Characterization of two regulators of aleurone mottling—summary of research initiated at the University of Wisconsin.

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Kernel pigmentation in certain accessions of open-pollinated varieties from the Four Corners region of Southwest US is exceptional in that mottled aleurone is true breeding. However, F1 kernels resulting from outcrosses with various stocks, including *r1* testers, are fully colored. A provisional test of inheritance was performed by crossing two such collections, Osage and Kokoma, to *R1-sc:124* in W22 background. F2 kernel progeny gave a 63:1 ratio of full color to dark and light mottled as expected for a three gene difference (Table 1). (See Stinard *et al.*, this MNL, for companion article.) This outcome would result if the accessions carried three recessive factors relative to the *R1-sc:124* stock: two regulators of mottling, provisionally termed *mot1* and *mot2*, and a responsive *r1* haplotype. The *r1* haplotype in these accessions proved to be of the *R1-d* class, which is subject to dilution by dominant modifiers (Stinard PS and Sachs MM, 2002. J Hered 93: 421-428).

An *r1-g mot1 mot2* tester was developed by backcrossing the Hopi mottled variants for four generations to a W22 inbred conversion of *r1-g(Stadler)*, a colorless seed and plant haplotype. The tester was selfed and crossed during each generation of introgression to a true breeding mottled stock to confirm the presence of the *mot* factors.

Twelve *R1-d* haplotypes isolated from geographic locations spread across western North America, plus the genetic stock *R1-d(Arapaho)*, were chosen to further characterize interaction between the *R1-d* class of haplotypes and the *mot* factors. The 13 *R1-d* stocks, each carried in W22 background, were crossed to the *r1-g mot1 mot2* tester to make an F1. These F1s (genotype *R1-d r1-g*; *Mot1 mot1*; *Mot2 mot2*) were then reciprocally crossed to the *r1-g mot1 mot2* tester. In backcrosses with the F1 as female, six of the haplotypes showed a ratio of 4 colorless (cl) to 3 dark mottled (DMT) to 1 light mottled (LMT) expected if three factors were segregating (Table 2). Only 3 ears out of 28 crosses had chi-square values showing deviation from this ratio. The remaining seven haplotypes did not segregate for an obvious light mottled class when the *R1-d* haplotype-carrying parent was used as a female, indicating a lack of sensitivity of the *R1-d* haplotype to the *mot* factors in female backcrosses. These crosses showed a 1:1 segregation for colorless to dark mottled (Table 3). Only 3 ears out of 34 had chi-square tests that showed significant deviation from a 1:1 ratio. However, the male backcrosses of all twelve haplotypes showed sensitivity of the *R1-d* haplotype to the *mot* factors.

Since we know from the male backcrosses that these *R1-d* haplotypes are capable of responding to the *mot* factors, imprinting or a dosage effect is indicated to have caused stronger expression of the aleurone color of these *R1-d* accessions when passed through the female in the presence of the *mot* factors. We call this the "strong imprinting response." On the other hand, the *R1-d* haplotypes responding to the *mot* factors when passed through the female can be said to have a null or weak response to imprinting on the female gametophyte, *i. e.* a "weak imprinting response." Although dosage effects have not been completely ruled out, we believe that imprinting differences are involved due to the effects of the *mot* factors on *R1-r(standard)* in classic imprinting experiments (data not shown). Therefore, we tentatively conclude that *R1-d* haplotype-specific imprinting responses in interaction with the *mot* factors are responsible for the differences observed in the female backcrosses.

In all crosses where *R1-d* is inherited through the male gametophyte (cross: [*r1-g mot1 mot2*] X [*R1-d r1-g*; *Mot1 mot1*; *Mot2 mot2*]), a wider variance in the color classes is observed than when *R1-d* is inherited through the female. A distinct medium mottling (MMT) class appears, and the dark mottled class is not as strong as when the *R1-d* haplotype is inherited from the female parent. This indicates that the "weak imprinting response" *R1-d* haplotypes do have a slight response to imprinting rather than a completely null response. Thus the *mot* factors can be used to differentiate between *R1-d* haplotypes that respond strongly and weakly to imprinting.

We hypothesize that one of the *mot* factors, arbitrarily named *mot2* on this basis, has a stronger effect on seed color than the other. The expected distribution of classes should be 4 cl (*r1-g r1-g*) : 2 DMT (*R1-d r1-g*; *Mot1 mot1*; *Mot2 mot2* or *R1-d r1-g*; *mot1 mot1*; *Mot2 mot2*) : 1 MMT (*R1-d r1-g*; *Mot1 mot1*; *mot2 mot2*) : 1 LMT (*R1-d r1-g*; *mot1 mot1*; *mot2 mot2*) if *mot2* has a stronger effect (see Figure 1). The chi-square tests for this ratio were non-significant for all but 4 out of 72 ears (data not shown). So we conclude that *Mot2* has a stronger effect on aleurone color than *Mot1*. Both imprinting-sensitive and insensitive *R1-d* haplotypes show light mottling of the aleurone when inherited through the male gametophyte in the presence of both *mot* factors, but full color when the wild type *Mot* alleles are present. This must be due to a compensation effect by the wild type *Mot* alleles that intensifies color (or prevents color reduction) even in the absence of imprinting, when *R1-d* is passed through the male gametophyte. At the same time, this shows that the segregation ratio of 1:1 for the *R1-d* imprinting-sensitive haplotypes when *R1-d* is passed through the female (Table 5) is not due to them not being able to respond to the *mot* factors, but rather imprinting provides an alternative route to increasing *r1* gene expression.

Another interesting aspect of the *mot* factors' effect on these geographic and tribal *R1-d* haplotypes is the differential effect on seed and seedling colors. The *R1-d* class of *r1* haplotypes typically shows strong pigmentation of the aleurone and also of the scutellum and germinating roots. We observed that medium mottled kernels from the male testcross, [*r1-g mot1 mot2*] X [*R1-d r1-g*; *Mot1 mot1*; *Mot2 mot2*], had colored scutella and produced seedling root color when germinated, but the light mottled kernels had colorless scutella and produced seedlings with green roots (Figure 2). We conclude that only one of the factors is needed to produce color in the scutellum and roots. This factor does not cause strong seed color and therefore must be *mot1*, since *mot2* was designated as the factor causing stronger seed color. We hypothesize that a color class distribution of both seed and seedling should be as follows: 1 LMT/Green : 1 MMT/Red : 1 DMT/Green : 1DMT/Red, where Green and Red refer to plant colors, if two *mot* factors are segregating but these two *mot* factors have different effects on plant color. These classes would correspond to: 1 LMT/Green = *R1-d r1-g*; *mot1 mot1*; *mot2 mot2*; 1 MMT/Red = *R1-d r1-g*; *Mot1 mot1*; *mot2 mot2*; 1 DMT/Green = *R1-d r1-g*; *mot1 mot1*; *Mot2 mot2*; and 1 DMT/Red = *R1-d r1-g*; *Mot1 mot1*; *Mot2 mot2*. This ratio was generally observed when kernels were germinated and plant colors were scored (Table 4). A few seedlings in two unexpected classes were also observed: LMT/Red and MMT/Green. One possible explanation for these unexpected classes is heterofertilization; other possibilities include kernel color misclassifications and the presence of other as yet uncharacterized modifiers. Chi-square tests of counts of seedlings grown from colored kernels of male testcrosses of four *R1-d* accessions, with two ears each, showed no deviation from the expected ratios for five out of the eight ears (Table 4).

In summary, the true-breeding mottled phenotype observed in Southwestern Native American accessions of maize results from the interaction of a permissive *R1-d* haplotype with two mottling factors, *mot1* and *mot2*. For mottling to occur, the *R1-d* haplotype must be homozygous, or heterozygous with a colorless *r1* haplotype, and *mot1* and *mot2* must be homozygous. The *R1-d* haplotypes studied can be grouped into two classes based on phenotype in the presence of *mot1* and *mot2* when crossed reciprocally with *r1-g mot1 mot2* testers: (1) Weak imprinting response *R1-d* haplotypes produce light mottled kernels in the presence of *mot1* and *mot2* regardless of whether the *R1-d* haplotype is transmitted through the male or female gametophyte. These are the *R1-d* haplotypes present in true-breeding light mottled lines. (2) Strong imprinting response *R1-d* haplotypes produce dark mottled kernels when transmitted through the female, but light mottled kernels when transmitted through the male in the presence of *mot1* and *mot2*. Table 5 summarizes the origins of the various *R1-d* haplotypes studied and their pattern of imprintability observed in combination with homozygous *mot1 mot2*. Note that an imprinting effect rather than an endosperm dosage effect was inferred on the basis of tests made using *R1-r(standard)*. More direct imprinting tests using *R1-d(Arapaho)* are in progress.

Four of the *R1-d* haplotypes characterized for imprinting response were analyzed molecularly by Walker and Panavas (2001. Genetics 159:1201-1215), two strong responders and two weak responders (Table 5). No differences were observed between the two types at the gross structural level—all four haplotypes showed the same structural features typical of *R1-d* haplotypes: a *q* gene, an intact *S2* gene, and a truncated *S1*

gene missing 5' noncoding sequences. The molecular basis for the difference in imprinting responses remains a question.

The *mot* factors themselves have differential effects on intensity of aleurone and plant color produced by all *R1-d* haplotypes studied. Seedlings grown from kernels carrying an *R1-d* haplotype and homozygous or heterozygous for the *Mot1* allele produce plant color regardless of *mot2* genotype; homozygous *mot1* seedlings are green regardless of *mot2* genotype. Kernels carrying an *R1-d* haplotype and the *Mot2* allele and homozygous for *mot1* are more darkly mottled than kernels carrying an *R1-d* haplotype and the *Mot1* allele and homozygous for *mot2*—this interaction is most evident when *R1-d* is transmitted through the male. These differential interactions are the basis for distinguishing between the two *mot* factors.

We used a bulk segregant analysis in an attempt to map the three factors involved in the aleurone mottling and seedling color effects. Seeds from 5 F2 ears of Navajo Robin's Egg Corn (NREC) crossed to *R1-sc:124* showing the 63:1 ratio of full color to mottled were germinated, plus one plant from each parental and one from F1 seed. Leaf discs from two to five of the F3 plants from each color class were pooled to produce a bulked DNA sample. The MaizeSNP50 Illumina Corn Chip was used for genotyping. Table 6 shows some statistics on data resulting from the analysis. We expected that polymorphic markers between bulked samples will be genetically linked to the color factors. Since these three factors act as recessive genes, we expect the colored pool to more likely show heterozygous calls for the markers in the linked region while the mottled pooled samples show homozygous calls, the same calls as for the mottling parent NREC. By comparing the 5 paired pools, F1 and 2 parental lines using excel filters, we found three regions linked to the mottling effect as expected. The *r1* gene position was confirmed by this analysis (Table 7) and is contained in the smaller interval detected on chromosome 10. *mot1* and *mot2* were located to two other segments on chromosomes 3 and 4. Which factor is located on which chromosome is not known, since this population was not large enough to allow their phenotypic discrimination. Their physical positions on chromosome 3 and chromosome 4 are shown in Table 7. Exact genetic positions are not provided, but the approximate size of each genetic interval is suggested.

Figure 1. Male backcross of a heterozygous *R1-d:Arapaho r1-g Mot1 mot1 Mot2 mot2* plant to an *r1-g mot1 mot2* tester. Colored kernels are in three classes: dark mottled, medium mottled and light mottled in a 2:1:1 ratio. Seedlings grown from medium mottled kernels were red, half of the seedlings grown from dark mottled kernels were red, and seedlings grown from light mottled kernels were green, indicating that the weak kernel mottling factor, *Mot1*, is responsible for induction of typical *R1-d* seedling color. Other *R1-d* geographic alleles showed variation in seedling pigmentation in response to the mottling factors.

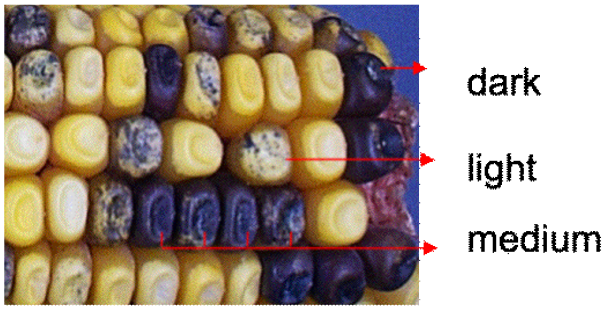

Figure 2. Seedling pigmentation phenotypes illustrating interactions between *R1-d* haplotypes and mottling factors *mot1* and *mot2*. (A) Green seedlings grown from *R1-d:Arapaho mot1 mot2* kernels. (B) Red seedlings grown from *R1-d:Arapaho Mot1 Mot2* kernels.

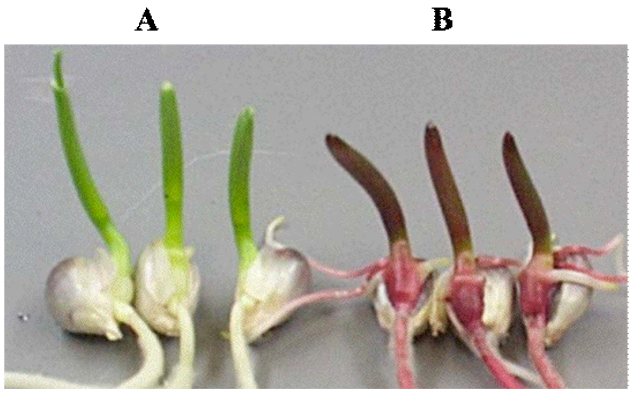

Table 1. Kernel counts from F2 ears of the cross of W22 *R1-sc:124* X Hopi mottled accessions. DMT = full colored and dark mottled. LMT = light mottled. Chi-square for 63 DMT : 1 LMT.

Source	*R1-d* haplotype	DMT	LMT	chi-square	significance
GB 871-1	Osage	452	9	0.455	NS
GB 871-2	Osage	570	11	0.413	NS
GB 872-1	Kokoma	446	6	0.162	NS
GB 872-2	Kokoma	456	6	0.209	NS
GB 872-3	Kokoma	454	11	1.950	NS
GB 872-4	Kokoma	446	10	1.179	NS
GB 872-5	Kokoma	448	8	0.109	NS
GB 872-6	Kokoma	533	10	0.275	NS

Table 2. Kernel counts of female backcrosses of *R1-d* haplotypes showing "weak imprinting response" (light mottled kernels segregating). Cross: [*R1-d r1-g*; *Mot1 mot2*; *Mot2 mot2*] X [*r1-g mot1 mot2*]. cl = colorless kernels. Chi-square for 4 cl : 3 DMT : 1 LMT.

Source	*R1-d* haplotype	PI number	cl	DMT	LMT	chi-square	significance
GB 645	Arizona-1	PI213729	206	186	54	3.453	NS
GB 645	Arizona-1	PI213729	202	161	39	3.221	NS
GB 645	Arizona-1	PI213729	233	186	41	5.83	NS
GB 645	Arizona-1	PI213729	147	102	29	1.46	NS
GB 645	Arizona-1	PI213729	172	141	48	0.817	NS
GB 652	Arizona-2	PI213738	207	158	65	2.718	NS
GB 652	Arizona-2	PI213738	244	195	113	32.464	P<.001
GB 652	Arizona-2	PI213738	171	142	45	0.806	NS
GB 652	Arizona-2	PI213738	178	143	36	2.29	NS
GB 651	Canada	PI214199	188	256	69	39.707	P<.001
GB 651	Canada	PI214199	115	96	21	3.147	NS
GB 651	Canada	PI214199	194	144	53	0.407	NS
GB 651	Canada	PI214199	185	136	47	0.056	NS
GB 651	Canada	PI214199	228	148	52	1.931	NS
GB 656	New Mexico-3	PI218150	166	111	41	0.918	NS
GB 656	New Mexico-3	PI218150	136	98	23	3.057	NS
GB 656	New Mexico-3	PI218150	160	130	50	1.961	NS
GB 656	New Mexico-3	PI218150	138	114	38	0.676	NS
GB 656	New Mexico-3	PI218150	158	142	44	2.473	NS
GB 658	New Mexico-5	PI218169	190	144	57	1.552	NS
GB 658	New Mexico-5	PI218169	121	130	34	8.322	P<.05
GB 658	New Mexico-5	PI218169	121	80	38	3.262	NS
GB 658	New Mexico-5	PI218169	210	153	58	0.701	NS
GB 658	New Mexico-5	PI218169	206	172	58	1.327	NS
GB 659	New Mexico-6	PI218173	160	115	50	2.59	NS
GB 659	New Mexico-6	PI218173	146	114	52	5.051	NS
GB 659	New Mexico-6	PI218173	156	97	38	2.178	NS
GB 659	New Mexico-6	PI218173	168	130	40	0.209	NS

Table 3. Kernel counts of female backcrosses of *R1-d* haplotypes showing "strong imprinting response" (no light mottled kernels segregating). Cross: [*R1-d r1-g*; *Mot1 mot2*; *Mot2 mot2*] X [*r1-g mot1 mot2*]. Chi-square for 1 cl : 1 DMT. (The few LMT kernels not included in chi-square tests.)

Source	*R1-d* haplotype	PI number	cl	DMT	LMT	chi-square	significance
GB	Iowa	PI217411	186	192	0	0.095	NS
GB	Iowa	PI217411	263	215	2	4.82	P<.05
GB	Iowa	PI217411	214	177		3.501	NS
GB	Iowa	PI217411	183	161	2	1.407	NS
GB	Iowa	PI217411	231	216		0.503	NS
GB653	N Dakota	PI213807	168	162	1	0.109	NS
GB653	N Dakota	PI213807	192	172		1.099	NS
GB653	N Dakota	PI213807	151	130	1	1.569	NS
GB653	N Dakota	PI213807	152	129		1.883	NS
GB653	N Dakota	PI213807	108	110		0.018	NS
GB	Oklahoma	PI213756	133	131		0.015	NS
GB	Oklahoma	PI213756	280	287		0.086	NS
GB	Oklahoma	PI213756	186	179		0.134	NS
GB	Oklahoma	PI213756	238	230	2	0.137	NS
GB	S Dakota 1	PI213779	194	179	2	0.603	NS
GB	S Dakota 1	PI213779	120	122		0.017	NS
GB	S Dakota 1	PI213779	209	221	3	0.335	NS
GB	S Dakota 1	PI213779	233	273	2	3.162	NS
GB650	Washington-1	PI217488	143	129		0.721	NS
GB650	Washington-1	PI217488	198	235	8	3.162	NS
GB650	Washington-1	PI217488	218	246	1	1.69	NS
GB650	Washington-1	PI217488	217	238	1	0.969	NS
GB650	Washington-1	PI217488	279	261	2	0.6	NS
GB660	Washington-2	PI217489	256	229	5	1.503	NS
GB660	Washington-2	PI217489	168	151	3	0.906	NS
GB660	Washington-2	PI217489	148	200	3	7.77	P<.01
GB660	Washington-2	PI217489	192	187		0.066	NS
GB660	Washington-2	PI217489	92	124		4.741	P<.05
GB	Arapaho		240	206		2.592	NS
GB	Arapaho		291	278	2	0.297	NS
GB	Arapaho		225	230		0.055	NS
GB	Arapaho		229	226		0.02	NS
GB	Arapaho		256	260	3	0.031	NS
GB	Arapaho		219	230		0.269	NS

Table 4. Seedling phenotypes for colored kernels from test crosses: [*r1-g mot1 mot2*] X [*R1-d r1-g*; *Mot1 mot1*; *Mot2 mot2*]. Chi-square for 1 LMT/Green : 1 MMT/Red : 1 DMT/Green : 1 DMT/Red. The few seedlings in unexpected classes were not included in chi-square calculations.

***R1-d* haplotype**	**PI number**	**imprinting**	**LMT/G**	**MMT/R**	**DMT/G**	**DMT/R**	**LMT/R**	**MMT/G**	**No Germination**	**chi-square**	**significance**
Arapaho		strong	63	53	56	64	5	12	2	1.458	NS
Arapaho		strong	57	50	87	62	2	8	2	12.156	P<.01
Canada	PI214199	weak	28	41	37	56	4	10	2	10.099	P<.01
Canada	PI214199	weak	38	39	56	49		3	5	4.857	NS
New Mexico-4	PI218157	weak	50	42	39	70	1	10		11.637	P<.01
New Mexico-4	PI218157	weak	50	51	64	43	1	10	3	4.423	NS
Washington-1	PI217488	strong	53	37	45	33	3	5	1	5.619	NS
Washington-1	PI217488	strong	58	64	76	70	6	16	2	2.687	NS

Table 5. Summary of *R1-d* haplotypes used in studies of *mot* factors, their origin (see Van Der Walt, W and Brink, RA. 1969. Geographic distribution of paramutable and paramutagenic *R* alleles in maize. Genetics 61:677-695), and pattern of imprintability in combination with homozygous *mot1 mot2*.

***R1-d* haplotype**	**PI number**	**Imprintability**
Arizona-1[1]	PI213729	weak
Arizona-2[1]	PI213738	weak
New Mexico-2	PI218143	weak
New Mexico-3	PI218150	weak
New Mexico-4	PI218157	weak
New Mexico-5	PI218169	weak
New Mexico-6	PI218173	weak
Canada[1]	PI214199	weak
New Mexico-1[1]	PI218170	strong
Oklahoma	PI213756	strong
Iowa	PI217411	strong
South Dakota-1[1]	PI213779	strong
South Dakota-2	PI213787	strong
North Dakota	PI213807	strong
Washington-1	PI217489	strong
Washington-2	PI217488	strong
Arapaho		strong

1 Haplotypes analyzed molecularly in Walker, EL and Panavas, T. 2001. Structural features and methylation patterns associated with paramutation at the *r1* locus of *Zea mays*. Genetics 159:1201-1215.

Table 6. Statistical summary of genotyping of parental and F1 samples with Maize50 Illumina plex.

Genotype	*Rsc:12 4 Mot1 Mot2 W22*	*R1-Navajo Robin's Egg Corn mot1 mot2*	[*R1-sc: 124/Navajo Robin's Egg Corn*] F1	Pool 732-9DK	Pool 732-9Mot	Pool 732-5DK	Pool 732-5Mot	Pool 732-4DK	Pool 732-4Mot	Pool 732-2DK	Pool 732-2Mot	Pool 732-1DK	Pool 732-1Mot
% heterozygous	1%	7%	36%	28%	23%	31%	18%	32%	25%	32%	23%	29%	23%
No. homozygous markers	48752	45164	31495	32684	35329	32027	37325	31880	33865	31812	34786	32278	35070
No. heterozygous markers	318	3378	17356	12884	10373	14147	8045	15061	11203	15183	10506	13114	10626
No. markers not scored	6056	6584	6275	9558	9424	8952	9756	8185	10058	8131	9834	9734	9430

Table 7. Looking at segregation patterns across multiple loci, one can define a region of confidence and a smaller region within the region of confidence where the presence of the locus most likely is. The *r1* locus is between positions 139,028,499 and position 139,122,292 on Chromosome 10. Therefore, the other two locations should correspond to mottling factors *mot1* and *mot2,* which were not distinguished in this analysis.

	Chr 3[a]		**Chr 4** [a]		**Chr 10** [a]	
Start of region of confidence	11,924,509		32,233		66,503,927	
Start of region most likely	21,279,187		32,233		136,523,828	
End of region most likely	102,621,860	~13 cM [b] - centromere	2,072,691	~7 cM [b]	141,117,052	~13 cM [b]
End of region of confidence	102,621,860		4,139,968		142,144,176	

[a] Bp position of markers on the physical assembly B73 RefGen_v2 sequence (www.maizegdb.org, www.maizesequence.org).
[b] Approximated genetic interval based on flanking marker information (data not shown).
Illumina MaizeSNP50 marker list and bp coordinates can be found at www.illumina.com/support/literature.ilmn.