middle). Silks grow by a meristematic activity at the base (RANDOLPH 1936); silk tips, which originate from earlier cell divisions, were more methylated than basal regions, again indicating a decrease with cell divisions (Figure 12, bottom two samples). All parts of a mature plant were chimeric for methylation (Figure 12, bottom), suggesting that such methylation changes occurred in all tissues.

This decrease could not, however, be cumulative with progressive cell division starting from the zygote. If this were so, silks and brace roots, which develop late in the life cycle, should be less methylated than tissues that develop earlier; the reverse is in fact true (Figure 12). The same argument applies to the similarity in methylation levels between ear leaf and pericarp. The data is more consistent with a progressive decrease in methylation with cell division that occur in all meristems, starting from a basal epigenetic state which may be similar for all meristems. This hypothesis should be testable using polymerase chain reaction (PCR)-based methods (*e.g.*, FROMMER *et al.* 1992) to quantitate methylation in tissue sections.

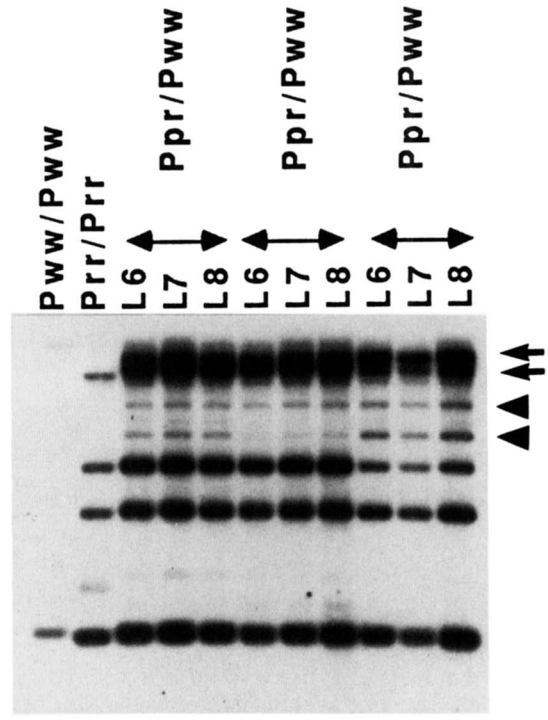


FIGURE 11.—Methylation in similar tissues of different plants. Three sibling plants of genotype *P-pr/P-ww* from the second generation of *P-pr-1* (defined in Figure 2) were used. The ear leaf (L7) and the preceding (L6) and succeeding (L8) leaves were excised from mature plants at anthesis, and DNA isolated from the basal 30 cm section of the blade after removal of the mid-rib. *Pst*I digests hybridized to probe 15 are shown. Bands arising from increased methylation in *P-pr* are indicated by triangles and arrows. The origin of bands corresponding to basal methylation is described in Figure 9, and these bands are unmarked. Of the two higher molecular weight bands shown by triangles, the lower of ~6 kb results from methylation of site 12 (sites numbered in Figure 5), and cutting at sites 10 and 14 (11 and 13 are always methylated); the higher of molecular weight ~8 kb arises from methylation of sites 9, 10 and 12, and cutting at sites 8 and 14. Further methylation of site 14 and cutting at 15 can be detected as a faint band of ~10 kb between the arrows and triangles. The two higher molecular weight bands indicated by arrows arise from methylation of 4, and cutting at 2 and 7, or from methylation of 1, 2 and 4, and cutting at 7 and a site outside the map on the left. These two sets of bands therefore indicate methylation in the left and right flanking regions. Note that methylation is very similar among the three leaves of each plant. The similar relative intensities of the two bands indicated by triangles among the three leaves of each plant, and the distinct differences between the three plants are clear. This also holds for the two bands shown by triangles, and is more clear in a lighter exposure (not shown). Here, the two bands are of similar intensities in the first three lanes, the lower band is slightly darker in the next three, and the higher band is darker in the next three.

This developmental analysis has been confirmed with three other restriction enzymes, and by analysis of similar sections of a sibling plant of the genotype *P-pr/P-pr* with all four enzymes. The magnitude of the methylation changes during development were modest in this plant as well, which contrasts with the large methylation differences between plants (Figure 8). Therefore, it is likely

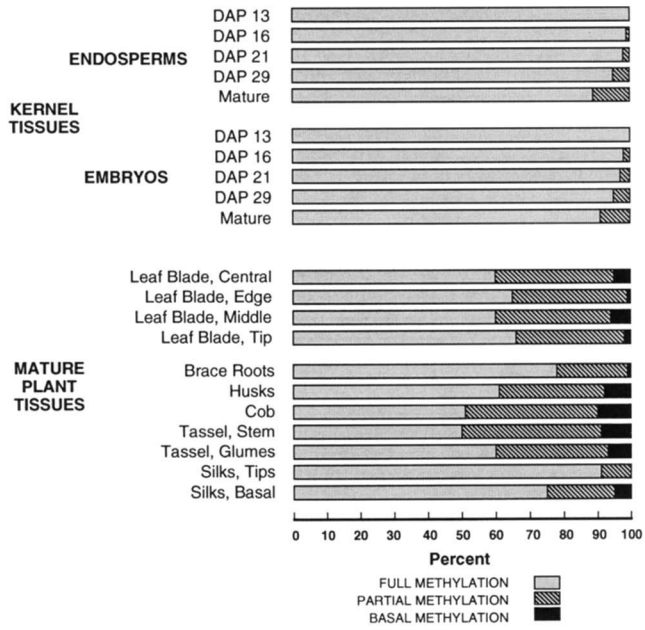


FIGURE 12.—Developmental changes in methylation. The bar chart represents quantitation of methylation as determined by *SalI* digests of DNA from various tissues. Stippling represents full methylation, striping represents partial methylation and filling represents basal methylation (as defined in Figure 6). For analysis of immature kernel tissue, a plant (sibling of the previous one) of the genotype *P-pr/P-pr* was self-pollinated, and 5–10 kernels were harvested from the ear at the indicated times after pollination. The plant used for isolation of plant tissues was of the genotype *P-pr/P-ww* from the third generation of *P-pr-1* (Figure 2). Silk tips were obtained from the initially visible tuft of average length ~10 cm, and basal regions of a similar length were dissected from sections adjacent to the silk attachment point. For leaf sections, a mature leaf was dissected as follows. A 30-cm region of the blade on one side of the mid-rib was dissected into two lengthwise sections of approximately equal width to give "central" and "edge" samples. The blade on the other side was dissected into a 12-cm basal section (not shown), a 13-cm "middle" section, and a 30 cm "tip" section. Note that kernel data from genotype *P-pr/P-pr*, which always shows high methylation levels, cannot be compared to plant data from genotype *P-pr/P-ww*, which usually shows lower methylation levels.

that individual plants start out with significant differences in their basal level of epigenetic modification. A decrease from this level may occur during development in each meristem; this can give rise to variegation when the decrease switches on a previously inactive gene. The extent of variegation may, therefore, depend on the basal modification level, and plant-to-plant variation in this level probably accounts largely for the variability in pigmentation. Some variability could also arise from differing rates of decrease of methylation in individuals. The basal state may be considered analogous to the "preset" state of a locus described by MCCLINTOCK (1964, 1965, 1967a,b). The ubiquitous developmental programs that were considered to control the eventual "setting" (*i.e.*, activation for expression) of a locus may be analogous to the epigenetic pathways which we hypothesize as controlling the changes in methylation and expression potential of *P-pr*. Large clonal sectors with sharp boundaries may represent early events that change the "preset" state, while sectors with diffuse boundaries may represent developmental modification of the programs that regulate the rate of change of this state.

## DISCUSSION

**Epimutation of the *P* gene:** Forward and reverse epimutations were defined previously (HOLLIDAY 1987) as increases and decreases, respectively, in the methylation of a gene, without an accompanying change in nucleotide sequence. By this definition, *P-pr* may be considered a product of forward epimutation of *P-rr*. This conclusion is based on the properties of the transmitted allele studied after one or more meioses have elapsed following the original epimutation; therefore, the nature of the initial event cannot be known with certainty. However, the fact that the phenotype of the original sectors (Figure 1) was similar to that of the transmitted alleles (Figures 8 and 10b) suggests that the transmitted allele is similar to the product of the somatic event.

Epimutations transmitted through meiosis have been identified for transposons in maize (BENNETZEN 1985; MARTIENSSEN *et al.* 1990; FEDOROFF 1989; BANKS *et al.* 1988). They have also been obtained by transformation with foreign or endogenous genes in fungi (*e.g.*, SELKER 1990b; RHOUNIN *et al.* 1992), animals (reviewed in LYON 1993; BESTOR 1993) and plants (reviewed in JORGENSEN 1993; MATZKE and MATZKE 1993). However, both transposition and transformation involve the insertion of a new genetic element into a locus. This may perturb local chromatin structure, or alternately, local chromatin organization may cause the element to adopt an unstable structure; because methylation is frequently associated with chromatin organization (SELKER 1990a), such interactions may render these elements more sensitive to epimutation than endogenous genes. Paramutation of the maize *R* gene can cause a heritable change in its methylation (M. ALLEMAN and J. L. KERMICLE, unpublished, in JORGENSEN 1993), and may therefore be defined as epimutation as well. However, paramutation at *R* is different in that it requires specific inducing alleles, and occurs almost invariably in their presence (BRINK 1973). In contrast to these cases, *P-pr* represents a spontaneous and heritable epimutation of a wild-type gene, and may therefore be the first example of its kind.

**Characteristics of epimutation:** It is clear that epimutation of *P-rr* is a mitotic event (Figure 1). The maize *B* gene also changes spontaneously to its paramutant allele *B′* in somatic tissue (COE 1966). Transmission frequency of epimutation is not low since 2 cases were transmitted from 6 single kernel sectors (Figure 1), under conditions where a maximum of 50% transmission would be

expected (ANDERSON and BRINK 1952; MORENO *et al.* 1992). The two other multikernel sectors (of 5 and 13 kernels) evidently did not result from similar events; if they did, an allele similar to *P-pr* should have been transmitted with considerably higher probabilities. The high transmission frequency may appear to contradict the suggestion that epigenetic defects would be largely corrected at meiosis (HOLLIDAY 1987). However, it could be argued that selection for colorless sectors biased representation, *e.g.*, by eliminating modest methylation changes that would not cause a noticeable change in phenotype. In this context, note that the multikernel sector covering 13 kernels had a phenotype of red crown, and orange (not colorless) sides, which may have resulted from a relatively modest change in expression potential of the gene. Kernels from this sector gave rise to only red or white ears in the next two generations.

Transmissible epimutation of *P-rr* is clearly a rare event, since only two isolates were recovered from $\sim 1 \times 10^6$ kernels. Because several factors listed below may have facilitated our recovery of these rare events, similar instances may have gone undetected for other genes, and possibly in other organisms. First, the phenotype of *P-rr* facilitated detection of late mitotic events (GREENBLATT 1985), and the extensive literature on their transmission (ANDERSON and BRINK 1952; ORTON and BRINK 1966; MORENO *et al.* 1992) enabled selection of appropriate sectored kernels. Second, the cell-level resolution provided by pigmentation suggested tests of developmental methylation changes (Figure 12), and models for their origin; without such resolution, the new allele may have been considered an unstable variant with poor expressivity. Third, the genetic background used, which displays high frequencies of somatic mutations (DAS *et al.* 1990a; DAS and MESSING 1993), recombination (DAS *et al.* 1991; O. P. DAS, M. C. P. TIMMERMANS and J. MESSING, unpublished data) and cytological aberrations (BENNER *et al.* 1992) may also be a major factor.

**Genetic properties of the product of epimutation:** The data presented show that the methylated state is penetrant for at least five generations (Table 2), and segregates as an allele of *P-ww* (Figure 2). The allele is of moderate stability since it can revert to full pigmentation. However, the revertant we tested did not lose methylation even after two additional generations (Figure 9), attesting further to the stability of transmission of the methylated state. Epimutation is likely to be a *cis*-acting change at *P*, since genetic analysis shows no requirement for unlinked factors either for methylation or for phenotype. Propagation in other backgrounds may, however, reveal factors that modify its properties. Genomic imprinting is associated with methylation for several animal genes (LYON 1993); in maize, imprinting has been demonstrated for the endosperm phenotype of the *R* gene (KERMICLE 1970), and for endosperm development (KERMICLE and ALLEMAN 1990). However, it has not been demonstrated for a plant phenotype, and accordingly, *P-pr* shows no effects of the gamete of origin. Unequal crossing over at meiosis between the tandem repeats flanking the gene is not greatly increased by methylation (no deletion alleles found in outcrosses of *P-pr/P-pr* to testers; frequency <1/299). Neither is somatic recombination between the repeats significantly increased (no sectors with loss of phenotype), as when *Ac* is present (ATHMA and PETERSON 1991). An earlier suggestion that recombination may be increased by meiotic pairing between homologous genes that differ in methylation (HOLLIDAY 1987) may be tested by measuring unequal crossing over in *P-pr/P-rr* heterozygotes. Such events are readily detected by loss of pigmentation upon outcrossing to *P-ww*.

**Epimutation and epigenetic pathways:** Animal genes whose expression correlates with methylation are usually demethylated in expressing tissue (reviewed in CEDAR and RAZIN 1990; RAZIN and CEDAR 1991). *P-pr* is unusual in that it is demethylated in all tissues. We have not detected any developmental differences in methylation for *P-rr*, indicating that these changes are specific to *P-pr*. Demethylation of *P-pr* (and its variegated phenotype) appear to be controlled in a manner reminescent of developmental or epigenetic pathways (WADDINGTON 1957). Similar mechanisms have been postulated in plants to regulate gene expression patterns (*e.g.*, BRINK 1964; MCCLINTOCK 1967a; FEDOROFF *et al.* 1989; MARTIENSSEN *et al.* 1990) and developmental transitions (POETHIG 1990; BURN *et al.* 1993). Canalized epigenetic pathways were hypothesized to buffer or insulate genes from switching from one developmental mode of expression to another as a result of environmental effects or mutation (WADDINGTON 1957). Epimutation of *P-rr* may have effected such a switch, because *P-rr* does not show such methylation changes. This possibility is also consistent with the observation that phenotypes on the parental sectored kernels resemble the *P-pr* phenotype (Figure 1); epimutation may have enabled the gene to respond to ongoing development in the mother plant in a manner similar to its response in progeny plants. Direct evidence for the existence of epigenetic pathways has been scant, and the clear phenotype and methylation of this new allele may help reveal their features.

These pathways may control *P* gene expression via methylation. However, the question whether methylation is a cause or a consequence of altered expression potential has not been resolved despite extensive study in animals (reviewed in CEDAR and RAZIN 1990; BIRD 1992; BESTOR 1993). Consequently, is is equally likely that these pathways regulate another property which coordinately affects both expression potential and methylation. Chromatin structure is a likely candidate for such a property, since it is often considered a common determinant of methylation and expression (SELKER 1990a; PFEIFER and RIGGS 1991). For maize, BRINK (1964)

and McClintock (1967a) have hypothesized that developmental changes in chromatin mediates paramutation and presetting, respectively. *P-pr* and *P-rr* provide two alleles of identical sequence and chromosomal location, which can be used to address some of these questions at a molecular level. For instance, the role of chromatin in methylation and expression can be assayed using PCR-based methods (Frommer *et al.* 1992, Pfeifer and Riggs 1991). Furthermore, methylation changes during development can be used to test if chromatin structure changes before or after methylation, analogous to the experiments on *X* chromosome inactivation that showed that gene inactivation preceded DNA methylation (Lock *et al.* 1987).

**Implications for *P* gene function:** Although other alleles of *P*, (*e.g.*, *P-wr* and *P-rw*) can differ in pigmentation in pericarp and cob (Anderson 1924; Coe *et al.* 1988) none of the alleles caused by *Ac* insertion into *P-rr* show variegation specific to one tissue (Orton and Brink 1966; Athma *et al.* 1992, Moreno *et al.* 1992). Also, deletion or mutation of the *P-rr* gene causes loss of pigmentation in both tissues (Athma and Peterson 1991; Grotewold *et al.* 1991a,b). In contrast, *P-pr* gives variegation in pericarp (Figures 1 and 8) but not in cob (not shown), and frequent clonal sectors that differ in pericarp variegation do not show underlying cob sectors. Methylation in *P-pr* correlates with pericarp, but not cob pigmentation, and pigmentation level in one tissue does not correlate with pigmentation in the other (not shown). While the data with *P-vv* and its derivatives suggest an absence of distinct determinants in *P-rr* that specify expression in the two tissues, our results argue for their presence. One possibility is that the nonrandomness of *Ac* insertion (Figure 5; Grotewold *et al.* 1991b; Athma *et al.* 1992; Moreno *et al.* 1992) may have prevented identification of such a site. Alternately, pigmentation in cob may be insensitive or differently sensitive to epigenetic states of the gene.

**Variability in methylation during development and between individuals as related to phenotype:** Increased methylation in *P-pr* appears to have resulted from a variable "spread" of methylation away from a region roughly overlapping the transcription unit to flanking regions on either side (Figure 5). The opposite trend of a spread into the gene would have been more similar to the spread of heterochromatin in position effect variegation in Drosophila. However, the underlying mechanisms (Brink 1964) could still be similar. Variability in the extent of the spread between individuals may be largely responsible for variation in pigmentation, and developmental changes account for variegation. Thus, with a high initial methylation level, more cell divisions have to elapse before the epigenetic program renders the gene functional, and only the kernel tips would show color. Conversely, with a very low methylation level (as in the revertant), expression potential may already be present, and developmental changes would not be detected as variegation. Intermediate cases would be the ones most likely to show marked variegation. The phenotypes of plants (Table 2) whose methylation has been determined is in agreement with this view.

Our results are consistent with the idea that methylation levels are largely similar among meristems of a given plant, and that methylation decreases as a function of cell division in each meristem. The decrease in methylation may have a stochastic component in that not all cell lineages originating from a single meristem would show similar changes at similar times. While this accounts for variegation, another distinct feature of the *P-pr* phenotype is the frequent presence of large sectors, covering up to half the ear. Kernels of such sectors display a consistent variegation pattern that differs from the variegation patterns on other kernels. Therefore, events that occur very early in development, when the precursors of the ear consist of only a few cells, likely determine the changes in methylation and expression potential of the gene during pericarp development, which occurs much later.

**Similarities to other maize genes and transposons:** A similar type of variegation has been recently described for the *Pl-Bh* allele of the maize *Pl* gene (Cocciolone and Cone 1993). This allele conditions "blotched" pigmentation in plant parts as well as in the aleurone layer of kernels. Here also, no significant nucleotide sequence differences were found between the *Pl* allele that gives full pigmentation in plant tissues and *Pl-Bh*. However, increased methylation of some *Hpa*II sites was found for the latter allele, whose methylation correlated inversely with *Pl* mRNA levels. It is possible that epigenetic pathways similar to those postulated for *P-pr* may control pigmentation in this case as well. Developmental changes in methylation and activity have also been shown for the maize transposons *Spm* (Banks and Fedoroff 1988) and *Mu* (Martienssen *et al.* 1990), and may also occur with *Ac* (Chomet *et al.* 1987). For *Mu*, a developmental decrease in activity associated with an increase in methylation has been postulated to occur progressively with increasing age of the shoot meristem (Martienssen *et al.* 1990). A detailed analysis of methylation changes of programmable *Spm* elements during development concluded that both increases and decreases in methylation occurred at specific stages. These stages included kernel development and inflorescence development, and early in plant development such that tillers are rendered different from the main stalk (Banks and Fedoroff 1988). In contrast to our results which showed largely similar methylation levels between embryos and endosperms and among plant tissues (Figure 12), this study showed marked methylation differences between embryos and endosperms, and among plant tissues. These studies on *Mu* and *Spm* may not be directly comparable to our results since different assays

(*e.g.*, phenotype suppression, transmission of activity, methylation and pigmentation patterns), tissues, loci and sequences are involved. However, it is notable that determinative events that occur very early in development appear to be involved in all cases.

**Similarities to other genetic phenomena:** Recently, a number of non-Mendelian phenomena have been described for transgenic plants (reviewed in JORGENSEN 1993; MATZKE and MATZKE 1993). Methylation changes have been associated with transgene silencing (*e.g.*, AMASINO *et al.* 1984; KILBY *et al.* 1992), and with cycling between active and silent states leading to non-Mendelian behavior of certain transgenes (KILBY *et al.* 1992; MEYER *et al.* 1992). Transgenes with homologous sequences can suppress each other's expression, which can correlate with methylation (MATZKE *et al.* 1989). In co-suppression, a transgene homologous to an endogenous gene suppresses the host copy, and is also suppressed in its own expression (NAPOLI *et al.* 1990; VAN DER KROL *et al.* 1990). Co-suppression can cause variable pigmentation patterns, which are sometimes heritable (JORGENSEN 1990, 1993). *P-pr* has some resemblance to such phenomena. At the phenotype level, somatic variability within a homogeneous tissue characterizes both, and both may originate from somatic events (JORGENSEN 1993). Developmental influences on expression are apparent in both cases (JORGENSEN 1993). Moreover, *P-pr* homozygotes from self-pollination of *P-pr/P-ww* plants display less pigmentation than sibling heterozygotes (Figures 8 and 10b); this could result from one copy of the methylated gene suppressing the other. We have also found that *P-pr* suppresses its parent, *P-rr*, such that in ears of *P-pr/P-rr* plants, variegated pigmentation is seen instead of the uniform phenotype of *P-rr* (O. P. DAS and J. MESSING, in preparation). Suppression varies among individuals, and between sectors of an ear, much like the phenotype of *P-pr* itself.

Paramutation in maize (COE 1966; BRINK 1964, 1973) has been considered a precedent for the suppression of one transgene by another (MATZKE and MATZKE 1993), and co-suppression (NAPOLI *et al.* 1990; VAN DER KROL *et al.* 1990). All these phenomena have some links to DNA methylation (JORGENSEN 1993; Matzke and MATZKE 1993). Paramutation of *R* may be a somatic phenomenon (BRINK 1973), and as mentioned earlier, paramutagenic *B′* is of somatic origin (COE 1966). Moreover, with normal *P* alleles (ANDERSON 1924; COE *et al.* 1988), as with non-paramutagenic *B* alleles (COE 1966), color is dominant to colorless, whereas with *P-pr*, and with paramutagenic *B′* alleles (COE 1966), the reverse can be true. Potential similarities between the interaction of *P-pr* and *P-rr* and paramutation can be established if the reduced pigmentation potential of *P-rr* in a heterozygote with *P-pr* is transmitted to the next generation.

The phenomena of repeat-induced point mutation (RIP) in *Neurospora* (SELKER 1990a,b) and methylation induced premeiotically (MIP) on repeated sequences in *Ascobulus* (RHOUNIM *et al.* 1992) also provide interesting precedents for our observations since much of the increased methylation in *P-pr* is associated with tandem repeats at the locus. In our case, methylation extends beyond the bounds of the repeats, but it is possible that the initiating event in epimutation is triggered by the presence of the repeats. In this context, it is notable that the region where increased methylation was observed with the *Pl-Bh* allele relative to *Pl* also overlaps a direct repeat that flanks the 3′ end of the gene (COCCIOLONE and CONE 1993). Here also, as with *P-pr*, some of the sites showing increased methylation appear to lie outside the repeated sequences.

As mentioned at the end of the results section, our results on methylation changes during development in *P-pr* can be interpreted in terms of the model of presetting of gene action hypothesized by MCCLINTOCK (1964, 1965, 1967a). It is notable that pericarp pigmentation patterns conditioned by this *P* allele resemble pericarp patterns obtained by MCCLINTOCK (1967b) arising from preset expression patterns of the *C2* gene. In addition, a lack of concordance between cob and pericarp phenotypes is seen in both cases. The preset patterns observed by MCCLINTOCK (1967b) resulted from a *C2* allele carrying a *dSpm* element that responded to an *Spm* element located elsewhere by a mechanism that did not involve transposition of the *dSpm* element. Preset expression patterns were occasionally heritable (MCCLINTOCK 1967a); in our case also, there appears to be some heritability of pigmentation levels. It is possible that studies on the inheritance behavior of pigmentation patterns conditioned by *P-pr* may shed light on this elusive phenomenon, which has been postulated to be of general significance (MCCLINTOCK 1967a).

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