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Germinal Derivatives of the *En* Controlling-Element System in Maize: Characterization of Colored, Pale and Colorless Derivatives of *a2-m**

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Summary. Several stable germinal derivatives, with varied phenotypic expression have been recovered from testcrosses of four different a2 mutable alleles (a2m-7 8018, a2m-6 8144, a2m-6 8140, and a2m-1 1511) under the control of the En controlling-element system. These are colored, pale, and colorless aleurone types; the former two represent mutations of a2-m→A2' type. The phenotypic variation among colored and pale derivatives has been confirmed by quantitative determination of anthocyanin content in the aleurone tissue of the kernels.

The data suggest that significant variation exists between phenotypically similar colored derivatives arising from different mutable alleles as well as from the same source allele. Every tested colored derivative is significantly different from the pale, colorless, and colored control (W22 color converted line). Genetic analysis of these colored derivatives suggests that all of them contain one or more En and, hence, they are characterized as permanent changes of a nonresponsive (nr) type. Their frequencies are variable among the testcrosses.

Similarly, the pale derivatives, which are clearly different from colored and colorless, also show significant differences in terms of anthocyanin content. Significant differences exist between pales arising from the same allele as well as between pales arising from different alleles. All the tested pales are significantly different from colorless and colored derivatives. Thus, the pales, as well as colored derivatives, represent a differential impairment of anthocyanin synthesis in the aleurone tissue. The genetic analysis confirmed that all the pales, except the pales of a2m-1 1511, lack the capacity to respond to En. These pale derivatives can be with or without En.

Qualitative differences among the colored, pale, and colorless types have been investigated by thin-layer chromatographic and spectroscopic techniques. The results suggest that there are no qualitative differences, in terms of anthocyanin pigments, between colored, pale, and colored control. All accumulate the same anthocyanins (namely, cyanidin-3-glucoside and pelargonidin-3-glucoside in appropriate proportions depending upon the Pr and pr constitution).

Introduction

Controlling-element systems in maize, such as Ac-Ds and En-I initiate mutation events at diverse loci (McClintock 1951a; Peterson 1975). The resultant phenotypic changes include widely diverse levels of genic expression from completely null (nonfunctional) to fully functional alleles. These are induced by the initial insertion of regulatory elements such as Ac or En (or Spm) at the designated locus or their respective control element Ds or I (Table 1; Peterson 1970, 1973).

McClintock (1951a, 1951b, 1963, 1965) discovered several new derivative alleles at the A, C and Wx loci that were under the control of different controlling-element systems (for example a-m, c-m, and wx-m). Varied stable derivatives of two of the controlling-element alleles at the A and C loci show significant differences in pigment intensity in the aleurone, whereas Wx' derivatives (from wx-m) show differences in iodine stain intensity of endosperm starch, presumably due to differences in the amylose-amylopectin ratio (McClintock 1963). Rhoades (1941), in studies of the Dt (dotted) system, found a series of new alleles with altered phenotypic expression at the A locus. It was reasoned that these derivatives might have originated as a result of the release of the affected gene from the control of Dt system.

Further studies with the Dt system revealed the occurrence of additional derivatives. Richardson (1956) found a broad array of derivatives of mutable a-pm under the control of Dt system, ranging from completely colorless to deep-colored aleurone types. Nuffer (1961) also described a number of new derivative alleles at the A locus under the control of Dt system with grades of phenotypic expression ranging from dilute to full-colored types.

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Within the En controlling-element system, there is a large number of independently induced controlling-element alleles, each originating from the dominant fully functional allele (Peterson 1975). Also, the En system generates phenotypically diverse derivatives at several loci controlling anthocyanin synthesis in the aleurone and plant tissue. A wide assortment of such derivatives with varied phenotypic expression (from null to full-colored aleurone) arose at the mutable a2 locus under the control of the En system (Peterson 1966). These derivative alleles, originating by mutational events of the a2-m → A2' type are differentiated on the basis of the intensity of anthocyanin pigmentation in the aleurone tissue of kernels. Fowler and Peterson (1974), studying the a2m(r-pa-pu) allele of the En system (a derivative allele of the original a2m-1 1511; Peterson 1968) demonstrated the differential phenotypic levels of several new derivative types ranging from colorless to full-colored with some intermediate color types. Some of these pale derivatives retained the capacity to respond to En, indicating the presence of the En responsive control element within or very close to the gene locus.

From the genetic analysis of such En-induced new allelic forms at three of the anthocyanin controlling loci (A, A2, and C) several general features of these alleles can be stated: 1) Each of these loci has the potential for generating a wide array of derivatives when under the control of the En controlling-element system: 2) From each locus, derivatives of all levels of phenotypic expression are possible, although the frequencies are allele specific: 3) All the derivatives recorded so far are induced by En, which can be located autonomously with respect to the involved locus or segregating independently: 4) Within any one locus, several mutable alleles have originated and are distinguished on the basis of their independent origin (Peterson 1975); these differ in the origination of derivative alleles (with some, differential types do not arise while with others diverse types frequently arise; thus, the allelic potential is unique for each mutable allele): 5) Such En-induced differential gene expression can occur in somatic or germinal tissue: 6) Newly derived alleles can be responsive to a regulatory element (En) or nonresponsive (nr): 7) No further mutations were recorded at most of the stable derivative nr alleles, indicating that these changes were fixed: 8) The derivative alleles, which presumably represent a permanent modification of gene action, exhibit a wide array of phenotypic expression.

Similar expression has been reported in other plants of novel variation arising from mutable alleles in the form of a wide assortment of distinctly different derivative alleles. Fincham and Harrison (1967) found several new alleles at the mutable *Pal-rec (Pallida recurrens)* locus in *Antirrhinum majus*, which show grades of flower pigmentation due to differential impairment of anthocyanin synthesis. Hondelmann (1959) reported the occurrence of a wide series of derivative alleles arising from mutable flower color gene g in Chinese aster plant (*Callistephus chinensis* Nees) ranging from completely colorless to full colored types and with intermediate cases.

Several attempts have been made earlier to clarify the nature of such derivatives in terms of qualitative and quantitative changes. Sager (1951) reported that no significant differences were observed among 40 Wx' derivatives of different mutable-wx alleles in terms of percentage amylose or amylose-amylopectin ratio. Nor did the derived Wx' show any significant differences with wild type. On the other hand, analysis of germinal derivatives of colored, pale, and colorless types arising from the a2m(r-pa-pu) allele revealed that there are significant differences between derivatives (Fowler and Peterson 1974). Fincham and Harrison (1967) found significant differences in anthocyanin content between derivatives and that certain of these derivatives accumulate greatly reduced quantities (2 and 12% of control) of pigment. No drastic qualitative changes in terms of altered accumulated products, however, have been observed between such derivatives either in the maize, *Antirrhinum majus*, or Chinese aster mutable systems.

The present paper is an analysis of the quantitative and qualitative characterization of some colored, pale and colorless derivatives of four different a2 mutable alleles to provide insight into the precise nature of the changes associated with these derivatives.

Materials and Methods

Gene symbols and the alleles discussed are described in Table 1. The origin of each of the mutable alleles, a2m-1 1511, a2m-6 8140, a2m-6 8144 and a2m-7 8018 (Fig. 1) has been discussed in a previous report (Peterson 1975).

Colored derivatives: a2m-7 8018: These originally were isolated from the following testcross of a mu-

Table 1. Designation of Symbols and Terms

Allele or element	Description or phenotype
A2	An anthocyanin producing complementary allele (chromosome 5) produces purple or red aleurone with Pr and pr alleles, respectively
a2	A recessive allele of A2; when homozygous recessive, produces colorless aleurone; does not respond to any regulatory element
En	Enhancer, a regulatory element necessary for mutability of En receptive alleles; can be located at the controlled locus or at a position independent of it; can exist in several states
I	Inhibitor, a control element associated with the locus; suppresses gene activity until changed by En
a2m(r)	A colorless derivative allele that responds (r) to En: With En it gives purple spots on a colorless background in the aleurone tissue of the kernels; used generally as En tester
A2'	Germinal mutation of a given mutable a2 allele to or toward full colored; does not respond to En
Bt	Round non-shrunken type kernels, designated as Bt kernels; located six to seven units proximal to a2 on the short arm of chromosome 5; recessive bt kernels are collapsed type, referred as bt kernels
Pr	With appropriate color genes, produces purple anthocyanin (cyanidin-3-glucoside); located on chromosome 5; recessive pr produces red anthocyanin (pelargonidin-3-glucoside) differing only in number of hydroxyl functions
a2m-1 1511	Original inception of mutability at a2 locus controlled by En system (autonomous): in the presence of En, gives colored spots on colorless background; also, several full colored and pale derivatives have been recovered from this allele; in the absence of En the aleurone is pale (Fig.1a)
a2m-7 8018	Coarse type originated from A2 under the control of En. Gives rise to several full colored derivatives under the control of En. In the absence of En, the aleurone is colorless (Fig.1b)
a2m-6 8144	Very fine clear medium hi. type (v.v.f.cl.m.hi.) controlled by En; gives pale and full-colored derivatives. The aleurone is colorless in the absence of En (Fig.1c)
a2m-6 8140	Originally very palish high (v.p.hi.) type, controlled by En; gives many pales and full-colored derivatives; the aleurone is colorless in the absence of En (Fig.1d)

Term	Definition
Regulatory element	Elements such as En and Ac that trigger control elements so that the locus under control becomes functional
Receptor element	Element such as I and Ds that suppress gene action when in cis position to the locus

table allele: a2m(coarse) Bt/a2m(pale) Bt × a2 bt/a2 bt. The genotype and pedigree number of the female parent and the frequency of the colored derivatives are given in Table 2 (columns 3 and 8). Plants were grown from these colored kernels and crossed by a known En tester (a2m(r) Bt/a2m(r) Bt) to identify the En content. Colorless kernels (a2m(r) Bt/a2m(r) Bt/a2 bt) were collected from these ears for analysis. All these derivatives are maintained in a uniform genetic background; W22-color-converted, homozygous for all the anthocyanin controlling genes, C, C2, R, A, A2, Bz, Bz2, and pr (hereafter identified as W22-Col).

Pale derivatives: a2m-6 8144. These originally were isolated as individual events from the following type of testcrosses: a2m(v.v.f.cl.m.hi) Bt/a2 bt × × a2 bt/a2 bt (Table 3, column 4). Several plants were grown from these kernels and recurrently crossed for two generations to the W22-Col inbred to ensure a uniform genetic background. Pale kernels were finally selected from the selfed ears (W22-Col/pale). The pales of a2m-6 8140 are selected in the following way: Plants grown from mutable kernels (a2m-6 8140; Fig. 1d) were crossed by an En tester: a2m Bt/a2 bt A Sh2/a-m Sh2 × am(r) Sh2/am(r) Sh2. Plants were grown from the variegated kernels (fine, hi. type of a mutable) and testcrossed by a2 bt/a2 bt (Table 3). Pale kernels were selected from these testcross ears, and plants grown from them were recurrently crossed to W22-Col for further analysis.

The isolation and characterization of pale derivatives of a2m-1 1511 were described by Peterson (1966) and Fowler and Peterson (1974).

En tester: The En tester (a2m(r) Bt/a2m(r) Bt) is used to confirm the presence of En: a2m(r) kernels are colorless in the absence of En; in the presence of En, the aleurone shows colored spots on a colorless background.

En line: The En line (a2 bt/a2 bt En/+) is used to test the responsiveness of the newly derived alleles to En. The plants grown from the derivatives were cross-

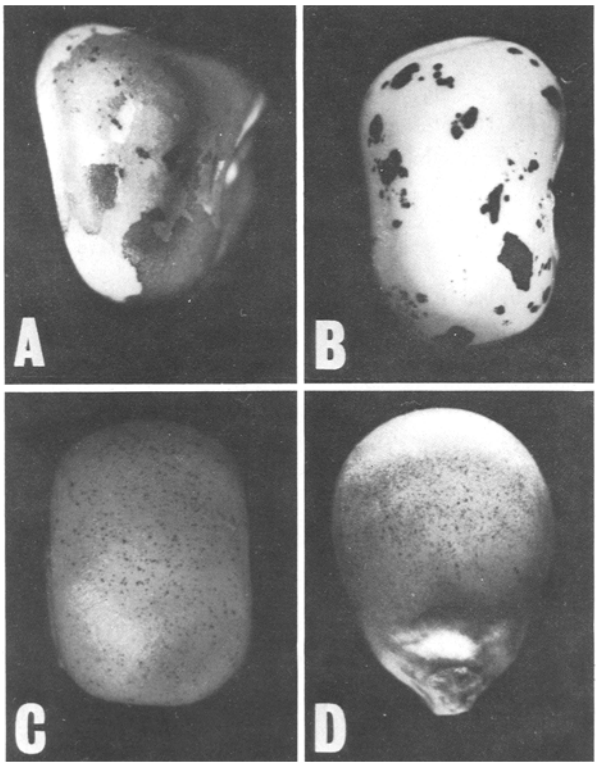

Fig. 1. Kernel types of four a2 mutable alleles.
A - a2m-1 1511: colored dots on colorless background;
B - a2m-7 8018: coarse type, large colored sectors on colorless background;
C - a2m-6 8144: very fine dots with medium high frequency on colorless background;
D - a2m-6 8140: very palish dots with high frequency on colorless background

ed by the En line as male parent. Also, these En plants at the same time are pollinated by an En tester (a2m(r) Bt/a2m(r) Bt) to confirm the presence of En in the En line.

Qualitative analysis of anthocyanin pigments

Replicate samples of kernels of desired genotypes were collected from segregating ears. The pericarp of these kernels was removed after presoaking in distilled water for 30 min. The aleurone layer was scraped carefully with a stainless-steel scalpel. The aleurone tissue was finely ground in a glass tube with a motor-driven porcelain pestle. The finely powdered aleurone was soaked in light petroleum ether overnight at room temperature, followed, after decanting, by diethyl ether. The diethyl ether solutions were decanted, and the residue was dried. The defatted residue was finally extracted with methyl alcohol, containing concentrated hydrochloric acid (0.1 ml conc. hydrochloric acid in 100 ml methyl alcohol). The methyl alcohol-HCl extractions were repeated thrice at room temperature. The combined extracts were concentrated under reduced pressure in a rotary flash evaporator.

The resulting crude pigment mixtures were spotted (2.5 cm from the edge) on Eastman Kodak Chromagram Cellulose Sheets (Cat. No. 13255, 160 μ thick, 20 × 20 cm). The chromatograms were developed in a solvent mixture of n-butyl alcohol:glacial acetic acid: water (4:1:5, upper layer; aged for 6 hrs before use) for ca. 2.5 hrs in an Eastman Chromagram developing apparatus. Rf values were calculated from at least five chromatograms. Pure cyanidin-3-glucoside, pelargonidin-3-glucoside, cyanidin chloride, and pelargonidin chloride were used as authentic controls on the same chromatograms.

The spots, which were clearly visible on the developed chromatograms, were cut and eluted with spectral grade methyl alcohol containing conc. hydrochloric acid. Solution spectra of chromatographically pure pigments were recorded in the visible region on Spectronic 505 (Bausch & Lomb, automatic recorder). Four to 5 ml of each replicated sample were used for each recording.

Table 2. The frequency of colored derivatives[1] in testcross progeny of plants of a2m(coarse)/a2m(pale) genotype: a2m(coarse) Bt/a2m(pale) × a2 bt/a2 bt

Cross No.	Pedigree No. of a2m(coarse) Bt/a2m(pale) Bt	Progeny: Number of Bt kernels					
		Cl[a]	Spotted	cl[b]	Pale	Total	% Cl/T
1	2	3	4	5	6	7	8
1	2 1401-1	29	197	142	166	534	5.4
2	2 1401-2	5	70	198	53	326	1.5
3	2 1403-3	13	32	58	39	142	9.1
4	2 1404-4	12	125	265	--	402	3.0
5	2 1401-5	3	131	26	--	160	1.9
6	2 1401-6	13	167	75	146	401	3.2
7	2 1402-1	8	76	52	70	206	3.9
8	2 1402-2	3	132	159	--	294	1.0
9	2 1402-3	16	116	53	101	286	5.6

[1] A2' (of a2m-7 8018)
[a] Cl = Colored
[b] cl = colorless

Table 3. Isolation of pales from testcrosses of a2m-6 8144 and a2m-6 8140 by a2 bt/a2 bt; a2m(v.v.f.cl.m.hi) Bt/a2 bt × a2 bt/a2 bt and a2-m Bt/A2 a-m sh2/am(r) Sh2 × a2 bt/a2 bt

Cross No. 1	Pedigree 2	Genotype and/or pattern 3	Progeny: Number of Bt kernels: Pale 4A	% total 4B	Spotted[a] 5	colorless 6	Total 7	Original source allele 8
1	2 1801-1	a2m(v.v.f.cl.m.hi) Bt/a2 bt	5	1.37	163	198	366	a2m-6 8144
2	2 1801-2	a2m(v.v.f.cl.m.hi) Bt/a2 bt	1	0.32	147	164	312	a2m-6 8144
3	2 1801-3	a2m(v.v.f.cl.m.hi) Bt/a2 bt	-	-	98	92	190	a2m-6 8144
4	2 1801-4	a2m(v.v.f.cl.m.hi) Bt/a2 bt	-	-	131	6	137	a2m-6 8144
5	2 1801-5	a2m(v.v.f.cl.m.hi) Bt/a2 bt	3	1.70	165	8	176	a2m-6 8144
6	2 1801-6	a2m(v.v.f.cl.m.hi) Bt/a2 bt	5	2.24	113	105	223	a2m-6 8144
7	2 1801-7	a2m(v.v.f.cl.m.hi) Bt/a2 bt	31	7.71	215	156	402	a2m-6 8144
8	0 7524	a2-m Bt/A2 a-m Sh2/am(r) Sh2	3	30.00	6[b]	-	9	a2m-6 8140
9	0 7540-1	a2-m Bt/A2 a-m Sh2/am(r) Sh2	31	48.44	33[b]	-	64	a2m-6 8140

[a] = Very fine clear dots with a medium high frequency on a colorless background
[b] = Medium fine dots with low frequency on a colorless background

Aglycones were obtained by the hydrolysis of glycosidic forms of the pigments with 2N HCl for an hour on a water bath. The hydrolysates were extracted with a small quantity of isoamyl alcohol. The concentrated solutions were subjected to thin-layer chromatography (TLC) and spectroscopy.

Quantitative determination of anthocyanin content

The anthocyanin content of aleurone tissue of colored, pale, and colorless derivatives were determined according to the method described by Reddy (1974).

From testcross or selfed ears, (as indicated in Table 5) two samples of twelve kernels each were collected. From each of the samples, two aliquots of 10 mg of aleurone were prepared and placed in 20 ml of spectroscopic grade methyl alcohol containing conc. hydrochloric acid (0.1 ml of HCl in 100 ml of methyl alcohol) and the mixture was left overnight at room temperature in the dark. Optical densities of the clear solutions were recorded on Spectronic-20 (Bausch & Lomb) at 530 nm. Methyl alcohol-HCl solution (0.1%) was used as the blank. Two recordings were made for each of these aliquot sample solutions and the mean O.D. values are computed as an average of four value of each original sample. The O.D. values of each aliquot was found to be quite reproducible with very little variability among the aliquots of a given sample.

Results

Three general types of germinal derivatives arise from testcrosses of a2m. They are full-colored, pale, and colorless types, which can readily be classified on the basis of visual distinctiveness.

Colored derivatives: Full-colored derivatives (A2') arise among the testcross progeny a2m(coarse) Bt/a2m(pale) Bt × a2 bt/a2 bt. These derivatives, which occur as independent single events, represent germinal mutations of a2m → A2' type. The frequencies of these derivatives are variable among progenies, and each occurs as an independent event, not in clusters. In a typical set of 9 testcrosses, the frequencies of the derivatives varied from 1 to 9.1% of the total Bt kernels (Table 2, column 8).

The En content of these full-colored derivatives was tested by crossing with the a2m(r) Bt/a2m(r) Bt male parent, and the results are described in Table 4. From a2m-7 8018, 19 independently occurring colored derivatives have been tested. Fifteen derivatives have an independent En, and the remaining four isolates have a linked En. Of these 15 derivatives with independently segregating En, 6 had a single En, 8 had two En, and 1 had more than two En. In as much as all these tested derivatives contain En, they are confirmed as non-responsive (nr) type. Similarly, all the tested colored derivatives of a2m-6 8144 contain one or more En.

The colored derivatives are varied in their phenotypic expression. This variation was tested by the quantitative determination of anthocyanin pigment present in the aleurone tissue of the kernels. Mean optical density (O.D.) and percentage control values are given in Table 5 (columns 4 and 5). Valid comparisons were made between colored derivatives of the same source allele, from different sources as well as of the colored control. These relationships are summarized in Table 6B. The four colored derivatives of a2m-7 8018 with mean O.D. values ranging from 0.373 to 0.493 can be grouped into A and B on the basis of Duncan's New Multiple Range Test: Group A constitutes 2 derivatives

Table 4. Test of En content[a]: Crosses of plants from colored and pale kernels; testcross ears to male En tester of a2m(r) Bt/a2m(r) Bt

Cross No. 1	Pedigree number of female parent 2	Phenotype of derivative 3	Source allele 4	Number tested 5		Number with En 6
1	4 4404	Colored	a2m-7 8018	2		2
2	4 4406	colored	a2m-7 8018	3		3
3	4 4409	colored	a2m-7 8018	7	= 19	7
4	4 4415	colored	a2m-7 8018	2		2
5	4 4417	colored	a2m-7 8018	5		5
6	1 3874	Pale	a2m-6 8140	6		5
7	1 3875	pale	a2m-6 8140	9		5
8	1 3875	pale	a2m-6 8140	5	= 33	0
9	1 3877	pale	a2m-6 8140	8		0
10	1 3879	pale	a2m-6 8144	5		5

[a] Pales of a2m 1 1511 respond to En by giving pale purple dots on colorless background in the aleurone, hence they are a2m(r) pale

Table 5. Quantitative determination of anthocyanin pigments from colored, pale and colorless derivatives of different a2 mutable alleles

Cross No. 1	Pedigree 2	Phenotype 3	Mean O.D.[a] 4	% control 5	Origin 6
1	4 2561/sib	Colored	0.588	100.00	W22-Color-converted (control)
2	4 4409-4/2335	Colored	0.375	67.24	a2m-7 8018
3	4 4409-1/2335	Colored	0.493	84.40	a2m-7 8018
4	4 4409-1/2335	Colorless	0.005	00.85	a2m-7 8018
5	4 4415-1/2340	Colored	0.373	63.79	a2m-7 8018
6	4 4415-1/2340	Colorless	0.005	0.85	a2m-7 8018
7	4 4474-4/2347	Colored	0.478	82.75	a2m-7 8018
8	4 4417-4/2347	Colorless	0.005	0.85	a2m-7 8018
9	308-78-7A ⊗	Pale	0.0715	12.00	a2m-6 8144
10	4 3048 ⊗	Pale	0.0445	6.80	a2m-6 8140
11	4 3048 ⊗	Colored	0.435	74.13	a2m-6 8140
12	4 3036 ⊗	Pale	0.0265	5.17	a2m-6 8140
13	4 3049 ⊗	Colored	0.403	68.96	a2m-6 8140
14	8 3825 ⊗	Pale	0.075	11.20	a2m-1 1511

[a] The O.D. values are not influenced by Pr-pr differences

(Table 5, column 4, crosses 2 and 5) with mean O.D. below 0.40 and, Group B, derivatives with mean O.D. above 0.40 (Table 5, column 4, crosses 3 and 7). The derivatives within Group A (with means 0.373 and 0.375) and Group B (with means 0.478 and 0.493) do not show significant differences from each other, but significant differences exist between group derivatives. Two of the tested colored derivatives of a2m-6 8140, with mean values 0.403 and 0.435 are not significantly different from each other; however, the former differ significantly from the Group B derivatives of a2m-7 8018. All the tested colored derivatives differ significantly from the colored control and other not full-colored derivatives ($F = 168.94^{**}$).

Pale derivatives: As with the full-colored derivatives, the pale derivatives isolated from testcrosses of different mutable alleles as single independent events vary in frequencies of origin (Table 3, column 4B). The En content of 33 pale derivatives of two different a2 mutable alleles (a2m-6 8144 and a2m-6 8140) are given in Table 4 (columns 5 and 6, crosses 6 to 10). Of these 33 tested pales, 15 showed the presence of En. When these pales were tested for the response to a known En by crossing with an En line (see Materials and Methods section) as male parent, no mutable kernels were recovered in the progeny, indicating that these are nonresponsive (nr) pales. These pales differ from the pale derivative of a2m-1 1511

Table 6. A comparison of full colored, pale and colorless derivatives of four different a2 mutable alleles (between the derivatives) and with W22-Col on the basis of optical density values

A. Analysis of variance

Source	D.F.	SS	MS	F
Total	27	1.295210	--	--
Treatments	13	1.287002	0.099000	168.94**
Error	14	0.008208	0.000586	--

B. Summary from Duncan's new multiple range test

Colored							
a2m-7 8018		a2m-6 8140		a2m-7 8018		W22-Color Converted Control
0.373	0.375	0.403	0.435	0.478	0.493	0.588

(Means underscored by the same solid line are not significantly different)

Table 7. Variability in pigment quantity of pale and colorless derivatives on the basis of optical density values

A. Analysis of variance

Source	D.F.	SS	MS	F
Total	13	0.0198036	--	--
Treatment	6	0.01078986	0.00179831	66.079**
Error	7	0.00019050	0.0000272142	

B. Summary from Duncan's new multiple range test

Colorless			Pale			
a2m-7 8018			a2m-6 8140		a2m-6 8144	a2m-1 1511
0.005	0.005	0.005	0.0265	0.0445	0.0715	0.0750

described by Fowler and Peterson (1974) that shows pale purple spots on a colorless background of aleurone tissue in the presence of En and therefore is considered as a2m(pale-mr) (responsive) type.

Quantitative determinations of anthocyanin content of these pale derivatives were made to verify the apparent phenotypic variation. The results are given in Table 5 (column 4, crosses 9, 10, 12, 14). The two pale derivatives of the same source mutable allele (a2m-6 8140), with mean values of 0.0265 and 0.0445, differ significantly from each other and also from pale derivatives of a2m-6 8144 and a2m-1 1511, with means 0.0715 and 0.0750, respectively (Table 7B). The pale derivative of a2m-6 8144, with a mean of 0.0715, does not differ significantly from the pale of a2m-1 1511, with a mean of 0.0750. Every pale tested in this study is significantly different from the colored control and colorless derivatives (Table 7). Some of these pales accumulate greatly reduced quantities of anthocyanin pigment (5 to 6% of control) in aleurone tissue of kernels.

Qualitative analysis of anthocyanin pigment

Results of the qualitative analysis of colored, pale, and colorless derivatives are presented in Table 8. No qualitative differences were observed between colored, pale, and control. The extracts of all colored derivatives show a strikingly uniform chromatographic pattern. Two clear spots, one magenta spot, with Rf value 40-42, and an orange-red, with average Rf

Table 8. Chromatographic and spectral characteristics of isolated anthocyanin pigments from the aleurone tissue of the colored and pale derivatives of four different a2 mutable alleles

S. No.	Cross	Derivative	Vis. Color	Rf × 100 × BAW	λ max. in nm MeoH-HCl
1	B 2561/sib	W22-Col.	Faint magenta	40	526
			orange red	50	512
2	4 4409-4/2335	Colored	Magenta	41	525
			pale orange red	50	510
3	4 4409-1/2335	Colored	Magenta	42	526
			orange red	50	512
4	4 4415-1/2340	Colored	Magenta	42	527
			pale orange red	51	513
5	4 4417-4/2347	Colored	Magenta	42	527
			pale orange red	51	513
6	308-78-7A ⊗	Pale	Faint magenta	42	525
			orange red	53	513
7	308-78-7A ⊗	Colored	V. faint magenta	41	526
			orange red	50	512
8	4 3048 ⊗	Pale	V. faint magenta	40	524
			orange red	50	512
9	4 3036 ⊗	Pale	Orange red	40	513
10	4 3049 ⊗	Colored	Faint magenta	40	526
			orange red	50	512
11	8 3825 ⊗	Pale	V. faint magenta	43	524
			orange red	52	513
12	8 3835 ⊗	Pale	V. faint magenta	42	526
			orange red	51	512
13	8 6640 ⊗	Pale	V. faint magenta	40	526
			orange red	52	512
14	Cyanidin-3-glucoside	-	Magenta	41	524
15	Pelargonidin-3-glucoside	-	Orange red	50	512

value 50-53, have been identified. The magenta spot gave an absorption maxima (λ max.) at 524-526 nm, whereas the orange-red spot gave a peak at 510-513 nm. These values agree with those of authentic co-chromatographed samples of cyanidin-3-glucoside and pelargonidin-3-glucoside (Table 8).

The magenta pigment on acid hydrolysis yielded cyanidin chloride whereas the orange-red pigment yielded pelargonidin chloride. It was concluded that all the pale derivatives tested accumulate mainly the same anthocyanin pigment as does the control; i.e., pelargonidin-3-glucoside and small amounts of cyanidin-3-glucoside. In contrast, the colored derivatives accumulate mainly cyanidin-3-glucoside and, in addition, small amounts of pelargonidin-3-glucoside. These differences are due, however, to the segregation of the pr allele. All the pales are of pr background, and the colored derivatives are of Pr constitution.

Colorless derivatives: The extracts of colorless kernels of a2m(r) Bt/a2m(r) Bt/a2 bt constitution did not show any colored spots on thin-layer chromatograms. Also, the spectrophotometric readings indicate that absolutely no anthocyanin pigments are present in the aleurone tissue.

Discussion

Each of the four mutable alleles a2m-7 8018, a2m-6 8144, a2m-6 8140, and a2m-1 1511 originated from an initial A2 allele from a common varietal source after the insertion of En. These mutable alleles, in turn, mutate to various stable, phenotypically diverse levels, and thus, each of them constitutes a source of derivative types. The derivatives included in this study constitute only a small sample because numerous stable derivative types have been recovered at A, A2, and C loci with varying frequencies. Also, the four mutable alleles considered in this study represent only a few cases of a large number of mutable alleles, which, in response to En, give rise to such derivatives with varied genetic potential (Peterson 1975).

The distinctiveness in phenotypic variation among the colored and pale derivatives of four different a2

mutable alleles has been confirmed by the quantitative determination of anthocyanin content in the aleurone. Several possible conclusions can be drawn from the present data: 1) Significant variation exists between colored derivatives, in terms of anthocyanin content of aleurone: 2) There can be significant differences between colored derivatives arising from the same source allele, indicating that the original allele does not represent a predetermined potential for derivative types (ex. group A and B of a2m-7 8018 Table 6B): 3) The colored derivatives arising from one source allele can be significantly different from the derivatives of other alleles (ex. a2m-7 8018 differ from derivatives of a2m-6 8140, Table 6B); in some cases, however, no significant differences have been observed between derivatives of different origin, and these observations indicate that the processes involved in the origin of such derivatives may not be identical; 4) Each of the colored derivatives included in this report is significantly different from the pales, colorless, and colored control; thus it can be concluded that all these colored derivatives represent some kind of differential impairment affecting quantitative levels of anthocyanin synthesis in the aleurone of kernels, although the exact nature of the En induced impairment of anthocyanin synthesis is not yet clear.

Similarly, the pales, which are distinctly different phenotypically from the colored, colorless, and colored control also show significant differences in anthocyanin content. It has been observed that significant differences exist between the pales arising from the same source (a2m-6 8140, Table 7B) as well as in pales arising from a different source. All the tested pales are significantly different from the colorless and colored types. Again, these intermediate allelic types represent a higher degree of impairment of anthocyanin synthesis because these accumulate relatively low quantities of anthocyanin pigment in the aleurone (as little as 6 to 13% control).

No qualitative differences were observed among these colored, pale, and colored controls in terms of anthocyanin pigments, within the limits of the techniques used (see Materials and Methods section). Each of these derivatives accumulates, in the aleurone, the same anthocyanin pigments; (namely, cyanidin-3-glucoside and pelargonidin-3-glucoside in appropriate proportions depending upon the Pr and pr constitution).

These results are in close agreement with the earlier observations on the newly derived allelic series of mutable *pal-rec* locus in *Antirrhinum majus* (Fincham and Harrison 1967) and mutable g locus in Chinise aster system (Hondelmann 1959). In both of these cases, the new series of allelic derivatives accumulate the same anthocyanin pigment in differing quantities, presumably due to the impairment of anthocyanin biosynthesis.

The precise mechanism by which these derivatives arise is not clear. Their relatively high frequency of occurrence, however, indicates that the mutational process involved here may be different from the phenomenon of spontaneous mutations occurring in loci unassociated with controlling elements. It has been suggested that the frequent occurrence of these diverse allelic types with differing phenotypic expression may be due to the changes in 'states' (McClintock 1965) of the gene locus or changes in the type of response of the control element to regulatory element. Also, it is demonstrated in several instances that many of the derivatives with altered genic expression induced by regulatory elements generally are associated with concomitant release of the gene locus from control by the system (Rhoades 1941; McClintock 1965). McClintock further stated that such releases of gene action from control may be associated with removal or modification of the control element. The present study confirms that the En controlling-element system generates both types of phenomena. The colored (a2m-7 8018) and pale (a2m-6 8144 and a2m-6 8140) derivatives represent instances in which the control element has been removed away from the locus in as much as none of the derived alleles responds to En. The pales of a2m-1 1511, however, are different. They represent a specific instance in which the control element still is located within the gene locus, but in a modified state, because these pales respond to En by giving pale purple spots on a colorless background in the aleurone.

Several similarities exist between maize and certain mutable bacterial systems in terms of occurrence of such new allelic derivatives with altered genic potential (Peterson 1970; Fowler and Peterson 1974). That random insertions of DNA segments of mutator phage (Mu) in several genes of host E. coli result in mutation and excisions of such inserted phage DNA

may result in restoration of wild-type gene action is well established (Bukhari and Zipser 1972; Bukhari 1975). Also, it is evident that Mu insertions can be random within a gene and that excisions can be exact or inexact, leading to the perfect wild type or pseudo-wild type respectively. The colored (a2m-7 8018) and pale derivatives (a2m-6 8144, a2m-6 8140) of mutable a2 gene may be reflections of the removal of the control element from the a2 locus in response to En. These excisions are presumably of inexact type because none of them is true wild type in terms of pigment quantity. As in bacterial systems, exact reversions to wild type may be rare in maize systems, and the excision phenomenon in both instances is controlled by a separate regulatory element (En, Dt, Spm, and Ac in maize and the X locus in E. coli; Bukhari 1975).

A wide assortment of allelic series at the R and A loci representing spontaneous variation in terms of anthocyanin pigmentation in aleurone as well as in certain plant tissue is well known in maize (Stadler 1946; Laughnan 1961). In both of these cases, the phenotypic variation is caused by the expression of different separable component alleles within the gene locus and none of them are associated with a controlling element system. No comparison was made between these cases of spontaneous variation at R and A loci and the phenotypically diverse derivatives of A2 because, firstly, there is no evidence of such separable components at the A2 locus, and, secondly, these derivatives are induced by a specific controlling element system.

Literature

Bukhari, A.I.: Reversal of mutator phage Mu integration. J. Mol. Biol. 96, 87-99 (1975)

Bukhari, A.I.; Zipser, D.: Random insertion of Mu-1 DNA within a single gene. Nature New Biol. 236, 240-243 (1972)

Fincham, J.R.S.; Harrison, B.J.: Instability at the Pa1 locus in *Antirrhinum majus*. II. Multiple alleles produced by mutation of the original unstable allele. Heredity 22, 211-224 (1967)

Fowler, R.G.; Peterson, P.A.: The a2m(r-pa-pu) allele of the En controlling element system in maize. Genetics 76, 433-446 (1974)

Hondelmann, W.: Untersuchungen an einem mutablen Blütenfarbgen von *Callistephus chinensis* Nees. Z. Vererbungsl. 90, 159-181 (1959)

Laughnan, J.R.: The nature of mutations in terms of gene and chromosome changes. In: Mutation and Plant Breeding, National Research Council Publication 891, p. 3-29 (1961)

McClintock, Barbara: Chromosome organization and genic expression. Cold Spring Harbour Symposium Quant. Biol. 16, 13-47 (1951a)

McClintock, Barbara: Mutable loci in maize. Carnegie Inst. Wash. Yearb. 50, 174-181 (1951b)

McClintock, Barbara: Further studies of gene control systems in maize. Carnegie Inst. Wash. Yearb. 62, 486-493 (1963)

McClintock, Barbara: The control of gene action in maize. Brookhaven Symp. Biol. 18, 162-184 (1965)

Nuffer, M.G.: Mutation studies at the A_1 locus in maize. I. A mutable allele controlled by Dt. Genetics 46, 625-640 (1961)

Peterson, P.A.: Mutable a_1 of the En system in maize. Genetics 46, 759-771 (1961)

Peterson, P.A.: Influence of mutable genes on induction of instability in maize. Iowa Acad. Sci. 70, 129-134 (1963)

Peterson, P.A.: Novel variation resulting from the effect of a regulatory controller system on a locus in maize. Genetics (Abstract) 1966

Peterson, P.A.: The origin of an unstable locus in maize. Genetics 59, 391-398 (1968)

Peterson, P.A.: Controlling elements and mutable loci in maize: Their relationships to bacterial episomes. Genetica 41, 33-56 (1970)

Peterson, P.A.: Unstable genetic loci as a probe in morphogenesis. Brookhaven Symp. Biol. 25, 244-261 (1973)

Peterson, P.A.: Controlling-elements: The induction of mutability at the A2 and C loci in maize. International Maize Symposium (1975). To be published by Wiley-Interscience, (ed.) D.B. Walden (in press)

Reddy, A.R.: Genetic and chemical studies of gene controlled intermediates in anthocyanin biosynthesis in maize. Ph.D. Thesis (unpublished), Osmania University, India (1974)

Rhoades, M.M.: The genetic control of mutability in maize. Cold Spring Harbor Symp. Quant. Biol. 9, 138-144 (1941)

Richardson, D.L.: An unstable compound locus, apm. Maize Genet. Coop. Newsl. 30, 111-113 (1956)

Sager, Ruth: On the mutability of the wx locus in maize. Genetics 36, 510-540 (1951)

Stadler, L.J.: Spontaneous mutation at the R locus in maize. I. The aleurone color and plant color effects. Genetics 31, 377-394 (1946)

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