Developmental Patterns of Chromatin Structure and DNA Methylation Responsible for Epigenetic Expression of a Maize Regulatory Gene

Owen A. Hoekenga,[1] Michael G. Muszynski[2] and Karen C. Cone

Division of Biological Sciences, University of Missouri, Columbia, Missouri 65211

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ABSTRACT

Epigenetic regulatory mechanisms heritably alter patterns of gene expression without changes in DNA sequence. Epigenetic states are often correlated with developmentally imposed alterations in genomic DNA methylation and local chromatin structure. *Pl-Blotched* is a stable epigenetic allele of the maize anthocyanin regulatory gene, *purple plant1 (pl)*. *Pl-Blotched* plants display a variegated pattern of pigmentation that contrasts sharply with the uniformly dark purple pigmentation of plants carrying the dominant *Pl-Rhoades* allele. Previously, we showed that the lower level of pigmentation in *Pl-Blotched* is correlated with lower *pl* mRNA levels and increased DNA methylation at some sites. To explore how DNA methylation, chromatin structure, and developmental stage might contribute to the expression of *Pl-Blotched*, we used methylation-sensitive restriction enzymes and DNaseI sensitivity assays to compare the methylation status and chromatin structure of *Pl-Blotched* and *Pl-Rhoades* at different stages in development. Both alleles exhibit developmentally sensitive changes in methylation. In *Pl-Blotched*, methylation of two diagnostic *Hpa*II/*Msp*I sites increases progressively, coincident with the juvenile-to-adult transition in growth. In seedlings, the chromatin encompassing the coding region of the gene is less sensitive to DNaseI digestion in *Pl-Blotched* than in *Pl-Rhoades*. Developmental maturation from seedling to adult is accompanied by expansion of this closed chromatin domain to include the promoter and downstream flanking sequences. We provide evidence to show that chromatin structure, rather than DNA methylation, is the primary epigenetic determinant for the phenotypic differences between *Pl-Blotched* and *Pl-Rhoades*.

EPIGENETICS is the term applied to describe patterns of gene expression caused by mechanisms other than changes in nucleotide sequence (Holliday 1987; Henikoff and Matzke 1997). Hallmark features of epigenetic regulation are somatic and/or germinal heritability, potential reversibility, developmental control, and changes in chromatin structure and/or DNA methylation. Most often, epigenetic effects result in down-regulating or silencing gene expression, principally at the level of transcription (Eden and Cedar 1994).

In plants, numerous processes are epigenetically regulated. These include changes in the activity of transposable elements (reviewed in Martienssen and Richards 1995), gametic imprinting (Kermicle and Alleman 1990; Chaudhuri and Messing 1994), paramutation (reviewed in Hollick *et al.* 1997), phenotypic variegation (Cocciolone and Cone 1993; Das and Messing 1994), and homology-dependent transgene inactivation (reviewed in Matzke *et al.* 1996; Kooter *et al.* 1999). In nearly all of these cases, changes in gene expression are associated with alterations in DNA methylation. Whether the altered expression, *e.g.*, effect on transcription, is caused directly by methylation or reflects another underlying feature, such as developmental state or chromatin structure, is not clear in most cases. However, some important correlations have been made.

The contribution of development to epigenetic regulation of DNA methylation is evident from analyses of maize transposable elements. Methylation and demethylation of *Activator* elements are correlated with developmental cycles of inactivity and activity of the elements (Chomet *et al.* 1987; Brutnell and Dellaporta 1994). Methylation of *Suppressor-mutator* elements increases during vegetative growth and during development of floral structures (Banks *et al.* 1988; Banks and Fedoroff 1989). Methylation of Robertson's *Mutator* elements occurs progressively as a clonal and heritable event that results in loss of element activity (Martienssen *et al.* 1990; Brown *et al.* 1994; Martienssen and Baron 1994).

An interrelationship among methylation, development, and chromatin structure is demonstrated by studies on several plant mutants. Arabidopsis plants expressing antisense DNA methyltransferase genes have drastic reductions in genomic cytosine methylation and display reduced size, alterations in heterochrony, changes in meristem identity or number, and floral abnormalities, including sterility (Finnegan *et al.* 1996; Ronemus *et al.*

Corresponding author: Karen Cone, 101 Tucker Hall, University of Missouri, Columbia, MO 65211. E-mail: conek@missouri.edu

[1] *Present address:* Boyce Thompson Institute for Plant Research, Ithaca, NY 14853.

[2] *Present address:* Pioneer Hi-Bred International, Johnston, IA 50131.

1996). Reduced levels of DNA methylation and accumulation of developmental defects are also characteristic of Arabidopsis plants carrying *ddm1*, a mutation in a gene encoding a protein with homology to chromatin remodeling proteins from yeast and animals (Vongs *et al.* 1993; Kakutani *et al.* 1996, 1999; Jeddeloh *et al.* 1999). These features of *ddm1* indicate that the ability to remodel chromatin is an important determinant for maintenance of genomic DNA methylation. A link between chromatin structure and DNA methylation is also associated with the variegated pericarp phenotype of kernels in maize plants carrying the *P-pr* allele of the maize *pericarp1 (p)* gene (Das and Messing 1994; Lund *et al.* 1995).

To further elucidate how epigenetic patterns of methylation and chromatin structure might be imposed through plant development, we compared *Pl-Blotched*, an epiallele of the maize anthocyanin regulatory gene *purple plant1* (*pl*), with *Pl-Rhoades*, the presumptive progenitor. Anthocyanins are red and purple flavonoid pigments synthesized in the aleurone layer of the endosperm and in many vegetative plant organs. The *pl* gene encodes a Myb-like transcription factor that activates the genes encoding the biosynthetic enzymes of the anthocyanin pathway (Cone *et al.* 1993a). Whereas the dominant *Pl-Rhoades* allele conditions uniform purple pigmentation in the vegetative and floral tissues of the plant (Cone *et al.* 1993b), the epiallele *Pl-Blotched* conditions variegated pigmentation (Cocciolone and Cone 1993). Pigmented cells occur in clusters that resemble clonal sectors, but within a sector, not every cell is pigmented to the same degree. The level of pigmentation is proportional to the level of *pl* mRNA (Cocciolone and Cone 1993). Weakly pigmented husks have less *pl* mRNA than heavily pigmented husks, and variegated husks from *Pl-Blotched* have less *pl* mRNA than uniformly purple husks from *Pl-Rhoades*. The unique phenotype of *Pl-Blotched* is not correlated with any large alterations in DNA sequence, but instead is correlated with a distinct pattern of DNA methylation (Cocciolone and Cone 1993; Hoekenga 1998). This pattern is typified by the methylation state of two CCGG *Msp*I/*Hpa*II restriction sites located 3′ of the coding region. In husk DNA, these sites are hypermethylated in *Pl-Blotched* relative to *Pl-Rhoades* (Cocciolone and Cone 1993).

To understand the relative contributions of methylation, chromatin structure, and development to epigenetic regulation of *Pl-Blotched*, we compared the methylation state and chromatin structure of *Pl-Blotched* and *Pl-Rhoades* by sampling leaves from different developmental stages. For the methylation survey, we used methylation-sensitive restriction enzymes, initially focusing on the two CCGG *Msp*I/*Hpa*II sites, but also including other enzymes to identify additional sites with developmentally-sensitive methylation patterns. We show that *Pl-Blotched* and *Pl-Rhoades* have distinct patterns of DNA methylation that change progressively through development. At the two CCGG sites in *Pl-Blotched*, methylation increases, coincident with the juvenile-to-adult transition in growth. To analyze chromatin structure, we used DNaseI sensitivity assays on nuclei isolated from seedlings and husks. We show that, relative to *Pl-Rhoades*, chromatin structure of the *Pl-Blotched* gene is closed in both juvenile and adult tissues. Because this chromatin difference appears to be meiotically transmitted, we conclude that chromatin structure is the primary determinant for the phenotypic differences between *Pl-Blotched* and *Pl-Rhoades*. Furthermore, we propose that changes in methylation imposed during development of the plant might serve to reinforce the chromatin state.

MATERIALS AND METHODS

Maize stocks and *pl* alleles: The *Pl-Rhoades* allele came from a McClintock stock and leads to deep purple pigmentation in vegetative tissues. The *Pl-Blotched* allele came from a stock obtained from the Maize Genetics Stock Center (University of Illinois, Urbana-Champaign) and leads to variegated pigmentation. Phenotypes and DNA sequences for these alleles have been described in detail previously (Cocciolone and Cone 1993; Cone *et al.* 1993a; Hoekenga 1998). GenBank accession numbers for *Pl-Rhoades* and for *Pl-Blotched* are L19494 and L13454, respectively.

Tissue sampling and DNA isolation: Seedlings (aboveground portions) were harvested at the three- to four-leaf stage. Later leaves were harvested just after they appeared in the whorl. Husks were harvested at anthesis. Replicate tissue samples were taken from two to four different plants of each genotype, *Pl-Rhoades* and *Pl-Blotched*. Genomic DNA was purified from the tissue samples by CsCl centrifugation as described (Cone *et al.* 1986).

Nuclei isolation and DNaseI sensitivity assays: Nuclei were isolated by published methods (Cone *et al.* 1993a) using 100–250 g fresh weight of inner husk leaves harvested at the time of silk emergence or three-leaf stage seedlings and stored at −80° until use. Intact nucleosomal structure was verified by micrococcal nuclease digestion (Bellard *et al.* 1989). For each DNaseI sensitivity assay, nuclei isolated from ~40 g of material were used per experiment. Replicate assays were performed: For husk nuclei, a total of nine assays were conducted on nuclei prepared at four different times. For seedling nuclei, two assays were conducted on one nuclei preparation. The nuclease sensitivity assay was adapted from published methods (Wurtzel *et al.* 1987; Bellard *et al.* 1989). Nuclease digestion buffer contained 50 mm Tris-HCl (pH 7.5), 10 mm $MnCl_2$, 50 μg/ml BSA, 340 mm sucrose, and 4 mm spermidine. A series of seven 16-μl aliquots of DNase I (GIBCO/BRL, Gaithersburg, MD), containing 0.064, 0.16, 0.32, 0.64, 1.28, 2.56, or 5.12 units of enzyme, was prepared using the nuclease digestion buffer as diluent and stored in an ice/water bath until use. Nuclei were thawed on ice and pelleted by spinning for 30 sec at maximum speed in a 4° bench top microfuge. The nuclear pellet was resuspended in 800 μl of nuclease digestion buffer and placed into an ice/water bath for 5 min. Nuclei (100-μl aliquots) were dispensed into the seven DNaseI-containing tubes and one tube with no added DNaseI to control for the activity of endogenous nucleases. This series of eight tubes was then incubated for 5 min in the ice/water bath. As controls for DNaseI digestion, chromatin-free CsCl-purified ("naked") DNAs were digested using only the two lowest DNaseI concentrations.

Reactions were stopped by the addition of 2 volumes of lysis

buffer: 350 mm NaCl, 50 mm EDTA, 1 mm Tris-HCl (pH 7.6), 7 m urea, and 2% sarkosyl. DNAs were isolated using repeated organic extraction, first with an equal volume of phenol and then with an equal volume of phenol:chloroform:isoamyl alcohol (100:100:1). Samples were precipitated with ethanol, resuspended in 10 mm Tris-HCl (pH 8.0), 10 mm EDTA (TE), extracted twice more with phenol:chloroform:isoamyl alcohol (100:100:1), precipitated with ethanol, and resuspended in TE. Samples were checked for yield and purity by measuring absorbance at 260 and 280 nm. Typical yield from each DNase I treatment was 30–60 μg DNA. Purified DNA samples were digested with restriction enzymes to generate reference sized fragments. A non-DNase I-treated CsCl-purified DNA was used to confirm this reference fragment. Gels were blotted as described below.

Restriction digests: For the developmental assay of DNA methylation, DNA samples from individual leaves were digested with *Msp*I or *Hpa*II overnight according to the manufacturers' specifications. For the broad analysis of methylation differences between seedling and husk DNA, samples were first digested overnight with *Eco*RV or *Hin*dIII to produce fragments with known sizes for each genotype, and a secondary overnight digestion was then performed with one of the following 48 methylation-sensitive restriction enzymes: *Acc*I, *Afl*II, *Afl*III, *Alu*I, *Apa*I, *Apa*LI, *Asp*718I, *Ava*II, *Bam*HI, *Ban*I, *Bbr*PI, *Bgl*II, *Bsa*I, *Bss*HII, *Bst*UI, *Cfo*I, *Cfr*10I, *Eag*I, *Eco*O109I, *Eco*RI, *Eco*RII, *Hae*II, *Hae*III, *Hha*I, *Hin*PI, *Hpa*II, *Hph*I, *Kpn*I, *Msp*I, *Mva*I, *Nar*I, *Nci*I, *Nhe*I, *Nsi*I, *Pml*I, *Pst*I, *Sal*I, *Sau*3AI, *Sau*96I, *Scr*FI, *Sfi*I, *Sgr*AI, *Spe*I, *Sst*I, *Stu*I, *Tha*I, *Xba*I, and *Xho*I. Nine other methylation-sensitive restriction enzymes were used for secondary digestion, but either failed to digest maize DNA or gave products that were unresolvable: *Ava*I, *Bsa*AI, *Bsa*BI, *Bsp*1286I, *Dde*I, *Eae*I, *Pvu*I, *Sin*I, and *Xma*III. For the nuclease sensitivity assays, DNaseI-treated DNAs were digested with *Hin*cII or *Nsi*I overnight. All restriction digests were fractionated on agarose gels, blotted to nylon membranes, and hybridized with radioactively labeled probes as described (Cone *et al.* 1986).

Probes: For the methylation analysis, *pl* probes were derived from the 3′ end of the gene. The 1.2-kb *Xho*I fragment, used for the CCGG methylation analysis, begins 50 bp upstream of the translational stop site. A smaller 0.62-kb *Bgl*II-*Nsi*I fragment, used for the methylation survey and for nuclease sensitivity assays, begins 175 bp downstream of the translational stop. Also used for nuclease sensitivity assays was a 0.8-kb *Hin*dIII fragment located ~7 kb upstream of the transcriptional start site.

Quantitation of hybridization signals: Bands were visualized using a phosphorimager (Fuji Medical Systems, Stamford, CT), and the hybridization signals were quantitated using MacBAS v2.31 (Fuji Film Co. Ltd. and Kohsin Graphic Systems, Tokyo, Japan). Intensities for hybridizing bands (constant area for each band) were recorded as pixels/mm^2. In assays of CCGG methylation, hybridization with the single-copy marker *npi223* was used as follows to correct for loading differences among lanes: An adjustment factor was obtained by dividing the intensity of the *npi223*-specific band in each sample by the intensity of the least intense *npi223*-specific band in the set of samples. Intensities of *pl*-specific bands were then normalized by dividing by the adjustment factor. For the broad methylation survey, hybridization intensities were measured for precursor and product bands and degree of methylation was calculated by adding intensities of product bands and dividing by sum of intensities of product and precursor bands.

For nuclease sensitivity assays, hybridization intensities were normalized to the amount of DNA loaded in each lane as follows: Ethidium bromide-stained gels were photographed using a UV-transilluminator (Fotodyne Incorporated, New Berlin, WI) and Polaroid MP4 land camera with instant film (B/W, 3000 ISO; Polaroid Corporation, Cambridge, MA). Prints were scanned at 600 dpi using a flatbed scanner (Astra 1200S flatbed scanner; UMAX Technologies, Fremont, CA). Images were cropped, converted to gray scale, inverted to negative images, and saved in PICT format (Adobe Photoshop; Adobe Systems, San Jose, CA). PICT files were imported into MacBAS v2.31 for evaluation. DNA concentrations were estimated using the average signal strength within each whole lane on the agarose gel. A loading adjustment factor was calculated by dividing the average signal strength for each lane by the highest signal strength obtained (lane with most DNA). Adjusted band intensities were obtained by multiplying the adjustment factor by the band intensity of each Southern hybridization product, as determined with MacBas imaging software. Finally, for normalization within a genotype, adjusted band intensities were divided by the adjusted band intensity of the sample with no added DNase I.

Characterization of juvenile and adult morphological traits: To distinguish juvenile from adult domains of the maize shoot, several epidermal morphological traits were scored on every leaf from eight *Pl-Blotched* plants (Poethig 1994). Leaves were considered to be juvenile if they had epicuticular wax, no epidermal macrohairs, and epidermal cells that stained purple with toluidine blue and had moderately crenulated cell walls. Adult leaves had no epicuticular wax, epidermal macrohairs, and epidermal cells that stained aqua with toluidine blue and had extremely crenulated cell walls. Transitional leaves had both juvenile and adult traits. For toluidine blue staining, small leaf sections (1–2 cm^2) were dissected from the tip, middle, and base of each leaf. Sections were fixed in a 3:1 ethanol:acetic acid solution overnight, stained with a 1:15 diluted toluidine stock solution (0.05% *O*-toluidine blue in 0.01 m sodium acetate, pH 4.4) for 48–72 hr, and viewed with a light microscope.

RESULTS

CCGG methylation state of *Pl-Blotched* differs in seedlings compared to husks: To explore whether developmental stage influences the degree of methylation of *Pl-Rhoades* or *Pl-Blotched*, genomic DNA isolated from seedlings and husks was digested with the methylation-sensitive isoschizomers *Msp*I and *Hpa*II and analyzed by Southern hybridization. Both enzymes recognize the sequence CCGG; *Msp*I cleaves if both cytosines are unmethylated or if the internal cytosine is methylated, whereas *Hpa*II cleaves only if both cytosines are unmethylated. By comparing the intensity of bands produced by *Hpa*II digestion to the intensity of the same-sized bands produced by *Msp*I digestion, the methylation state of CCGG sites giving rise to those bands can be inferred.

Digestion of *Pl-Rhoades* and *Pl-Blotched* DNA with *Msp*I or *Hpa*II and hybridization with a DNA fragment from the 3′ end of the gene produces seven bands defined by nine CCGG sites (Figure 1, A and B). For six of these sites, methylation status does not vary with development, as judged by comparing hybridization patterns for seedling and husk DNA within a genotype. Four sites (open arrows in Figure 1B) can be digested by *Hpa*II, producing fragments of 1.1 and 7.0 kb; this indicates that these sites are at least partially unmethylated. Two sites (shaded

arrows) are not digested by *Hpa*II, but can be partially digested by *Msp*I, as judged by the presence of faint 1.5- and 4.5-kb bands in *Msp*I lanes but not in *Hpa*II lanes; this indicates that some fraction of these sites is methylated on the internal cytosine. The methylation state of one site (solid arrow) could not be determined because its 1.1-kb product comigrates with another fragment produced by cleavage at other sites.

For two additional CCGG sites, methylation is more complex. These sites (solid lollipops in Figure 1B) define three fragments of 1.8, 0.7, and 0.4 kb. For the 1.8-kb fragment, hybridization intensity is stronger in *Msp*I digests than in *Hpa*II digests for all samples, indicating that the site giving rise to this fragment is partially methylated at the internal cytosine. In *Pl-Blotched* husk DNA (lanes 7 and 8), the intensity difference is more pronounced, suggesting that the site giving rise to the 1.8-kb band is more heavily methylated in DNA from husks than in *Pl-Blotched* seedling DNA or in the DNA from either tissue in *Pl-Rhoades*. For the 0.7- and 0.4-kb fragments, hybridization intensities are nearly equal in *Hpa*II and *Msp*I digests of seedling and husk DNAs for *Pl-Rhoades*. However, for *Pl-Blotched*, intensities of hybridization for these fragments are markedly different in seedling *vs.* husk DNA. In seedlings, the intensities of the *Msp*I and *Hpa*II fragments are approximately equal. By contrast, in husks, the difference in intensities between *Msp*I and *Hpa*II fragments is pronounced. In addition, the intensities of husk 0.7- and 0.4-kb fragments are lower than the seedling fragment intensities in both *Msp*I and *Hpa*II digests. Taken together, these results indicate that the sites giving rise to the 1.8- and to the 0.7- and 0.4-kb fragments are more methylated in *Pl-Blotched* husk DNA than in seedling DNA.

CCGG methylation of *Pl-Blotched* changes through development: The difference in methylation between *Pl-Blotched* seedlings and husks implies that development might play some role in regulating methylation. In maize, the vegetative meristem produces leaves such that those at the base of the plant, which emerge first, display juvenile traits, whereas leaves that emerge later toward the top of the plant display adult traits (Poethig 1994). This pattern provides an opportunity to examine methylation as the plant progresses from juvenile (seedling) to adult (higher leaves and husks) developmental stages.

To measure the changes in methylation of *Pl-Blotched* and *Pl-Rhoades* during plant maturation, DNA was isolated from seedlings and from individual leaves harvested just as they emerged from the whorl. DNA methylation patterns for all samples were determined by

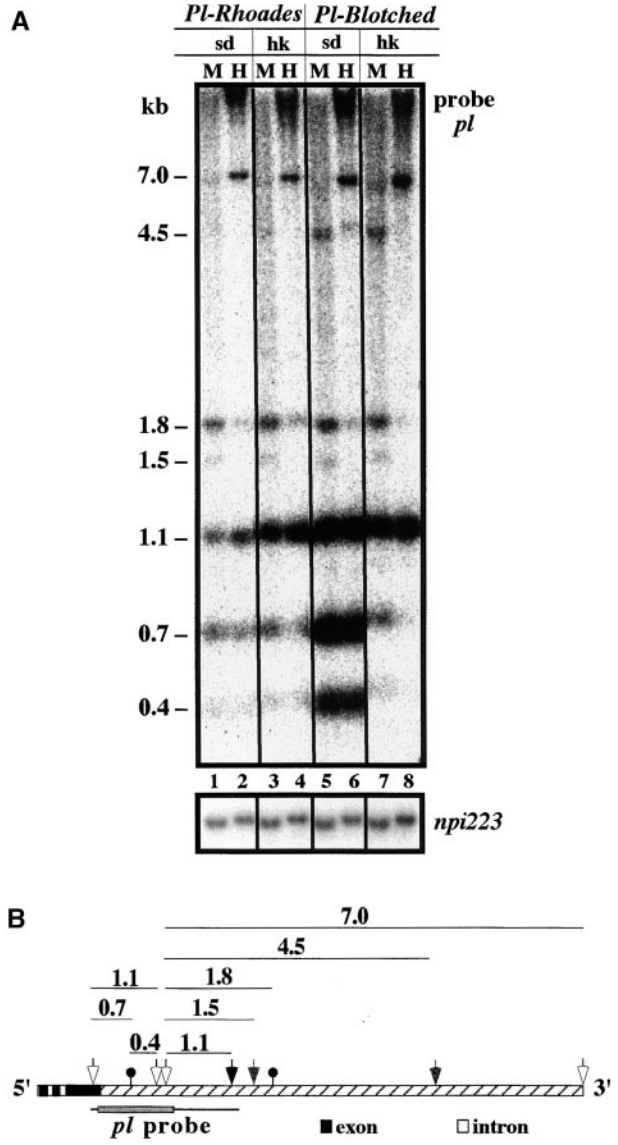

Figure 1.—Analysis of cytosine methylation of genomic DNA from *Pl-Rhoades* and *Pl-Blotched* seedlings and husk tissue. (A) Southern blot of genomic DNA from homozygous *Pl-Rhoades* and *Pl-Blotched* seedlings (sd) and husks (hk). DNA samples were digested with the restriction enzymes *Msp*I (M) and *Hpa*II (H), fractionated on agarose gels, blotted to nylon membranes, and hybridized with a *pl*-specific probe. Sizes of hybridizing fragments in kilobases are indicated at left. Hybridization of the same blots with an unrelated single-copy probe, *npi223*, controlled for DNA load and complete digestion. (B) Diagram showing the location of the *Msp*I/*Hpa*II sites in the 3′ flanking region of *Pl-Rhoades* and *Pl-Blotched*. Restriction fragments and their sizes are shown above the schematic representation of the gene. Exons and introns are indicated with solid and open boxes, respectively; downstream region is indicated with diagonal hatching. Arrows indicate sensitivity of indicated sites to digestion by *Hpa*II and *Msp*I: open arrow, sensitive to *Hpa*II digestion; shaded arrow, resistant to *Hpa*II digestion and partially resistant to *Msp*I digestion; solid arrow, not possible to determine sensitivity. Lollipops indicate sites that are differentially methylated in *Pl-Rhoades* and *Pl-Blotched* as discussed in the text. The probe (1.1-kb *Xho*I fragment, stippled box) is part of a tandem duplication (thin line). Numerous *Msp*I/*Hpa*II sites located in the coding region are not shown because their methylation status was not measurable in this experiment.

digestion with *Msp*I and *Hpa*II and Southern hybridization. Hybridization intensities were quantitated using a phosphorimager and corrected for loading differences by normalizing to hybridization intensity of the single-copy probe, *npi223*, as described in materials and methods. Relative methylation estimates were calculated as the ratio of *Msp*I intensity / *Hpa*II intensity for each band in a sample. Both cytosines in the sequence CCGG are substrates for methylation in plants. If the sites giving rise to a specific size band are unmethylated in all the molecules digested, then the intensity of that band in *Msp*I and *Hpa*II digests would be equal and the ratio of those intensities would be one. If the site is methylated at the internal cytosine in some molecules, the *Hpa*II band will decrease in intensity whereas the intensity of the *Msp*I band will not change. Therefore, the ratio of *Msp*I / *Hpa*II intensities will increase in value. Larger ratios indicate higher levels of methylation on internal cytosines.

Relative methylation as a function of developmental stage was assessed for the two diagnostic CCGG sites (solid lollipops in Figure 1B). At the 3′-most site, methylation was assessed by measuring the *Msp*I / *Hpa*II ratio for the 1.8-kb band (Figure 2A). In all tissues sampled from *Pl-Rhoades* plants, the *Msp*I / *Hpa*II ratio for this band is relatively constant (shaded bars). For *Pl-Blotched* plants, however, the ratios increase with developmental stage (hatched bars). At the seedling and 5-leaf stages, the ratio is approximately the same as seen in all stages of *Pl-Rhoades* plants. Beginning with leaf 7, the ratio is progressively higher with a plateau in leaves 13 and above.

At the more 5′ CCGG site, enzyme cleavage generates two bands, 0.7- and 0.4-kb. Methylation status of this site was determined by measuring the *Msp*I / *Hpa*II ratios for both bands. Because the pattern obtained was the same for both, only the data for the 0.7-kb band are presented. The ratios for the 0.7-kb band are lower than for the 1.8-kb band, but the profiles are similar. In *Pl-Rhoades* leaves, the *Msp*I / *Hpa*II ratios are constant, and in *Pl-Blotched* leaves, the ratios progressively increase.

Together, these results indicate that methylation on internal cytosines of both CCGG sites is constant in all leaves of *Pl-Rhoades* plants. By contrast, in *Pl-Blotched*, methylation at internal cytosines of both sites increases from the bottom (juvenile stage) to the top (adult stage) of the plants.

The methylation patterns of the CCGG sites are not equivalent: Expressing results as an *Msp*I / *Hpa*II ratio masks the dramatic differences in hybridization intensity of individual *Msp*I and *Hpa*II bands such as those seen for the 0.7-kb band in *Pl-Blotched* DNA from seedlings *vs.* husks (Figure 1A, compare lanes 5 and 6 to lanes 7 and 8). To obtain a clearer idea of how methylation of *Pl-Blotched* increases with development, *Hpa*II band intensities were quantitated and plotted against developmental stage. *Hpa*II will cleave only if both cytosines

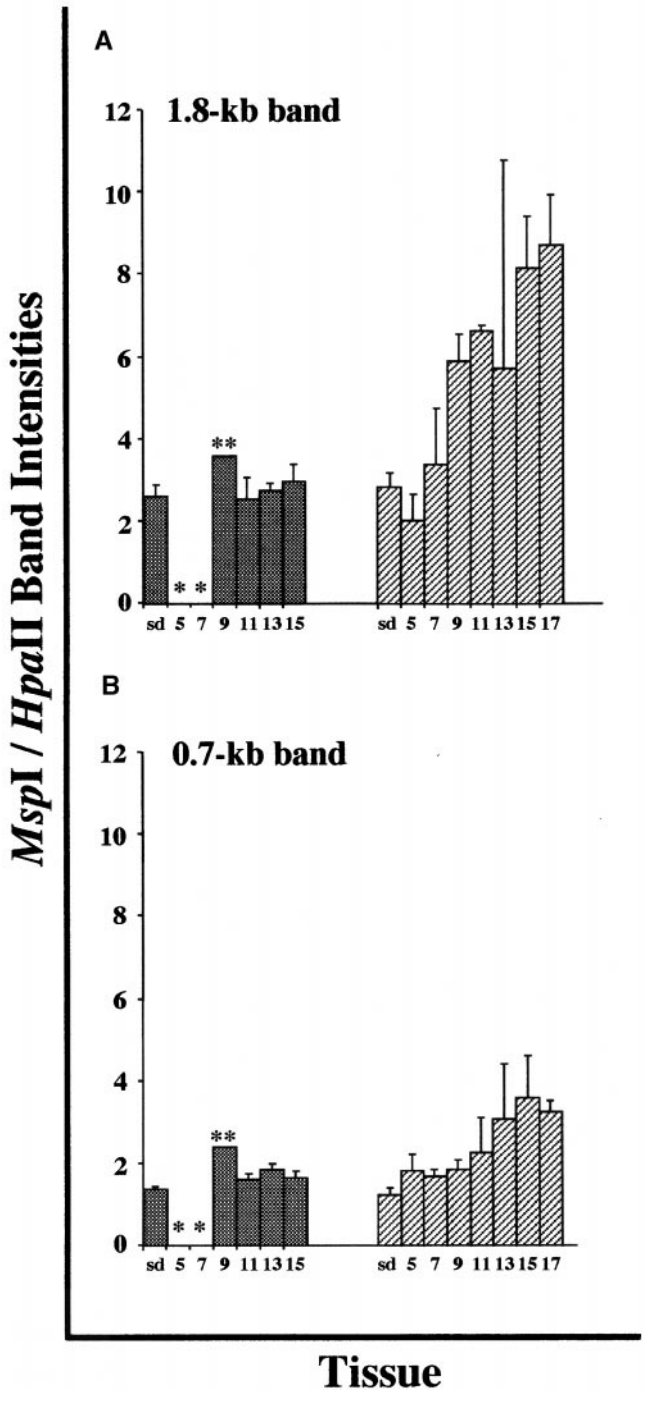

Figure 2.—Ratios of *Msp*I to *Hpa*II band intensities through development. Stippled bars represent ratios for *Pl-Rhoades* plants; hatched bars represent *Pl-Blotched*. Tissues sampled included seedlings (sd) and odd-numbered leaves. Ratios are the mean of replicates sampled from different plants (n = 2–4, except * designates n = 0 and ** designates n = 1); standard deviations are indicated by error bars. (A) Ratios for the 1.8-kb band. (B) Ratios for the 0.7-kb band.

are unmethylated. Consequently, a reduction in band intensity reflects an increase in total methylation, *i.e.*, methylation at internal, external, or both cytosines.

In *Pl-Blotched* plants, *Hpa*II band intensities for both

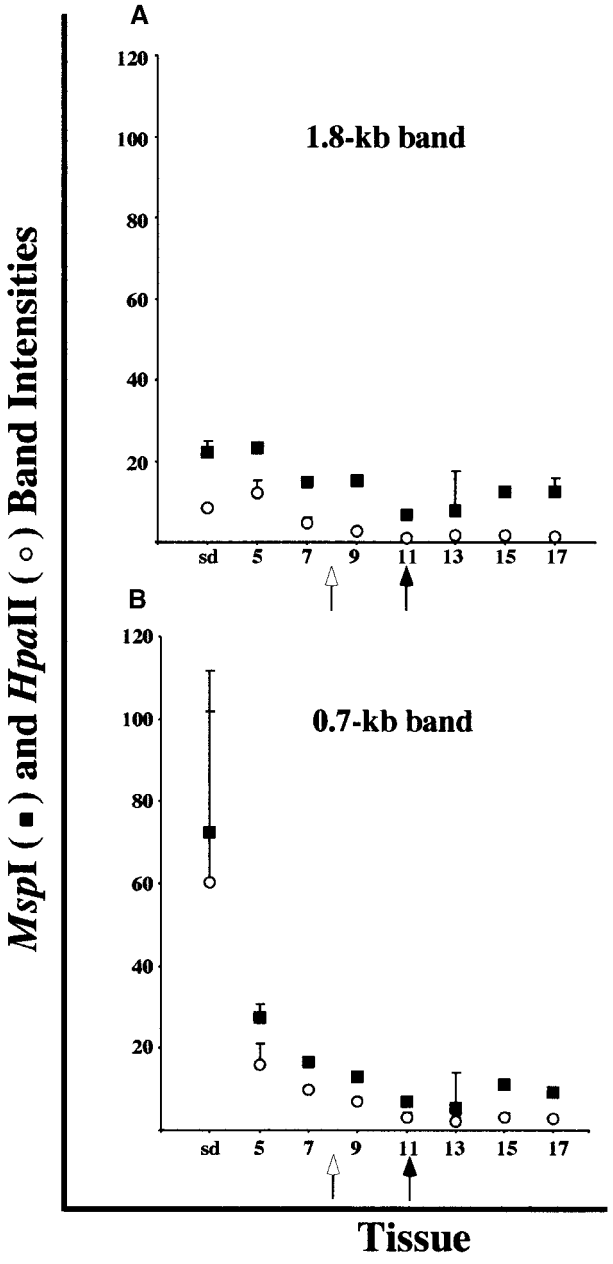

Figure 3.—*Msp*I and *Hpa*II band intensities through development in *Pl-Blotched*. Tissues sampled were as described in Figure 2. Band intensities are the mean of replicates sampled from different plants (n = 2–4); standard deviations are indicated by error bars. *Msp*I band intensities are indicated with solid squares and *Hpa*II band intensities are indicated with open circles. Arrows indicate position of first transitional leaf (open arrow) and first adult leaf (solid arrow). (A) Intensities for the 1.8-kb band. (B) Intensities for the 0.7-kb band.

the 1.8- and 0.7-kb bands were highest in seedlings and leaf 5 (Figure 3, A and B, open circles). In seedlings, *Hpa*II intensities for the 0.7-kb band varied widely from seedling to seedling (range in intensities: 24.1–108.7, n = 4), although the reason for this variation is unclear. Beginning with leaf 7, intensities for both bands dropped dramatically, such that in leaves 11 and above, the *Hpa*II intensities were ~2.5- to 3-fold lower than those for the same bands in *Pl-Rhoades* leaves (data not shown). This result reflects an apparent switch in CCGG methylation status for *Pl-Blotched* through development, with lowest methylation in seedlings and leaf 5, increased methylation in leaves 7 through 11, and a plateau of highest methylation in later leaves.

An increase in methylation at a given CCGG site in *Pl-Blotched* could reflect methylation on internal, external, or both cytosines. If methylation occurs primarily on the internal cytosine, then the site will be cut by *Msp*I. This pattern should result in relatively high *Msp*I/*Hpa*II ratios, and the difference between *Msp*I and *Hpa*II intensities (*Msp*I–*Hpa*II) should reflect the amount of methylation on the internal cytosine. If methylation occurs on external cytosines or both cytosines, then the site will not be cut by either *Msp*I or *Hpa*II. This pattern should produce a profile of *Msp*I digestion that closely parallels the profile of *Hpa*II digestion, with relatively low *Msp*I/*Hpa*II ratios and small differences between *Msp*I and *Hpa*II intensities.

To ask which of these patterns of methylation occurs at the two CCGG sites in *Pl-Blotched*, *Msp*I and *Hpa*II band intensities were compared for the 1.8- and 0.7-kb bands. As noted earlier, comparison of *Msp*I/*Hpa*II ratios revealed higher ratios through development for the 1.8-kb band than for the 0.7-kb band (Figure 2). Similarly, comparison of the differences between mean *Msp*I and *Hpa*II band intensities at each developmental stage shows the differences are greater for the 1.8-kb band than for the 0.7-kb band (Figure 3, A and B). The differences between the means is statistically significant [paired two-sample t-test for means: $T = 4.5155$; $P(T \leq t)$, two-tailed = 0.00196]. This result suggests that at the 3′-most CCGG site giving rise to the 1.8-kb band there is more methylation on the internal cytosine. Conversely, the relatively small differences between *Msp*I and *Hpa*II band intensities for the 0.7-kb band suggest that the more 5′ CCGG site undergoes methylation on the external cytosine. The differential methylation patterns of these two sites suggest that there are distinct mechanisms for methylating cytosines in the CG context (internal cytosines) as opposed to cytosines in the CNG context (external cytosines).

Change in CCGG methylation of *Pl-Blotched* coincides with the juvenile-to-adult developmental transition: The developmental change in methylation status for *Pl-Blotched* prompted us to ask if this change was coincident with the transition from juvenile to adult growth. This phase transition is characterized by alterations in specific morphological traits including presence or absence of epicuticular wax, production of epidermal macrohairs, epidermal cell wall crenulation, and color of epidermal cell staining with toluidine blue (Poethig 1994). These traits were assessed for all leaves from eight *Pl-Blotched* plants. Four plants had 17 leaves, three had 18 leaves, and one died after producing 15 leaves. The

region of phase transition is delineated by arrows in Figure 3 (open arrow, first transitional leaf; solid arrow, first adult leaf). Leaves 1 through 7 expressed only juvenile traits. Leaves 8 through 10 were transitional and expressed both juvenile and adult traits. Leaves 11 and above expressed only adult morphological traits. In all plants (except the one that died and produced no reproductive structures), the ear was subtended by leaf 12, and husks expressed adult traits. Comparison of phase-specific traits for each leaf with methylation status revealed a general pattern in which methylation profiles of the hypermethylated CCGG sites in *Pl-Blotched* (Figure 3, A and B) tend to follow the phase transition in these plants. The lowest methylation levels are seen in juvenile leaves 1 through 5, and the highest methylation levels are seen in leaves 11 and above. Intermediate levels of methylation are characteristic of leaves at or near the transitional boundaries, *i.e.*, leaves 7 through 9. This correlation raises the intriguing possibility that developmental programming of *Pl-Blotched* methylation may be coordinated by the same signals that govern the juvenile-to-adult transition in the maize plant.

Developmentally sensitive changes in methylation occur at other sites: In cases of epigenetic regulation that involve changes in DNA methylation, the patterns of methylation are diverse in terms of density and distribution of methylated cytosines. For example, in *Arabidopsis thaliana*, an epiallele of the *superman* gene exhibits a dense pattern of methylation close to the start of transcription (Jacobsen and Meyerowitz 1997). By contrast, in the *P-pr* epiallele of the maize *P* locus, the density of methylation is more sparse, although there also seems to be regional specificity in the sites of methylation (Das and Messing 1994).

To ask if the developmentally-sensitive change in methylation revealed by the two downstream *Hpa*II/*Msp*I sites is paralleled at other sites in and around the *Pl-Blotched* and *Pl-Rhoades* genes, we surveyed the methylation status of many restriction sites distributed in coding and flanking regions. Seedling leaves were assayed as representative juvenile tissue, and husks were assayed as representative adult tissue. The strategy for the survey was to evaluate the ability of a methylation-sensitive restriction enzyme to digest a precursor DNA fragment into smaller products. The ratio of precursor to product would reflect the degree of methylation of the precursor DNA. Genomic DNAs were digested first with a methylation-insensitive restriction enzyme, such as *Hin*dIII, to generate a precursor fragment of known size. Samples then were digested with a methylation-sensitive restriction enzyme that had at least one recognition site within the precursor fragment. The methylation state of cytosines (or adenines) within the recognition site was calculated from relative yield of fragments with sizes characteristic of precursor and products. Fully methylated sites would yield only precursor-sized fragments; unmethylated sites would yield only product-sized fragments; and partially methylated sites would yield both precursor- and product-sized fragments. Methylation status was judged to be sensitive to development, if digestion of seedling and husk DNA samples did not produce equivalent results.

The methylation status of 141 methylatable residues (133 cytosines and 8 adenines) in coding and flanking sequences was evaluated using 48 methylation-sensitive restriction enzymes, and the data are summarized in Figures 4 and 5. For 101 residues, methylation state did not differ between *Pl-Blotched* and *Pl-Rhoades*. A cluster of 22 cytosines associated with a CpG-like island upstream of the coding region was completely methylated in *Pl-Blotched* seedling and husk DNA. In *Pl-Rhoades*, these residues were nearly fully methylated in seedling DNA (only a trace of product-sized fragments was detected) and fully methylated in husks (no product-sized fragments were detected). Another 16 sites showed methylation changes through development in at least one of the alleles. All of these developmentally sensitive sites were located outside the coding region in 5′ and 3′ flanking sequences. Of the 5 upstream sites, 4 were located in or near a degenerate *doppia* transposable element that forms a portion of the promoter for both genes (Figure 4). The downstream sites spanned a region of ~3 kb. Five developmentally sensitive sites were distinct for *Pl-Blotched* and 3 were distinct for *Pl-Rhoades*.

The direction and extent of change in methylation during development varied among the sites (Figure 5). In *Pl-Blotched*, 13 residues were differentially methylated between seedling and husk; 12 of these showed a methylation increase. Similarly, in *Pl-Rhoades* 9 of 11 differentially methylated sites showed a methylation increase in husks. Comparison of methylation levels between genotypes at seedling and husk stages revealed that in *Pl-Blotched* seedlings, the level of methylation for 15 of 16 sites was less than or equal to the level of methylation in *Pl-Rhoades* seedlings. In *Pl-Blotched* husks, the methylation level at 9 sites was higher than or equal to methylation in *Pl-Rhoades* husks.

Chromatin structure differs in *Pl-Blotched* and *Pl-Rhoades*: To ask if the developmentally sensitive changes in DNA methylation in *Pl-Blotched* and *Pl-Rhoades* are tied to chromatin structural differences between the two alleles, DNaseI sensitivity assays were conducted on nuclei isolated from seedlings and husks of both genotypes (Ferl 1985; Wurtzel *et al.* 1987; Bellard *et al.* 1989). In these assays, regions of DNA that are sensitive to DNaseI digestion are indicative of open chromatin that is presumed to be accessible to RNA polymerase and is thus transcriptionally competent (Ferl 1985; Felsenfeld 1992). Regions of DNA that are resistant to DNase I digestion are closed and are assumed to be less accessible to RNA polymerase and therefore less transcriptionally competent.

To test nuclease sensitivity, nuclei from *Pl-Blotched* and *Pl-Rhoades* were incubated with a range of DNaseI

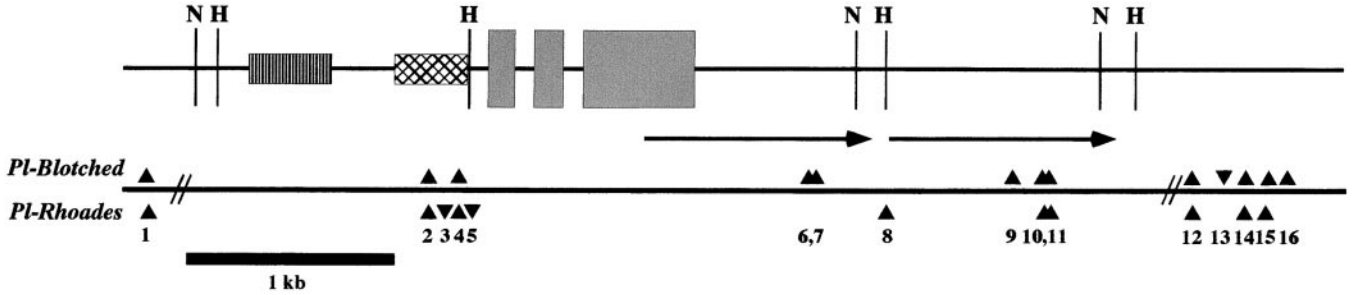

Figure 4.—Locations of developmentally sensitive sites of DNA methylation in *Pl-Rhoades* and *Pl-Blotched*. Top shows gene organization with coding region and promoter elements shown as boxes: shaded, protein coding regions; cross-hatching, *doppia* element; vertical hatching, CpG island. A tandem duplication is indicated by horizontal arrows and restriction sites are indicated by H, *Hin*cII, and N, *Nsi*I. Numbers designate developmentally sensitive methylation sites. ▲, sites in which methylation is higher in husk DNA than in seedling DNA; ▼, sites in which methylation is higher in seedling DNA than in husk DNA.

concentrations for a constant period of time. As a control, chromatin-free CsCl-purified (naked) DNA was also treated with DNaseI. DNA was subsequently purified and digested with restriction enzymes to generate reference-sized fragments. Digests were blotted to nylon membranes and hybridized with probes from *pl*. Nuclease sensitivity was measured by a decrease in the hybridization signal in the reference fragments as DNaseI concentration was increased.

Figure 7 shows the results of a representative experiment in which nuclei were assayed from seedlings (Figure 7, A and B) and from husks (Figure 7, C and D). In A and C, the photographs of the ethidium bromide-stained gels show that approximately equivalent amounts of DNA were loaded for *Pl-Rhoades* and *Pl-Blotched*. These images also show that high molecular weight DNA began to disappear at the same DNaseI concentration for both tissue types and genotypes, confirming that all sets of nuclei received equivalent DNaseI treatment. In this experiment, the DNaseI-treated DNAs were digested with *Hin*cII and hybridized with probe A (Figure 6). Digestion with *Hin*cII generates two DNA

Site	Position	Enzyme	Sequence	Degree of Methylation (0% ○ ◔ ◑ ◓ ◕ ● 100%) *Pl-Rhoades* sd	*Pl-Rhoades* hk	*Pl-Blotched* sd	*Pl-Blotched* hk
1	1856	*Spe*I	**AC**TAGT	◔	< ◓	◔	< ◔
2	3024	*Sfi*I	GGCC**A**C	◔	< ◓	◔	< ◓
3	3109	*Kpn*I	GGT**A**CC	◓	> ◔	◔	= ◔
4	3172	*Stu*I	AGG**CC**T	◓	< ●	◓	< ◕
5	3243	*Sal*I	GT**C**G**A**C	◓	> ◔	◑	= ◑
6	4948	*Msp*I	**C**CGG	◕	= ◕	◑	< ◕
7	4949	*Hpa*II	**CC**GG	◕	= ◕	◓	< ●
8	5224	*Hin*cII	GTTA**A**C	◔	< ◑	◔	= ◔
9	5825	*Pst*I	**C**TGCAG	◑	= ◑	◑	< ◓
10	5988	*Sau*3AI	GAT**C**	◑	< ◓	◔	< ◑
11	6022	*Spe*I	**AC**TAGT	◑	< ◓	◔	< ◑
12	6966	*Bam*HI	GGAT**C**C	◓	< ◕	◓	< ◕
13	~7000	*Sau*96I	GGN**C**C	◕	= ◕	◓	> ◑
14	~7300	*Msp*I	**C**CGG	◔	< ◑	◑	< ◕
15	~7500	*Nhe*I	GCTAG**C**	◔	< ◑	◔	< ◓
16	~8000	*Sau*96I	GGN**C**C	◔	= ◔	◔	< ◑

Figure 5.—Features of developmentally sensitive sites of DNA methylation in *Pl-Rhoades* and *Pl-Blotched*. Position corresponds to the nucleotide sequence for *Pl-Blotched* (GenBank accession no. L13454). Within a restriction site, methylation of the residues indicated by boldface, underlined lettering is reported to interfere with digestion (Nelson *et al.* 1993); however, exactly which residues were methylated is uncertain because methylation status of individual residues was not measured *in vivo*. Symbols are used to depict methylation changes with development from juvenile (sd, seedling) to adult (hk, husk) stage: <, methylation increase; >, methylation decrease; =, no change in methylation. Degree of methylation at each stage is indicated by partially filled to filled circles.

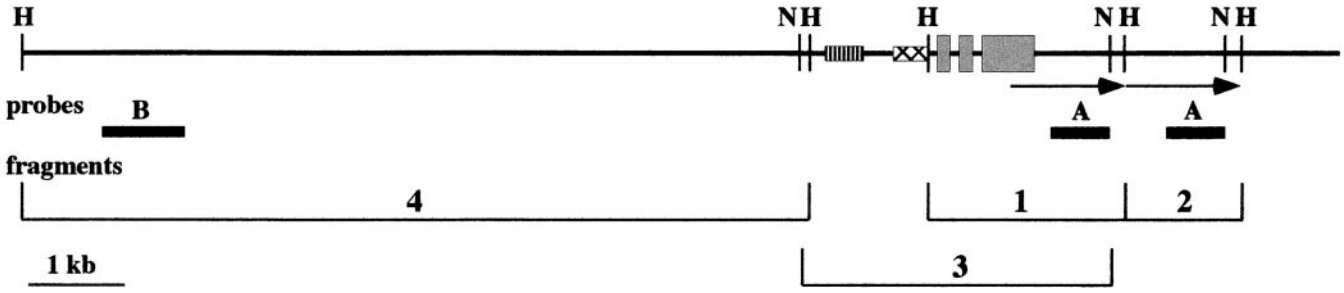

Figure 6.—Diagram of genic region of *Pl-Rhoades* and *Pl-Blotched* showing fragments assayed in nuclease sensitivity assays. Orientation of the gene is 5′ to 3′, left to right. Shaded boxes, protein coding regions; cross-hatched box, *doppia* element; vertically hatched box, CpG island. Fragments 1, 2, and 4 are produced by *Hin*cII (H) digestion. Fragment 3 is produced by *Nsi*I (N) digestion. Probe A is a 0.6-kb *Bgl*II-*Nsi*I fragment that hybridizes to a tandemly duplicated region indicated by double arrows. Probe B is a 0.8-kb *Hin*dIII fragment.

fragments of 2.1 and 1.2 kb, labeled 1 and 2, respectively, in Figure 7, B and D, that hybridize with probe A. A 3.3-kb fragment in *Pl-Rhoades* and in husk DNA from *Pl-Blotched* results from incomplete digestion, due to variable methylation of the A in the GTTGAC recognition site common to the 2.1- and 1.2-kb fragments. Fragment 1 contains the coding region and immediate downstream flanking region; fragment 2 contains the far downstream flanking region (Figure 6). For all four types of nuclei, the intensity of fragments 1 and 2 decreased with increasing DNaseI concentration. Smaller, diffusely hybridizing fragments, presumably the products of digestion, appeared below the reference fragments. However, the pattern of disappearance of fragments 1 and 2 was not the same for *Pl-Rhoades* and *Pl-Blotched*.

To compare the disappearance of fragments 1 and 2 as a function of DNaseI concentration, hybridization intensities were quantitated and graphed (Figure 8). In *Pl-Blotched* seedling nuclei, the reduction in band intensity for fragment 1 was small (Figure 8A, squares). By comparison, the intensity of the same fragment for *Pl-Rhoades* was rapidly reduced to a much lower level (Figure 8A, circles). At 5.2 units of DNaseI, the relative intensities in *Pl-Blotched* and *Pl-Rhoades* differed by about fourfold. By contrast, for fragment 2, the susceptibility to DNaseI was similar in both genotypes, and band intensities at 5.2 units of DNaseI were nearly equal (Figure 8C). Taken together, these results indicate that fragment 1, which encompasses the coding region and adjacent downstream sequences, is fourfold more sensitive to DNaseI treatment in *Pl-Rhoades* than in *Pl-Blotched* seedlings. This indicates that within this region of *Pl-Blotched* the chromatin has a more closed structure, whereas within the same region around *Pl-Rhoades*, the chromatin is more open. This differential sensitivity to DNaseI is not seen for the more downstream fragment 2, suggesting that there may be a defined 3′ boundary to the differences in chromatin structure between *Pl-Blotched* and *Pl-Rhoades* in seedlings.

To map the upstream boundary of the chromatin structure difference, two enzyme/probe combinations were used: *Nsi*I/probe A and *Hin*cII/probe B. When DNA from DNaseI-treated nuclei was digested with *Nsi*I and hybridized with probe A, two fragments were detected. The larger *Nsi*I fragment (fragment 3 in Figure 6) encompasses a portion of fragment 1, but extends 1.1 kb farther upstream. Fragment 3 contains a CpG island and a degenerate *doppia* transposable element that is part of the promoter. The nuclease sensitivity of this fragment was equivalent in *Pl-Rhoades* and *Pl-Blotched* seedlings (Figure 8E). When DNA from DNaseI-treated nuclei was digested with *Hin*cII and hybridized with probe B, an 8-kb *Hin*cII fragment (labeled 4 in Figure 6) extending 7 kb upstream of the coding region also displayed equal nuclease sensitivity in *Pl-Rhoades* and *Pl-Blotched* seedlings (Figure 8G).

Together, these results indicate that *Pl-Rhoades* and *Pl-Blotched* have different chromatin structures in seedlings, but the difference is localized to a region that includes 50 bp upstream, the coding region, and 700 bp downstream. Within this region, *Pl-Blotched* is two- to fourfold less sensitive to DNase I digestion than is *Pl-Rhoades*.

To ask if this chromatin structure difference was evident in adult tissues, a parallel set of experiments was conducted using nuclei isolated from husks. The results are summarized in Figure 8, B, D, F, and H. In husk nuclei, fragment 1 was about twofold less sensitive to DNaseI digestion in *Pl-Blotched* than in *Pl-Rhoades* at the highest concentration of enzyme used. The pattern of disappearance for fragments 2 and 3 was very similar to that for fragment 1, indicating reduced susceptibility in *Pl-Blotched* compared to *Pl-Rhoades* (Figure 8, D and F). Fragment 4 in *Pl-Blotched* was less susceptible to digestion than *Pl-Rhoades* at low enzyme concentrations, but at 5.2 units of DNaseI, the susceptibility was equivalent for both genotypes (Figure 8H). This type of pattern might be predicted if only a portion of fragment 4,

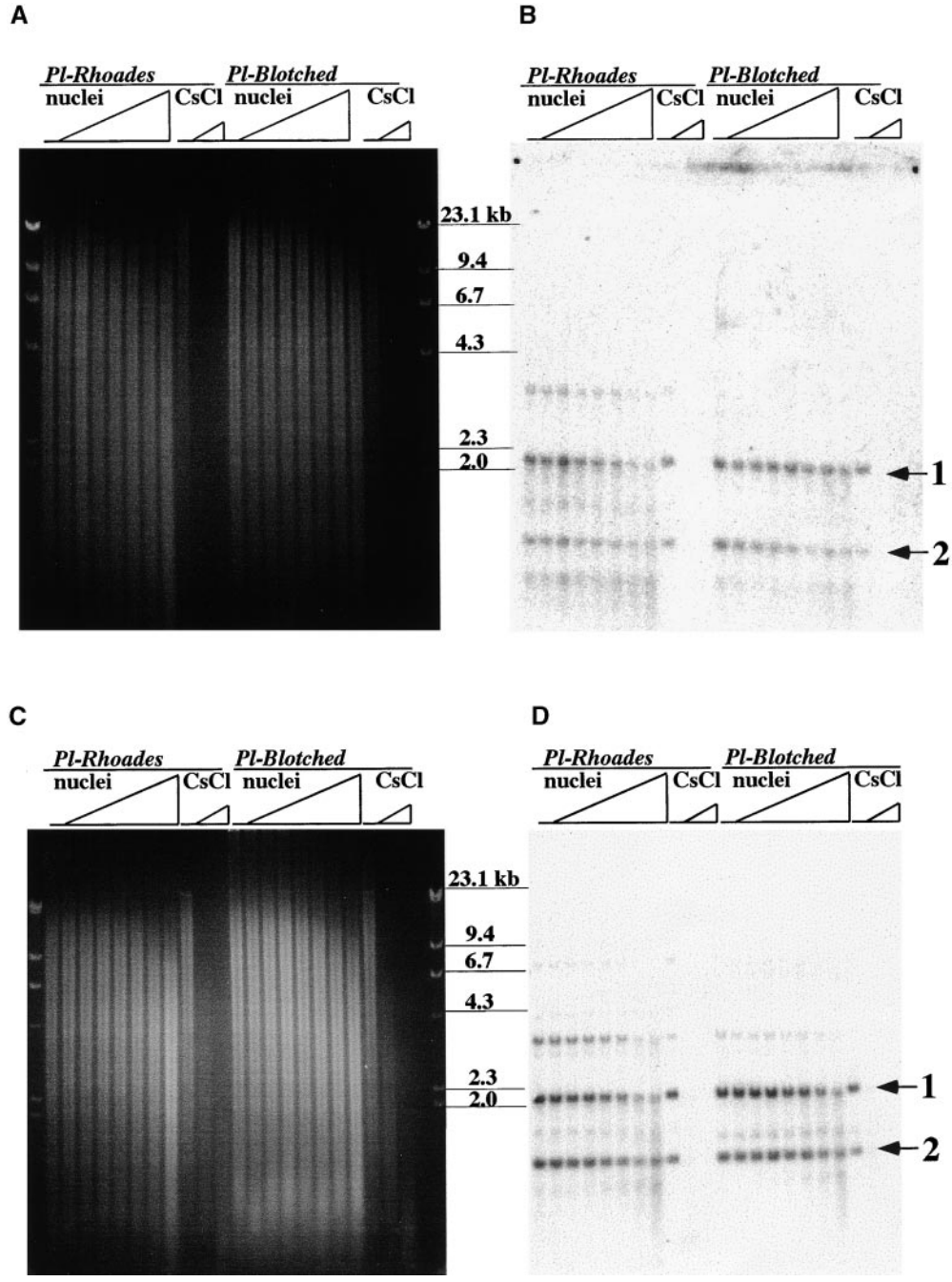

Figure 7.—Representative DNaseI sensitivity assay of seedling and husk nuclei from *Pl-Rhoades* and *Pl-Blotched*. (A and C) Ethidium bromide-stained gels of DNA digested with *Hin*cII after treatment of nuclei with increasing concentrations of DNaseI. Lanes, from left: 1, λ *Hin*dIII molecular size marker; 2–9, DNaseI-treated DNA from *Pl-Rhoades* nuclei; 10–12, DNaseI-treated CsCl-purified naked DNA from *Pl-Rhoades*; 13–20, DNaseI-treated DNA from *Pl-Blotched* nuclei; 21–23, DNaseI-treated CsCl-purified naked DNA from *Pl-Blotched*; 24, λ *Hin*dIII molecular size marker. (B and D) Corresponding blots of gels hybridized with probe A (see Figure 6). Nuclei were treated with 0, 0.064, 0.16, 0.32, 0.64, 1.28, 2.56, and 5.12 units of DNaseI. CsCl-purified DNA was treated with 0, 0.064, and 0.16 units of DNaseI. Bands indicated by arrows numbered 1 and 2 correspond to 2.1- and 1.1-kb bands as described in text and depicted in Figure 6.

perhaps the 3′ region, is included in a closed chromatin configuration.

The results of the chromatin structure experiments indicate that overall *Pl-Blotched* has a more closed chromatin domain than does *Pl-Rhoades*, but the size of the closed chromatin domain varies with developmental stage. In seedlings, the domain is restricted to the coding region and closely flanking sequences (fragment 1), whereas in husks, the closed chromatin domain is larger and extends at least 2 kb downstream of the coding region into sequences encompassed by fragment 2 and at least 1.4 kb upstream into sequences encompassed by fragment 3 and part of fragment 4.

DISCUSSION

The *Pl-Rhoades* and *Pl-Blotched* alleles are distinguished from one another by differences in two hallmark indicators of epigenetic regulation, DNA methylation and chromatin structure. In *Pl-Blotched*, chromatin structure is more closed than in *Pl-Rhoades*, and the size of the closed chromatin domain expands during develop-

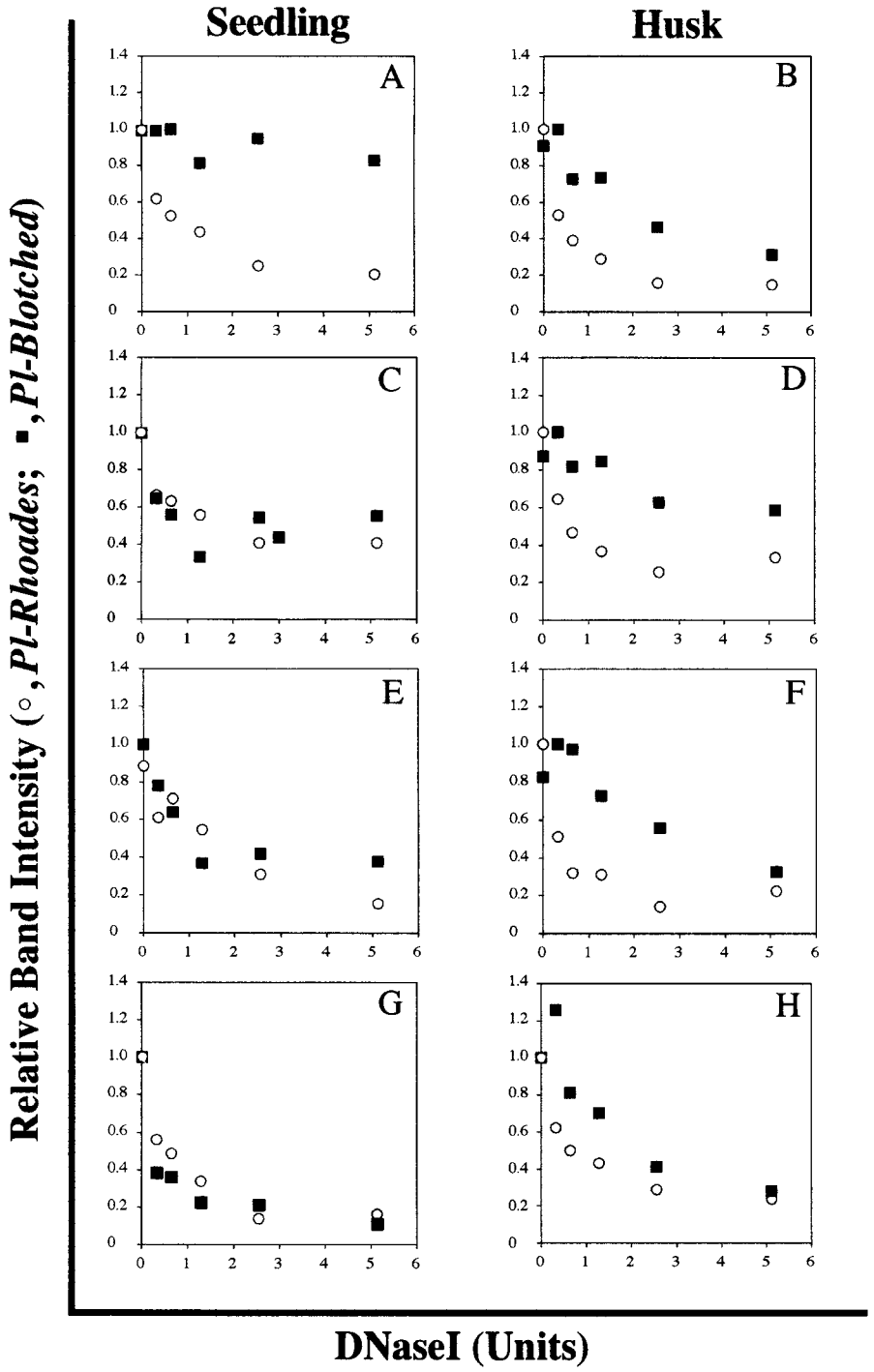

Figure 8.—Quantitative analysis of disappearance of *Pl-Rhoades* and *Pl-Blotched* DNA fragments in nuclease sensitivity assays. Relative band intensity, calculated from scans of DNA blots, is plotted against amount of DNaseI used to treat nuclei from *Pl-Blotched* (solid squares) and *Pl-Rhoades* (open circles) seedlings and husks (left and right, respectively). DNA fragments assayed, depicted in Figure 6, were as follows: fragment 1, A and B; fragment 2, C and D; fragment 3, E and F; fragment 4, G and H.

ment. This change in chromatin configuration is accompanied by changes in DNA methylation that coincide with the shift in developmental phase from juvenile to adult growth.

DNA methylation is frequently correlated with epigenetic silencing of gene expression in plants (reviewed in Finnegan *et al.* 1998; Kooter *et al.* 1999). Whether methylation is a primary effector or a secondary effect of silencing is debatable in many cases. Three lines of evidence argue *against* DNA methylation as the primary explanation for the differences in phenotype of *Pl-Blotched* and *Pl-Rhoades.* First, in seedlings, where many sites in *Pl-Blotched* are less methylated than in *Pl-Rhoades* (Figures 4 and 5), one might predict higher expression in *Pl-Blotched.* However, pigmentation and *pl* mRNA are virtually undetectable in *Pl-Blotched* seedlings, whereas *Pl-Rhoades* seedlings are visibly pigmented and *pl* mRNA is readily detected (P. Cooper and K. Cone, unpublished results). Second, in *Pl-Blotched* husks, there are no differences in DNA methylation between heavily pig-

mented and sparsely pigmented sectors from the same plant, even though *pl* mRNA levels differ dramatically in such sectors (Cocciolone and Cone 1993; M. Muszynski, unpublished results). Finally, the DNA methylation patterns imposed through development in both *Pl-Blotched* and *Pl-Rhoades* are not transmitted through meiosis. Methylation acquired as the plant matures and produces reproductive structures (tassels and ears) is not retained in the next generation; seedlings always exhibit the juvenile pattern methylation. This apparent "resetting" of methylation state is reminiscent of the cycles of developmental demethylation / methylation imposed on some mammalian genes as a result of genomic imprinting. For instance, in the germline of male mice, the *Xist* gene is demethylated at the onset of meiosis, and this status is retained in mature spermatozoa (Norris *et al.* 1994). Only later, in early embryonic development, is *Xist* methylated.

The results reported here suggest that chromatin structure plays a primary role in controlling the epigenetic state of *Pl-Blotched*. In both juvenile and adult tissues, *Pl-Blotched* chromatin structure is consistently more nuclease-resistant than in *Pl-Rhoades*. This implies that the meiotically heritable ground state of chromatin in *Pl-Blotched* is closed. The "degree" of closure varies in seedlings and husks, and this variation can be correlated with differences in gene expression both within a *Pl-Blotched* plant and between *Pl-Blotched* and *Pl-Rhoades* plants. Within a *Pl-Blotched* plant, the sequences encompassing the coding region of *Pl-Blotched* are most resistant to DNaseI in seedlings, the stage where *pl* mRNA and pigmentation are not detectable (Figure 8A). In *Pl-Blotched* husks, the chromatin in this coding region is not as DNaseI-resistant, but nevertheless is still "closed" relative to the chromatin of *Pl-Rhoades*. Furthermore, the size of the closed chromatin domain has expanded to encompass upstream and downstream flanking regions. These observations are consistent with results of expression studies (Cocciolone and Cone 1993). *Pl-Rhoades*, the allele with consistently open chromatin, expresses higher levels of both *pl* mRNA and pigment than *Pl-Blotched*, the allele with consistently closed chromatin.

The relationship among methylation, chromatin structure, developmental stage, and gene expression in *Pl-Blotched* exhibits similarities to, and differences from, regulatory mechanisms that operate to control epigenetic expression in other plant systems. The density of developmentally sensitive methylation sites in *Pl-Blotched* is lower than the density of methylated sites that distinguish the maize *P-pr* epiallele from its progenitor *P-rr* (Das and Messing 1994). The distribution of sites also differs in that most of the sites in *Pl-Blotched* are outside the coding region, whereas methylation in *P-pr* occurs at sites both in and out of the coding portion of the gene. The pattern of methylation in *Pl-Blotched* differs dramatically from methylation of the Arabidopsis *superman* epiallele in both density and distribution of sites, although the density difference is probably magnified by the different techniques used for assaying methylation state in the two systems (Jacobsen and Meyerowitz 1997). The *superman* gene was examined using a genomic sequencing method capable of assessing methylation at all cytosines; *Pl-Blotched* (and *P-pr*) was analyzed using methylation-sensitive restriction enzymes, thus confining methylation detection to those cytosines and adenosines that occur at enzyme recognition sites.

The relationship of development to methylation and chromatin structure may be different for *P-pr* and *Pl-Blotched*. The variegated phlobaphene pigmentation in *P-pr* pericarp, cob, and husks is correlated with increased DNA methylation, decreased DNaseI hypersensitivity, and decreased *p* transcript levels (Das and Messing 1994; Lund *et al.* 1995). Changes in methylation of *P-pr* during development were assayed by examining the extent of methylation in numerous organs and tissues within a single plant. Unlike the situation in *Pl-Blotched*, methylation levels among different leaves in *P-pr* plants were comparable, as were levels between leaves and pericarps. Within any specific organ or tissue there was a modest decrease in methylation through development. These observations prompted a model invoking an epigenetic pathway in which methylation decreases progressively as a function of cell division in the individual meristems and organ primordia of the plant.

In the *Mutator*-induced *hcf106* chloroplast mutant of maize, loss of *Mutator* activity is correlated with a pattern of transposable element methylation that is stable both mitotically and meiotically—a feature not characteristic for methylation of *Pl-Blotched*. In *hcf106*, methylation occurs in clonal sectors of cells that can span several nodes and leaves of a single plant (Martienssen *et al.* 1990; Martienssen and Baron 1994). The clonal nature of these sectors suggests that methylation occurring in individual cells of the developing shoot apex is propagated mitotically to descendant cells, which then become incorporated into upper nodes of the plant. If these nodes include those giving rise to the ear or tassel, then the methylated state can be passed meiotically to progeny, leading to stable lines in which the *Mutator* elements are inactivated. The stability of this methylation pattern is underscored by the results of meristem culture experiments in which shoot apices were excised from *hcf106* plants at a time in development when the *Mutator* elements had become methylated. When these apices were cultured so that the developmental phase was reset to the juvenile stage, methylation was retained (Irish and Jegla 1997).

Stable inheritance of methylation state is also a characteristic of *A. thaliana* plants carrying the *ddm1* mutation, which causes a drastic reduction in genomic cytosine methylation (Vongs *et al.* 1993; Kakutani *et al.* 1996). In *ddm1* plants, the hypomethylated state of genomic sequences is inherited through both mitotic and

meiotic cell divisions (Kakutani *et al.* 1999). Even when the *ddm1* mutation is segregated away, remethylation is slow and requires multiple generations to become reestablished. Recent studies demonstrating that the *ddm1* gene encodes a SWI2/SNF2-like protein imply that chromatin remodeling is integral for regulating genomic DNA methylation (Jeddeloh *et al.* 1999).

The relationship between methylation and chromatin organization suggests that one possible role for methylation in regulating *Pl-Blotched* might be to reinforce the closed chromatin state. In seedlings, the closed chromatin domain is centered on the coding region, but in husks, the closed domain expands to encompass upstream and downstream flanking regions, which include some of the sites of developmentally induced increases in DNA methylation. In animal systems, methylated cytosines can serve as substrates for methyl-CpG-binding proteins. Some of these proteins are found as complexes with histone deacetylases and function as key components of the remodeling machinery responsible for chromatin condensation (reviewed in Bird and Wolffe 1999). In *Pl-Blotched*, developmentally imposed changes in methylation might serve to recruit chromatin remodeling machinery, leading to reorganization and expansion of the closed chromatin domain.

A tie between methylation and chromatin remodeling may also help unravel the mechanisms that control another unique aspect of the *Pl-Blotched* phenotype. Unlike most *pl* alleles, *Pl-Blotched* leads to pigmentation in the aleurone layer of the endosperm. The pattern of pigmentation is variegated and results from ectopic expression of *Pl-Blotched* mRNA in the aleurone (Cocciolone and Cone 1993). Pigment levels can be up- or down-regulated by unlinked modifier loci, which act in a tissue-specific manner to control accumulation of *pl* mRNA (M. Muszynski and K. Cone, unpublished results). Recent descriptions in vertebrates of multiple forms of methyl-CpG-binding proteins with different tissue-specific distributions and different affinities for methylated DNA (Bird and Wolffe 1999) suggest intriguing and testable hypotheses for discovering the identity of tissue-specific modifiers of *Pl-Blotched* pigmentation.

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