

# Progressive Loss of DNA Methylation Releases Epigenetic Gene Silencing From a Tandemly Repeated Maize *Myb* Gene

Rajandeep S. Sekhon<sup>1</sup> and Surinder Chopra<sup>2</sup>

Department of Crop and Soil Sciences and Plant Biology Graduate Program, The Huck Institutes of the Life Sciences, Pennsylvania State University, University Park, Pennsylvania 16802

Manuscript received October 6, 2008

Accepted for publication November 7, 2008

## ABSTRACT

Maize *pericarp color1* (*p1*) gene, which regulates phlobaphene biosynthesis in kernel pericarp and cob glumes, offers an excellent genetic system to study tissue-specific gene regulation. A multicopy *p1* allele, *P1-wr* (white pericarp/red cob) is epigenetically regulated. Hypomethylation of *P1-wr* in the presence of *Unstable factor for orange1* (*Ufo1*), leads to ectopic pigmentation of pericarp and other organs. The *Ufo1*-induced phenotypes show incomplete penetrance and poor expressivity: gain of pigmentation is observed only in a subset of plants carrying *Ufo1* mutation, and the extent of pigmentation is highly variable. We show that *Ufo1* induces progressive hypomethylation of *P1-wr* repeats over generations. After five generations of exposure to *Ufo1*, a 30–40% decrease in CG and CNG methylation was observed in an upstream enhancer and an intron region of *P1-wr*. Interestingly, such hypomethylation correlated with an increase in penetrance of the *Ufo1*-induced pigmentation phenotype from ~27 to 61%. Expressivity of the *Ufo1*-induced phenotype also improved markedly as indicated by increased uniformity of pericarp pigmentation in the later generations. Furthermore, the poor expressivity of the *Ufo1* is associated with mosaic methylation patterns of the *P1-wr* upstream enhancer in individual cells and distinct *P1-wr* gene copies. Finally, comparison of methylation among different tissues indicated that *Ufo1* induces rapid CG and CNG hypomethylation of *P1-wr* repeats during plant development. Together, these data indicate that the poor penetrance and expressivity of *Ufo1*-induced phenotypes is caused by mosaicism of methylation, and progressive mitotic hypomethylation leads to improved meiotic heritability of the mutant phenotype. In duplicated genomes like maize, loss of an epigenetic regulator may produce mosaic patterns due to redundancy of epigenetic regulators and their target sequences. We show here that multiple developmental cycles may be required for complete disruption of suppressed epigenetic states and appearance of heritable phenotypes.

METHYLATION of cytosine residues in DNA is a highly conserved epigenetic mark in most eukaryotic organisms. Historically, DNA methylation has been proposed to be a defense mechanism adopted by genomes against selfish DNA elements (GOLL and BESTOR 2005). Indeed, transposons and other retroelements are generally methylated and loss of methylation at these elements leads to increased transposition (MIURA *et al.* 2001; SINGER *et al.* 2001; KATO *et al.* 2003; ZHANG *et al.* 2006). In addition to its role in protecting genome integrity, DNA methylation has also been implicated in the regulation of gene expression (BIRD 2002; ZHANG *et al.* 2006; ZILBERMAN *et al.* 2007). Generally, increased cytosine methylation leads to transcriptional silencing and, conversely, loss of methylation results in increased transcriptional activity (CHAN *et al.* 2005; GOLL and BESTOR 2005; ZILBERMAN

*et al.* 2007). Plasticity of DNA methylation during the life cycle of an organism, and heritability of methylation patterns over generations are crucial for normal development (SCHOB and GROSSNIKLAUS 2006). Genetic manipulations that alter DNA methylation cause various developmental abnormalities in plants (FINNEGAN *et al.* 1996; RONEUMUS *et al.* 1996; CAO and JACOBSEN 2002), mice (LI *et al.* 1992; OKANO *et al.* 1999), humans (HANSEN *et al.* 1999; XU *et al.* 1999), and fungi (FOSS *et al.* 1993; MALAGNAC *et al.* 1997).

Unlike in mammals, where most cytosine methylation marks are erased during an early embryonic stage and reestablished later (reviewed in OKANO *et al.* 1999; BIRD 2002), plants perpetuate this modification during gametogenesis and embryogenesis (BENDER 2004). Another unique difference between plants and mammals lies in their reproductive biology: while germ and somatic cells do not share a common lineage in mammals, plant germ cells are derived from somatic cells (RICHARDS 2006). This provides a mechanism through which epigenetic marks (including DNA methylation) accumulating during the life cycle of plants can

<sup>1</sup>Present address: Great Lakes Bioenergy Research Center, University of Wisconsin, Madison, WI 53706.

<sup>2</sup>Corresponding author: Department of Crop and Soil Sciences, 116 Agricultural Sciences and Industries Bldg., University Park, PA 16802. E-mail: sic3@psu.edu

be transmitted to the next generation. Therefore, cytosine methylation modifications provide a new source of genetic diversity in plants.

Although recent work has shed light on the importance of DNA methylation in diverse processes, its role in regulation of tissue-specific gene expression patterns is not clear (GEHRING and HENIKOFF 2007). The maize flavonoid biosynthetic pathway is well suited to address this question because: (1) genes encoding structural enzymes and transcription regulators have been genetically and molecularly characterized; (2) multiple natural alleles and epialleles of these genes have been identified; and (3) many of these alleles produce distinct tissue-specific pigmentation patterns. Biosynthesis of phlobaphenes—brick red flavonoids that accumulate in floral organs including pericarp, tassel and cob glumes, and silk—is regulated by an R2R3 Myb transcription factor encoded by *pericarp color1* (*p1*) (COE *et al.* 1988; STYLES and CESKA 1989). Remarkably, *p1* has >100 natural alleles and epialleles, many of which can be easily distinguished on the basis of their expression in the floral organs (BRINK and STYLES 1966; COCCIONE *et al.* 2001). The *p1* alleles are identified by their pigmentation in pericarp and cob glumes; *e.g.*, *P1-rr* produces red pericarp and red cob glumes, whereas *P1-wr* conditions white pericarp and red cob glumes.

Expression patterns of *P1-rr* and *P1-wr* are intriguing; both specify red cob but *P1-wr* fails to produce any pigmentation in pericarp. The two alleles share high (>99.0%) DNA sequence similarity in their coding, proximal promoter, and upstream promoter regions (CHOPRA *et al.* 1998). *P1-rr* is composed of a single gene unit flanked by a 5.2-kb sequence, whereas *P1-wr* consists of six or more copies of a complete 12.6-kb gene unit arranged in a head-to-tail fashion. Furthermore, *P1-wr* is densely methylated in the promoter and coding region as compared to *P1-rr*. On the basis of these differences and characterization of transgenic plants carrying promoter and coding region of *P1-rr* and *P1-wr*, the unique expression pattern of *P1-wr* has been attributed to epigenetic mechanisms (COCCIONE *et al.* 2001). The nature of these epigenetic mechanisms, however, remains largely unknown.

We have characterized *Unstable factor for orange1* (*Ufo1*), a *trans*-acting dominant modifier of *P1-wr*. *P1-wr; Ufo1* plants show ectopic accumulation of phlobaphenes in pericarp and other organs including husk, sheath, silks, and tassel glumes. *Ufo1*-induced phenotypes are not completely penetrant such that only a subset (~27%) of F<sub>1</sub> progeny (*P1-wr; Ufo1*) plants shows gain of pericarp pigmentation (CHOPRA *et al.* 2003; this study). In addition, *Ufo1*-induced phenotypes exhibit poor expressivity as the pericarp pigmentation ranges from a red scar at the silk attachment point to uniform dark pigmentation. Such incomplete penetrance and poor expressivity of *Ufo1* does not seem to be back-

ground dependent as we observed similar levels of penetrance in other genetic backgrounds including W22, T232, and B73 inbred lines (R.S. Sekhon and S. Chopra, unpublished results). Phenotypes produced by *P1-wr; Ufo1* plants are meiotically unstable as a subset of the progeny reverts back to a normal *P1-wr* expression pattern. Interestingly, the gain of phlobaphene pigmentation in pericarp is associated with hypomethylation of *P1-wr* repeats and, importantly, there is a direct correlation between the extent of hypomethylation and levels of pericarp pigmentation (CHOPRA *et al.* 2003).

This study was aimed at understanding the mechanism(s) responsible for incomplete penetrance and poor expressivity of the *Ufo1*-induced epigenetic regulation in tissue-specific gene expression. We studied the *Ufo1* and *P1-wr* interaction for multiple generations and found that each generation of such interaction leads to gradual increase in penetrance and expressivity of the *Ufo1*-induced phenotypes. Interestingly, such improvement in penetrance and expressivity was accompanied by progressive loss of *P1-wr* methylation. Furthermore, detailed profiling of methylation of a regulatory region of *P1-wr* indicated that poor expressivity of the *Ufo1*-induced phenotypes is due to somatic mosaicism of methylation. Such mosaicism appears to be contributed by differential *P1-wr* methylation in individual cells and distinct *P1-wr* gene copies. Finally, we also observed that the presence of *Ufo1* leads to rapid loss of *P1-wr* methylation during plant development. On the basis of these observations, we propose that the function of wild-type *ufo1* is to maintain a stable chromatin state at the *P1-wr* repeat complex. Extended exposure to *Ufo1* leads to cumulative destabilization of the *P1-wr* epigenetic state over generations resulting in improved penetrance and expressivity of ectopic pigmentation phenotypes. Overall, our study shows that, in highly complex and duplicated genomes like maize, redundancy of epigenetic regulators and their target sequences may hinder the phenotypic effects expected from the loss of an epigenetic regulator. Thus, prolonged absence of such regulatory elements for multiple developmental cycles may be needed to produce noticeable phenotypes.

## MATERIALS AND METHODS

**Maize stocks:** A maize stock carrying the *Ufo1* mutation in the *P1-wr* background was obtained from Derek Styles, University of Victoria, Canada. This mutation was introgressed into an inbred line 4Co63 (genotype *p1-wr c1 r-r*) as described previously (CHOPRA *et al.* 2003). Herein, the homozygous *Ufo1* stock in the 4Co63 background is referred to as *p1-wr; Ufo1*.

**Genetic crosses:** *p1-wr; Ufo1* plants were crossed to *P1-wr* (W23 inbred) to obtain F<sub>1</sub> progeny (Figure 2A). For developing the backcross population, F<sub>1</sub> plants (*P1-wr[W23] × p1-wr; Ufo1*) with high levels of *Ufo1*-induced pigmentation on leaf sheath, husk, and tassel glumes were backcrossed to *P1-wr[W23]* (Figure 2A). Since gain of pericarp pigmentation is the most reliable marker for the presence of *Ufo1*, highly expressing BC<sub>1</sub>F<sub>1</sub> ears were selected on the basis of uniform

dark orange pericarp pigmentation. The BC<sub>1</sub>F<sub>1</sub> plants grown from these selected ears were subjected to subsequent cycles of backcrossing and selection to develop the later populations. To develop the testcross progeny, BC<sub>6</sub>F<sub>1</sub> plants showing *Ufo1*-induced uniform pericarp pigmentation phenotype were crossed to the *p1-wu[4Co63]* inbred line and progeny plants were scored for pericarp pigmentation (Figure 2B). Homozygosity of the BC<sub>6</sub>F<sub>1</sub> plants for the *P1-wr* allele was established by a PCR assay that differentiates between *P1-wr* and *p1-wu[4Co63]* alleles (not shown). All the populations discussed here were grown at the Agronomy Farm, Pennsylvania State University, and in the winter nursery at Puerto Rico.

**DNA and RNA purification and gel blot hybridizations:** Plant genomic DNA was isolated using a modified CTAB method (SAGHAI-MAROOF *et al.* 1984). For comparison of DNA methylation among different leaves, DNA was isolated from alternate leaves of a plant at the time of tassel emergence. For all other experiments, the fifth leaf from the base of plants at 5- to 6-leaf stage was harvested for DNA extraction. Restriction enzyme digestions were performed using enzymes, reagents, and incubation conditions from Promega (Madison, WI). The restricted DNA was fractionated on 0.8% agarose gels, transferred to nylon membranes, and the gel blots were sequentially hybridized with  $\alpha^{32}\text{P}$ -dCTP labeled DNA fragments. For RNA gel blot analysis, total RNA was isolated from various tissues using lithium chloride buffer [100 mM lithium chloride, 100 mM Tris HCl (p.H. 8.0), 10 mM EDTA, 1% SDS] and precipitated with 4 M lithium chloride (BARLOW *et al.* 1963). Total RNA was fractionated on denaturing gels, blotted onto nylon membranes, and hybridized with  $\alpha^{32}\text{P}$ -dCTP probe fragments as described previously (BODDU *et al.* 2004). DNA fragments F15 and F8B belonging to an upstream regulatory region and second intron, respectively, were used as probes in Southern blot analysis (LECHELT *et al.* 1989; GROTEWOLD *et al.* 1991; SEKHON *et al.* 2007). Probe fragments used for Northern analysis were obtained from the plasmids pC2 containing a maize *C2* cDNA (WIENAND *et al.* 1986), pChi1 containing a maize *Chi1* cDNA (GROTEWOLD and PETERSON 1994), and pZmA1 with a maize *DFR* cDNA (SCHWARZ-SOMMER *et al.* 1987). Plasmid DNA was prepared using Strataprep plasmid miniprep kit (Stratagene, La Jolla, CA) following the manufacturer's directions. The Southern and Northern blots were stripped by washing for 15 min in boiling solution of 0.1% SDS before rehybridization.

**Quantification of flavan-4-ols:** Flavan-4-ols are colorless precursor compounds that polymerize to form red phlobaphene pigments (STYLES and CESKA 1977). These compounds have absorption maxima of 564 nm. Relative accumulation of flavan-4-ols was estimated by spectral analysis following previously published method (WATERSON and BUTLER 1983; GROTEWOLD *et al.* 1998). Briefly, pericarp and cob tissues were collected at 20 days after pollination and immediately frozen in liquid nitrogen. Two hundred milligrams of tissue from each genotype was ground in a mortar with a pestle and transferred to a 15-ml centrifuge tube containing 1 ml of 30% HCl/70% butanol (v/v) solution. Samples were incubated for 1 hr at 37°, spun at 14000  $\times g$  for 10 min, and the supernatant was collected in a 2-ml tube. Absorbance was measured using UV Mini 1240 spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). The absorbance value of these extracts at 564 nm ( $\lambda_{\text{max}}$ ) was used as an estimate of flavan-4-ols. Two independent samples from one plant were subjected to this analysis and mean and standard deviation of the replicates is presented. Flavan-4-ols are heat labile and boiling leads to elimination of the 564-nm peak that is diagnostic of these compounds (GROTEWOLD *et al.* 1998). Thus, the extracts were boiled for 10 min and spectral measurements of the boiled

samples were recorded to confirm elimination of the 564-nm peak.

**Genomic bisulfite sequencing:** DNA methylation of the upper DNA strand from a 333-bp upstream promoter region and a 312-bp intron 2 region of *P1-wr* was assayed by genomic bisulfite sequencing. Eight micrograms of leaf genomic DNA was completely digested with suitable restriction enzymes that cut outside the regions of interest. The digested DNA was treated with sodium bisulfite following a previously published protocol (JACOBSEN *et al.* 2000) with minor modifications (SEKHON *et al.* 2007). Regions of interest were PCR amplified using *P1-wr* primers specifically designed to amplify DNA modified with sodium bisulfite (supplemental Table 1). The resulting PCR products were gel purified, cloned using TOPO TA cloning kit (Invitrogen, Carlsbad, CA), and 20–30 clones/ligation/genotype were sequenced to obtain the bisulfite modified sequence data.

## RESULTS

**Penetrance of *Ufo1*-induced phenotypes correlates with *p1*-regulated phlobaphene biosynthesis:** *P1-wr*  $\times$  *Ufo1* interaction results in a gain of pigmentation in pericarps and cob glumes of a subset of progeny plants (CHOPRA *et al.* 2003), and pigmentation levels in individual plants are quite variable (Figure 1A). We tested whether such phenotypic differences are due to differential expression of *p1*-regulated pathway genes and/or relative levels of flavan-4-ols, compounds that polymerize to produce phlobaphenes. While only trace amounts of flavan-4-ols were present in pericarp of F<sub>1</sub> (*P1-wr/p1-wu; Ufo1/ufo1*) plants that did not show pericarp pigmentation (hereafter called nonexpressers), substantial levels were detected in sibling plants showing *Ufo1*-induced gain of pericarp pigmentation (hereafter called expressers) (Figure 1B). Steady state transcript levels of three *p1*-regulated structural genes *c2*, *chi1*, and *a1* were compared in various floral and vegetative tissues of sibling F<sub>1</sub> plants. In nonexpressers, only silk tissue had appreciable amounts of transcripts of the three structural genes (Figure 1C). Conversely, the expressers had a significant increase in transcript accumulation of these genes in pericarp, silk, and husk. Overall, these results indicate that phenotypic differences among sibling expresser and nonexpresser plants are a result of differential activity of the *p1*-regulated phlobaphene biosynthetic pathway.

**Inheritance of *Ufo1*-induced pigmentation phenotype improves over generations:** We have previously shown that *Ufo1* exhibits incomplete penetrance and poor expressivity (CHOPRA *et al.* 2003). *Ufo1* induces varying degrees of hypomethylation from *P1-wr* repeats and that the extent of hypomethylation is directly correlated with the level of pericarp pigmentation (CHOPRA *et al.* 2003). Thus, it is possible that the variability of *P1-wr* expression due to such differential methylation patterns is manifested as poor expressivity and, eventually, incomplete penetrance of pigmentation phenotype. Furthermore, *Ufo1* appears to be a weak

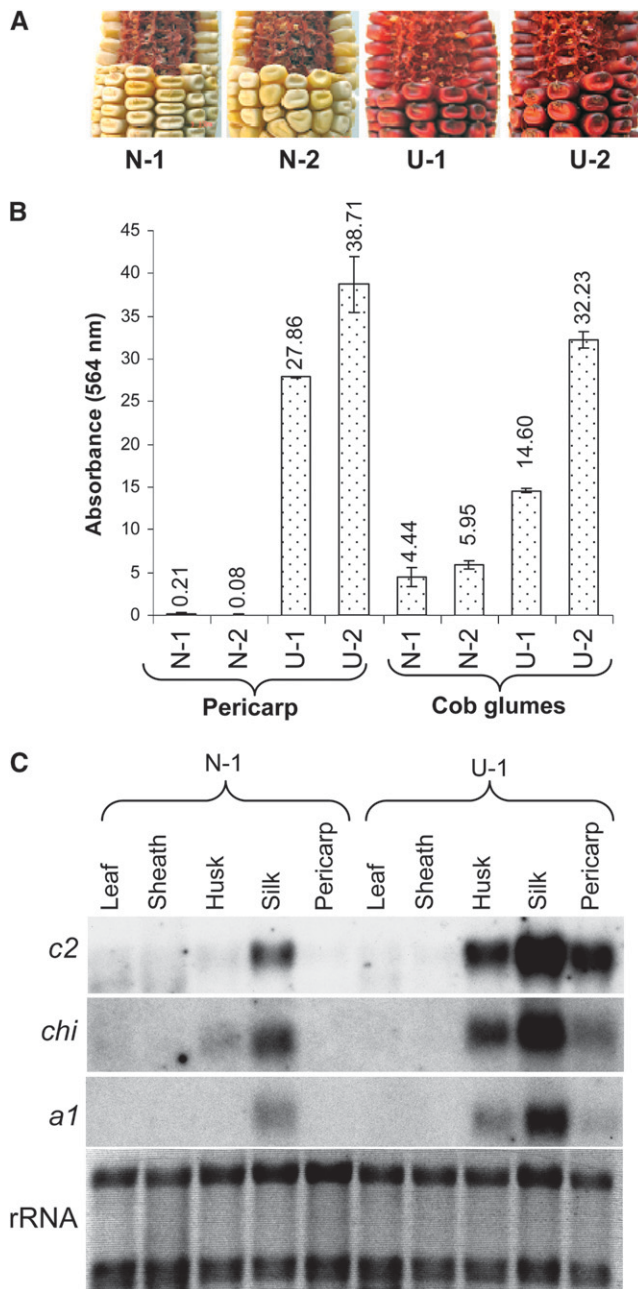
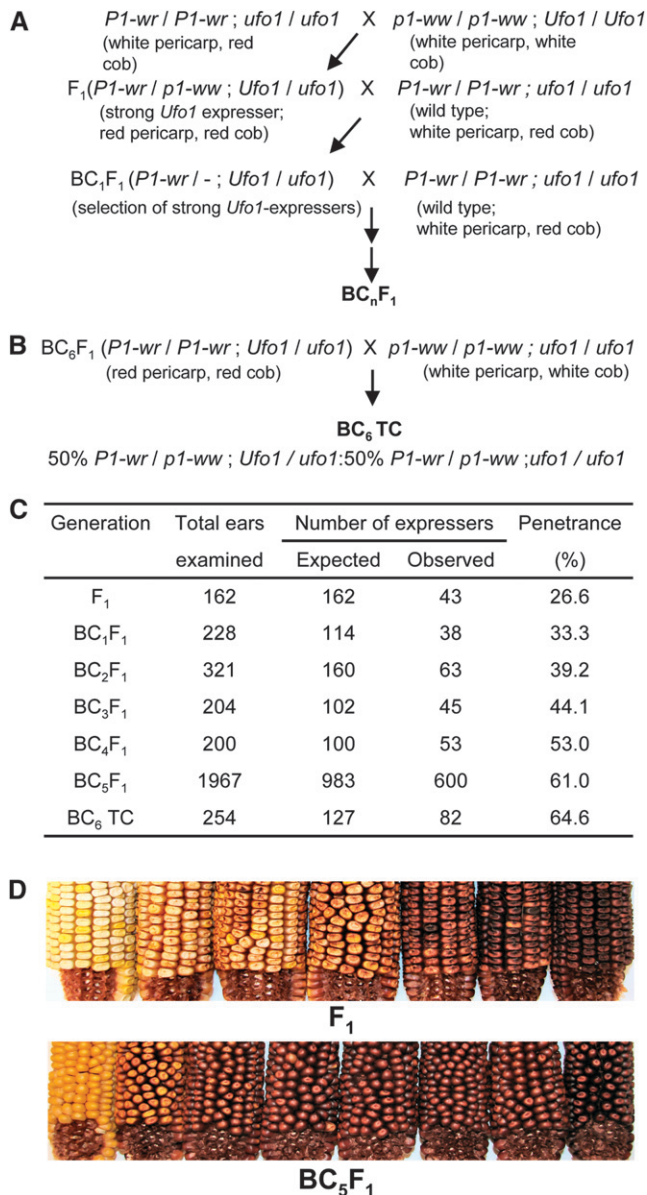


FIGURE 1.—*Ufo1*-induced upregulation of phlobaphene biosynthesis in floral and vegetative organs. (A) Gain of pericarp and cob pigmentation in the  $F_1$  ( $PI-wr/PI-wr$ ;  $ufo1/ufo1 \times p1-wr/p1-wr$ ;  $Ufo1/Ufo1$ ) individual plants. Shown here are representative ears from sibling  $F_1$  plants that either lack pericarp pigmentation (nonexpressers, N-1 and N-2), or show *Ufo1*-induced pericarp pigmentation (expressers, U-1 and U-2). (B) Pericarp and cob pigmentation levels of ears in A correlate with accumulation of flavan-4-ols. Levels of flavan-4-ols are based on relative absorbance (564 nm) of extracts from tissues of individual plants (see MATERIALS AND METHODS). (C) Steady-state transcript levels in floral and vegetative tissues. Three *p1*-regulated structural genes of the phlobaphene biosynthetic pathway were assayed. Gel blot carrying total RNA isolated from different tissues of two sibling plants shown in A was sequentially hybridized with probes of genes shown on the left.

modifier that is not able to induce complete hypomethylation of *PI-wr*. However, in such a scenario, prolonged exposure of *PI-wr* to *Ufo1* might cause increased destabilization of the *PI-wr* epigenetic state and lead to improved heritability of the pericarp pigmentation phenotype. To test this idea, we looked at penetrance of *Ufo1* in  $F_1$ , five backcross populations, and a testcross population (see Figure 2, A and B for details). In the  $F_1$  generation,  $\sim 27\%$  of *PI-wr*; *Ufo1* plants showed gain of pericarp pigmentation instead of 100% expected from a dominant monogenic trait (Figure 2C). Additionally, the *Ufo1*-expressing  $F_1$  plants exhibited a range of pericarp pigmentation indicating poor expressivity (Figure 2D). Remarkably, the penetrance of *Ufo1* showed an incremental improvement after each backcross (Figure 2C). For example, of 983  $BC_5F_1$  plants expected to show *Ufo1*-induced gain of pericarp pigmentation, 600 exhibited the phenotype indicating 61% penetrance in this generation. Expressivity of *Ufo1*-induced phenotypes also improved upon backcrossing as evident from relatively uniform pericarp pigmentation in the  $BC_5F_1$  (Figure 2D). To further confirm if such improved penetrance and expressivity persists in later generations, we testcrossed strong expressers of  $BC_6F_1$  generation with *p1-wr*[4*Co63*] (Figure 2B). Interestingly, of 127 plants expected to carry *Ufo1* mutation, 82 showed uniform pericarp pigmentation, indicating 65% penetrance of *Ufo1* in this population. In conclusion, these results indicate that extended exposure of *PI-wr* to *Ufo1* leads to improved inheritance of pericarp pigmentation phenotype.

***Ufo1* causes progressive loss of DNA methylation of *PI-wr* over generations:** *Ufo1*-induced gain of pigmentation is associated with decreased DNA methylation of *PI-wr* repeats, and such hypomethylation in individual *PI-wr*; *Ufo1* plants is variable and shows a direct correlation with level of pericarp pigmentation (CHOPRA *et al.* 2003; SEKHON *et al.* 2007). We tested if improved penetrance and expressivity of gain of pigmentation phenotypes after multiple generations of exposure to *Ufo1* could be due to progressive hypomethylation of the *PI-wr* multicopy complex. DNA methylation of  $F_1$  and  $BC_4F_1$  plants was compared by digesting leaf genomic DNA with *HpaII* and hybridizing the gel blot with a *p1* fragment 15 as a probe (Figure 3A). In *PI-wr*, probe 15 hybridized to a prominent band of  $\sim 7.9$  kb, a weak band of  $\sim 12.0$  kb, and three smaller size bands of  $\sim 0.6$ ,  $0.5$ , and  $0.4$  kb (Figure 3B). In  $F_1$  expresser plants, three new fragments of  $\sim 3.6$ ,  $3.0$ , and  $2.3$ -kb size were observed, indicating hypomethylation of additional *HpaII* sites. In  $BC_4F_1$ , interestingly, the number and intensity of *HpaII* bands increased: a total of seven bands were observed in the *Ufo1*-expressing individuals and intensity of these bands was higher than that observed in the  $F_1$  expressing plants. The low molecular weight bands were either absent or very light in the nonexpresser plants. These results suggested that *PI-wr*



**FIGURE 2.**—Penetrance and expressivity of gain of pericarp pigmentation phenotype improves upon extended exposure of *P1-wr* to *Ufo1* for multiple generations. (A and B) Crossing scheme used to develop the  $F_1$  and  $BC_5F_1$  and  $BC_6$  testcross ( $BC_6TC$ ) progenies. (C) Penetrance of *Ufo1* in different populations. Expected phenotypes are based on a hypothetical assumption that *Ufo1* has complete penetrance. In the  $BC_nF_1$  population, 50% of the progeny are expected to carry *Ufo1* (genotype:  $P1-wr / - ; Ufo1 / ufo1$ ) and show *Ufo1*-induced gain of pericarp pigmentation. In the  $BC_6TC$  population, all the individuals will be heterozygous at the *p1* locus ( $P1-wr / p1-ww$ ) and show 1:1 segregation for the *ufo1* locus ( $Ufo1 / ufo1 : ufo1 / ufo1$ ). Thus, 50% of the  $BC_6TC$  individuals are expected to show gain of pericarp pigmentation. Penetrance was calculated as percentage of observed/expected *Ufo1*-expressers. (D) Pericarp and cob pigmentation in representative  $F_1$  and  $BC_5F_1$  progeny plants.

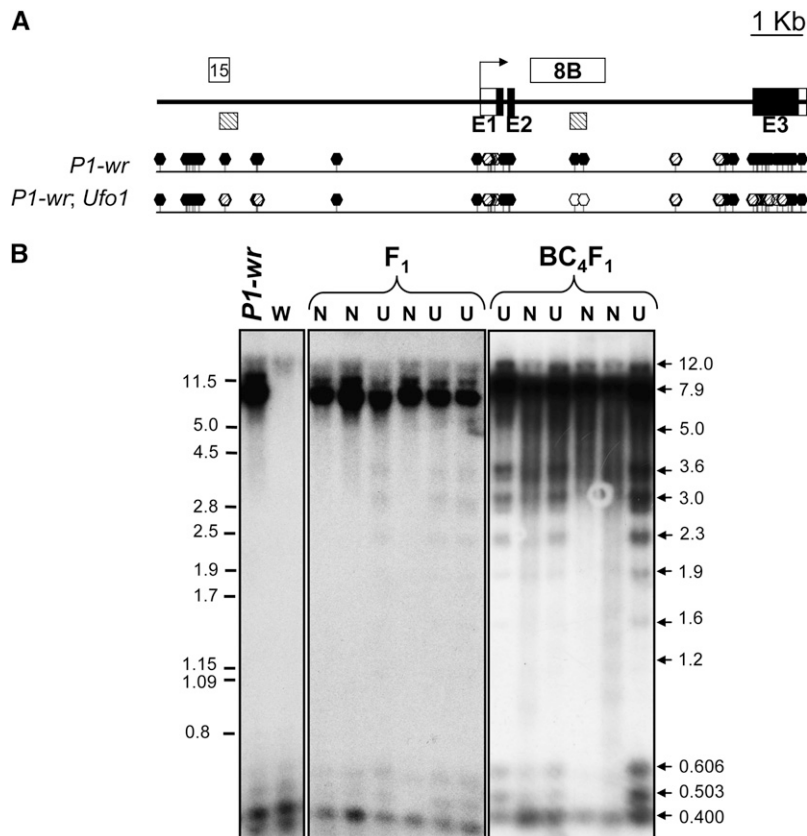
repeat complex is more hypomethylated in  $BC_4F_1$  as compared to  $F_1$  generation.

To determine the exact position and context of cytosines undergoing *Ufo1*-induced methylation mod-

ifications, we performed genomic bisulfite sequencing. Two regions were chosen for this assay: a 333-bp fragment from a distal enhancer region, and a 312-bp region from the 5' end of intron 2 of *P1-wr* (see Figure 3A for location of these regions). Both regions undergo significant methylation modifications during *P1-wr*  $\times$  *Ufo1* interaction and have been considered important for upregulation of *P1-wr* expression (CHOPRA *et al.* 2003; SEKHON *et al.* 2007; ROBBINS *et al.* 2008). In the distal enhancer region, overall CG and CNG methylation was very high ( $\sim 95\%$ ) in *P1-wr* as well as in the  $F_1$  expressers (Figure 4A). Expresser plants in the  $BC_4F_1$  generation, however, had significantly lower levels of CG (64%) and CNG (50%) methylation. Interestingly, CHH methylation levels were very low in all three genotypes. Individual CG and CNG sites also showed a similar trend; methylation levels were comparable for *P1-wr* and  $F_1$  plants but showed sizeable decrease in the  $BC_4F_1$  generation (supplemental Figure 1, A and B). In summary, while *Ufo1* did not induce noticeable changes in CG and CNG methylation of the *P1-wr* distal enhancer region in  $F_1$  plants, significant hypomethylation was observed in the  $BC_4F_1$  generation.

Likewise, in the intron 2 region, cumulative CG and CNG methylation levels did not show striking differences between *P1-wr* and  $F_1$  plants. CG methylation was high in *P1-wr* (96%) as compared to  $F_1$  (82%) (Figure 4B), while CNG methylation in  $F_1$  (81%) was more than that in *P1-wr* (73%). In  $BC_4F_1$ , however, CG and CNG methylation levels dramatically decreased to 48% and 40%, respectively. CHH methylation levels in the intron 2 region were very low in all three genotypes. The methylation pattern of individual CG sites resembled that of overall methylation in this region; methylation of most CG sites was highest in *P1-wr*, slightly decreased in  $F_1$ , and was lowest in the  $BC_4F_1$  generation (supplemental Figure 2A). Methylation of individual CNG sites was more variable; while some sites showed hypermethylation in the  $F_1$  generation, others were hypomethylated as compared to *P1-wr* (supplemental Figure 2B). In the  $BC_4F_1$  generation, however, all CNG sites were less methylated as compared to *P1-wr* and  $F_1$  plants. Overall, combined data from DNA gel blot analysis and genomic bisulfite sequencing indicated that long-term exposure of *Ufo1* leads to increased *P1-wr* hypomethylation in the distal enhancer and second intron.

***Ufo1* induces rapid hypomethylation of *P1-wr* during plant development:** In the presence of *Ufo1*, decrease of *P1-wr* methylation from the  $F_1$  to the  $BC_4F_1$  generation indicated that the hypomethylated state was meiotically transmitted. To test if *Ufo1*-induced *P1-wr* hypomethylation progresses mitotically during plant development, we compared methylation of different leaves. Leaf genomic DNA of expresser and nonexpresser  $BC_5F_1$  plants was digested with *HpaII* and the resulting gel blot was probed with the *p1* distal enhancer fragment 15. *Ufo1*-induced hypomethylation of *P1-wr* in these plants



**FIGURE 3.**—Progressive loss of DNA methylation of *P1-wr* in the presence of *Ufo1*. (A) Gene structure and methylation map of *P1-wr*. Solid line represents a complete 12,596-bp single *P1-wr* repeat. Transcription start site is indicated by a bent arrow. Boxes superimposed on the solid line represent three exons marked as E1, E2, E3; blank regions of E1 and E3 represent UTRs. Probe fragments are shown at the top of the gene structure. Striped boxes at the bottom of the gene structure represent two regions subjected to genomic bisulfite sequencing (see RESULTS). Methylation map of *HpaII* sites in *p1* sequence of *P1-wr* and *P1-wr; Ufo1* plants is shown at the bottom of the gene structure. Open, striped, and solid hexagons represent unmethylated, partially methylated, and completely methylated *HpaII* sites, respectively. (B) Gel blots showing DNA methylation differences between *F1* and *BC4F1* plants (see Figure 2 for scheme used to develop these populations). Leaf genomic DNA of respective plants was digested with *HpaII* and gel blots were hybridized with *p1* probe fragment 15. Abbreviations are: N, *Ufo1* nonexpressing plants carrying wild-type *P1-wr* (phenotype: white pericarp, red cob); W, plants carrying null *p1-wr* allele (phenotype: white pericarp, white cob); U, *Ufo1*-expressing plants (phenotype: red pericarp, red cob). Molecular weight marker bands are shown in kilobase pairs (kb) on right. Bands hybridizing with the probe are indicated by arrows on the right side along with their sizes in kilobases.

is shown by the presence of three fragments (indicated by arrows in Figure 5A). Interestingly, intensity of these bands varied among different leaves of the expresser plant: while barely detectable in first, third, and fifth leaves, these bands had higher signal intensity in the seventh, ninth, and eleventh leaves. Similarly, hybridization of the same blot with probe 8B (intron 2 region) also showed incremental loss of methylation (compare the intensity of two bands marked by arrows in Figure 5B). Thus, in the expresser plants, the loss of methylation progresses from the bottom leaf to the terminal leaf. These data indicated that *P1-wr* exhibits developmental hypomethylation in the presence of *Ufo1*. The small-size bands indicative of *Ufo1*-induced *P1-wr* hypomethylation were not observed in nonexpressers (data not shown) suggesting an absence of developmental *P1-wr* hypomethylation in the absence of *Ufo1*.

To further confirm the developmental hypomethylation of *P1-wr* sequence and to identify the sequence context undergoing such modifications, bisulfite sequencing data was compared between the fifth leaf and pericarp, two organs representing distinct developmental stages. Since the *Ufo1*-induced phenotypic changes are clearly evident in pericarp, and the fifth leaf is a nonexpressing tissue for *p1*, the developmental hypomethylation is expected to be higher in pericarp tissue. For this comparison, we chose the 333-bp region from the distal enhancer (see Figure 3A for location). Bisulfite

assay showed that, even in the wild-type *P1-wr*, this region had less CG and CNG methylation in pericarp as compared to leaf, indicating hypomethylation during development (Figure 6). This result is consistent with the fact that, although the *P1-wr* allele does not induce phlobaphene pigmentation in the pericarp, *p1* transcripts do accumulate in this tissue albeit at lower levels as compared to cob glumes (CHOPRA *et al.* 1996). Interestingly, in the *F1* expresser plants, the hypomethylation in CG and CNG context was more drastic in pericarp as compared to leaf. In summary, these data show that DNA methylation decreases during differentiation of cells from the fifth leaf, an organ that represents an early developmental stage and lacks detectable *p1* expression, to pericarp tissue, which represents a later stage and accumulates detectable levels of *p1* transcripts. Furthermore, presence of *Ufo1* causes a rapid change in the epigenetic state of *P1-wr* during development, which is consistent with increased *p1* transcription in pericarp and a correlative gain of phlobaphene pigmentation (CHOPRA *et al.* 2003).

***Ufo1* induces increased mosaicism of *P1-wr* methylation:** Variable somatic expression and poor penetrance of *Ufo1*-induced pericarp pigmentation could be due to differential methylation patterns of *P1-wr* in different gene copies and/or cells. Such mosaicism of *P1-wr* methylation may affect expression in individual cells thereby producing uniformly pigmented, variegated, or

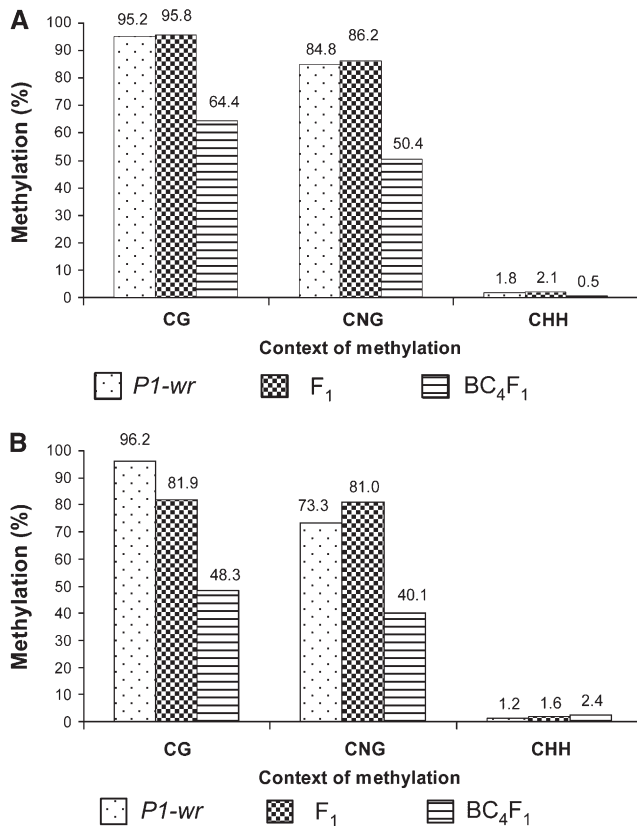


FIGURE 4.—Bisulfite data showing *Ufo1*-induced progressive hypomethylation of two distinct regions of *PI-wr*. Cumulative methylation in CG, CNG (N is any nucleotide), and CHH (H is A, C, or T) context in a 333-bp distal enhancer (A), and a 312-bp intron 2 region (B) is shown (see Figure 2A for location of these regions). The genotypes assayed were wild-type W23 carrying the homozygous *PI-wr* allele (designated as *PI-wr*), and *F*<sub>1</sub> and *BC*<sub>4</sub>*F*<sub>1</sub> progenies showing *Ufo1*-induced pericarp pigmentation phenotype (see Figure 2 for crossing scheme used to develop these populations). For each genotype, overall methylation in each context was calculated by dividing the number of methylated cytosines by total number of cytosines in the context in all the clones.

nonpigmented pericarp phenotypes. Additionally, the methylation state of *PI-wr* in the cell contributing to gamete formation may decide penetrance and thus the pericarp pigmentation of the progeny. Analysis of CG and CNG sites in individual clones of the 333-bp distal enhancer region of pericarp of *PI-wr* and *F*<sub>1</sub> expresser plants revealed a very interesting methylation pattern (Figure 7). Of 24 clones derived from *PI-wr* pericarps, 1 was hypomethylated, 2 showed a mosaic pattern characterized by a mixture of methylated and nonmethylated sites, while 21 clones were highly methylated. An interesting change in methylation pattern was observed in *F*<sub>1</sub> pericarps; of 26 clones examined, 9 were hypomethylated, 7 showed a mosaic pattern, while 10 were hypermethylated. These results show that the presence of *Ufo1* leads to substantial increase in the number of *PI-wr* DNA molecules with partial or complete hypomethylation. It should be noted that such hypomethylated

clones could be contributed by different *PI-wr* copies, individual cells, or both. The presence of some indels (see positions −4984, −4977, −4900, and −4899 in clones RBS287 and RBS286, and positions −4900 and −4899 in clones RBS300 and RBS 281) indicates that these clones are derived from different copies. To summarize, *PI-wr*; *Ufo1* plants show enhanced methylation mosaicism that is contributed by differential *PI-wr* methylation in individual cells and distinct gene copies.

## DISCUSSION

The white pericarp/red cob phenotype of *PI-wr* has been attributed to the hypermethylated state of its tandemly repeated copies (CHOPRA *et al.* 1998; COCCIOLOONE *et al.* 2001). The presence of *Ufo1* induces varying levels of hypomethylation of the *PI-wr* repeat complex and a corresponding gain of pericarp pigmentation. However, the extent of *Ufo1*-induced pigmentation is highly variable and shows poor penetrance. In maize, such poor penetrance has been attributed to the redundancy of the genes controlling/contributing to the phenotype (SATO *et al.* 1999; VEGA *et al.* 2002). Furthermore, epigenetic states of eukaryotic genes are collectively maintained by DNA methylation and chromatin modifications, two redundant and mutually dependent epigenetic marks that are usually imposed by RNA-based mechanisms (reviewed in BENDER 2004; TARIQ and PASZKOWSKI 2004; KLOSE and BIRD 2006). Due to redundancy, loss of one of these components may partially be compensated by the other and could, therefore, lower the severity of mutant phenotype. However, loss of one of the components may produce small changes in epigenetic states that are cumulative over generations. This indeed is evident from studies involving *mop1* (*mediator of paramutation1*), an RNA-dependent RNA polymerase (RdRP) in maize, and *DDM1* (*DECREASE IN DNA METHYLATION1*), a SWI2/SNF2-like protein in Arabidopsis (JEDDELOH *et al.* 1999; ALLEMAN *et al.* 2006). Mutations in these genes lead to slow but cumulative “decay” of epigenetic states of their target sequences and it takes several generations before phenotypic and molecular change are observed (VONGS *et al.* 1993; KAKUTANI 1997; WOODHOUSE *et al.* 2006). As proposed in these studies, progressive improvement in penetrance of *Ufo1* in this study may also be due to redundancy epigenetic marks.

Partial maintenance of epigenetic state over multiple mitotic divisions can generate somatic cell lineages with either silent or active gene expression state and thus produce variegation patterns (reviewed in RICHARDS 2006). Cellular mosaicism and range of DNA methylation at the population level is manifested as poor expressivity and penetrance, respectively (MATZKE and MATZKE 1991; 1998; MCGOWAN *et al.* 1989). Herein, we present the evidence that the poor expressivity of *Ufo1*-induced phenotypes could be due to a strikingly distinct

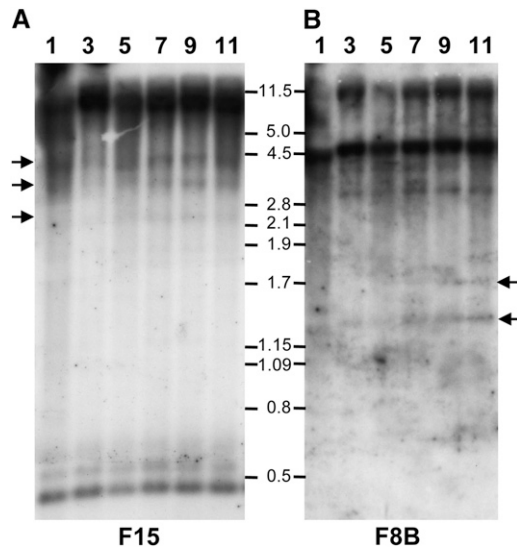


FIGURE 5.—Mitotic progression of *Ufo1*-induced hypomethylation of *PI-wr* during plant development. Shown here is a comparative methylation pattern of leaves at different developmental stages. Genomic DNA from different leaves of a BC<sub>5</sub>F<sub>1</sub> expresser plant was digested with *Hpa*II and hybridized with a *pI* promoter fragment 15 (A) and an intron fragment 8B (B). Numbers on the top of lanes represent leaves, 1 being the first leaf and 11 being the flag leaf. Molecular weight marker bands in kilobase pairs are shown in the middle of the two blots.

methylation profile of a *PI-wr* regulatory region in individual pericarp cells and gene copies (Figure 7). Depending upon the *PI-wr* epigenetic state in the somatic lineages, the pericarps may be uniformly pigmented, variegated, or colorless. This line of thinking is supported by the observation that *PI-wr; Ufo1* plants show a range of methylation that correlates with the extent of pericarp pigmentation (CHOPRA *et al.* 2003; SEKHON *et al.* 2007). Thus our study shows that, in tandemly duplicated genes like *PI-wr*, the differential epigenetic state of regulatory regions of individual gene copies can also add to mosaicism of epigenetic states. In complex and redundant genomes like maize wherein approximately one-third of the genes are tandemly duplicated (MESSING *et al.* 2004), this adds another layer of complexity in epigenetic regulation of gene expression.

Epigenetic states change during the development of an organism as reported for *Pl-Blotched* allele of *purple plant1* (*pl1*) gene and Robertson's *Mutator* elements in maize (MARTIENSSSEN *et al.* 1990; HOEKENGA *et al.* 2000). It is known that such fine tuning of epigenetic states regulates development- and tissue-specific gene expression in higher eukaryotes (POETHIG 1990; LU *et al.* 2008). Indeed, comparison of leaf and pericarp methylation showed that *PI-wr* expression is developmentally regulated (Figure 6). The leaf used for this comparison represents an adult vegetative stage while the pericarp is associated with reproductive phase (POETHIG 1990; 2003). We found that such a developmental change

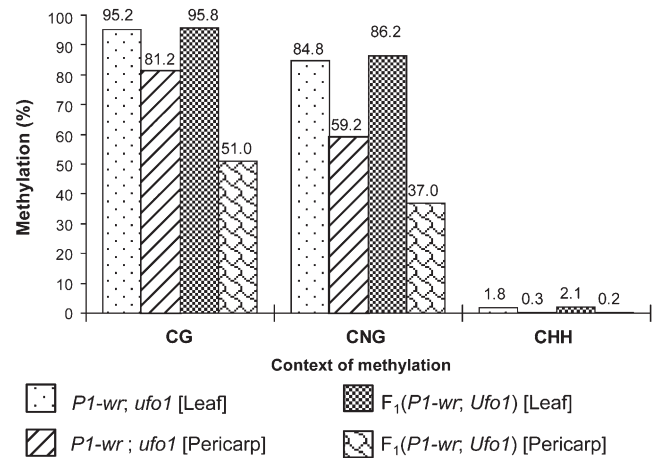


FIGURE 6.—*PI-wr* methylation differences among tissues belonging to vegetative and reproductive phases of development. Methylation of a 333-bp distal enhancer region of *PI-wr* (see Figure 2A for location) in fifth leaf and pericarp was assayed by genomic bisulfite sequencing. Genotypes compared were homozygous *PI-wr/PI-wr; ufo1/ufo1* (designated as *PI-wr; ufo1*) and an F<sub>1</sub> generation of the cross *PI-wr/PI-wr; ufo1/ufo1* × *p1-wr/p1-wr; Ufo1/Ufo1* (designated as *PI-wr; Ufo1*). Calculations used to make this figure and other details are the same as for Figure 4.

from leaf to pericarp is associated with loss of CG and CNG methylation. This observation is consistent with the *PI-wr* expression pattern; while leaf does not accumulate detectable *pI* mRNA, appreciable levels of transcripts are found in the pericarp (CHOPRA *et al.* 1996). However, pericarps of *PI-wr* are not pigmented possibly because a threshold level of *pI* mRNA required for activation of downstream genes and thus phlobaphene pigmentation is not achieved (CHOPRA *et al.* 2003). In the presence of *Ufo1* mutation, there is a rapid destabilization of *PI-wr* epigenetic states as indicated by reduced CG and CNG methylation. This observation is consistent with increased transcription of *pI* and *pI*-regulated genes and the appearance of pericarp pigmentation in *PI-wr; Ufo1* plants.

In plants, DNA methylation modifications accumulating during the life cycle can be transmitted to the next generation because plant germ cells are derived from somatic cells. In maize, the megaspore mother cell (MMC) is derived from the pericarp precursor cells, although the nature of cell lineages that lead to MMC formation is debatable (GREENBLATT 1985; ZHANG 1999). Depending upon the methylation state of *PI-wr* in the pericarp cell involved in gamete formation, any of the methylation patterns can be represented in the egg cell and subsequently in the progeny (Figure 7). Increased hypomethylation of the *PI-wr* complex due to extended exposure to *Ufo1* may result in higher numbers of cells carrying hypomethylated *PI-wr*. This in turn may lead to an increased chance of gametes being derived from cells with such a hypomethylated state of *PI-wr* resulting in improved inheritance of the

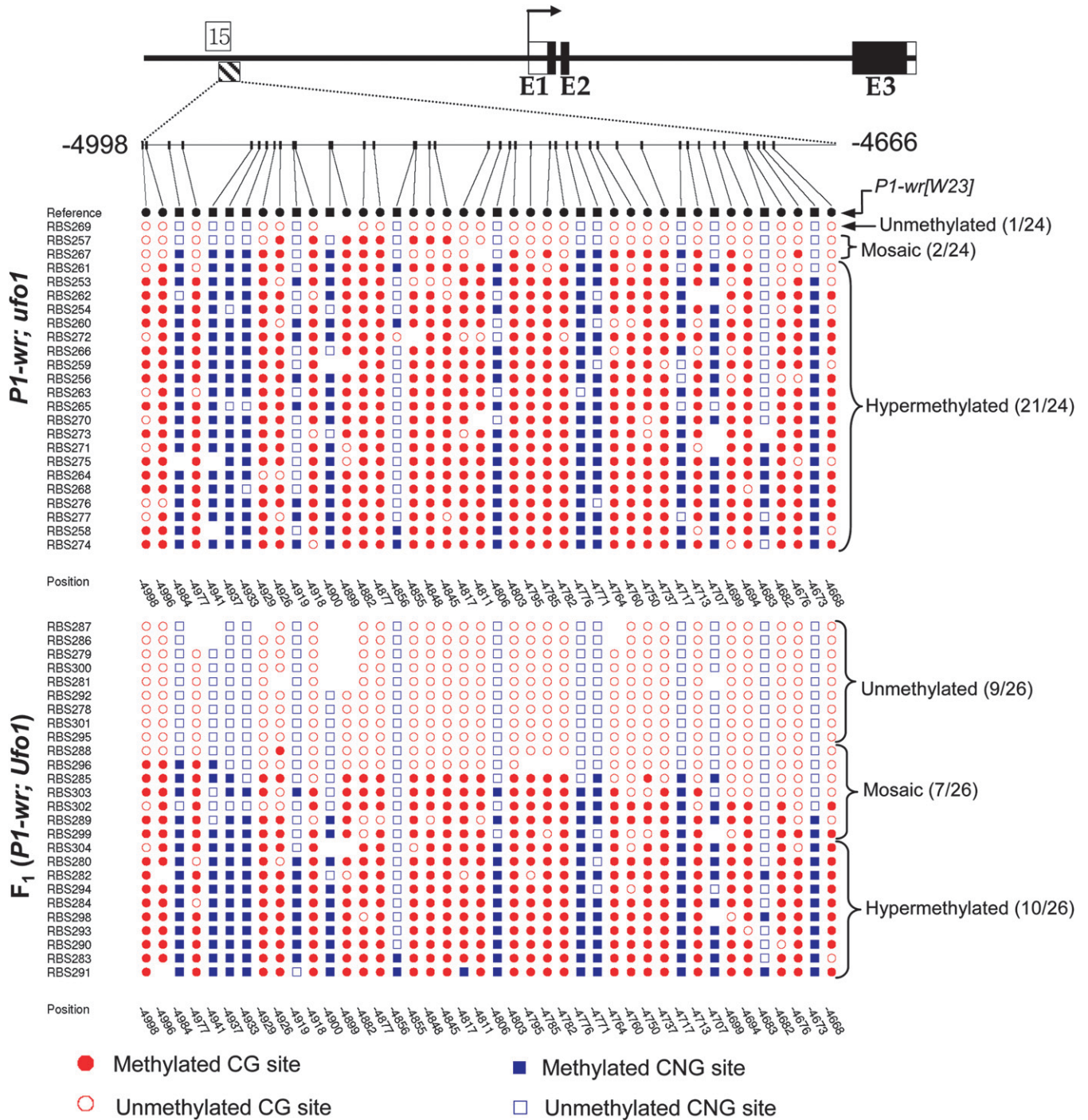


FIGURE 7.—*Ufo1*-induced somatic variability of *P1-wr* methylation at CG and CNG sites in pericarp tissue. Shown here is the methylation state of CG (ovals) and CNG (squares) sites in individual clones obtained by cloning a 333-bp distal enhancer region of *P1-wr*. Genotypes compared were homozygous *P1-wr/P1-wr; ufo1/ufo1* (designated as *P1-wr; ufo1*) and an  $F_1$  generation of the cross *P1-wr/P1-wr; ufo1/ufo1  $\times$  *p1-wr/p1-wr; Ufo1/Ufo1* showing *Ufo1*-induced pericarp pigmentation (designated as *P1-wr; Ufo1*). Gene structure of *P1-wr* is shown at the top and represents a complete 12,596-bp single *P1-wr* repeat. Transcription start site is indicated by a bent arrow. Boxes superimposed on the solid line represent three exons marked as E1, E2, E3; white portions of E1 and E3 represent UTRs. Probe fragment 15 is shown at the top of the gene structure. The striped box at the bottom of the gene structure represents the 333-bp distal enhancer region subjected to genomic bisulfite sequencing; coordinates of the region and density of CG and CNG sites is shown in the blown-up image. The first row (indicated by black ovals and squares) represents the position of CG and CNG sites in the region, respectively. Clones with all the CG and CNG sites lacking methylation are designated as unmethylated while those with most of these sites methylated are referred to as hypermethylated. Mosaic clones are those having a mixture of methylated and unmethylated CG and CNG sites. Absence of some CG and CNG sites in some of the cells indicates the presence of sequence polymorphisms among *P1-wr* copies.*

pigmented phenotype. Furthermore, it may also reduce the disparity of epigenetic states of *PI-wr* in the pericarp cells and gametes, thereby contributing to enhanced meiotic inheritance of the mutant phenotype.

Since recurrent selection of individuals with intense and uniform pigmentation was performed, one could argue that such selection lead to accumulation of modifiers resulting in increased penetrance and expressivity. Indeed, in maize, poor penetrance of certain mutant phenotypes has also been attributed to the suppressors/enhancers present in the background (SATO *et al.* 1999; VEGA *et al.* 2002). Therefore, the improved penetrance of the *Ufo1*-induced pericarp pigmentation phenotype in BC<sub>5</sub>F<sub>1</sub> as compared to the F<sub>1</sub> population could be due to accumulation of favorable modifiers. However, a testcross of BC<sub>6</sub>F<sub>1</sub> should produce individuals with genetic makeup as well as phenotypic penetrance identical to the F<sub>1</sub> population. Since the testcross progeny also showed high (~65%) penetrance of *Ufo1*-induced pigmentation phenotype, it seems that the increased penetrance here is not solely due to accumulation of modifiers.

*Ufo1* mutation has not been cloned and thus the molecular nature of this modifier remains elusive. On the basis of this study and past studies (CHOPRA *et al.* 2003; SEKHON *et al.* 2007; ROBBINS *et al.* 2008), it seems that a function of *ufo1* (wild type) is to maintain a tissue-specific expression pattern of *PI-wr* by modulating its chromatin state during development. Disruption of the *PI-wr* chromatin state in *Ufo1* (mutant) background results in ectopic expression in pericarp and other tissues. Once the epigenetic state of the *PI-wr* locus is destabilized in the *Ufo1* mutant background, it may either go back to its normal state and produce wild-type phenotype or maintain the altered expression pattern. Selection of plants showing pericarp pigmentation phenotype helps in perpetuation of the altered *PI-wr* chromatin state and the absence of the *ufo1* function in these plants causes further destabilization of the epigenetic state over generations. To test this hypothesis, the chromatin state of the *PI-wr* repeat complex needs to be studied through chromatin immunoprecipitation analysis.

We thank David Braun and Michael Robbins for helpful suggestions and critical reading of the manuscript. This work was supported by a National Science Foundation award MCB-0619330, a Hatch 4154 award to S.C., and dissertation award from the College of Agricultural Sciences and Research Assistantships from the Department of Crop and Soil Sciences and Plant Biology Graduate Program (to R.S.S.).

#### LITERATURE CITED

- ALLEMAN, M., L. SIDORENKO, K. MCGINNIS, V. SESHADRI, J. E. DORWEILER *et al.*, 2006 An RNA-dependent RNA polymerase is required for paramutation in maize. *Nature* **442**: 295–298.
- BARLOW, J. J., A. P. MATHIAS, R. WILLIAMSON and D. B. GAMMACK, 1963 A simple method for the quantitative isolation of undegraded high molecular weight ribonucleic acid. *Biochem. Biophys. Res. Commun.* **13**: 61–66.
- BENDER, J., 2004 Chromatin-based silencing mechanisms. *Curr. Opin. Plant Biol.* **7**: 521–526.
- BIRD, A., 2002 DNA methylation patterns and epigenetic memory. *Genes Dev.* **16**: 6–21.
- BODDU, J., C. SVABEK, R. SEKHON, A. GEVENS, R. NICHOLSON *et al.*, 2004 Expression of a putative flavonoid 3'-hydroxylase in sorghum mesocotyls synthesizing 3-deoxyanthocyanidin phytoalexins. *Physiol. Mol. Plant Pathol.* **65**: 101–113.
- BRINK, R. A., and E. D. STYLES, 1966 A collection of pericarp factors. *Maize Genet. Coop. News Lett.* **40**: 149–160.
- CAO, X., and S. E. JACOBSEN, 2002 Locus-specific control of asymmetric and CpNpG methylation by the DRM and CMT3 methyltransferase genes. *Proc. Natl. Acad. Sci. USA* **99**(Suppl 4): 16491–16498.
- CHAN, S. W., I. R. HENDERSON and S. E. JACOBSEN, 2005 Gardening the genome: DNA methylation in *Arabidopsis thaliana*. *Nat. Rev. Genet.* **6**: 351–360.
- CHOPRA, S., P. ATHMA, X. G. LI and T. PETERSON, 1998 A maize Myb homolog is encoded by a multicopy gene complex. *Mol. Gen. Genet.* **260**: 372–380.
- CHOPRA, S., P. ATHMA and T. PETERSON, 1996 Alleles of the maize P gene with distinct tissue specificities encode Myb-homologous proteins with C-terminal replacements. *Plant Cell* **8**: 1149–1158.
- CHOPRA, S., S. M. COCCIOLOONE, S. BUSHMAN, V. SANGAR, M. D. McMULLEN *et al.*, 2003 The maize *Unstable factor for orange1* is a dominant epigenetic modifier of a tissue specifically silent allele of *pericarp color1*. *Genetics* **163**: 1135–1146.
- COCCIOLOONE, S. M., S. CHOPRA, S. A. FLINT-GARCIA, M. D. McMULLEN and T. PETERSON, 2001 Tissue-specific patterns of a maize Myb transcription factor are epigenetically regulated. *Plant J.* **27**: 467–478.
- COE, E. H., M. G. NEUFFER and D. A. HOISINGTO, 1988 The genetics of corn, pp. 81–258 in *Corn and Corn Improvement*, edited by G. F. SPRAGUE and J. DUDLEY. American Society of Agronomy, Madison, WI.
- FINNEGAN, E. J., W. J. PEACOCK and E. S. DENNIS, 1996 Reduced DNA methylation in *Arabidopsis thaliana* results in abnormal plant development. *Proc. Natl. Acad. Sci. USA* **93**: 8449–8454.
- FOSS, H. M., C. J. ROBERTS, K. M. CLAEYS and E. U. SELKER, 1993 Abnormal chromosome behavior in *Neurospora* mutants defective in DNA methylation. *Science* **262**: 1737–1741.
- GEHRING, M., and S. HENIKOFF, 2007 DNA methylation dynamics in plant genomes. *Biochim. Biophys. Acta* **1769**: 276–286.
- GOLL, M. G., and T. H. BESTOR, 2005 Eukaryotic cytosine methyltransferases. *Annu. Rev. Biochem.* **74**: 481–514.
- GREENBLATT, I. M., 1985 The pericarp of maize: a major tool to study transposable elements, pp. 405–417 in *Plant Genetics*, edited by M. FREELING. Alan R. Liss, New York.
- GROTEWOLD, E., P. ATHMA and T. PETERSON, 1991 Alternatively spliced products of the maize P gene encode proteins with homology to the DNA-binding domain of myb-like transcription factors. *Proc. Natl. Acad. Sci. USA* **88**: 4587–4591.
- GROTEWOLD, E., M. CHAMBERLIN, M. SNOOK, B. SIAME, L. BUTLER *et al.*, 1998 Engineering secondary metabolism in maize cells by ectopic expression of transcription factors. *Plant Cell* **10**: 721–740.
- GROTEWOLD, E., and T. PETERSON, 1994 Isolation and characterization of a maize gene encoding chalcone flavonone isomerase. *Mol. Gen. Genet.* **242**: 1–8.
- HANSEN, R. S., C. WIJMEGA, P. LUO, A. M. STANEK, T. K. CANFIELD *et al.*, 1999 The DNMT3B DNA methyltransferase gene is mutated in the ICF immunodeficiency syndrome. *Proc. Natl. Acad. Sci. USA* **96**: 14412–14417.
- HOEKENG, O. A., M. G. MUSZYNSKI and K. C. CONE, 2000 Developmental patterns of chromatin structure and DNA methylation responsible for epigenetic expression of a maize regulatory gene. *Genetics* **155**: 1889–1902.
- JACOBSEN, S. E., H. SAKAI, E. J. FINNEGAN, X. CAO and E. M. MEYEROWITZ, 2000 Ectopic hypermethylation of flower-specific genes in *Arabidopsis*. *Curr. Biol.* **10**: 179–186.
- JEDDELOH, J. A., T. L. STOKES and E. J. RICHARDS, 1999 Maintenance of genomic methylation requires a SWI2/SNF2-like protein. *Nat. Genet.* **22**: 94–97.
- KAKUTANI, T., 1997 Genetic characterization of late-flowering traits induced by DNA hypomethylation mutation in *Arabidopsis thaliana*. *Plant J.* **12**: 1447–1451.

- KATO, M., A. MIURA, J. BENDER, S. E. JACOBSEN and T. KAKUTANI, 2003 Role of CG and non-CG methylation in immobilization of transposons in *Arabidopsis*. *Curr. Biol.* **13**: 421–426.
- KLOSE, R. J., and A. P. BIRD, 2006 Genomic DNA methylation: the mark and its mediators. *Trends Biochem. Sci.* **31**: 89–97.
- LECHELT, C., T. PETERSON, A. LAIRD, J. CHEN, S. L. DELLAPORTA *et al.*, 1989 Isolation and molecular analysis of the maize *P* locus. *Mol. Gen. Genet.* **219**: 225–234.
- LI, E., T. H. BESTOR and R. JAENISCH, 1992 Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* **69**: 915–926.
- LU, Y., T. RONG and M. CAO, 2008 Analysis of DNA methylation in different maize tissues. *J. Genet. Gen.* **35**: 41–48.
- MALAGNAC, F., B. WENDEL, C. GOYON, G. FAUGERON, D. ZICKLER *et al.*, 1997 A gene essential for de novo methylation and development in *Ascombolus* reveals a novel type of eukaryotic DNA methyltransferase structure. *Cell* **91**: 281–290.
- MARTIENSSSEN, R., A. BARKAN, W. C. TAYLOR and M. FREELING, 1990 Somatic heritable switches in the DNA modification of *Mu* transposable elements monitored with a suppressible mutant in maize. *Genes Dev.* **4**: 331–343.
- MATZKE, M. A., and A. J. MATZKE, 1991 Differential inactivation and methylation of a transgene in plants by two suppressor loci containing homologous sequences. *Plant Mol. Biol.* **16**: 821–830.
- MATZKE, M. A., and A. J. MATZKE, 1998 Epigenetic silencing of plant transgenes as a consequence of diverse cellular defence responses. *Cell. Mol. Life Sci.* **54**: 94–103.
- MCGOWAN, R., R. CAMPBELL, A. PETERSON and C. SAPIENZA, 1989 Cellular mosaicism in the methylation and expression of hemizygous loci in the mouse. *Genes Dev.* **3**: 1669–1676.
- MESSING, J., A. K. BHARTI, W. M. KARLOWSKI, H. GUNDLACH, H. R. KIM *et al.*, 2004 Sequence composition and genome organization of maize. *Proc. Natl. Acad. Sci. USA* **101**: 14349–14354.
- MIURA, A., S. YONEBAYASHI, K. WATANABE, T. TOYAMA, H. SHIMADA *et al.*, 2001 Mobilization of transposons by a mutation abolishing full DNA methylation in *Arabidopsis*. *Nature* **411**: 212–214.
- OKANO, M., D. W. BELL, D. A. HABER and E. LI, 1999 DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* **99**: 247–257.
- POETHIG, R. S., 1990 Phase change and the regulation of shoot morphogenesis in plants. *Science* **250**: 923–930.
- POETHIG, R. S., 2003 Phase change and the regulation of developmental timing in plants. *Science* **301**: 334–336.
- RICHARDS, E. J., 2006 Inherited epigenetic variation—revisiting soft inheritance. *Nat. Rev. Genet.* **7**: 395–401.
- ROBBINS, M. L., R. S. SEKHON, R. MEELEY and S. CHOPRA, 2008 A mutator transposon insertion is associated with ectopic expression of a tandemly repeated multicopy Myb gene pericarp color1 of maize. *Genetics* **178**: 1859–1874.
- RONEMUS, M. J., M. GALBIATI, C. TICKNOR, J. CHEN and S. L. DELLAPORTA, 1996 Demethylation-induced developmental pleiotropy in *Arabidopsis*. *Science* **273**: 654–657.
- SAGHA-MAROOF, M. A., K. M. SOLIMAN, R. A. JORGENSEN and R. W. ALLARD, 1984 Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proc. Natl. Acad. Sci. USA* **81**: 8014–8018.
- SATO, Y., N. SENTOKU, Y. MIURA, H. HIROCHIKA, H. KITANO *et al.*, 1999 Loss-of-function mutations in the rice homeobox gene *OSH15* affect the architecture of internodes resulting in dwarf plants. *EMBO J.* **18**: 992–1002.
- SCHOB, H., and U. GROSSNIKLAS, 2006 The first high-resolution DNA “methylome”. *Cell* **126**: 1025–1028.
- SCHWARZ-SOMMER, Z., N. SHEPHERD, E. TACKE, A. GIERL, W. ROHDE *et al.*, 1987 Influence of transposable elements on the structure and function of the *Al* gene of *Zea mays*. *EMBO J.* **6**: 287–294.
- SEKHON, R. S., T. PETERSON and S. CHOPRA, 2007 Epigenetic modifications of distinct sequences of the *p1* regulatory gene specify tissue-specific expression patterns in maize. *Genetics* **175**: 1059–1070.
- SINGER, T., C. YORDAN and R. A. MARTIENSSSEN, 2001 Robertson’s *Mutator* transposons in *A. thaliana* are regulated by the chromatin-remodeling gene *Decrease in DNA Methylation (DDM1)*. *Genes Dev.* **15**: 591–602.
- STYLES, E. D., and O. CESKA, 1977 The genetic control of flavonoid synthesis in maize. *Can. J. Genet. Cytol.* **19**: 289–302.
- STYLES, E. D., and O. CESKA, 1989 Pericarp flavonoids in genetic strains of *Zea mays*. *Maydica* **34**: 227–237.
- TARIQ, M., and J. PASZKOWSKI, 2004 DNA and histone methylation in plants. *Trends Genet.* **20**: 244–251.
- VEGA, S. H., M. SAUER, J. A. ORKWISZEWSKI and R. S. POETHIG, 2002 The early phase change gene in maize. *Plant Cell* **14**: 133–147.
- VONGS, A., T. KAKUTANI, R. A. MARTIENSSSEN and E. J. RICHARDS, 1993 *Arabidopsis thaliana* DNA methylation mutants. *Science* **260**: 1926–1928.
- WATERSON, J. J., and G. L. BUTLER, 1983 Occurrence of an unusual leucoanthocyanidin and absence of proanthocyanidins in sorghum leaves. *J. Agric. Food Chem.* **31**: 41–45.
- WIENAND, U., U. WEYDEMANN, U. NIESBACH-KLOSSEN, P. A. PETERSON and H. SAEDLER, 1986 Molecular cloning of the *c2* locus of *Zea mays*, the gene coding for chalcone synthase. *Mol. Gen. Genet.* **203**: 202–207.
- WOODHOUSE, M. R., M. FREELING and D. LISCH, 2006 The *mop1* (*mediator of paramutation1*) mutant progressively reactivates one of the two genes encoded by the *MuDR* transposon in maize. *Genetics* **172**: 579–592.
- XU, G. L., T. H. BESTOR, D. BOURC’HIS, C. L. HSIEH, N. TOMMERUP *et al.*, 1999 Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. *Nature* **402**: 187–191.
- ZHANG, P., 1999 Molecular characterization of myb-homologous transcription factors of the flavonoid pathway in *Zea mays*. Ph.D. Thesis, Iowa State University, Ames, IA.
- ZHANG, X., J. YAZAKI, A. SUNDARESAN, S. COKUS, S. W. CHAN *et al.*, 2006 Genome-wide high-resolution mapping and functional analysis of DNA methylation in *Arabidopsis*. *Cell* **126**: 1189–1201.
- ZILBERMAN, D., M. GEHRING, R. K. TRAN, T. BALLINGER and S. HENIKOFF, 2007 Genome-wide analysis of *Arabidopsis thaliana* DNA methylation uncovers an interdependence between methylation and transcription. *Nat. Genet.* **39**: 61–69.

Communicating editor: D. VOYTAS