Imprinting of *R-r*, paramutation of *B-I* and *Pl*, and epigenetic silencing of *MuDR/Mu* transposons in *Zea mays* L. are coordinately affected by inbred background

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Summary

The extent of imprinting at *R-r*, frequency of paramutation at *B-Intense* and *Pl*, and epigenetic silencing of *Mu* transposons were evaluated in the W23 and A188 inbred lines of maize. All types of epigenetic phenomena affecting these loci of the anthocyanin pathway occurred more frequently in the W23 inbred line. Absence of down-regulation was dominant in F1 hybrid progeny. Identical alleles programme lower anthocyanin accumulation in A188 than in W23, and A188 plants develop more rapidly than W23. The possibilities that specific genetic factors, intrinsic gene expression levels and/or the rapidity of the life cycle modulate epigenetic gene controls are discussed.

1. Introduction

The transcriptional output of all loci depends on *trans*-acting factors required for RNA production. In addition the alleles of some loci acquire quasi-stable activity states that act *in cis* to modify the expected phenotype by modulating transcription (Wolffe & Matzke, 1999; Chandler *et al*., 2000). These epigenetic expression states yield a range of phenotypes from a single allele – a potentially rich source of variation. Such variation can contribute profoundly to phenotypes observed in natural species: the bilaterally symmetrical flowers of *Linaria vulgaris* switch to radial symmetry when the *Lcyc* gene is transcriptionally silenced and heavily methylated (Cubas *et al*., 1999). As with many examples of epigenetic modification, 'reversions' occur sporadically that restore the standard phenotype.

Epigenetic phenomena are currently classified on the basis of their stability, allele requirements and involvement of DNA methylation. Imprinting is a phylogenetically widespread process in which the parental origin of an allele determines the extent of expression in the next generation; imprinted alleles are restored to full expression capacity during organismal development (Wolffe & Matzke, 1999). Imprinting typically reflects differential methylation of an allele in the maternal and paternal gametes (or their progenitor cells). For example, imprinting of the *medea* locus of *Arabidopsis* requires the maintenance DNA methylation activity contributed by DDM1 (Vielle-Calzada *et al*., 1999).

Paramutation, in contrast to imprinting, is a mitotically and meiotically heritable alteration in the expression of particular alleles caused by other alleles of the same locus (Brink, 1973). Paramutation violates Mendel's first law that alleles are unmodified in a heterozygote. Recently, paramutation of susceptible maize *r1* alleles was shown to correlate with methylation, which is centred on a short promoter segment similar to the *doppia* transposon (Walker, 1998). Paramutation of affected *R* alleles is stably inherited for many generations, but reversions to higher activity do occur. The *b* locus is a functional duplicate of *r1* (Chandler *et al*., 1989); it also undergoes paramutation, but the decreased expression of the resulting *B′* alleles is permanent and does not involve detectable methylation changes (Patterson *et al*., 1993). Paramutation of *B-I* in a homozygote can occur spontaneously (Patterson *et al*., 1995); however, the standard assay is in a *B-I//B′* heterozygote. On test crossing *B-I//B′* to *b*, all progeny are *B′*. The new *B′* allele (old *B-I* allele) is stably altered, and it has acquired secondary paramutation, the ability to paramutate naïve *B-I* alleles with 100% efficiency (Patterson *et al*., 1993). A third case of paramutation

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involves the *Pl* locus (Hollick *et al*., 1995; Hoekenga *et al*., 2000), which encodes a myb-related protein that in concert with *B* or *R* directs anthocyanin pigmentation (Cone *et al*., 1993). The *Pl-Rhoades* allele confers dark purple pigmentation, but it can spontaneously paramutate to *Pl′-mahogany*. Diverse *Pl′-mah* alleles vary in anther pigment intensity and exhibit high-efficiency secondary paramutation (Hollick *et al*., 1995).

Transposon silencing is a third case of epigenetic control. Somatic mutability of *Mu* transposon-induced alleles is frequently lost (Walbot, 1986). The multi-copy, dispersed *Mu* and transposase-encoding *MuDR* elements of maize are coordinately methylated coincident with silencing (Chandler & Walbot, 1986; reviewed in Walbot & Rudenko, 2001). Once silenced, inactive *MuDR*/*Mu* elements rarely reactivate spontaneously (Walbot & Stapleton, 1998). Introduction of a transcriptionally active copy of *MuDR* (Lisch *et al*., 1995) or a MURA transposase transgene (Raizada & Walbot, 2000) can reactivate somatic instability and demethylation of *Mu* terminal inverted repeats, presumably by binding to a motif within the terminal inverted repeats of this family of transposons (Benito & Walbot, 1997). Transposon silencing is distinct from imprinting and paramutation: the frequency of inactivation and reactivation is highly variable and dispersed *Mu* elements are involved. Furthermore, the *MuDR* transcription units are both a target for silencing and encode the MURA transposase that acts *in trans* to restore element activity.

The precise mechanisms underlying imprinting, paramutation and transposon silencing remain elusive, and these processes are currently viewed as separate phenomena. As discussed above, methylation is inconsistently involved, but it is hypothesized to follow rather than cause transposon silencing (Fedoroff & Chandler, 1994; Walbot & Rudenko, 2001). Furthermore, in *mom* mutants, highly methylated *Arabidopsis* alleles are transcriptionally reactivated although methylation levels remain high (Amedeo *et al*., 2000). Given the variable requirement for methylation, alteration in chromatin configuration is the favoured hypothesis to explain the imposition and maintenance of the lowered transcription observed from imprinted and paramutated alleles and epigenetically silenced transposons (Wolffe & Matzke, 1999). Tests to determine whether these three processes are co-regulated could elucidate whether there are shared regulatory components.

2. Materials and methods

(i) *Maize strains*

A fully purple W23 inbred line (*R-r C1 B-Intense Pl* with functional alleles of the structural genes for pigment synthesis) and a near-colourless A188 inbred line (*r-r c1 b pl* plus functional structural genes) were the starting material. It is likely that the *Pl* allele is *Pl-Rhoades* (Hollick *et al*., 1995) but this has not been proven; the derivative *Pl* alleles are therefore referred to as *Pl′*, although they have the phenotypes described for *Pl′-mah* alleles. The A188 stock originally had weak pigmentation restricted to the anthers (from the functional–*r* component of the compound *r-r* locus). The dominant, functional alleles of the four regulatory loci in the purple W23 stock were introduced into A188 as described in Bodeau & Walbot (1995). After a minimum of 4 backcrosses, the resulting lines are $>93\%$ A188 with A188 cytoplasm. J. Kermicle (University of Wisconsin, Madison, WI) provided separately derived *r-g* and *R-r*:*standard* maize lines in the A188 inbred. The Maize Genetics Cooperation Stock Center provided *B Pl*, *b Pl* and *b pl* stocks in an unspecified background; these lines are listed as Coop in the text. The *bz2::Mu1-mu1* (McLaughlin & Walbot, 1987), *bz2::MuDR-mu4* (Hershberger *et al*., 1991) and *bz1::Mu1-mu1* (Britt & Walbot, 1991) alleles were derived from Robertson's purple Mutator line (*R-r C1 B Pl*) crossed to *bz1* or *bz2* in the W23 inbred; mutable alleles were propagated by repeatedly crossing into the W23 background for at least 7 generations. When *bz1* and *bz2* tester lines in the A188 background were available, the mutable alleles were introgressed into this background for four generations. Except as noted, materials were grown at Stanford, CA.

(ii) *Anthocyanin measurements*

One square centimetre blocks of tissue were pooled from 10 phenotypically identical individuals in a family; the tissue pools were weighed and then macerated briefly and extracted overnight in 20 ml of methanol acidified with 1% HCl (v/v). The supernatant was cleared by centrifugation at 300 ***g*** for 5 min. Absorbance was measured in a plate reader spectrophotometer (BioRad); highly pigmented samples were diluted with acidified methanol to reduce peak absorption at $A_{530\text{nm}}$ to $<0{\cdot}3$, well within the linear range. Duplicate or triplicate biological samples were each measured twice.

Visual inspection for paramutation in adult leaves and stem was performed weekly. In the W23 background, *B-I Pl* plants are purple-black in all visible tissues (Patterson *et al*., 1993); spontaneous paramutation to *B′* results in mainly green tissue with occasional light purple blotches as is found in directed paramutation. In the A188 background, *B-I Pl* plants are less intensely purple than the W23 coloration. The tassel stem tissue and the glumes (bract-like organs surrounding the flowers) were inspected for pig-

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mentation at the beginning of pollen shed. Anthers were scored on 2 days for plants showing any evidence of *Pl* paramutation by assessing pigment intensity on 10–20 newly emerging anthers as suggested by Hollick *et al*. (1995).

The extent of imprinting at *R-r* transmitted through pollen was scored on a numerical scale, with 1 equalling no pigment (*r-g* kernel reference) and 5 equalling uniform purple colour as found with female transmission of *R-r*. Two people each scored a minimum of 14 ears of each genotype for imprinting, and the values were averaged.

(iii) *Morphometric analysis*

B-I Pl purple lines in the W23 and A188 backgrounds were grown in contiguous rows in 1997 and 1999. At weekly intervals, 20 individuals were evaluated for plant height, the number of visible leaves, and the length and width of juvenile leaf 8 and adult leaves 11 and 14. The dates when the tassel was first visible, of pollen shed and of silk emergence were also recorded for each plant. Average values are reported. Similar measurements were made on *bz2 B-I Pl* individuals in the two inbred lines; results were similar but are not shown.

3. Results

(i) *Frequency of spontaneous paramutation of B-I and of Pl-Rhoades depends on the background*

Although directed paramutation involving an inducing and responding allele is about 100% effective (Patterson *et al*., 1995), spontaneous paramutation in the W23 background was reported in 1–15% of homozygous *B-I* (Patterson *et al*., 1995) or *Pl-Rhoades* (Hollick *et al*., 1995) plants. Details on the timing of spontaneous paramutation were not reported. As shown in Table 1, in a population of 144 more than 40% of the *B-I Pl* homozygotes exhibited paramutation at one or both loci. Approximately 10% of the individuals were affected in adult stem and leaf (sheath and blade) tissues. A much higher frequency of paramutation was observed within the tassel, where 24% had a *B′* phenotype of green rather than purple tissue, 10% had the *Pl′* phenotype of reddish rather than purple, and a few individuals were *B′ Pl′* (green tassel, yellow anthers). These results indicate that paramutation occurs preferentially late in the life cycle in the W23 background and independently in *B* and *Pl*. Cumulatively, the frequency of paramutation of *B-I* was 33% and that of *Pl* was about half as frequent (14%) in this experiment. Paramutation of *B-I* was assessed in four prior years, with an overall frequency of 24·8% (Table 2).

Table 2. *Frequency of B-I paramutation in the W23 background*

Year[a]	*n*	B′ (%)
1991	90	10 (11·1)
1994	35	9 (25·7)
1995	70	13 (18·6)
1996	31	7 (22·5)
Total	226	56 (24·8)

[a] Materials were grown at Stanford, CA, except in 1995 when the scoring was done on Molokai, HA.

A subset of *B′ Pl′*, *B′* and *Pl′* individuals were self-pollinated and outcrossed as pollen parent to *b pl* tester lines. In all cases, the progeny exhibited the paramutant phenotype (data not shown). In most self-pollinations, the sheath tissue surrounding the ear was *B-I Pl* in phenotype; however, progeny exhibited the *B′ Pl*, *B-I Pl′* or *B′ Pl′* phenotype of the tassel. Therefore, the spontaneous paramutation events are both heritable and exhibit 100% secondary paramutation, because the new *B′* and *Pl′* alleles acquired

Table 1. *Frequency of paramutation at B-I and Pl in the W23 and A188 inbred backgrounds assessed in vegetative and floral tissues*

Phenotypic expression	Organs affected	W23 ($n = 144$)	A188 ($n = 303$)	[A188 × W23] ($n = 66$)
B-I Pl	Uniform in plant	83 (57·6%)	293 (96·7%)***	60 (90·9%)***
B′ Pl	Adult stem and leaves	11 (7·6%)	0	1 (1·5%)
B-I Pl′	Adult stem and leaves	2 (1·4%)	0	0
B′Pl′	Adult stem and leaves	2 (1·4%)	0	0
B′ Pl	Tassel stem tissue	30 (20·8%)	10 (3·3%)***	5 (7·8%)***
B-I Pl′	Tassel and anthers	11 (7·6%)	0	0
B′ Pl′	Tassel and anthers	5 (3·5%)	0	0

The maintenance of full pigmentation in the entire plant or within the tassel is significantly different ($P < 0{\cdot}001$) in the W23 background compared with either A188 or the A188 (egg parent) × W23 hybrid, based on a 2 × 2 contingency test and the χ^2 analysis.

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Table 3. *Anthocyanin content of adult leaf blade tissues*

Family	Nuclear background	Allele status	Anthocyanin content[a]
Q1	W23	*B-I Pl*	0·191 ± 0·01
Q2	A188	*B-I Pl*	0·088 ± 0·006
Q3	W23 × A188	*B-I Pl*	0·092 ± 0·01
Q7	A188 × W23	*B-I Pl*	0·072 ± 0·04
Q10	W23 × Coop	*B-I Pl*	0·293 ± 0·01
Q11	A188 × Coop	*B-I Pl*	0·189 ± 0·01
Q1	W23	*B′ Pl*	0·008 ± 0·001
Q6	W23 × A188 (*b*)	*B′/b Pl*	0·002 ± 0·001
Q9	W23 × Coop	*B′/b Pl*	0·043 ± 0·001

[a] Absorbance of a methanolic extract at 530 nm reported in units per gram of tissue. The standard error is shown.

the ability to down-regulate a *B-I* allele or *Pl* allele in the heterozygotes generated by self-pollination. Acquisition of this key characteristic indicates that spontaneous paramutation generates paramutagenic alleles indistinguishable from those resulting from directed paramutation (Patterson *et al*., 1993, 1995).

In contrast to the high frequency of paramutation in the W23 background, the same alleles were almost immune from this epigenetic regulation in the A188 inbred line. Spontaneous paramutation of *B-I* to *B′* in A188 was restricted to the tassel stem tissue, and the frequency was 6-fold lower than in W23 tassels. The new *B′* alleles in the A188 background were heritable and capable of secondary paramutation (data not shown). No instances of *Pl′* were observed in 303 plants scored. Given the lower intrinsic pigmentation level in A188, it is possible that some subtle changes in *B-I* or *Pl* might have been overlooked in the adult leaves.

(ii) *Anthocyanin pigment content depends on genotype and inbred line background*

An intensely purple W23 stock was used to derive an A188 line with the identical anthocyanin regulatory alleles: the helix-loop-helix factors *R-r* and *B-Intense* and the myb-type factors *Pl* and *C1* (Bodeau & Walbot, 1995). As these dominant alleles were introgressed into the A188 background, the intensity of anthocyanin pigmentation decreased. As shown in Table 3, after the fourth generation of introgression the resulting line had approximately half (0·088 $A_{530\mathrm{nm}}$ units) the leaf blade pigmentation level of the W23 line (0·191 units). As expected (Coe et al., 1988), near-isogenic lines containing *pl* had reduced pigment levels (data not shown). Crossing either the A188 or W23 *B-I Pl* lines to a *B Pl* Coop background nearly doubled leaf anthocyanin. Collectively, these results suggest that anthocyanin accumulation is modulated by unknown factors in these inbred lines.

Spontaneous paramutation of *B-Intense* to *B′* reduced leaf pigment approximately 20-fold in the W23 background (Table 3). Crossing a paramutated *B′* allele in W23 to the *b Pl* tester stock in A188 resulted in a further 4-fold reduction in pigment; in contrast, crossing to the *b Pl* Coop background, increased anthocyanin content 5-fold compared with the *B′* W23 level. These results confirm that anthocyanin accumulation, whether programmed by a *B-I* or *B′* allele, depends on the inbred line in which it is measured. F1 hybrids between W23 and A188 show intermediate pigment levels (Table 3) but are quite similar to the A188 parent in paramutation behaviour (Table 1). Therefore, the control of pigment level and frequency of paramutation appear to be dominant factors present in the A188 background.

(iii) *Imprinting at R-r is lower in the A188 background*

An important historic example of imprinting involves the *r1* locus of maize. Functional *R* alleles encodes a basic helix–loop–helix protein that is required for the transcriptional activation of the anthocyanin biosynthetic pathway (Ludwig *et al*., 1989); when the standard *R-r* allele is transmitted through the female gametophyte, the aleurone epidermal layer of triploid progeny endosperm is intensely and uniformly pigmented. In contrast, the standard *R-r* allele is imprinted in the male parent such that pollen transmission results in colourless aleurones with sectors of purple pigmentation. These sectors reflect somatic activation of *R* expression during endosperm development (Kermicle & Alleman, 1990). The pollen-derived *R-r* allele is expressed fully in subsequent generations if transmitted through the egg parent, indicating that no permanent alteration in expressivity was conditioned by transmission through pollen in a previous generation.

Seed coloration, regulated by the *R-r* and *C1* alleles, is very dark purple in the W23 background but lavender purple in the A188 line (Table 4). This intensity difference parallels what was observed in leaf pigmentation (Table 3). W23 *R-r C1* lines crossed as pollen parent to various *r-g C1 b pl* testers exhibit classic imprinting (Kermicle, 1970). In a visual scoring system (see Section 2), the imprinting score ranged from 2·5 (approximately 40% purple sectors on a colourless field) to 4·0 (75% purple); in all cases, mottling was evident at a glance. In contrast the A188 lines averaged 4·25 (approximately 85% purple), and many individual kernels appeared to be uniformly purple until examined microscopically.

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Table 4. *Impact of inbred background on seed coloration programmed by an imprinted* R-r *allele*

Inbred line and genotype of pollen	*r-g C1 b pl* tester background	Anthocyanin imprinting	Colour intensity score[a]
W23 *B-I Pl R-r C1*	A188	2·50 ± 0·3	Purple
W23 *B′ Pl R-r C1*	A188	3·25 ± 0·3	Purple
W23 *B-I Pl R-r C1*	W23	3·75 ± 0·5	Purple
W23 *B′ Pl R-r C1*	W23	2·75 ± 0·3	Purple
W23 *B-I Pl R-r C1*	[W23 × K55] hybrid	3·75 ± 0·5	Purple
W23 *B′ Pl R-r C1*	[W23 × K55] hybrid	4·00 ± 0·3	Purple
A188 *B-I Pl R-r C1*	A188	4·25 ± 0·5	Lavender
A188 *B-I Pl R-r C1*	W23	4·00 ± 0·5	Lavender
A188 *B-I Pl R-r C1*	[W23 × K55] hybrid	4·50 ± 0·5	Lavender

[a] Colourless *r-g B-I pl* tester is 1, a score of 2 is a mainly colourless field up to 25% purple (strong imprinting), 3 equals up to 50% purple (moderate imprinting), 4 equals up to 75% purple (near full colour, light imprinting), and 5 equals fully purple (no imprinting). Standard deviation is indicated. For additional analysis, the W23 *B-I* and *B′* values were combined. In pairwise comparisons using a 2 × 2 contingency test, pollen transmission of *R-r* in a W23 background to the A188 or W23 or [W23 × K55] hybrid *r-g* testers resulted in significantly less coloration ($P < 0·05$) than transmission of the same allele in A188 pollen onto these tester lines.

Table 5. *Frequency of spontaneous loss of somatic mutability in the aleurone*

	Crossed as ear parent		Crossed as pollen parent	
Reporter allele[a]	W23	A188	W23	A188
bz2::Mu1-mu1	21/50 (42%)***	1/48 (2%)	39/50 (78%)***	3/48 (6%)
bz2::MuDR-mu4	9/44 (20%)*	2/39 (5%)	27/39 (69%)***	2/39 (5%)
bz1::Mu1-mu1	7/50 (14%)*	1/43 (2%)	21/50 (42%)***	2/43 (5%)

[a] Plants heterozygous for each reporter allele were crossed reciprocally to either *bz2* or *bz1* tester lines in the W23 or the A188 backgrounds. Half the progeny kernels should be somatically mutable with numerous purple spots; ears with fewer than 20% such spotted kernels were scored as losing Mutator activity. The differences between W23 and A188 are significant for all allele comparisons using a 2 × 2 contingency test. For W23, the differences between ear and pollen parentage are very significant ($P < 0·001$) for *bz2::Mu1-mu1* and *bz2::MuDR-mu4* and highly significant ($P < 0·01$) for *bz1::Mu1-mu1*.

(iv) *Somatic mutability of Mu reporter alleles is preserved more often in the A188 background*

Persistence of somatic instability at three anthocyanin reporter alleles was examined in the W23 background (Table 5). In 50 reciprocal crosses of *bz2::Mu1-mu1/bz2* to a *bz2* tester, 42% of the ear progeny and 78% of the pollen progeny were losing Mutator activity; as previously reported, spontaneous loss of mutability is more frequent through the pollen (Walbot, 1986). *MuDR/Mu* transposon silencing was lower in the *bz2::MuDR-mu4/bz2* and *bz1::Mu1-mu1/bz1* stocks, but there was the same large bias for loss through pollen. In contrast to W23, somatic excision was preserved in nearly all A188 individuals with all three reporter alleles, and there was no significant difference between pollen and ear transmission (two by two contingency test).

In F1 hybrids between A188 and W23, loss of Mutator activity was similar to the A188 parent: 5% through the ear and 6% through the pollen in a population of 43 reciprocal crosses. No significant difference was detected when the F1 hybrid was constructed by crossing W23 by A188 as pollen parent (data not shown).

(v) *Morphometric comparison of W23 and A188*

B-I Pl purple lines in the W23 and A188 backgrounds were planted side-by-side in the 1999 summer field and exhibited significant differences in most measures

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Table 6. *Morphometric analysis of W23 and A188*

Character	A188 *B-I* *Pl-Rhoades*	W23 *B-I* *Pl-Rhoades*
Days to leaf 8 emergence	20±2**	30±4
Days to leaf 11	34±3*	42±5
Days to leaf 14	45±5	51±5
Number of leaves	14±1**	18±2
Day when tassel visible	53±3**	69±4
Height (m)	1·1±0·1**	1·6±0·3
Day when pollen shed	59±3**	76±4
Day when silks visible	62±3**	78±5

Standard deviations in units appropriate to the measurement are reported. The Kolmogorov–Smirnov two-sample test was applied to the individual data sets: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

of plant development (Table 6). In particular, the rate of leaf emergence was faster in A188, and only 14 leaves were produced compared with 18 in the W23 background. The leaves of A188 were also approximately 20% narrower and shorter (data not shown). The year 1999 was a cool growing season at Stanford, but A188 produced pollen and silks in 59 and 62 days, respectively; field notes from prior years indicate flowering more typically initiates after 50–53 days in A188 lines. In contrast, W23 required an additional 16–17 days to reach the reproductive stage; plants shed pollen after 76 days, and the silks emerged in 78 days. In warmer years, reproduction commonly occurred after 66–70 days in the W23 background, but was still about 2 weeks slower than in the A188 lines.

Discussion

It is not surprising that regulatory alleles determine quantitatively different phenotypic outcomes in different backgrounds, such as the 4-fold difference in pigment accumulation in adult leaf blades (Table 3) and a visible difference in seed colour: dark purple for W23 and lavender purple for A188. Crosses to the Coop *B Pl* line increased anthocyanin accumulation in both hybrids, further evidence that there are genetic factors beyond those currently identified which influence pigmentation intensity. Genetic variation in these factors, which govern the transcription or action of the proteins encoded by the anthocyanin regulatory genes, probably exists between W23 and A188.

The key result of this study was that three epigenetic processes are similarly affected by inbred background. Spontaneous paramutation of *B-I* and *Pl* are frequent in W23, imprinting of *R-r* is routine, and loss of Mutator activity occurs at a high frequency with several *Mu* reporter alleles. In contrast, in an A188 background the identical alleles have 6-fold less (*B*) or no (*Pl*) paramutation, imprinting is much less severe, and loss of Mutator activity is 4- to 20-fold less frequent. The *B′* alleles of both W23 and A188 are heritable and exhibit secondary paramutation, suggesting that they have undergone similar epigenetic alterations. Also, once Mutator activity is epigenetically silenced in either the W23 (Walbot & Stapleton, 1998) or A188 (data not shown) backgrounds, reactivation is rare. Thus, the outcomes of epigenetic changes are similar in two inbred lines that exhibit very different frequencies of these events.

Hollick *et al.* (1995) hypothesized that *B-I* and *Pl-Rhoades* could be unusual states of high allele activity resulting from human selection. That is, the paramutated status of *B′* and *Pl-mah* could be the more stable expression states of these loci. It is intriguing that the intrinsically lower expression level of *B-I* and *Pl* in A188 is less susceptible to paramutation than the higher expression states of identical alleles in the W23 background. The lower seed pigmentation conditioned by *R-r* in A188 is also correlated with less imprinting during pollen transmission. Many instances of transgene silencing are correlated with high transcriptional activity, which is proposed to invoke RNA-mediated epigenetic silencing (reviewed by Ding, 2000; Fagard & Vaucheret, 2000), which in turn is probably modulated by chromatin changes. When appropriate assays are available it will be interesting to determine whether *B-I*, *Pl* and *R-r* alleles adopt different chromatin configurations in the W23 and A188 inbred lines that result in lower intrinsic expression and a lower frequency of epigenetic changes, including Mutator silencing.

In a recent review, Chandler *et al.* (2000) described three new loci relevant to the relationship between transcriptional activity and paramutation. Recessive alleles of *mop1* (*mediator of paramutation*), *rmr1* (*required to maintain repression*) and *rmr*4 result in higher levels of transcription of *B′* and cause heritable change *Pl′* to *Pl-Rhoades* expression status. *mop1* prevents establishment of *B* paramutation and can reactivate methylated *Mu* elements. The impact of recessive mutations of these loci on imprinting at *R* was not reported. Analysis of allelic differences in W23 and A188 for these newly identified loci could provide a genetic explanation for the results reported here. However, low imprinting, paramutation, and transposon silencing phenotypes of A188 appear to be under dominant genetic control, suggesting that an additional factor(s) contributes.

Developmental differences between W23 and A188 could be a contributing factor. A188 is an unusual inbred that readily forms embryogenic callus in tissue culture (Bodeau & Walbot, 1995); it is one of the parents in the HiII hybrid (A188 × B73) that is commonly used for generating transgenic maize (Armstrong & Green, 1985). In contrast, embryos of W23 do not form embryogenic callus (Bodeau &

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Walbot, 1995). A188 is a short-stature, early flowering line that makes fewer organs and develops more quickly than W23 (Table 6). It is possible, therefore, that plant growth rate or even chronological time alters epigenetic events. Recently, Hoekenga *et al*. (2000) demonstrated that *Pl-Blotched*, an epi-allele of *Pl*, has a more condensed chromatin configuration than *Pl-Rhoades*; furthermore, the closed chromatin state was extended at the transition from seedling to adult growth. Environmental effects have also been noted on paramutation when temperature and light are manipulated in maize seedlings (Mikula, 1995). It is possible that the length of time in the seedling state and/or the duration of adult growth contribute to the imposition, maintenance and transmission of epi-alleles. Furthermore, silencing of *MuDR*/*Mu* elements is progressive, increasing in frequency during growth; late in development, such large sectors occur that apical meristem cells are assumed to be affected (Martienssen & Baron, 1994). These observations are echoed in the higher frequency of Mutator activity maintenance in the ear, which is derived from node 10–12, compared with the tassel, which is derived after production of the last leaf. A188, with far fewer leaves and a much more rapid development, could finish organ differentiation in advance of an age-dependent epigenetic loss of Mutator activity.

The hypothesis that developmental rate and duration contribute to epigenetic alterations should be tested by analysing additional inbred lines that differ in the rate of development. Crosses between distinctive inbred lines can result in hybrid vigour, a confounding factor. For example, flowering time of F1 hybrids of W23 and A188 is accelerated relative to W23, and the hybrids exhibit epigenetic behaviours more similar to the A188 parent. This yields the appearance of dominant genetic control, but could result from developmental changes. The interplay between genetic and developmental controls is complex, because self-pollination of F1 hybrids and/or backcrossing yields few individuals resembling either A188 or W23 in the frequency of epigenetic changes.

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