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An *En (Spm)*-system insertion partly reduces the color-suppressing potency of the dominant *C-I* allele in maize

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Summary. Two mutable *C-I* alleles, *C-Im*857059 and *C-Im857062* of the *Enhancer-Inhibitor* (*En-I*) or *Suppressor-mutator* (*Spm*) transposable-element system, were shown to express a sectored phenotype (colorless sectors on a colored background). This sectoring is a consequence of an *I* receptor element at the *C-I* allele responding to an independently segregating, transactive *En* element. The *I* element insertion results in the partial reduction of the suppressive potency of the normal *C-I* alleles. A wide range of suppressive potencies of these two *C-Im(r)* alleles was found when tested against other *C* alleles, including *C-S* and *C*. Though each of the *C-Im(r)* alleles has a standard *I* element, there is a significant difference in the suppressive potencies of the two *C-Im(r)* alleles, which possibly indicates a different position of the *I* insert in the coding region affecting the *C-I* transcript.

Key words: Maize – Transposable elements – Anthocyanin – Regulatory gene – Partial suppression

Introduction

The genetic control of anthocyanin pigmentation in the aleurone tissue of maize represents a eukaryotic gene interaction between many dispersed loci in the genome that produce an observable phenotype. At least 15 loci are included in anthocyanin biosynthesis. The *C* (*Colored*) locus plays a regulatory role and, therefore, represents an important feature of this interaction. Substantive proof that this locus is a regulatory one has been derived from genetic (Chen and Coe 1977; Coe 1962, 1964; Hutchison 1922), biochemical (Dooner 1980, 1983; Dooner and Nelson 1979), and molecular analyses (Cone et al. 1986; Paz-Ares et al. 1986, 1987). These studies have shown that the *C* locus regulates the function of other structural genes that code for the different enzymes in the anthocyanin biosynthetic pathway. The *C* locus is tissue-specific in this regulatory role in that it conditions pigmentation in only two tissue, the aleurone and scutellum of the maize kernel. The duplicate locus for *C*, namely *P1*, regulates anthocyanin production in other plant parts such as leaves, stem, anthers, etc., but not in the aleurone and scutellum.

The *C* locus has a number of allelic forms; these include numerous and diverse alleles such as *C-I* (*Color-Inhibtor*), the dominant suppressor of anthocyanin pigmentation; *C-S* (*Colored-Super*); *C* (varied Colored alleles), which conditions anthocyanin; *c-p* (*colored-positive*), the conditioned, colorless allele that produces pigment in germinating kernels only in the presence of light; and the *c-n* (*colored-null*) or *c*, which is the completely recessive, colorless allele. In the order of dominance to recessiveness, they can be arranged as *C-I*, *C-S*, *C*, *c-p*, and *c*. Of these, two different sets of alleles, the *C-I*, on the one hand, and the *C-S* and *C*, on the other hand, assume importance becaue of their antagonistic functions, one suppressing the pigmentation and the other promoting it. Several hypotheses have been proposed to explain the complexity of the *C* locus and the dominant-suppresive effect of the *C-I* alleles over the *C* allele in interactions between these two alleles (Coe 1964; Paz-Ares et al. 1987; Peterson and Leleji 1974).

The two transposable-element-induced mutant *C-I* alleles were characterized to determine the effects of the transposable-element insertion on the dominant-suppresive function of the *C-I* allele and to determine the interaction of these mutable alleles with the other alleles at the *C* locus.

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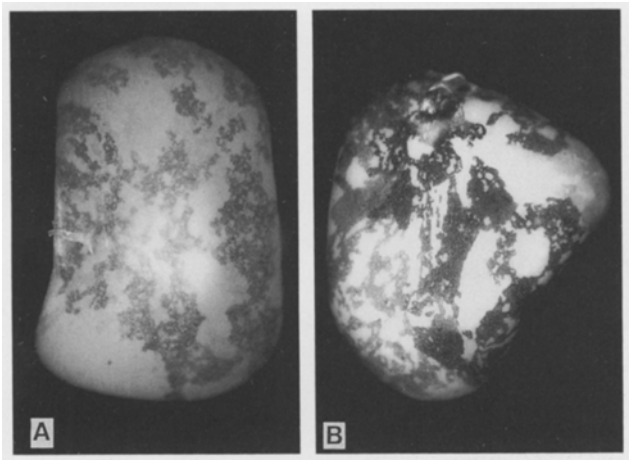

Fig. 1 A and B. The *C-Im* mutant kernels: **A** *C-Im857059*; **B** *C-Im857062*

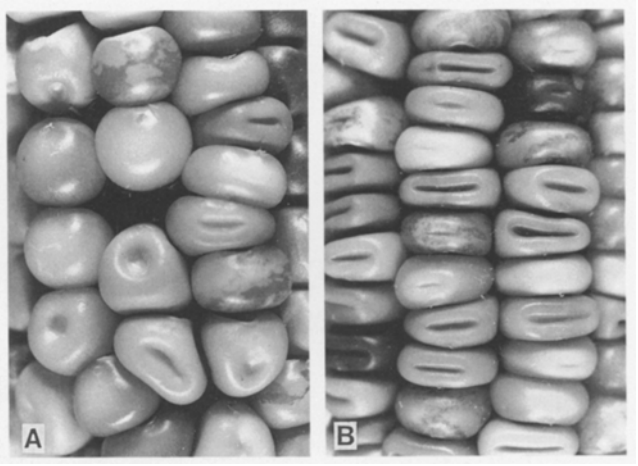

Fig. 2 A and B. Testcross ears of the *C-Im* mutants: **A** *C-Im-857059*; **B** *C-Im857062*

Materials and methods

Origin of the C-I mutable alleles

The two mutable *C-I* alleles studied in this report, *C-Im857059* and *C-Im857062*, originated as independent events from crosses in isolation plots in wich the females plants were homozygous for the dominant *C-I Sh Bz Wx* condition, with one or more *En* elements segregating. The male plants in these isolation plots were a *C sh bz wx* tester line, containing a standard *C* alleles that does not respond to *En*.

[(*C-I Sh Bz Wx*/*C-I Sh Bz Wx En*/−) × (*C sh bz wx*/*C sh bz wx*)]. This cross yielded several exceptional kernel types, *C-I** (*C-I**/*C sh bz wx*), phenotypically identified by colorless sectors on a colored background (Fig. 1 A, B). These variegated, aleurone types were found as single events on an otherwise fully colorless F_1 ear of each cross. Plants from these exceptional kernels were tested to confirm the inheritance, stability, and phenotypic expression of each allele. The rate of origin of these mutable kernels was approximately 15×10^{-6}.

Out of the several exceptional kernel types, two mutable kernels were selected and designated as *C-Im857059* and *C-Im857062*. Both dissplayed a sectored phenotype – colorless sectors on a colored background (Fig. 1 A, B).

Quantitative determination of anthocyanin content

The anthocyanin pigments were extracted in methyl alcohol solution containing 0.1% HCl from the aleurone layers (Reddy and Peterson 1976). Equal amounts (dry weight) of aleurone layers from bulk samples of three different ears were taken for each of the 26 crosses (genotypes of the progeny of these crosses are listed in Table 4), and the anthocyanin pigment content was presented as mean optical density (OD) values. The OD values were recorded at 530 nm. The lines used in the 26 crosses were mostly isogenic, other than at the *C* locus in each set of crosses.

Results

C-Im857059 and C-Im857062: their inheritance and progeny in test crosses

In crosses of these mutants with the *C sh bz wx* tester

(*C-Im Sh Bz Wx*/*C sh bz wx*) × (*C sh bz wx*/*C sh bz wx*) (cross 1)

sectored kernels having the appearance of the parental phenotype appeared among the progeny, thus establishing the heritability of these mutants (Fig. 2 A, B; Table 1). Colorless, pale, and colored kernels also were among the progeny. As will be demonstrated later, the colorless and pale-colored kernel types lack a transactive *En* element. The colored class represents crossovers (*C* and *Sh* are three map units apart) or mutations at the *C-Im* allele. The bronze, sectored (*C-Im sh bz wx*) type was included with the bronze, shrunken class. The frequency of the shrunken class (*sh*) is approximately one-half (Table 1).

Control of mutability by En

In the *C-Im857059* mutant, the presence of *En* and its relation to the mutability was determined by using the *wx-m8* reporter allele in crosses with sectored types, as shown in cross 2:

(*C-Im Wx*/*C wx-m8*) × (*C wx-m8*/*C wx-m8*). (cross 2)

Because the only *wx* allele (i.e., *wx-m8*) in the cross is a reporter for *En*, all sectored kernels that are *wx* should be *wx*-mutable (Fig. 3). This is verified in Table 2, column 2. The colorless kernels that are *wx*-mutable (Table 2, column 6) do have an *En* element, but are not sectoring or are changed derivatives of the sectored class that no longer respond to *En*. The *C-I* pale kernels (Table 2, columns 3 and 4) are without *wx* mutability, indicating that these *C-I* types lack *En*. The paleness is from the *C-Im*/*C* genotype.

The colored class includes the *C*-containing chromosome with the linked *wx-m8* allele, and the occurrence of *wx* mutability is an indication of the segregation of *En* and its reaction on the reporter allele.

The distribution of *En* clearly correlates with the mutability of *C-Im*; the presence of *En* leads to the sectoring pattern and its absence leads to the pale pattern. The *En* element activates the *I* receptor element at the *C-I* allele.

Table 1. Allelism test and test of inheritance of mutability. The cross: (*C-Im Sh Bz Wx/C sh bz wx*) × (*C sh bz wx/C sh bz wx*)

Mutable allele *C-Im85*	*Sh* (round)								Total[a] *Sh*	*sh* (shrunken)					Total *sh*	Total per ear
	Sec.		cl.		Pale	Br.	Cl.			Br.	Sec.	cl.	Pale	Cl.		
	1	%[b]	2	%[c]	3	4	5	%[d]	6	7	8	9	10	11	12	13
7059																
−1	38	(57.57)	19	(28.78)	0	1	8	(12.12)	66	66	0	0	0	2	68	134
−2	24	(60.00)	10	(25.00)	2	0	4	(10.00)	40	33	0	0	0	0	33	73
−3	48	(59.25)	24	(29.62)	2	1	6	(7.40)	81	74	0	1	0	1	76	157
−4	45	(72.28)	8	(12.90)	0	0	9	(14.50)	62	66	0	0	0	0	66	128
−5	40	(45.97)	30	(34.48)	0	0	7	(8.04)	77	83	0	0	1	1	85	162
−6	32	(65.30)	10	(20.40)	1	0	6	(12.24)	49	45	0	0	0	0	45	94
−7	17	(48.57)	10	(28.57)	5	0	3	(8.57)	35	36	0	0	0	0	36	71
−8	27	(65.85)	8	(19.51)	1	0	5	(12.19)	41	38	0	0	0	0	38	79
−9	23	(45.09)	19	(35.84)	5	0	6	(11.32)	53	50	0	1	0	0	51	104
−10	59	(83.09)	13	(15.11)	5	1	8	(9.30)	86	86	0	0	0	0	87	173
7062																
−1	40	(60.60)	23	(34.84)	1	0	2	(3.03)	66	70	0	0	0	1	71	137
−2	30	(41.09)	39	(52.00)	0	0	6	(8.00)	75	73	0	0	0	0	73	148
−3	47	(44.76)	51	(48.57)	3	1	3	(2.85)	105	103	0	1	0	0	104	209
−4	53	(50.00)	37	(34.90)	0	0	16	(15.09)	106	89	0	1	0	1	91	197
−5	67	(46.52)	71	(49.30)	3	1	2	(1.38)	144	143	0	0	1	2	146	290
−6	75	(52.08)	65	(44.82)	0	0	5	(3.44)	145	123	0	0	0	1	124	269
−7	36	(48.00)	30	(38.96)	0	0	11	(14.28)	77	75	0	0	0	0	75	152
−8	84	(45.90)	87	(47.54)	1	1	10	(5.41)	183	177	0	0	1	2	180	363
−9	71	(42.51)	84	(50.29)	4	0	8	(4.79)	167	153	0	0	0	1	154	321
−10	48	(46.60)	50	(48.54)	4	0	1	(0.97)	103	107	0	0	0	0	107	210

[a] χ^2 values for the segregation of 1*Sh*:1*sh*. $P(\chi^2 > 3.84) < 0.05$, not significant in all cases
[b] Percentage of sectored among total round kernels
[c] Percentage of colorless among total round kernels
[d] Percentage of colored among total round kernels
Abbreviations: Sec. = Sectored; cl. = Colorless; Cl. = Colored; Br. = Bronze

Table 2. Summary of results showing the correlation of the presence of *En* with the mutability of *C-Im857059*. The cross: (*C-Im(r) Wx/Cwx-m8 En*) × (*Cwx-m8/Cwx-m8*)

Serial number	Progeny[a]								No. of *En*[b] present	No. of ears[c] examined
	Sectored		Pale		Colorless		Colored			
	wx	*wx-m*	*wx*	*wx-m*	*wx*	*wx-m*	*wx*	*wx-m*		
	1	2	3	4	5	6	7	8	9	10
1	−	+	+	−	+	+	+	+	1	6
2	−	+	+	−	+	+	+	+	2	7
3	−	+	+	−	+	+	+	+	3	3
4	−	+	+	−	+	+	+	+	4	1

[a] Only half of the progeny kernels are presented; the other half would be *Wx*
[b] The number of *En* elements present is dependent on the ratio of sectored versus pale kernels; one element when the ratio is 1:1, two when 3:1, three when 7:1, and four when 15:1
[c] The average number of kernels per ear is 313

This can be described as the *C-Im(r)* receptor allele responding to *En* trans-active signals.

The sectored kernels of the *C-Im857062* allele from Table 1 were crossed with *a-m(r)/a-ml En*-receptor alleles (cross 3), and the resulting F_1 sectored and colored

(*C-Im Sh Bz Wx/C bh bz wx*) × (*a-m(r) Sh2/a-ml sh2*) (cross 3)

progeny was backcrossed again with *a-m(r)/a-ml* (cross 4a, b):

(*C-Im Sh Bz Wx/C Sh Bz Wx A Sh2/a-m(r)* or *a-mlsh2*) × (*a-m(r) Sh2*/a-ml sh2) (Sectored) (cross 4a)

(*C sh bz wx/C Sh Bz Wx A Sh2/a-m(r) Sh2* or *a-mlsh2*) × (*a-m(r) Sh2/a-ml sh2*) (Colored). (cross 4b)

Table 3. Summary of results for confirming that *En* independently triggers the mutability of *C-Im(r)857059* and *C-Im(r)857062*

Mutable allele	Number of ears examined		
	Total	With sectored kernels	Without sectored kernels
A. With *En*			
C-Im(r)857059	24	24	0
C-Im(r)857062	25	24	1
B. Without *En*			
C-Im(r)857059	21	0	21
C-Im(r)857062	24	0	24

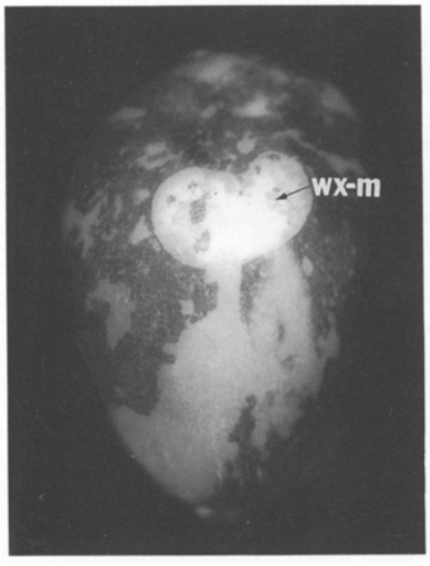

Fig. 3. *C-Im857059* sectored kernel showing the mutability of *wx-m8* to *Wx*

Although the sectored kernel progeny showed *En* activity (*a-m(r)* or *a-ml* spotting) in 29 out of 29 ears, only 8 of the 29 progeny ears from colored F_1 kernels showed spotting (data not shown). If *En* was carried by the line and was unrelated to the mutability at *C-Im-857062*, then it would be expected that all the crosses involving the colored F_1 kernels would show *En* activity like the sectored sib kernels. This indirect evidence suggests that the mutability at *C-Im-857062* might be under the control of the *En* two-element system. The proposed assessment of the *C-Im(r)* alleles can be tested in a reconstitution test.

Reconstitution test

When the colorless kernels (Table 1) and the pale kernels (*C-I* pale) (Table 2) are crossed with the *wx-m8* allele (the kernels containing the *wx-m8* allele are from the same parental ear) with and without *En* (crosses 5 and 6),

$$(C\text{-}Im(r)/C) \times (C\ Sh\ Bz\ wxm\text{-}8/C\ Sh\ Bz\ wx\text{-}m8\ En/-) \quad \text{(cross 5)}$$

$$(C\text{-}Im(r)/C) \times (C\ Sh\ Bz\ wxm\text{-}8/C\ Sh\ Bz\ wx\text{-}m8\ +/+ \quad \text{(cross 6)}$$

sectored kernels would be an indication of the reconstitution of mutability. Such mutability occurred only with crosses containing *En* (Table 3), supporting the claim that *En* activates the *I* element at the *C-Im(r)* allele. The sectoring is colored to colorless and indicates the restoration of *C-I* inhibition after the excision of the *I* element from the locus (Fig. 4A–D). The single deviant ear of the *C-Im(r)857062* allele containing no sectored kernels (Table 3) could be because the *C-Im(r)* allele had become a non-responsive allele [*C-Imn(r)*] in this instance.

No other transposable-element systems that were tested such as *Ac*, *Dt*, *Bg*, *Fcu*, *Mrh*, *Mut*, and *Uq*, except

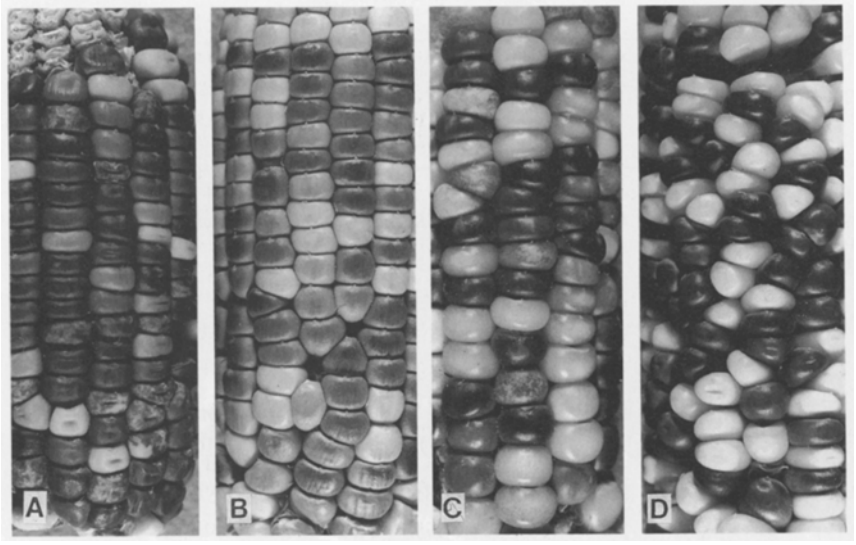

Fig. 4A–D. Reconstitution crosses of the *C-Im* mutants: **A** *C-Im(r)857059* with *En* showing sectored kernels; **B** *C-Im(r)857059* without *En* with no sectored kernels; **C** *C-Im(r)857062* with *En*; **D** *C-Im(r)857062* without *En*

Table 4. Allelic interaction as shown by aleurone pigmentation in different combinations of *C-I* and *C-Im(r)* alleles with *C-S, C,* and *c* alleles

Cross no.	Genotype[a]	Phenotype	Mean OD[b]
1	*C-I C-I* C^1	colorless	0.009 a
2	*C-Im(r)59 C-Im(r)59* C^1	light pale	0.148 c
3	*C-Im(r)62 C-Im(r)62* C^1	v. light pale to colorless	0.065 b
4	*C-I* C^1 C^1	colorless	0.010 a
5	*C-Im(r)59* C^1 C^1	med pale	0.381 f
6	*C-Im(r)62* C^1 C^1	light pale	0.182 c
7	*C-I C-I* C^2	colorless	0.007 a
8	*C-Im(r)59 C-Im(r)59* C^2	med pale	0.420 g
9	*C-Im(r)62 C-Im(r)62* C^2	light pale	0.265 e
10	*C-I* C^2 C^2	colorless	0.015 a
11	*C-Im(r)59* C^2 C^2	dark pale	0.610 j
12	*C-Im(r)62* C^2 C^2	med pale	0.404 g
13	*C-I C-I C-S*	colorless	0.009 a
14	*C-Im(r)59 C-Im(r)59 C-S*	colored	0.670 k
15	*C-Im(r)62 C-Im(r)62 C-S*	colored	0.580 i
16	*C-I C-S C-S*	dark pale	0.623 j
17	*C-Im(r)59 C-S C-S*	colored	0.913 m
18	*C-Im(r)62 C-S C-S*	colored	0.865 l
19	*C-I C-I c*	colorless	0.007 a
20	*C-Im(r)59 C-Im(r)59 c*	colorless	0.011 a
21	*C-Im(r)62 C-Im(r)62 c*	colorless	0.005 a
22	*C-I c c*	colorless	0.006 a
23	*C-Im(r)59 c c*	colorless	0.009 a
24	*C-Im(r)62 c c*	colorless	0.012 a
25	*c c* C^1	colored	0.211 d
26	*c* C^1 C^1	colored	0.459 h

[a] C^1 allele originated from *C sh bz wx* tester stock, and C^2 allele is derived from line *C* stock

[b] Mean ODs followed by different letters significant at $P=0.05$ (Student-Newman-Keul's test)

the *En* system, induced mutability at both the *C-Im(r)* alleles (Jayaram 1988).

Anthocyanin pigments in these insert-laden C-I alleles

The *C-Im(r)* alleles were crossed and heterozygotes were obtained with several *C* alleles [*C-S*, *C* (line *C* source), *C sh bz*, and a recessive *c* allele] (Table 4). The original *C-I* allele from which the *C-Im(r)* alleles originated was used in control combinations. These combinations were made to test the inhibitory potency of *C-Im(r)*.

The different combinations 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 OD values are given in Table 4. All the control combinations with the original *C-I* allele, except *C-I C-S C-S*, are colorless and have negligible or no anthocyanin pigment content. The combination involving the *C-Im(r)* allele shows various phenotypes, from colorless to dark colored, having a wide range of anthocyanin pigment contents. The OD values also differ with respect to the two different *C-Im(r)* allelic combinations, with the *C-Im(r)857059* combinations showing comparatively higher OD values.

Discussion

Each of the alleles, *C-Im857059* and *C-Im857062,* arose as independent events. Their variegated phenotype (sectoring, colored to colorless in the aleurone layer) in the presence of a transactive *En* is an indication that these alleles possess an insertion of an *I* receptor element at the original *C-I* allele (Peterson 1985). This indication of a response to *En* makes these *C-I* receptors or *En* reporter alleles, and as such they can be referred to as *C-Im(r)857059* and *C-Im(r)857062.*

The higher anthocyanin pigment content in combinations involving the mutant alleles (Table 4) indicates that the *I* element insertion at these alleles has a significant effect in reducing the suppressive potency of the *C-I* alleles in heterozygotes with other alleles at the *C* locus. The *C-Im(r)857059* allelic combinations show comparatively higher OD values than those of *C-Im(r)857062* combinations. This indicates a difference in suppressive potency between the two mutants, the *C-Im(r)857059* having less potency. This could be due to the difference in the position or composition of the *I* element insert at the *C-I* allele, because both these mutable alleles arose as independent events. Also, the reduction in the suppressive potency rather than complete loss of suppressive activity can be observed when OD values in the two sets of crosses with numbers 2 and 3 versus 25 and 5 and 6 versus 26 (Table 4) are compared. If there is a complete inactivation of the *C-I* allele due to *I* element insertion, the *C-I m(r)* allele might act as a *c* allele.

The dominant action of the *C-I* allele in inhibiting color formation in the aleurone has been the subject of considerable speculation, both from genetic and molecular observation. With the cloning and sequencing of the *C* gene and many of the applicable alleles associated with the *C-I* effect, it was possible to identify the putative protein with its representative domains. There is a basic amino terminus and an acidic carboxy terminus, as deduced from theoretical considerations of the cDNA (Cone et al. 1986; Paz-Ares et al. 1986, 1987). Transcriptional activation is thought to be involved with the acidic domain (Paz-Ares et al. 1987). However, the transcript of the *C-I* allele is not complete enough to produce a wild-type protein because of a frame shift mutation and an internal deletion, and results in the absence of the acidic domain at the carboxy terminus (Paz-Ares et al. 1990). Because the *C-I* gene product still has an intact basic

domain, the DNA binding capacity is retained and thus can compete for the same site of action as the protein encoded by the *C* allele, resulting in a lack of color activation and, effectively, color inhibition. Consequently, anthocyanin pigment production in aleurones heterozygous for the *C-I* allele is a consequence of the balance between the quantitative of both proteins produced by the *C-I* and *C* alleles. However, this hypothesis is yet to be proven, and the absence of the acidic domain might not have anything to do with the inhibitory potency of the *C-I* allele in interactions with the their *C* alleles.

The reduced potency of the *C-Im(r)* alleles can be explained in two ways. One possibility is that the transcript still might be produced, but in an altered form, and that the insert gets spliced off during the processing of the transcript. There are several instances in which such a mechanism is shown to be operating (Hwa et al. 1987; Wessler et al. 1986, 1987). Alternatively, the *I* element insertion at the *C-Im(r)* alles might be within or near the region that codes for the basic domain of the protein product. Thus, the insertion might affect the DNA binding capacity of the protein which, in turn, reduces the suppressive potency. Molecular analysis of these mutants might explain the interaction of the *C-I* allele with the other alleles at the *C* locus.

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