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GENETIC AND MOLECULAR ANALYSIS OF CANALIZATION AT THE MAIZE *r1* LOCUS

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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List of Abbreviations

ANOVA: Analysis of Variance

BSA: Bovine Serum Albumin

DNA: Deoxyribonucleic acid

EDTA: Ethylenediaminetetraacetic acid

EMS: Ethyl methyl sulfonate

5-meC: 5-methyl cytosine methylation

L or (L): *R-sc:86-17*(*L*)

RdDM: RNA directed DNA Methylation

RT-PCR: Reverse Transcription Polymerase Chain Reaction

Abstract

GENETIC AND MOLECULAR ANALYSIS OF CANALIZATION AT THE MAIZE *r1* LOCUS

By Jennifer Haley Derkits, B.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biology at Virginia Commonwealth University.

Virginia Commonwealth University, 2013

Major Advisor: William B. Eggleston, Jr.
Associate Professor, Department of Biology

Canalization, the stability of phenotypes in the presence of a specific genotype and environment is important for trait selection and understanding gene expression pathways. The goal of this study was to investigate mechanisms involved in the loss of canalization at the maize *r1* locus. A specific lightly colored *R-sc* derivative, *R-sc:86-17*(*L*), was mutagenized with ethyl methyl sulfonate (EMS), and the progeny screened for restoration of dark seed color and canalization. Putative revertants/mutants were mapped relative to the *r1* locus, tested for complementation to each other to identify a minimum number of genes involved in canalization, tested for *cis* silencing of the *r1* gene *Lc*, analyzed to determine the genetic basis for loss of canalization and *r1* gene expression, analyzed for changes in molecular structure at the *r1* locus, and analyzed for changes in cytosine methylation patterns. At least two complementation groups were found to be responsible for increased seed color, one at or near *r1* and one elsewhere in the genome. Phenotypic analysis revealed highly variable kernel pigmentation, possible incomplete

penetrance and a novel class termed *Nc* enhancers. *Nc* enhancers were characterized by light to heavy mottling. Southern blot hybridization analysis demonstrated that 19 out of 20 putative EMS-induced mutants were unchanged in the molecular structure of genes *Sc*‖*nc1, Nc2,* and *Nc3.* One mutant resulted in the loss of one of the *Nc* genes. The loss of *Lc* expression was found to be rare in *R-sc:86-17*(*L*) *Lc,* and restoration of seed color in *R-sc:86-17(L)/P* heterozygotes was found to have resulted from recombination between *Sc*‖*nc1* and *P* with loss of *Nc2* and *Nc3*. Analysis of cytosine methylation patterns revealed low levels of methylation in the 5' region of *Sc* and high levels in the 5' region of the *Nc* genes and in the 3' ends of *Sc*‖*nc1, Nc2* and *Nc3* in the homozygous putative EMS-induced mutants as previously found for *R-sc:86-17*(*L*). Four out of the twenty EMS-induced mutants tested displayed altered methylation patterns, with changes at both the 5' and 3' regions of the *r1* genes. These findings suggest that the genes or elements involved in the canalization and/or restoration of seed color of *R-sc* are located at the *r1* locus, at the 3' end of the *Sc* gene and that at least one additional site in the genome is involved in seed color expression of *r1*.

Introduction

Epigenetics, Canalization and Habituation

Changes in gene expression which occur in the absence of altered DNA sequences are termed epigenetic. The term epigenetic also describes changes in gene expression during development, which was the first use of the term (Dupont *et al*. 2010). The phenotypes affected by epigenetic changes can be heritable through meiosis (Mosher and Melnyk 2010). Examples include: *X*-chromosome inactivation, genomic imprinting, gene silencing, and paramutation, all of which occur in many organisms (Salathia and Queitsch 2007; Mosher and Melnyk 2010).

The term canalization refers to the ability of a phenotype to be buffered against genetic and/or environmental perturbations over time (Waddington 1942). Canalized phenotypes are expressed in certain environments with a specific genotype, allowing for natural selection to take place (Sato and Siomi 2010). Hsp 90 (chaperone heat shock protein) in *Drosophila* has been suggested to play a role in canalization. When Hsp 90 is functional, genetic variation is observed over a normal range and cryptic (Figure 1a) (Sato and Siomi 2010). When the function of Hsp 90 is reduced or eliminated, (Waddington 1953; Rutherford and Lindquist 1998) an increase in phenotypic variation is observed which may be selected upon (Figure 1b) (Sato and Siomi 2010). Sato and Siomi (2010) suggested that reduction in the Hsp 90 pathway result in transposon-mediated mutagenesis which causes traits such as eye and body color to become more variable (Figure 2). Key components in canalization are: a) changes in phenotype can be assimilated to

produce new static phenotypes and b) that there is a major change in the genetics or environment to cause a change in phenotype (Debat and David 2001).

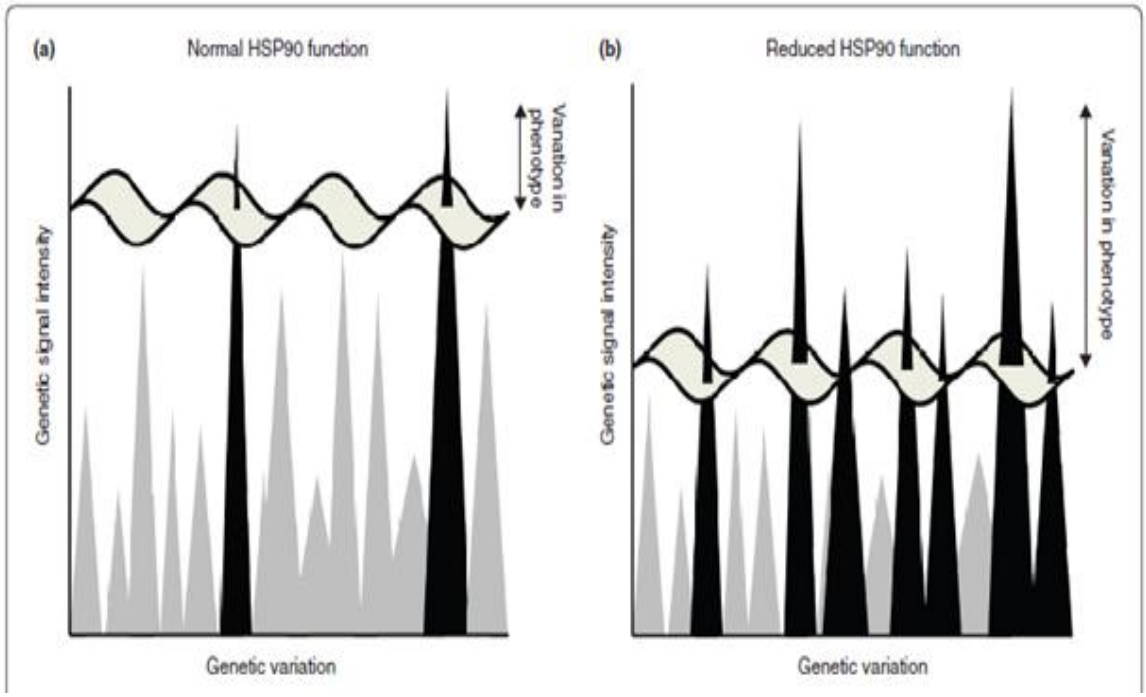


Figure 1: Effect of normal and reduced function of Hsp 90 on phenotypic variation. a: Above wavy line is the phenotypic observed variation. Normal changes are seen as "within normal variation." b: When HSP 90 function is reduced, higher amounts of genetic phenotypic variation are observed and can be selected upon. From Soto and Siomi (2010).

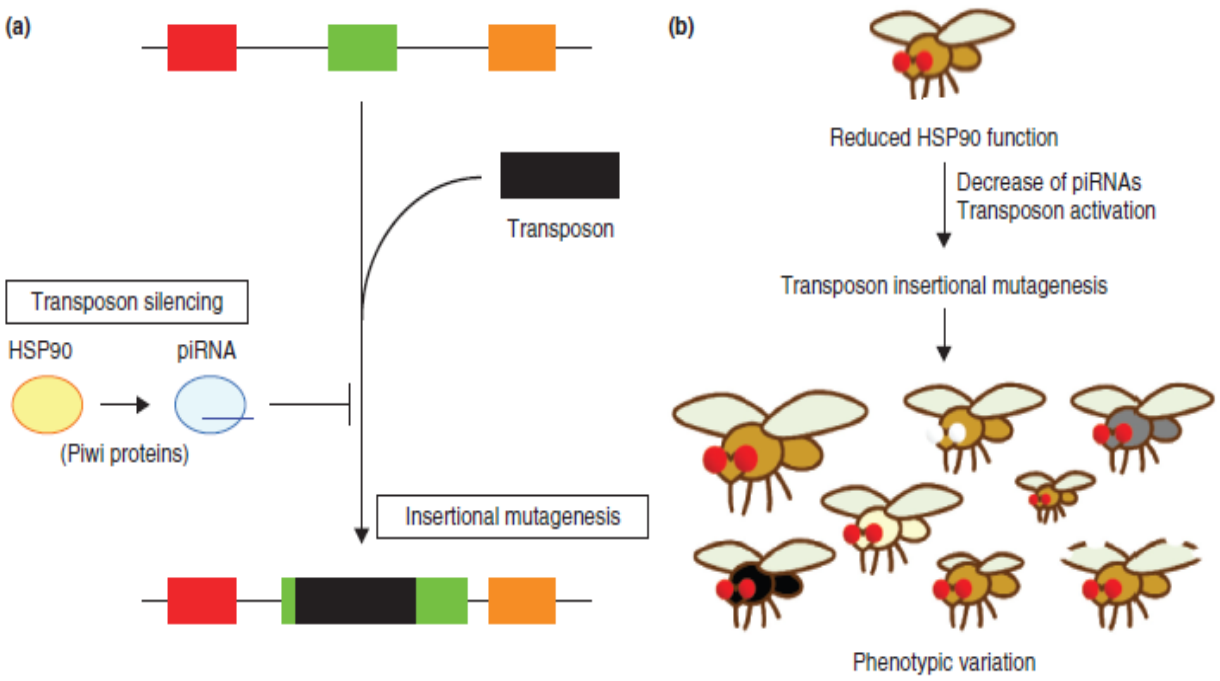


Figure 2: Hsp 90 functions in the suppression of transposon-mediated 'canonical' mutagenesis via the piRNA pathway. (a) Activated transposons can move from one site in a genome to another by the process of transposition with the potential to induce *de novo* gene mutations by insertion. Hsp 90 could be involved in the control of transposition by affecting piRNA biogenesis. (b) Reduced Hsp 90 function leads to transposon-mediated insertional mutagenesis and the induction of morphological variability, such as variation in eye and body color. From Sato and Siomi (2010).

Another term which addresses changes in phenotypes is habituation (Meins 1989). Habituation results from gradual changes in cell heritability, that occur more frequently than classical mutations, and are reversible (Meins 1989). Meins described habituation based on reduction in

the levels of cytokines, hormones, and vitamins over multiple generations in tobacco which led to observable changes in phenotype (Meins 1989).

Although the mechanism is unclear, the behavior of one allele of the *r1* locus in maize, and the focus of this work, is similar to habituation or observed gradual phenotypic changes. *The R gene family*

The *R* gene family contains over 100 alleles found from diverse geographic regions (Ludwig *et al*. 1989). Members of the *R* gene family reside on the long arm of chromosome *10* at the *r1* locus, and in a small minority of strains at the *lc1*/*sn1* locus 4 mu distal to *r1*. Examples of *r1* genes include *Lc, Sn:bol3, S, P, Sc, Nc, R-ch:Hopi,* and *Nj* (Ludwig *et al*. 1989; Dooner *et al*. 1991; Alleman and Kermicle 1993; Consonni *et al*. 1993; Eggleston *et al*. 1995; Li *et al*. 2001; Petroni *et al*. 2001; Hichri *et al*. 2011) (Table 1). Molecular analyses have shown that naturally occurring *r1* alleles can have from zero to five *r1* genes. Complete and/or incomplete *R* genes have been observed (Eggleston *et al*. 1995; Walker *et al*. 1995). *r1* alleles activate anthocyanin (red and purple pigments) expression in a wide array of tissues including the aleurone of the kernel, scutellum, pericarp, root, husks, silks, leaves, anthers, coleoptile, leaf basis (ligule), and glumes (Stadler 1948; McWhirter and Brink 1962; Consonni *et al*. 1993; Tonelli 1994; Kermicle 1997; Petroni *et al*. 2000). Anthocyanins serve a variety of functions including attraction of pollinators, protection against insects, protection from phytopathogens, protection from UV light, and adaption to cold stress (Petroni *et al*. 2000; Hichri *et al*. 2011). Individual *r1* alleles can have anthocyanin expression in none, a subset of, or all of the tissues noted. Since the

1950's, the *r1* locus has been used to study gene regulation, transposable elements, paramutation, imprinting, gene structure, and cancer (Eggleston *et al*. 1995; Pilu 2011).

MYB, MYC and the WD40 Complex

Three gene families [*r1* (*red color1*)/*b1* (*booster 1*); *c1 (colorless 1)/pl1 (purple leaf 1)* and *pac1 (pale aleurone color1)*] act together as transcriptional activators of the structural genes *C, Chi, F, A,* and *Bz* that encode proteins directly involved in the biosynthesis of anthocyanin (Ludwig *et al*. 1989; Consonni *et al*. 1993; Petroni *et al*. 2001; Carey *et al*. 2004; Hichri *et al*. 2011). The maize *r1* and *b1* loci are duplicate loci located on chromosomes *10* and *2* respectively, and both encode proteins that have an amino acid sequence containing the basic helix-loop-helix (bHLH) domain, homologous to the *MYC* family of transcription factors (Ludwig *et al*. 1989; Consonni *et al*. 1993; Hichri *et al*. 2011). The bHLH proteins are found in numerous organisms including yeast, *Arabidopsis*, and rice (Hichri *et al*. 2011). Hichri *et al*., (2011) report that these proteins, depending on the target promoter, may act alone to bind DNA or as a dimer with the MYB oncoproteins. In maize, *c1* and *pl* are duplicate loci located on chromosomes *9* and *6* respectively, and encode proteins with homology to *MYB* transcription factors (Cone *et al*. 1986 and 1993; Petroni *et al*. 2000). *MYB* transcription factors are responsible for DNA binding, dimerization, and regulating target gene expression (Hichri *et al*. 2011). The *MYC* and *MYB* transcription factors work in a ternary complex with WD40 proteins enabling the complete biosynthesis of anthocyanin (Petroni and Tonelli 2011). For example, full activation of anthocyanin aleurone in maize requires the WD40 gene *pac1* to be present. *pac1* mutants display reduced pigmentation in the seed (Carey *et al*. 2004). A WD40 gene was recently found to be

integrated into the *Mu* transposable element *Mu13* (Tan *et al*. 2011). *Mu13* is transpositionally active and contains two open reading frames encoding the WD40 protein, and is found exclusively in W22 and Robertson's Mutator line (Tan *et al*. 2011). The *r1/b1*: *c1/p1*: WD40 complex present in maize is also found in *Arabidopsis.* In maize the complex is required for complete anthocyanin biosynthesis (from isoflavones through proanthocyanidins), while in *Arabidopsis*, the early anthocyanin biosynthesis genes are independent of the complex which is only required for late stage biosynthesis (from flavonols through proanthocyanidins) (Petroni and Tonelli 2011).

Table 1: Select *r1* gene expression patterns in maize.

Gene name	Location of pigmentation in maize	Citation	Haplotypes
S	aleurone	Walker *et al*. 1995	*R-r:standard*
P	roots, anthers, coleoptile	Walker *et al*. 1995	*R-r:standard*
Sc	aleurone, coleoptile tip	Eggleston *et al*. 1995	*R-stippled*
Nc	near colorless aleurone	Eggleston *et al*. 1995	*R-stippled*
R-ch:Hopi	scutellum, aleurone, pericarp, root, coleoptile, leaf basis, glume, and pericarp	Petroni *et al*. 2000	*R-cherry:Hopi*
Nj	aleurone of the seed, scutellum, root, and anthers	Consonni *et al*. 1993	*R-nj:Cuda*
lc1	root, scutellum, coleoptile, leaf basis, pericarp, and glume	Ludwig *et al*. 1989	*R-r:1172 Lc*
sn1	root, scutellum, coleoptile, leaf basis, pericarp, and glume	Tonelli *et al*. 1994	*Sn:bol3*

*Single genetic units (Ludwig *et al*. 1989; Tonelli *et al*. 1994; Petroni *et al*. 2000)

Composition of r1 haplotypes

Many *r1* alleles are comprised of multiple *r1* genes (Li *et al*. 2001). The individual *r1* genes in the "haplotypes" can be in a forwards or backwards orientation and can be close together or several hundred kilobase pairs apart (Eggleston *et al*. 1995; Walker *et al.* 1995; Li *et al*. 2000). Examples of genetically complex *r1* alleles include: *R-stippled* (*R-st)*, *R-marbled* (*R-mb)* and *R-r standard* (*R-r)* and their derivatives (Kermicle 1984; Eggleston *et al*. 1995; Panavas 1999).

Origin and composition of R-sc:86-17pale

As shown in Figure 3, *R-sc:86-17pale* is directly derived from *R-stippled (R-st).* *R-st* is composed of four *r1* genes: *Sc* and three copies of the *Nc* gene (*Nc1*, *Nc2*, and *Nc3*) (Eggleston *et al*. 1995). The allele is somatically and germinally unstable, producing spotted seeds, due to excision of the transposable element *I-R* (*inhibitor of R*) from the *Sc* gene (Eggleston *et al*. 1995). Germinal reversion of *R-st* to *R-sc* (self-colored with full seed pigment) occurs as a result of unequal recombination between the *r1* genes in *R-st* and by *I-R* excision (Kermicle 1970; Eggleston *et al*. 1995). Ashman (1960) studied *R-st* and recovered 99 *R-sc* independent mutants, one of which was *R-sc:86,* the progenitor to *R-sc:86-17pale.* *R-sc:86* arose from unequal cross over and contains three *r1* genes (Eggleston *et al*. 1995). McWhirter (1961) studied 83 out of the 99 *R-sc* mutants (*R-sc:53-138*) for paramutagenicity and germinal stability and found that the alleles varied greatly in the ability to induce paramutation (McWhirter 1961). From controlled matings of the *R-sc* alleles, 17 independent phenotypic mutations were found. Among these, a single ear arising from *R-sc:86* contained a cluster of 7 unstable brownish kernels, including *R-sc:86-17pale* (McWhirter 1961).

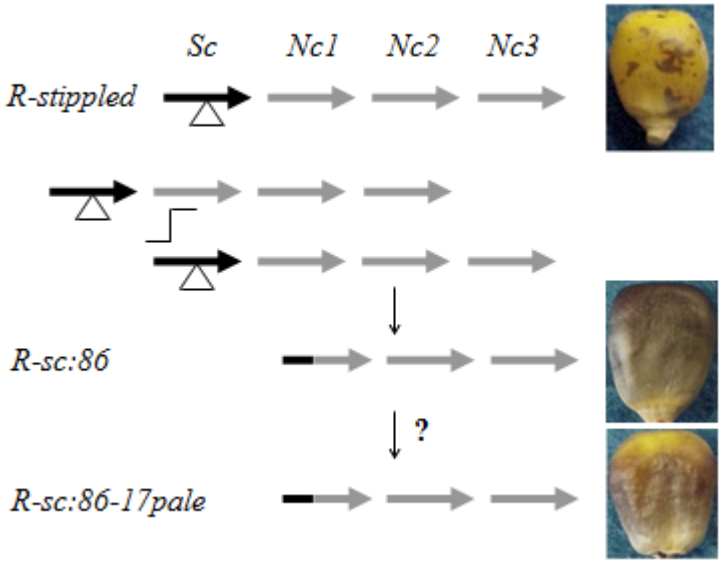


Figure 3: Origin and structure of *R-sc:86-17pale*. Arrows represent *r1* genes and direction of transcription. Centromere is to the left. Black arrows represent the *Sc* gene, shaded arrows the *Nc* genes and triangles are the *I-R* transposable element. Not to scale. Kernel phenotypes are shown to the right. From W. Eggleston pers. comm.

Due to the unusual phenotypic kernel variation on homozygous ears, McWhirter chose *R-sc:86-17pale* for further study. Starting with homozygous plants, McWhirter repeatedly selected for the darkest or lightest seeds over five generations of self-pollination. He also reversed selection midway in both the light and dark direction (W. Eggleston pers. comm.). The final states of these selections were found to be semi-stable, and based on the original change from *R-sc:86* to *R-*

sc:86-17pale, potentially indicate a loss of canalization of the *R-sc:86-17pale* allele (Figure 4). The selections by McWhirter of *R-sc:86-17* and *R-sc:86-17pale* (semi-stable phenotypes, which are reversible) could also potentially be attributed to habituation as the change is gradual and arose over several generations due to selection and is reversible.

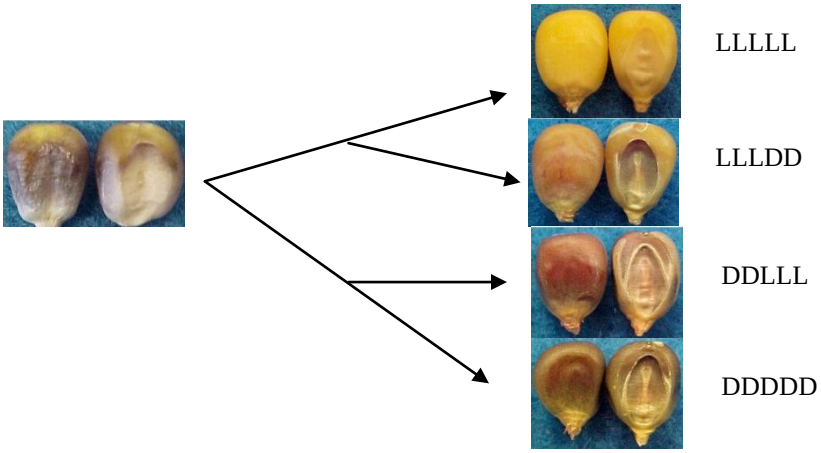


Figure 4. Kernel phenotypes of *R-sc:86-17pale* and putative epiallelic derivatives. All materials were maintained and are shown as homozygotes. Beginning with *R-sc:86-17pale* (left), selection regimens from top to bottom, were: LLLLL, DDLLL, LLLDD and DDDDD. L's denote selecting, planting and self-pollinating the lightest kernels and D's, the darkest (W. Eggleston pers. comm.)

McWhirter determined that *R-sc:86-17* was weakly paramutagenic (McWhirter 1960). To test whether the altered expression of *R-sc:86-17pale* resulted from self-paramutation (gene silencing), the darkest (*R-sc:86-17*(*D*)) and lightest (*R-sc:86-17*(*L*)) sublines were tested for paramutagenicity and the ability to effect each other's expression, *R-sc* expression, and *Lc*

expression in *trans. R-sc:86-17*(*L*) was also tested for the ability to silence *R-sc:124* and *R-sc:86.* In no case was there evidence of heritable or non-heritable gene silencing by *R-sc:86-17*(*L*) of other *r1* alleles or *Lc* (W. Eggleston pers. comm). These results suggest that the pathway involved in silencing *R-sc:86-17(L)* is not related to the siRNA or miRNA pathways. If siRNA or miRNA had caused the original change from *R-sc:86* to *R-sc:86-17pale*, then *R-sc:86-17(L)* would have silenced *r1* genes and *Lc* when in *trans* (W. Eggleston pers. comm.).

Structure and 5-meC patterns of R-sc:86-17 derivatives

To test whether *R-sc:86-17* and its derivatives resulted from a structural change at *r1*, Eggleston (pers. comm.) showed that the DNA structure of *R-Sc:86, R-sc:86-17(L),* and L-D variants of *R-sc: 86-17pale* are identical by Southern blot methods, using restriction enzymes insensitive to 5-methyl cytosine patterns (5-meC). Significant differences were detected in *r1* gene 5-meC patterns between *R-sc:86*, *R-sc:86-17*, and *R-sc:86-17*(*L-D*) (Figure 5) using restriction enzymes sensitive to 5-meC. Specific differences in methylation were found in 4-6 leaf seedlings with an increase in methylation at the 3' regions and a decrease at the 5' end of *Sc* from *R-Sc:86* to *R-Sc:86-17pale* and its' derivatives, indicating an epigenetic rather than a genetic basis for the observed differences in phenotypes (W. Eggleston, unpubl. obs.). Higher 3' 5-meC levels were correlated with decreased kernel color (W. Eggleston, unpubl. obs.).

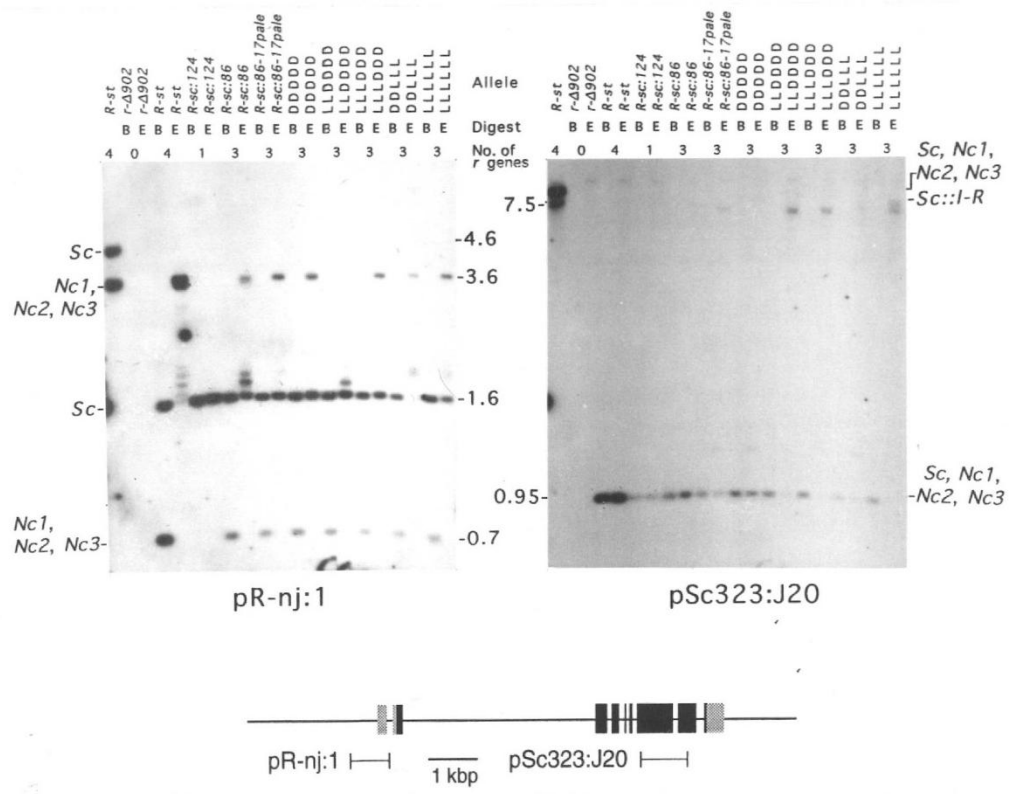


Figure 5. Southern blot analysis of 5-meC patterns of *R-st* derivatives. DNA from seedlings homozygous for alleles noted was digested with *Hin*dIII (methylation insensitive) alone (left lane) or in combination with the isoschizomers *Eco*RII (E) (sensitive) or *Bst*NI (insensitive). Left panel shows hybridization with pR-nj:1 and right panel, with pSc323:J20. Above each lane is the allele, enzyme and number of *r1* genes present. Probes are shown on canonical *r1* gene on which transcription begins to left, thin line indicates introns, black boxes exons and grey boxes 5' and 3' UTRs, based on comparison of the *Sc* sequence (M. Alleman, J. Kermicle and W. Eggleston, pers. comm.) with *Lc* (Ludwig *et al.* 1989, 1990; S. Ludwig, L. Habera and S. Wessler, pers. comm. to W. Eggleston). L's and D's as in Figure 4. *R-sc:124* contains only the single *r1* gene *Sc*||*nc3* (Eggleston *et al*. 1995).

Mapping the genetic basis for R-sc:86-17(L)

To test the genetic and molecular basis for loss of kernel color and canalization of *R-sc:86-17(L)* Eggleston (pers. comm.) made *R-sc:86-17(L)* heterozygous with *r-r:n142* (yellow seed allele, red plants parts, containing a single *P* gene) and performed testcrosses (Figure 6). In initial tests, six heritable full color revertants were observed. DNA isolation and Southern blot analysis showed that the *Sc||nc1* gene had recombined with *P* to create *Sc||p* (loss of *Nc2* and *Nc3*) with fully colored seeds in all six full-color revertants. These results indicate that the lesion responsible for *R-sc:86-17pale* is at or near the 3' end of *Sc||nc1* gene in *R-sc:86* (W. Eggleston pers. comm.).

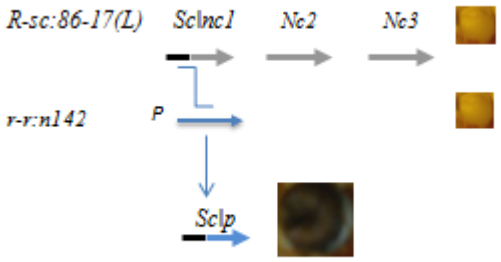


Figure 6. Origin and structure of full-color revertants from *R-sc:86-17(L)*/ *r-r:n142* heterozygotes. Arrows as in Figure 3. Blue arrow represents *P* gene (W. Eggleston pers. comm.). See Table 1 for expression patterns.

Objectives of the present study

R-sc:86-17pale was found not only to have initially reduced *Sc* expression, but also have an unstable phenotype. Generated after five generations of habituation, when selecting for lightest seed color, *R-sc:86-17*(*L*) is an excellent model to determine how and where canalization was lost at *r1*. While the prior methylation pattern observations are consistent with current siRNA models (Erhard *et al*. 2013), previous genetic tests show a lack of *trans* silencing of *r1* alleles and *Lc.* These data are not consistent with several *trans*-based silencing mechanisms, including siRNA or miRNA. Alternative models include processes involving transposon systems (the maize genome is over 85% transposons) or novel mechanisms of the RNA-directed DNA methylation (RdDM) pathway (Erhard *et al*. 2013).

This thesis will focus on whether the reduction in gene expression and habituation of *R-sc:86-17pale* was due to loss of narrow expressivity/ loss of canalization at *r1*. To address this *R-sc:86-17(L)* was mutagenized with EMS, which causes transition mutations (G:C to A:T) (W. Eggleston pers. comm.), to restore seed color to determine how many genes are potentially involved in the restoration of canalization and identify location(s) responsible for the lesion(s) (at or near *r1* or somewhere else in the genome). Additionally the ability of *R-sc:86-17(L)* to silence *Lc* in *cis* was tested.

To address the above objectives homozygous putative EMS-induced mutants were phenotypically evaluated and mapped relative to the *r1* locus and to each other in order to identify *cis*- and *trans*-sites involved in canalizing *r1*. The putative mutants were analyzed by Southern blot analysis to determine structure and 5-meC patterns. Six additional full-color

revertants from *R-sc:86-17(L)/P* heterozygotes were analyzed by Southern blot analysis for structure and methylation to determine structure and further localize lesions associated with *R-sc:86-17pale. R-sc:86-17(L) Lc* was analyzed phenotypically and by Southern blot analysis to determine whether the reduced expression at the *r1* locus affects *Lc* expression in *cis* and the molecular basis for the loss of expression.

Materials and Methods

All tests were performed using inbred maize line W22 unless otherwise noted.

Table 2: Select *r1* haplotypes

Haplotype	Location of pigmentation in maize	Citation
R-sc:86	full-color purple/black aleurone and green plant color	Eggleston *et al*. 1995
R-sc:86-17	full-color purple/black aleurone and green plant color with a cluster of 7 lighter non-purple/black kernels	McWhirter, pers. comm to W. Eggleston
R-sc:86-17(L)	yellow aleurone and green plant color (lightest of the five generations from *R-sc:86-17*)	McWhirter, pers. comm to W. Eggleston
R-sc:124	full-color purple/black aleurone and green plant color	McWhirter 1961
R-st	stippled (spotted) black aleurone and green plant color	Eggleston *et al*. 1995
R-nj:Cuda	crown aleurone color, scutellum, root, and anthers	Dellaporta *et al*. 1988
r:Δ902	colorless aleurone (lack of *r1* genes) with green and yellow plant color	Eggleston *et al*. 1995
g1 R-g:8 pale	yellow plant parts and light pale aleurone	Kermicle 1984
R-r:1172 Lc	full-color black aleurone with red plant parts, scutellar node, pericarp, and ligules	Pers. comm. W. Eggleston
r-r:*n142*	yellow aleurone, red plant parts	Walker *et al*. 1995

See Figure 7 for molecular structures of select *r1* haplotypes and Table 1 for gene expression patterns.

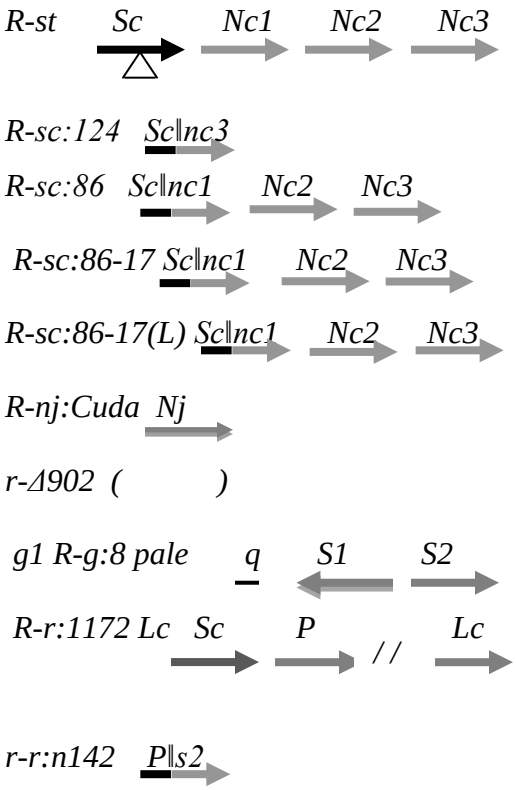


Figure 7: Molecular structure of select *r1* haplotypes. Symbols as in Figure 3.

Phenotypic Color Scale: Due to the observed kernel variability among the derivatives of *R-sc:86-17(L)*, a phenotypic color scale was created to systematically identify and quantitate different pigmentation states. Representative kernels were screened on a range from lightest to darkest for growing locations in Virginia and Florida and for *y1+* and *y1-* genetic backgrounds: 1-2, no color or very light (light pale); 3-4, some color (medium pale); 5, solid color and slightly darker than medium (dark pale); and 6, dark color, but not as dark as the progenitor *R-sc:86*. Kernel color scales are shown in Figure 8.

In addition to the color scoring, an Agtron reflectometer was used to determine mean reflectance values for each allele measured as described in Alleman and Kermicle (1993). Kernels from homozygous putative EMS-induced mutants were cleaned to get rid of the glumes and four independent reflectance measurements taken. Statistical analyses were performed using John's Macintosh Project (JMP®).

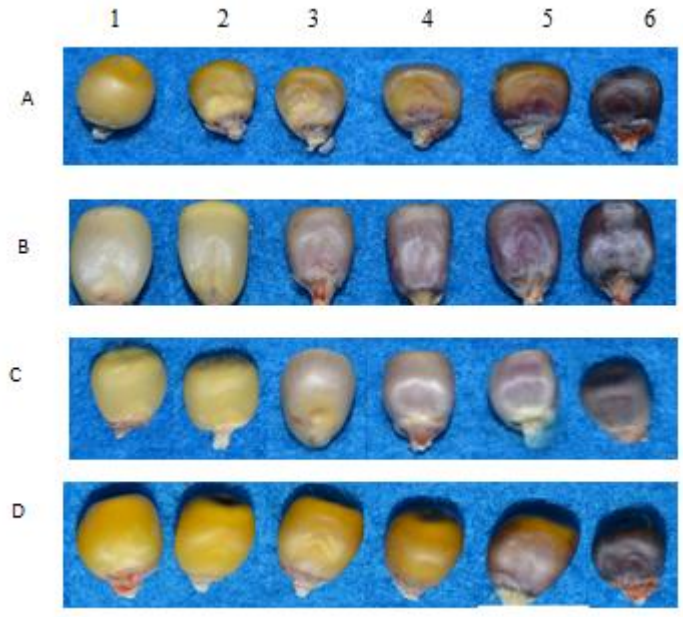


Figure 8: Phenotypic color variation scale. Used for determining effects of *r1 y1+* and *y1-* genetic backgrounds. A: *y1+/y1+* background, Ashland, VA. B: *y1-/y1-* background, Ashland, VA. C: *y1-/y1-* background, Homestead, Fla. D: *y1+/y1+* background, Homestead, Fla.

EMS Mutagenesis of R-sc:86-17(L):

In 2004 and 2007, ethyl methyl sulfonate (EMS) was used to treat pollen of *R-sc: 86-17(L),* to create point mutations, as described in Neuffer (1994) (W. Eggleston pers. comm.). In 2004 *y1+/y1+* pollen were treated, and in 2007 *y1-/y1-* pollen were treated to be able to aid in identifying possible pollen contaminants. Plants grown from the resulting seeds were self-pollinated, and screened for the presence of approximately 1/4 more darkly pigmented seeds expected from recessive mutations that caused increased seed color. No apparent dominant restorations of seed color were noted (W. Eggleston pers. comm.). Resulting EMS-associated mutant alleles were termed 05 and 08 respectively. In subsequent generations, the darkest kernels were self-pollinated to generate homozygous EMS-induced mutants (Figure 9) and to identify pleiotropic effects. The putative homozygous EMS-induced mutants were germinated in the laboratory for DNA isolation and Southern blot analysis.

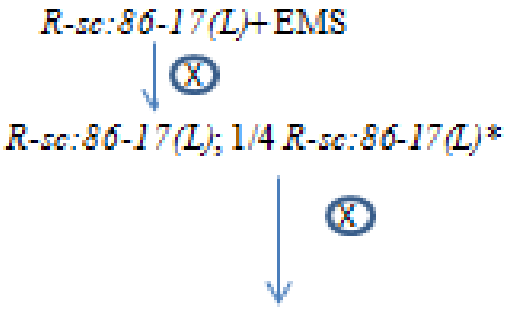


Figure 9: Screen for EMS-induced mutations of *R-sc:86-17(L)* resulting in mutants with darker seed color. *Indicate putative mutants resulting in darker seed color. Does not imply location.

Mapping mutations relative to the r1 locus

Mapping recessive putative mutants relative to *r1* was performed by crossing homozygous putative EMS-induced mutant alleles to *R-nj:Cuda* (due to the unique phenotype of *R-nj:Cuda*) (Figure 10). The F_1 ears were then self-pollinated and the F_2 progeny ears visually screened to determine the ratio of *Nj* to light colored kernels (no change sibs of *R-sc:86-17*(*L*)) and/or dark kernels (enhancers of *R-sc:86-17*(*L*)). It was expected that if the element(s) which caused loss of canalization are located at or near *r1*, a 3 Navajo (*Nj/Nj*; *Nj*/(*L*)*): 1 darker kernels ((*L*)*/(*L*)*) phenotypic ratio would result, behaving as a mono-hybrid cross. If the enhancing element(s) are located away from *r1*, a 12 (*Nj-)*: 3(*L*-): 1(**/**) phenotypic ratio was expected, behaving as a

di-hybrid cross (*= recessive mutation at or near *r1*; **=recessive mutation not at *r1* with *R-sc:86-17*(*L*); (*L*)=*R-sc:86-17*(*L*)). P values were calculated based on α=0.05.

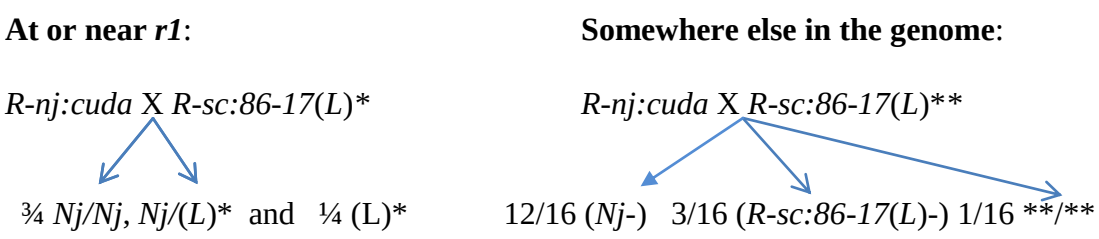


Figure 10: Mapping of putative EMS-induced mutants relative to *r1*. *R-nj:Cuda* is a dominant single *r1* gene. * indicates darker seed color, spotting, or mottling at or near *r1*; **indicates darker seed color, spotting, or mottling away from *r1*.

Complementation tests of putative EMS-induced mutations that increase kernel color

In order to determine how many genes are responsible canalization at *r1*, complementation tests (allelism) were performed on homozygous putative EMS-induced mutant alleles by intercrossing. The resulting F_1 ears were visually screened for color variation and presence/absence of mutant phenotypes when compared to no change phenotypic sibling ears grown in parallel. If two recessive mutations are in the same gene (allelic) then the progeny ears are expected to produce a mutant phenotype (darker seed color). However, if the two recessive mutations are in different genes, the resulting progeny fail to complement resulting in a light-brown kernel (no change) phenotype (Figure 11). Dominant alleles or alleles suspected to not be *R-sc:86-17*(*L*) will not be included in the complementation test.

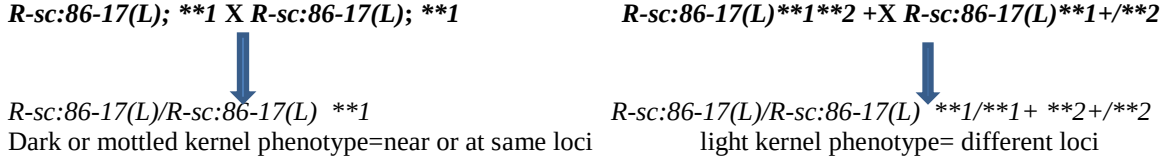


Figure 11: Complementation tests of homozygous putative EMS-induced mutants. ******1=homozygous EMS mutant 1, ******2=homozygous EMS mutant 2; "+" functional dominant or wild type phenotype, based on no change sib.

Southern blot hybridization analysis of gene structure and 5-meC patterns

Kernels from *R-sc:86, R-sc:124, r-Δ902, R-sc:86-17(L) Lc, R-sc:86-17(L)/r-r:n142, R-sc:86-17*(*L*) *Lc* losses, and the putative mutants for *R-sc:86-17(L)* were grown in the laboratory (approximately n=5) for DNA isolation. DNA was extracted from juvenile (3-4 leaves) plants with 1:1 phenol: chloroform, cleaned, and re-suspended to 0.5 µg/µL as described in Eggleston *et al*. (1995). For structural analyses, approximately 5 µg of DNA was digested with the methylation insensitive restriction enzymes *Hin*dIII. To determine and compare 5-meC methylation patterns, the methylation sensitive restriction enzyme *Eco*RII (CNG) was used in conjunction with *Hin*dIII. As a control, select DNAs were digested with *Bst*N1, an isoschizomer of *Eco*RII, which is not blocked by 5-meC, and *Hin*dIII. DNA was size fractionated by electrophoresis through 1% agarose gels in 0.5XTBE (89 mM Trisborate, 89 mM Boric acid, 2

mM EDTA pH 8.0). Digested samples were then stained with ethidium bromide and viewed under UV illumination. The DNA was transferred to nitrocellulose paper (Hybond-XL^{TM}, GE Healthcare) by the method of Southern (1975), UV cross-linked @ 1200 µJ/cm^2 and baked under vacuum for 2 hours @ 80°C. DNA was hybridized to probes labeled with ^{32}PdCTP (Perkin-Elmer) (Eggleston *et al*. 1995). Membranes were pre-hybridized at 42°C for 1- 4 hours in hybridization solution (50% formamide (v/v), 5X SSC (1 X = 150 mM sodium chloride,15mM sodium citrate), 0.2%SDS, 2X Denhardt's solution [l X = 0.02% (w/v) Ficoll, polyvinylpyrrolidone and BSA] and 50 pg/ml sheared herring sperm DNA) before addition of heat denatured labeled DNA fragments. Hybridizations were carried out overnight at 42°C**.** Filters were post-hybridized by a room temperature rinse in 2 X SSC followed by two 10-min washes in 2 X SSC, 0.2% SDS at 55°C and two 10-min washes in 0.1 X SSC, 0.2% SDS at 55°C. Membranes were then auto radiographed for 3-7 days, with intensifier screen at -80°C with x-ray film (GeneMate). Probes used in this analysis contained regions from the 5' and 3' ends of the *r1* transcription unit. pR-nj:l, which was cloned from *R-Nj***,** contains a region of the promoter (Dellaporta *et al.* 1988). The clone pSc323:J20 was recovered from *R-sc:m323* and contains a fragment from the 3' end of the *r1* transcription unit (Kermicle *et al.* 1989; Alleman and Kermicle 1993). Taken verbatim with minor changes from Eggleston *et al*. (1995).

Extended mapping of the genetic basis for R-sc:86-17(L)

Seed from six full-color derivatives recovered from *R-sc:86-17*(*L*)/*r-r:n142* heterozygotes by W. Eggleston (pers. comm.) were germinated in the laboratory for DNA isolation and Southern blot analysis with *Hin*dIII, to test for structure.

Test for loss of Lc expression in cis with R-sc:86-17(L)

R-sc:86-17(L) Lc; wx+ seedlings crossed to *g1 R-g:8pale; wx-* (Eggleston unpubl. obs) were germinated for approximately one week at 80-85ºF, under continuous fluorescent lighting, using trays with wetted newspaper. The resulting seedlings were then visually and individually screened for loss of *Lc* expression indicated by a lack of red roots, red scutellar node, and red coleoptile. Seedlings were then transplanted to the field to rescreen for loss of *Lc* expression. Of those families that survived, kernels were then visually screened for *wx-* segregation and grown in the laboratory for DNA isolation and Southern hybridization.

Three to four families each of *R-sc:86-17(L) Lc*, *R-sc:86-17(D) Lc*, *R-sc:86 Lc*, *R-sc:86-17(L) lc*, *R-sc:86-17(D) lc*, *R-r 1172 Lc*, *r-Δ902 Lc* heterozygous with *r-g:8pale; wx-* were grown in the field. The resulting mature plants were then visually screened for loss or reduction of *Lc* expression (W. Eggleston pers. comm.) and compared (α=0.05) using JMP®.

RESULTS

Phenotypic variation among putative EMS-induced mutants

Thirty-one self-pollinated putative homozygous EMS-induced mutants were analyzed for darker seed color and all but five alleles were categorized as light pale, medium pale, dark pale, dominant *R*, or recessive *Sc* enhancers (Table 3). Twelve alleles were found to be false (light) selections, resulting in multiple generations of light seed color. The putative mutants were found to be highly variable in seed color from year to year and within a growing location. In terms of kernel pigmentation, ears grown in Florida resulted in a "Navajo" like pigmentation pattern on the crown (Figure 12), this was not observed on ears grown in Virginia.

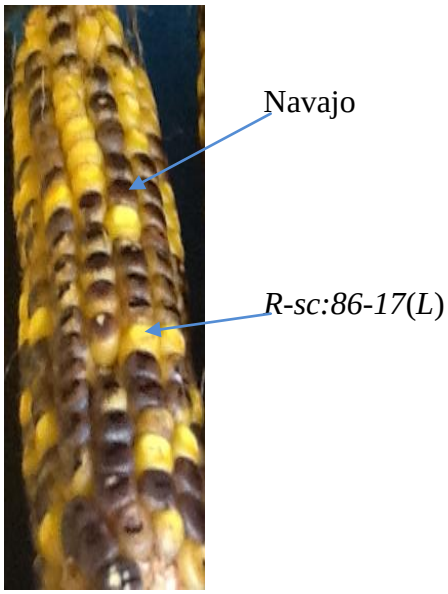


Figure 12*:* Representative ear of F_2- *R-nj:Cuda* crossed to putative homozygous EMS-induced mutant.

Table 3: Putative EMS-induced mutants

Allele	Intercrossed	Homozygous Phenotype: *R-sc:86-17(L)*
05-058-2	Yes	Dominant *Sc*
05-026-15	Yes	Light
05-116-7	Yes	Recessive *Sc**
05-032-24	Yes	Light/Medium**
05-040-19	Yes	Recessive *Sc*
05-025-29	Yes	Dark
05-053-6	Yes	Medium
08-105-11	Yes	No change sib, control
08-146-1	Yes	Light
08-157-13	Yes	Light, Medium
08-185-13	Yes	Recessive *Sc*
08-187-8	Yes	Medium
08-111-12	Yes	Light, Medium*
08-127-13	Yes	Light*
08-128-9	Yes	Light
08-105-12	Yes	Light, Medium
08-128-10	Yes	Medium
08-146-2	Yes	Light*
08-151-16	Yes	Medium
08-173-7	Yes	Light, dominant *Nc* enhancer
08-177-6	Yes	Light, does not grow well*
08-178-1	Yes	Light
05-013-24	Yes	Dominant *Sc*
08-113-8	Yes	Light, Dark*
08-155-13	Yes	Light
05-028-21	Yes	Medium
05-098-10	Yes	Recessive *Nc* enhancer
05-034-21	Yes	Recessive *Nc* enhancer
05-052-16	Yes	Dominant *Nc* enhancer
08-175a-5	Yes	Recessive *Nc* enhancer, Light
08-158-16	Yes	Dominant *Nc* enhancer

Segregating defective kernels (DEK) or lethal, **Wilty phenotype

Both locations resulted in a consistent aleurone background seed color. Seven homozygous putative EMS-induced mutant alleles were found to be segregating for defective kernels (DEK's), one allele segregated for plants with wilty leaves, dwarf height, and late development (Figure 13). Three alleles were found to be developmentally delayed. Although the background seed color was found to be consistent in each growing location (VA. or Fla.) kernel mottling or anthocyanin deposition was found to be highly variable.

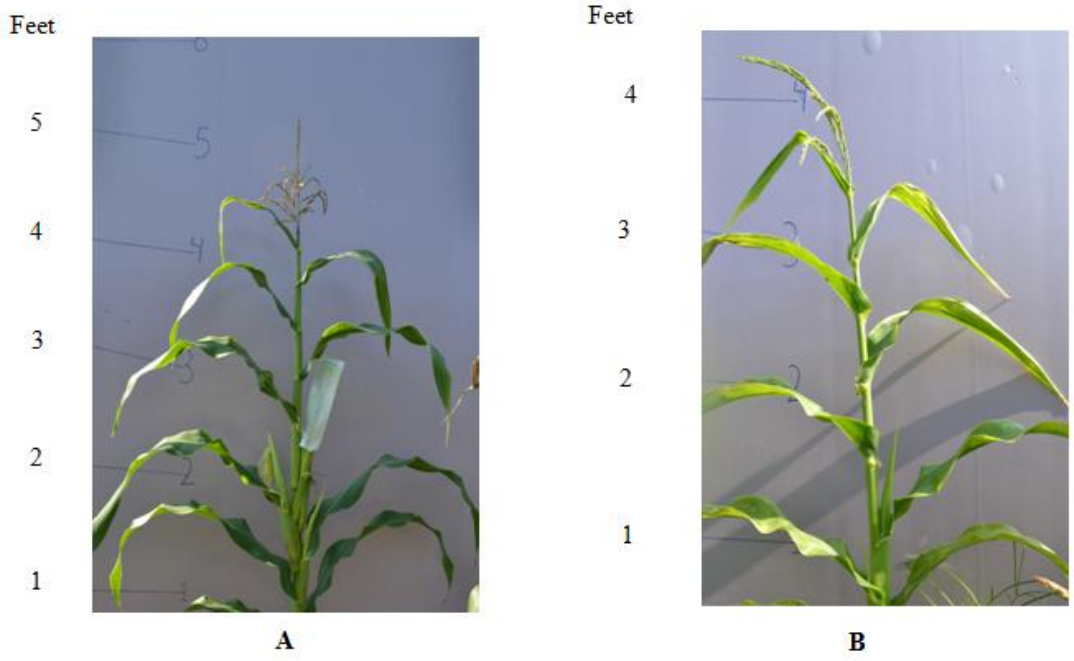


Figure 13: Pleiotropic effects of mutant *05-032-24* on plant height and water transpiration. (A) Typical plant height close to 6" and normal leaf structure (B): Aberrant plant height and leaf structure.

In addition to the *Sc* enhancers, an unusual class was found in five of the homozygous putative EMS-induced mutants conferring irregular lightly mottled to heavily mottled kernel

pigmentation, rather than solid seed color (Figure 14: A-E). This class was termed *Nc* enhancers as irregular mottling is associated with *Nc* gene expression (W. Eggleston pers. comm.). The mottling phenotype shows variable pigmentation with distinct borders. Both the *Sc* and *Nc* enhancers were found to be highly variable, dosage dependent, possibly incompletely penetrant, and in some cases dominant when crossed to *r-Δ902*.

Seed color differences between Sc and Nc enhancers

To determine whether there was a statistical difference between seed color pigmentation of the *Sc* enhancers (darker seed color) and *Nc* enhancers (mottled or spotted seed pigmentation) the Agtron reflectometer was used to measure kernel pigmentation levels. Twenty-six putative mutants were categorized according to the phenotypic color scale and assigned a category of light pale, medium pale (included medium to dark pale), *Nc* enhancer, or *Sc*. The alleles were then tested, by the Agtron (n=4) and analyzed using JMP®. A significant difference ($p<0.001$) was observed for the means between all groups: light pale- 50.36 (s.d. 3.62), medium pale-34.48 (s.d. 5.71), mottled-46.32 (s.d. 1.50), and R- 14.5 (s.d. 0.50). The mean reflectance value for the no change control sibs was 44.41 (s.d. 2.86) (Figure 15). Although a significant difference was observed between the groups, the *Nc* enhancer and medium to dark pale groups are more highly variable than light pale or *Sc*. Due to this variability, overlap was seen with at least one allele of the *Nc* enhancer group and the medium to dark pale *Sc* enhancer group.

A. *05-098-10* maps elsewhere in the genome (Recessive *Nc* enhancer)

B. *05-034-21* maps elsewhere in the genome (Recessive *Nc* enhancer)

C. *05-052-16* maps at or near *r1* (Dominant *Nc* enhancer)

D. *08-175a-5* maps at or near *r1* (Recessive *Nc* enhancer)

E. *08-158-16* maps elsewhere in the genome (*Nc* enhancer)

F. *05-058-2* dominant *Sc* contaminant

G. *05-013-24* dominant *Sc* contaminant

H. *08-157-13* maps at or near *r1* (hyper variable *Sc* enhancer)

I. *08-151-16* not mapped-crosses with *r-g*:Δ902 are mottled when transmitted from female

J. *08-187-8* maps elsewhere in the genome; dominant medium pale when crossed to *r-g:Δ902* as the female

K. *08-185-13* maps elsewhere in the genome; dominant medium to dark pale when crossed to *Δ902* as female and when transmitted as the male; crossed to *r-g*:Δ902 results in several mottled kernels

L. *05-053-6* maps at or near *r1*; dominant medium pale when crossed to *r-g:Δ902*

M. *08-185-13* maps at or near *r1*, mottles when crossed to *r-g:Δ902*.

N. *08-105-12* not mapped; dominant medium to dark pale when crossed to *r-g:Δ902* male or female.

Figure 14: Available homozygous putative EMS-induced mutants not included in complementation tests of *Sc* enhancers (A-N).

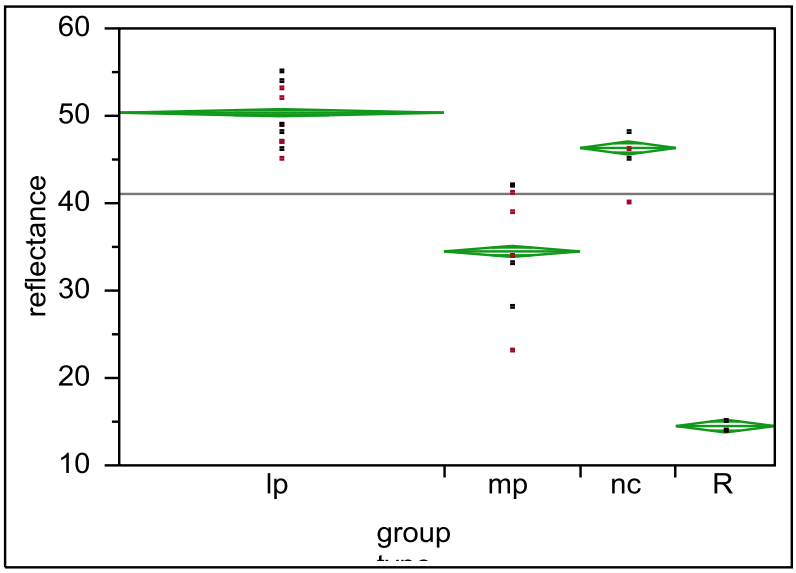


Figure 15: One-way ANOVA between reflectance and kernel color classification for *Sc* and *Nc* enhancers. Lp=light pale, mp=medium to dark pale, nc= *Nc* enhancer, and *R=Sc*

Mapping putative EMS-induced mutants relative to r1

During the screening of M1 (05 and 08 alleles) EMS-induced mutants, twenty-six putative ears were identified as having approximately one-fourth darker seed color (*Sc* enhancer) (W. Eggleston unpubl. obs.). Through subsequent generations thirty-one mutants were identified as

containing darker seed color, including the original twenty-six and five additional alleles. The five additional alleles were identified as segregating for more than one phenotype. For example: in 2009 the single allele *08-151-16* segregated for two phenotypes: medium pale and light pale. These two segregating phenotypes were maintained and selected for though all generations (Table 3). Eighteen of the thirty-one alleles were crossed to *R-Nj:Cuda* resulting in 10 putative mutants mapping to or near *r1* and eight mapping elsewhere in the genome (Table 4). These results suggest that the sequence(s) involved in the restoration of seed color are found within or near *r1* genes and outside *r1*. Two strong *Sc* enhancer alleles identified during the M1's, as restoring seed color (*05-058-2* and *05-013-24*) (Figure 14: F-G) were found to be dominant *Sc* contaminants due to phenotypic and molecular analyses (below).

Table 4: Mapping and complementation data for putative EMS-induced mutants

Gene type	Map to *r1*	Map elsewhere	Un-mapped	Total
Sc enhancer	8	5	13	26
Nc enhancer	2	3	0	5
Total	10	8	13	31

Complementation tests of putative EMS-induced mutants

The thirty-one homozygous putative EMS-induced mutants were intercrossed to determine complementation. Alleles unable to be included in the complementation test included: two dominant *Sc* contaminants (*05-058-2* and *05-013-24*), one non-*R-sc:86-17(L)* allele (*05-028-21*), two dominant mottled *Nc* enhancers (*05-052-16* and *08-158-16*), three dominant medium to dark pale alleles (*08-187-8*, *08-185-13*, and *05-053-6*) and one hyper-variable *r1* allele (*08-157-13*) (Figure 14: F-H and J-M). Two unmapped alleles *08-151-16* and *08-105-12* were found to be dominant Sc enhancers when crossed to *r-Δ902* (Figure 14: I and N). Intercrossing of the *Sc* and *Nc* enhancers resulted in one complementation group located at or near *r1*, and a second complementation group mapping elsewhere in the genome, thirteen alleles were unmapped (Table 4). Controls included *r-Δ902*, and the no change control *y1-/y1-* allele *08-105-11* (Tables 5-9). Due to developmental delays, semi-lethality and semi-sterility not all of the putative EMS-induced mutants were able to be crossed.

Table 5: Results for putative EMS-induced mutant intercrosses including *Sc* enhancers only

	05-026-15	*05-116-7*	*05-032-24*	*05-040-19*	*05-025-29*	*08-105-11*
05-026-15	X					
05-116-7		X	-			
05-032-24			X			
05-040-19	+	-	+	X		
05-025-29					X	
08-105-11		-	-	-	-	
08-146-1		-				
08-111-12						
08-127-13						
08-128-10						
08-146-2						
08-177-6						
08-178-1						
08-113-8						
08-155-13						

+ Allelic; - non-Allelic; *08-105-11* is no change sibling control

Table 6: Results for putative EMS-induced mutant intercrosses including *Sc* enhancers only

	08-146-1	*08-111-12*	*08-127-13*
05-026-15			
05-116-7	-		
05-032-24	+		
05-040-19	+		
05-025-29			
08-105-11	-	-	-
08-146-1	X	-	
08-111-12	-	X	
08-127-13			X
08-128-10	-	-	
08-146-2	-	-	
08-177-6		-	
08-178-1	-		
08-113-8			
08-155-13			

+ Allelic; - non-Allelic; *08-105-11* is no change sibling control

Table 7: Results for putative EMS-induced mutant intercrosses including *Sc* enhancers only

	08-128-10	*08-146-2*	*08-151-16*	*08-173-7*	*08-177-6*	*08-178-1*	*08-113-8*	*08-155-13*
05-026-15								
05-116-7	-		-				+	
05-032-24								
05-040-19								
05-025-29								
05-053-6							-	
08-105-11	-	-	-	-	-	-	-	-
08-146-1	-	-	-	-		-		
08-185-13								
08-187-8	-	-		-				
08-111-12	-	-	-	-	-			
08-127-13				-				
08-128-9	-	-	-	-				
08-105-12	-	-	-	-				
08-128-10	X	-	-	-	-			
08-146-2	-	X	-					
08-151-16	-	-	X					-
08-173-7	-			X		-		
08-177-6	-				X	-		
08-178-1				-	-	X		
08-113-8							X	
08-155-13								X

+ Allelic; - non-Allelic; *08-105-11* is no change sibling control

Table 8: Results for putative EMS-induced mutant intercrosses including *Sc* and *Nc* enhancer males crossed to only *Nc* enhancer females

	05-026-15	*05-116-7*	*05-098-10*	*05-032-24*	*05-034-21*	*05-040-19*
05-098-10	-	+	X	-	-	-
05-034-21	+	-	-	-	X	-
08-175a-5						
	05-025-29	08-105-11	08-175a-5	08-146-1	08-111-12	08-127-13
05-098-10	-		-	-		
05-034-21			-	+		
08-175a-5			X			
	08-128-10	08-146-2	08-177-6	08-178-1	08-113-8	08-155-13
05-098-10						
05-034-21						
08-175a-5						

+ Allelic; - non-Allelic; *08-105-11* is no change sibling control

Table 9: Results for putative EMS-induced mutant intercrosses including *Sc* and *Nc* enhancer females crossed to *Nc* enhancer males only

	05-098-10	*05-034-21*	*08-175a-5*
05-026-15	-		-
05-116-7	-	+	-
05-032-24		-	-
05-040-19	-	-	-
05-025-29			
08-105-11			
08-146-1			
08-111-12			
08-127-13			
08-105-12			
08-128-10			-
08-146-2			
08-177-6	-		
08-178-1	-		
08-113-8			
08-155-13			
05-098-10	X	-	-
05-034-21	-	X	-
08-175a-5			X

+ Allelic; - non-Allelic; *08-105-11* is no change sibling control

Structural analysis of the Sc and Nc enhancers

Genomic DNA for 20 homozygous putative EMS-induced mutants was digested with *Hin*dIII (Table 10). The *r1* probes pRnj:1 and pSc323:J20 were used to hybridize to the 5' and 3' regions, respectively (Figures 16 and 17). Controls used were *R-sc:86* and *08-105-11* (no change control sib). Southern blot analysis revealed the presence of two pRnj:1 *r1* hybridizing fragments at the 5' end of the genes: 4.1kb and 3.5kb representing *Sc‖nc1* and *Nc2, Nc3* respectively in *R-sc:86*. Nineteen of the twenty mutants showed no detectable change from *R-sc:86* or *R-sc:86-17*(*L*) in terms of hybridizing fragment size, number and relative intensity. One of the mutant alleles, *08-146-2,* revealed a fainter 4.1kb fragment, representing the *Nc* genes, at the 5' end when compared to *R-sc:86* and *08-105-11* (Figure 17). Analysis of the 3' end revealed a large fragment at 7.5kb representing *Sc‖nc1*, *Nc2*, and *Nc3* in *R-sc:86*, *08-105-11*, and mutants. Results indicated no large structural changes in the 3' end of the *r1* genes. In three cases there was insufficient DNA to observe the fragments and these samples were not included in the analysis.

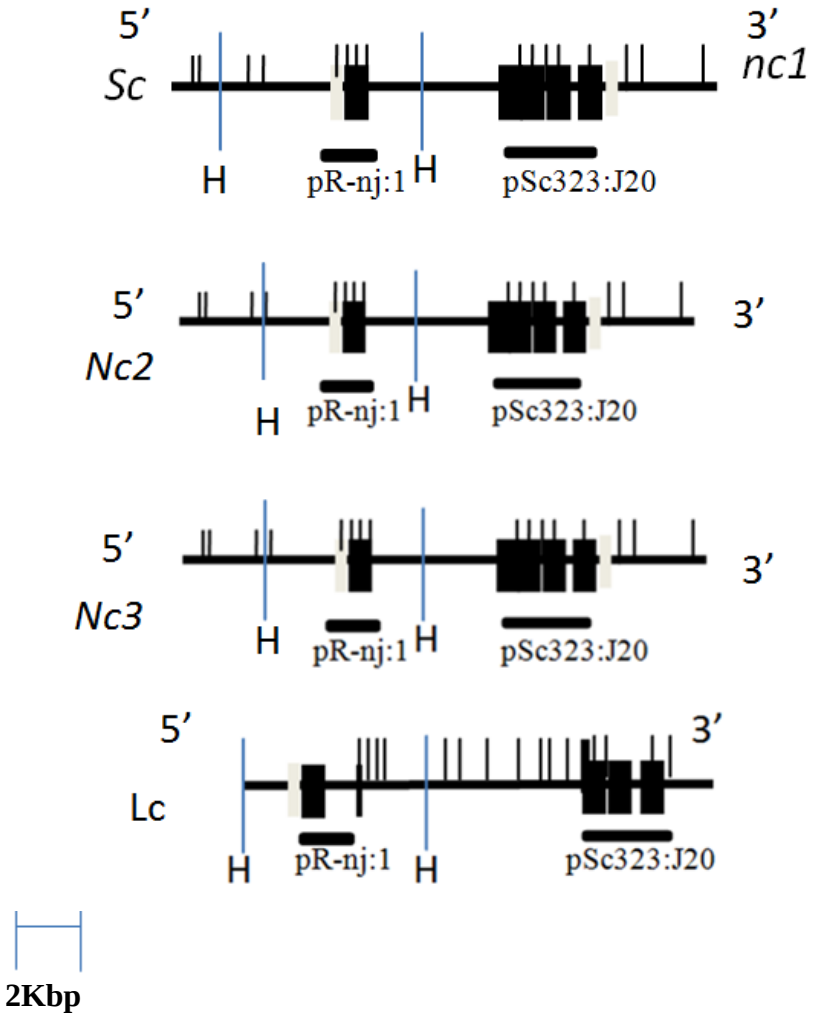


Figure 16: Restriction map of *Sc*, *Nc*, and *Lc* genes. Genes are stacked from top (proximal gene) to bottom (distal gene) to highlight monomorphic and polymorphic restriction sites. The structure of *Lc* is based on the *Lc* cDNA and sequence (Eggleston *et al*. 1995; Ludwig *et al.* 1989) and Southern blot restriction map analysis of genomic DNA. Boxed area, locations of probes used in Southern blot hybridizations are shown at the bottom. Restriction sites are as follows: H, *Hin*dIII; half bars = *Eco*RII**.** Sites based on Southern blot analysis of genomic DNA (Eggleston *et al*. 1995).

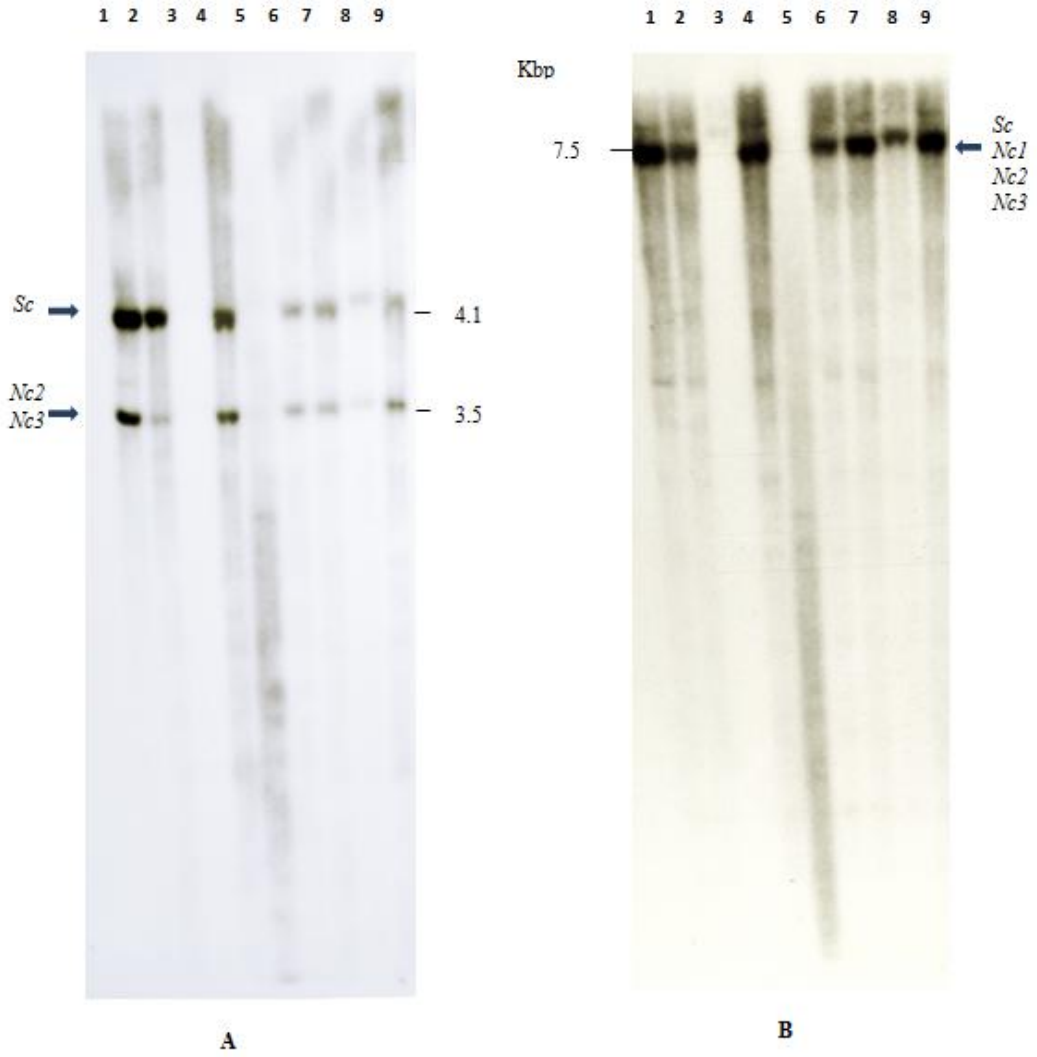


Figure 17: Southern blot analysis of putative EMS-induced mutants. Genomic DNA was digested with *Hin*dIII and sequentially hybridized with pR-nj:l (A) and pSc323:J20 (B). Lane 1: *R-sc:86*; Lane 2: *08-146-2*; Lane 3: *08-151-16*; Lane 4: *08-157-13*; Lane 5: *08-158-16*; Lane 6: *08-158-16* (mottled); Lane 7: *05-098-10*; Lane 8: *05-032-24*; Lane 9: *05-034-21*. Lanes 3 and 5 contain insufficient and degraded DNA. Putative EMS-induced mutants appear similar in structure to *R-sc:86* within loading differences. *08-146-2* has lost at least one of the *Nc* genes as indicated by the change in intensity of the *Nc* fragment.

Table 10: Putative EMS-induced mutant mapping and Southern blot data

Allele	Mapped	Maps to *r1*	P value	*Sc* gene	*Nc* genes	Methylation 5' region (*Sc*,*Nc*)	Methylation 3' region (*Nc*)
05-058-2	Yes	No	**	-	+	N/A	N/A
05-026-15	Yes	No	**	N/A	N/A	N/A	N/A
05-116-7	Yes	Yes	*	N/A	N/A	N/A	N/A
05-032-24	Yes	Yes	*	+	+	Low, High	High
05-040-19	Yes	Yes	*	N/A	N/A	N/A	N/A
05-025-29	Yes	Yes	*	N/A	N/A	N/A	N/A
05-053-6	Yes	Yes	*	N/A	N/A	N/A	N/A
08-105-11	Yes	Yes	*	+	+	Low, High	High
08-146-1	Yes	No	**	+	+	Low, High	High
08-157-13	Yes	Yes	**	+	+	Low, High	High
08-185-13	Yes	No	**	+	+	Low, High	High
08-187-8	Yes	No	**	+	+	Low, High	High
08-111-12	No	---	----	N/A	N/A	N/A	N/A
08-127-13	No	---	----	N/A	N/A	N/A	N/A
08-128-9	No	---	----	N/A	N/A	N/A	N/A
08-105-12	No	---	----	N/A	N/A	N/A	N/A
08-128-10	No	---	----	+	+	Low, High	High
08-146-2	No	---	----	+	+	partial, Low	High
08-151-16	No	---	----	+	+	Low, High	High
08-173-7	No	---	----	+	+	Low, High	High
08-177-6	No	---	----	N/A	N/A	N/A	N/A
08-178-1	No	---	----	+	+	Low, High[1]	Low[2]
05-013-24	No	---	----	+	-	Low, N/A	N/A
08-113-8	No	---	----	+	+	partial, High	High
08-155-13	No	---	----	N/A	N/A	N/A	N/A
05-028-21	Yes	Yes	*	+	+	Low, High	High
05-098-10	Yes	No	**	+	+	Low, High	High
05-034-21	Yes	No	**	+	+	Low, High	High
05-052-16	Yes	Yes	*	+	+	Partial, High	High
08-175a-5	Yes	Yes	*	+	+	Low, High	High
08-158-16	Yes	No	**	+	+	Low, High	High

*=p values >0.05; **=p values <0.025; + indicates presence of the gene(s) by pR-nj:1 and pSc323:J20; N/A=not available; 1=one of the duplicates displayed partial methylation of *Sc* at the 5' end. 2=one of the duplicates displayed methylation at the 3' end of the *Nc* genes.

5-meC patterns of the Sc and Nc enhancers

Genomic DNA for twenty homozygous putative EMS-induced mutants was digested with the methylation insensitive enzyme *Hin*dIII and methylation sensitive enzyme *Eco*RII. The controls used were *R-sc:86* digested with *Hin*dIII alone, with *Bst*N1, and with *Eco*RII; *r-Δ902* (*Hin*dIII alone); and *08-105-11* (no change control sib) digested with *Hin*dIII and *Eco*RII. Southern blot analysis revealed the presence of two 5' end *r1* hybridizing fragments: 3.5kb and 1.6kb, representing *Nc2* and *Nc3;* and *Sc‖nc1* respectively (Figure 18). These results indicate lower methylation at the 5' end of *Sc* and higher methylation in the *Eco*RII sites of the *Nc* genes as previously detected (Figure 5) and found to be consistent with *R-sc:86* and *08-105-11* digested with *Hin*dIII and *Eco*RII. Two alleles, *08-113-8* and *05-052-16,* revealed limited partial methylation in the *Eco*RII sites of *Sc* (Figure 23-D Lanes 2-5). Analysis of the 3' end revealed a large smeared fragment at 7.5kb representing the 3' end of *Sc‖nc1, Nc2,* and *Nc3* (single fragment) (Figure 19). The smearing in all methylation data presented is most likely due to non-specific hybridization (W. Eggleston pers. comm.). In one allele (*08-178-1*), in one of the duplicates, the 3' end of the genes was unmethylated as observed in the digestion of *Hin*dIII and *Eco*RII which revealed a smaller fragment at ~0.95kb (Figure 23) (Table 9). If *Eco*RII or *Bst*N1 digests fully then a similar sized fragment was expected (Figure 5). In two of the four gels analyzed, the *Bst*N1 fragment was unobserved indicating the gels were overrun.

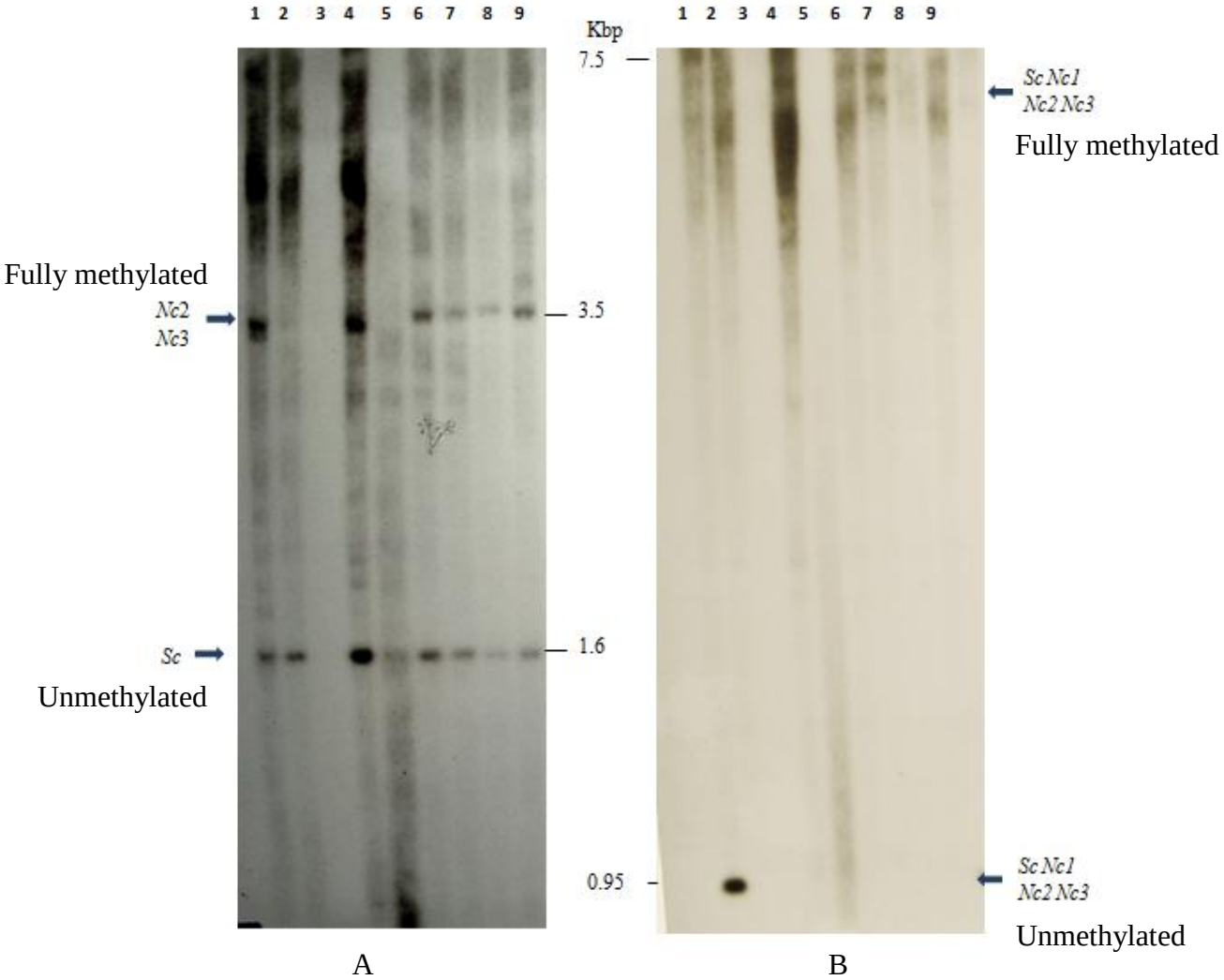


Figure 18: Southern blot analysis of 5-meC patterns of putative EMS-induced mutants. Genomic DNA was digested with *Hin*dIII and *Eco*RII, and sequentially hybridized with pR-nj:l (A) and pSc323:J20 (B). Lane 1: *R-sc:86*; Lane 2: *08-146-2*; Lane 3: *08-151-16*; Lane 4: *08-157-13*; Lane 5: *08-158-16*; Lane 6: *08-158-16* (mottled); Lane 7: *05-098-10*; Lane 8: *05-032-24*; Lane 9: *05-034-21*. Lanes 3 and 5 contain insufficient and degraded DNA respectively. The doublet, representing the *Nc* genes, in *R-sc:86* is indicative of a *Eco*RII site located near a *Hin*dIII site. The 0.95kb indicates complete digestion of *Eco*RII and that region is unmethylated.

Structural analysis of full-color revertants from R-sc:86-17(L)/r-r:n142 heterozygotes

Genomic DNA was made from six full-color revertants. One sample of each of the six alleles was digested with the methylation insensitive enzyme *Hin*dIII. Southern blot analysis of the full-color revertants revealed the presence of one *r1* hybridizing fragment at the 5' end: 4.1kb representing *Sc* (Figure 19). Analysis of the 3' end revealed a 3.8 kb fragment similar to what was previously found in the six initial full-color revertants as the 3' region of *P* (W. Eggleston pers. comm.). These results further support the previous conclusion indicating that the lesion responsible for *R-sc:86-17pale* is in or near the 3' end of the *Sc||nc1* gene in *R-sc:86*.

5-meC patterns of full-color revertants of R-sc:86-17(L)/r-r:n142 heterozygotes

One sample of each of the six alleles, noted above, was digested with the methylation insensitive enzyme *Hin*dIII and methylation sensitive enzyme *Eco*RII. Southern blot analysis of the revertants revealed the presence of one *r1* hybridizing fragment at the 5' end: 1.6kb representing low methylation at *Sc* (Figure 20). Analysis of the 3' end revealed a large smeared fragment and a lack of the 0.95 fragment at the 3' end of the *P* as was previously observed in the six initial full color revertants (data not shown) (W. Eggleston pers. comm.). These results are consistent with no methylation at the 3' end of the *P* gene, as no 0.95kb fragment was observed.

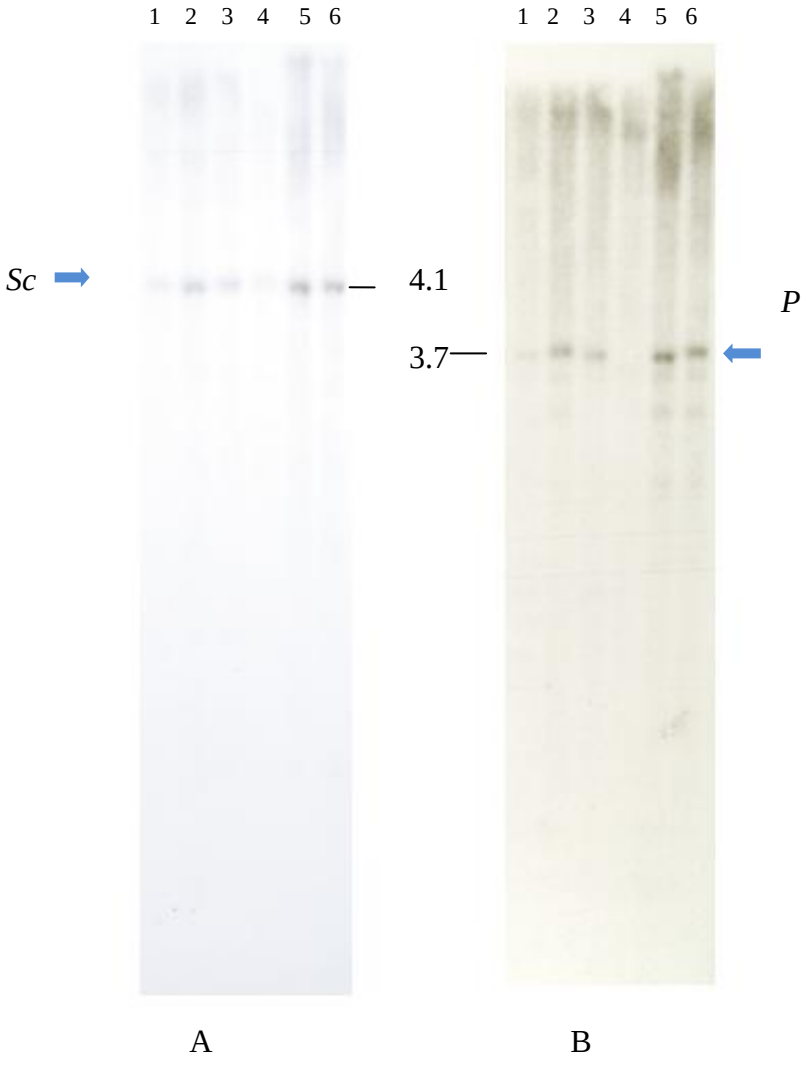


Figure 19: Southern blot analysis of full-colored revertants from *R-sc:86-17*(*L*)/*r-r:n142*. Genomic DNA was digested with *Hin*dIII and sequentially hybridized with pR-nj:l (A) and pSc323:J20 (B). Lane 1-6: *R-sc:86-17(L)/P*. Hybridizing fragments for Nc genes are not present indicating a cross-over event between the 5' end of *Sc* and the 3' end of *P*.

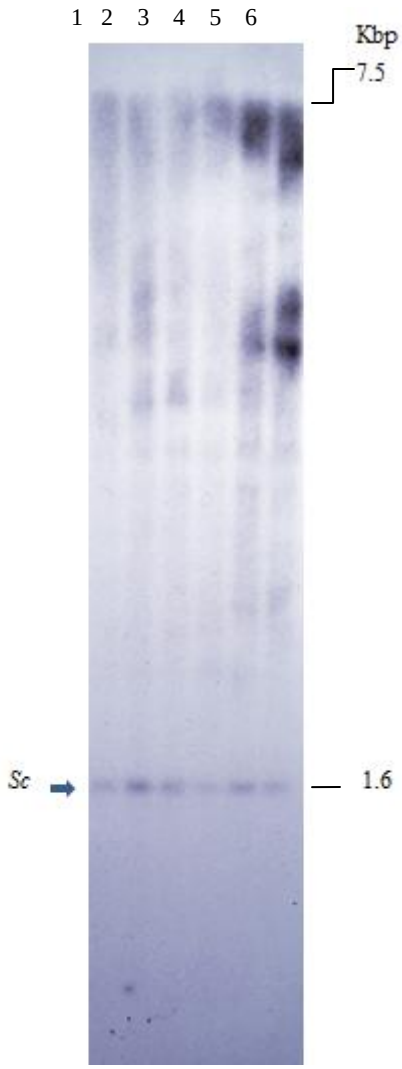


Figure 20: Southern blot analysis of 5-meC patterns of full-colored revertants from *R-sc:86-17*(*L*)/*r-r:n142* heterozygotes. Genomic DNA was digested with *Hin*dIII and sequentially hybridized with pR-nj:l. Lane 1-6: *R-sc:86-17(L)/P*. No hybridizing fragments were observed with the pSc323:J20 probe.

Lc losses from R-sc:86-17(L) Lc

Eight thousand-two hundred and thirty one kernels representing six families were grown to the coleoptile stage and visually screened for loss of *Lc* expression indicated by lack of red roots, red coleoptile, and red scutellar node. One black full color kernel was observed, but did not germinate, all other kernels were yellow. Only a subset was screenable due to heavy mold contamination, resulting in a lack of germination, and the entire number screened was not recorded. From among the six families thirty-eight (n=6-ED516; n=7-ED515; n=6-ED504; n=7-ED492; n=7-ED482; and n=5-ED501) were then transplanted to the field. Families ED492 and ED482 had previously lost *Lc* expression and the other families retained *Lc* expression. Three mature plants from ED492 (*Lc* loss) were recovered and used to collect all DNA representative of this family. DNA was then purified from from three families (ED516, ED504 and ED492; ED482 did not germinate).

In addition 3-4 families were visually screened for loss or depletion of *Lc* representing *R-sc:86-17(L) Lc*, *R-sc:86-17(D) Lc*, *R-sc:86 Lc*, *R-sc:86-17(L) lc*, *R-sc:86-17(D) lc*, *R-r 1172 Lc*, and *r-Δ902 Lc*. No *Lc* expression was observed in the *R-sc:86-17(L) lc*, *R-sc:86-17(D) lc*. High Lc expression was observed in the controls *r-Δ902 Lc*, *r-r:n19 Lc* (a single *r1* gene and *Lc*) and *R-sc:86-17(D) Lc*. Variation between moderate to high *Lc* expression was observed for *R-r:1172 Lc* (source of *Lc*) and *R-sc:86 Lc*. A significant difference was found between the loss of *Lc* expression and positive *Lc* expression ($p<0.001$) (Figure 21). No significant difference was found between *R-sc:86-17*(*L*) *Lc* and *r-r:n19 Lc* ($p=1.000$). A significant difference was observed between *R-sc:86-17*(*L*) *Lc* and *R-sc:86 Lc*, *R-r 1172 Lc* ($p<0.0051$, $p<0.001$

respectively). A significant difference was also observed between *R-sc:86-17*(*L*) *Lc* and *R-sc:86-17 lc* and *R-sc:86-17*(*D*) l*c* ($p<0.001$). The difference between *R-r 1172 Lc*, *R-sc:86 Lc* and *R-sc:86-17*(*L*) *Lc* is most likely due to the variability of color between the plants in *R-r 1172 Lc* ($\mu=4$, s.d.=0, $\mu=2.46$, s.d. 1.24; $\mu=3.46$, s.d. 0.89 respectively). These results indicate that there is no loss of *Lc* expression when in *cis* with *R-sc:86* and its' derivatives.

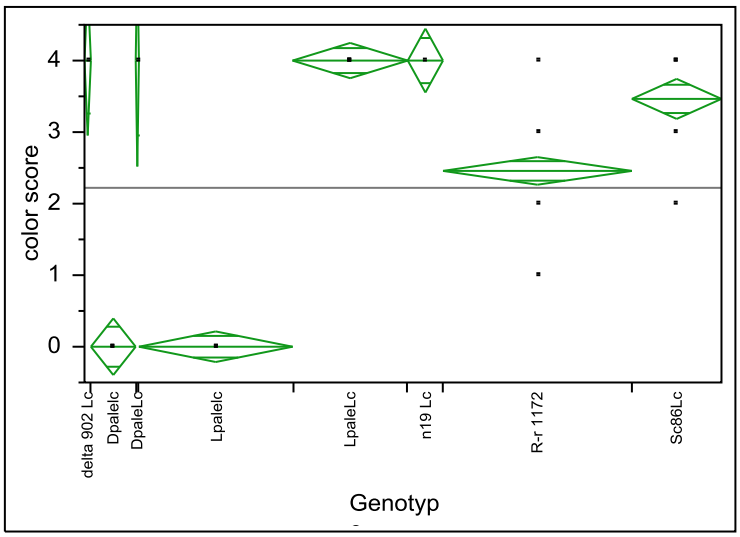


Figure 21: One-way ANOVA of *Lc* expression of *R-sc:86 Lc* derivatives and controls in mature plants. Color score: 0=no color on nodes, prop roots, or leaf color, 1= light prop root color, no color in nodes or leaves, 2= prop root color, light color in 1-3 lower nodes, no leaf color, 3= color in prop roots and >3 nodes, light leaf color in 1-3 leaves, 4= color in prop roots and > 3 nodes, light leaf color in >3 leaves and 5= dark color in prop roots and >5 nodes and color in > 3 leaves.

Structural analysis of Lc losses from R-sc:86-17(L) Lc

Genomic DNA for *R-sc:86-17(L) Lc* and the three samples from the putative *Lc* losses were digested with the methylation insensitive enzyme *Hin*dIII. Southern blot analysis of *R-sc:86-17*(*L*) *Lc* revealed the presence of three *r1* hybridizing fragments at the 5' end: 4.1kb, 3.5kb, and 3.9kb representing *Sc*‖*nc1, Nc2, Nc3,* and *Lc* (Eggleston *et al.* 1995). The three samples from ED492 were all missing the observed fragments from the *Lc* control; however fragments of 3.3kb and 2.5kb were observed (Figure 22). Analysis of the 3' end revealed two fragments in the *Lc* control at 7.5Kb and 5.6Kb representing *Sc*‖*nc1*, *Nc2*, and *Nc3* (single fragment) and *Lc*. The *Lc* loss samples contained no hybridizing *r1* fragments in the 3' region (Data not shown). ED492 resulted from a molecular change (loss of the *Lc* gene) rather than an epigenetic change.

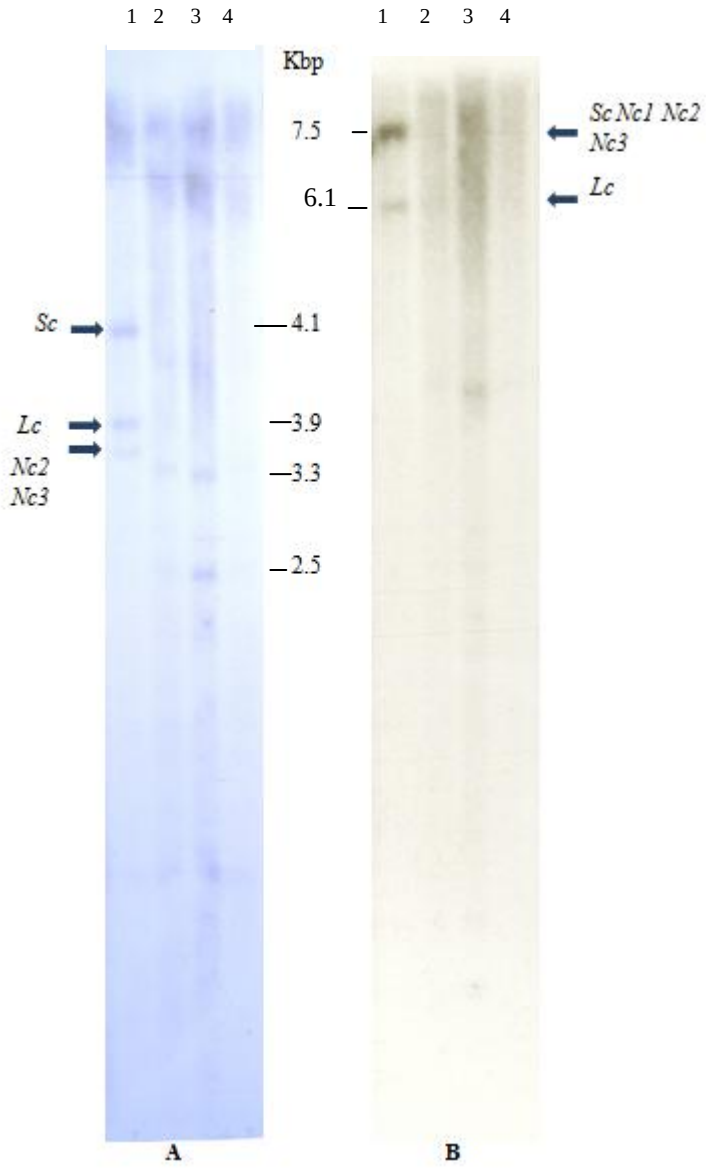


Figure 22: Southern blot analysis of putative *Lc* losses from *R-sc:86-17(L) Lc*. Genomic DNA was digested with *Hin*dIII and sequentially hybridized with pR-nj:l (A) and pSc323:J20 (B). Lane 1: *R-sc:86-17(L) Lc*; Lane 2-4: *R-sc:86-17(L) Lc*- (n=3 from a single family). There are no hybridizing fragments to *Sc*, *Lc*, or the *Nc* genes in the *Lc* loss samples, indicating a physcial loss of the genes.

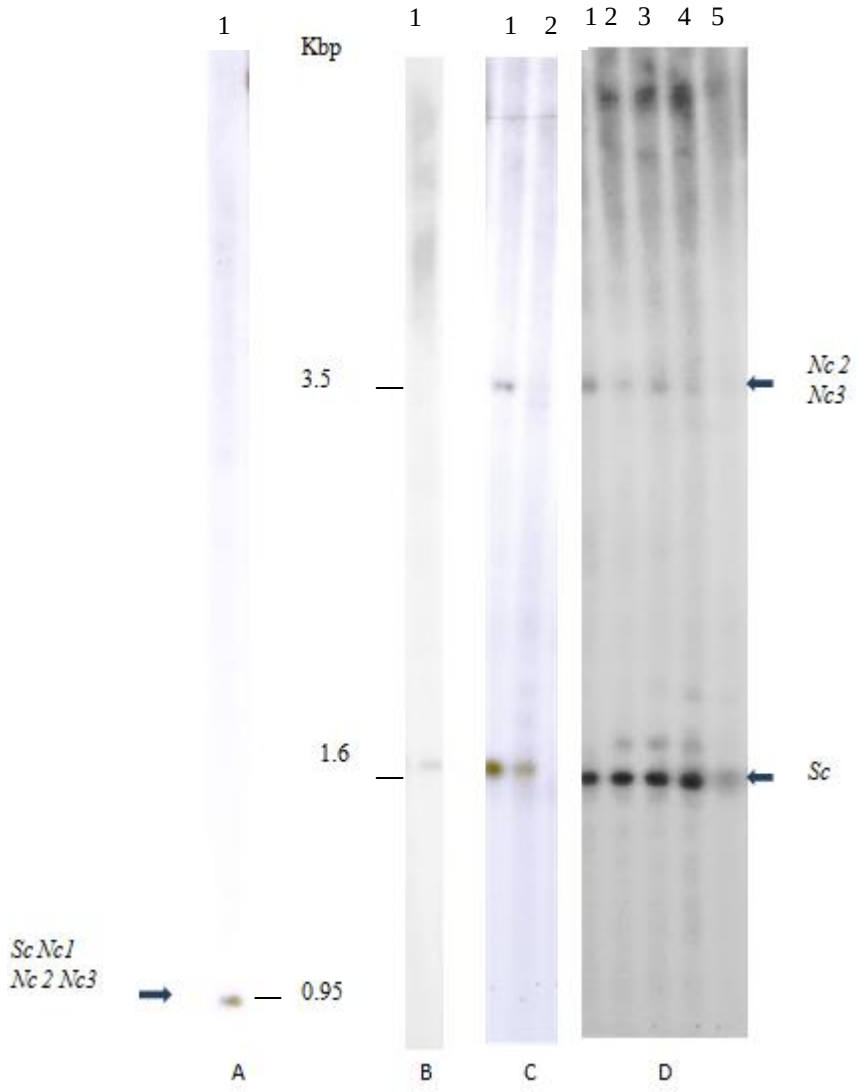


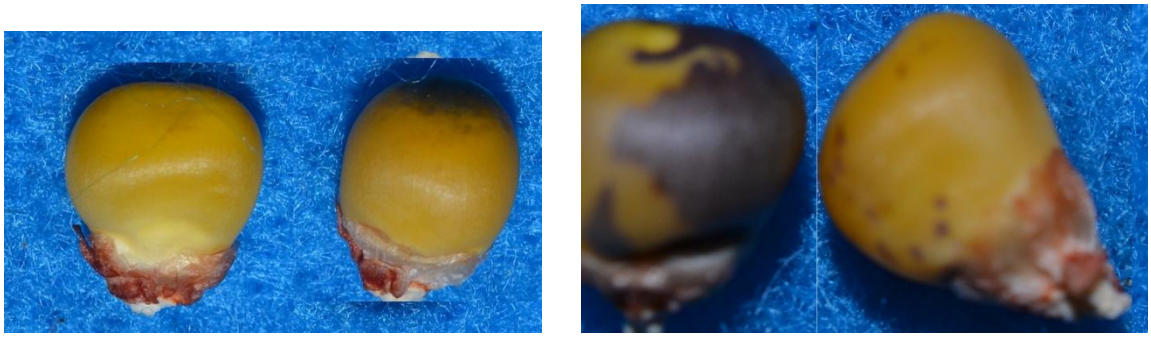
Figure 23: Southern blot analysis of 5-meC patterns of atypical putative EMS-induced mutants. Genomic DNA was digested with *Hin*dIII, and *Eco*RII and sequentially hybridized with pSc323:J20 (A) and pR-nj:l (B,C and D). Lane 1-A: *08-178-1* (un-methylated genes); Lane 1-B: *08-146-2* (loss of at least one *Nc* gene); Lane 1-C: *08-178-1* (typical); Lane 2-C: *08-178-1* (partial methylation of *Nc* genes)*;* Lane 1-D: *05-034-21* (typical); Lane 2-3 D: *05-052-16* (partial methylation of *Sc*); Lane 4-5-D: *08-113-8* (partial methylation of *Sc* in lane 4, lane 5 has less DNA).

Discussion

Anthocyanin deposition in the kernels of Sc and Nc enhancers

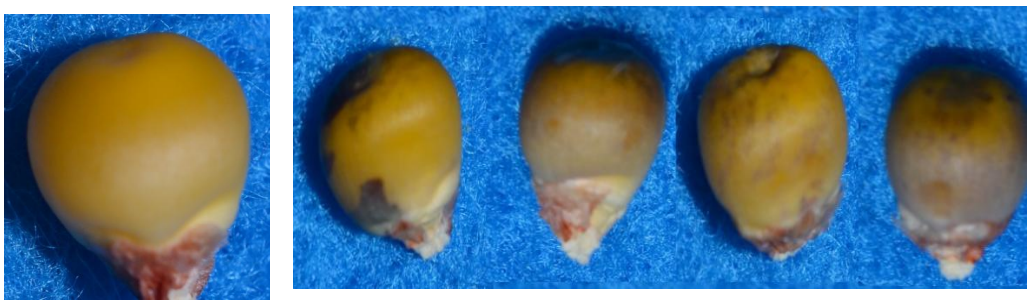
The pigmentation pattern of the *Sc* enhancers revealed a stable, but highly variable phenotype from year to year and within a growing location. Due to the kernel variability a phenotypic scoring system and reflectometer were used to categorize the alleles. Although a significant difference ($p<0.001$) was found between all means of the groups tested, it should be noted that overlap was observed between the *Nc* enhancer and the medium-dark pale *Sc* enhancer groups. This overlap is most likely due to the variability in kernel color. The kernel colors observed on the homozygous EMS-induced putative mutants of *R-sc:86-17(L)* is different than what is normally expected at the *r1* locus when the *Sc* gene is present and transcriptionally active (Eggleston *et al*. 1995). When *Sc* is present and functional, the result is a full-color black seed. Unless there is a known element such as *I-R* (as in *R-stippled*) or *Ds* insertion, or excision, then little variation in color is normally observed in the absence of disease (Alleman and Kermicle 1993; Eggleston *et al*. 1995). Part of the observed variability in kernel phenotype could be due to the selection scheme of the initial *Sc* enhancers (e.g. the darker the earliest seeds (05), the darker the observed seeds in subsequent generations (06-12)). This high variability in kernel color was also observed in the *Nc* enhancers and had an effect on the amount or level of mottling observed.

Figure 24: EMS mutant *08-128-9* (*Sc* enhancer) crossed to *08-105-12* (*Sc* enhancer).



A B

Figure 25: Kernel Phenotypes for the intercross of *05-040-19* (*Sc* enhancer) to *05-032-24* (*Sc* enhancer) (A): typical phenotype; (B): atypical patches and mottling.



A B

Figure 26: Complementation test of EMS-induced mutants *05-040-19* (*Sc* enhancer) crossed to *05-098-10* (*Nc* enhancer). (A): typical kernel phenotype; (B) atypical mottling and patches on kernels.

Figure 27: Complementation test of EMS-induced mutants *05-116-7* (*Sc* enhancer) crossed to *08-113-8* (*Sc* enhancer) resulting in atypical kernel mottling and patches.

Genes involved in restoring seed color

To determine how many elements and the location of these elements involved in canalizing *R-sc*, complementation and mapping relative to *r1* tests were conducted. The complementation and mapping data indicate that more than one gene is involved in restoring seed color of the *r1* locus. Out of eighteen mapped putative EMS-induced mutant alleles, ten mapped at or near *r1*, and eight alleles mapped elsewhere in the genome.

The mottling observed in the *Nc* enhancer alleles was also observed in the progeny of intercrosses between *Sc* enhancers to *Nc* enhancers and to each other (Figures 24-27). The level of mottling was found to correspond to the level of color observed on the kernel (e.g. the darker the kernel, the more mottling). Two of the five *Nc* enhancers were found to be dominant when crossed to *r-Δ902* (Figure 14). For example a dominant *Nc* enhancer allele (i.e. *05-052-16*) when crossed to *Sc* and *Nc* enhancer alleles resulted in ~10% of the kernels as spotted or mottled, versus all the kernels indicating partial dominance or incomplete penetrance. Also in crosses between the Sc enhancers, mottling was observed in the progeny in various levels and some kernels contained "patches" of color (Figures 25-27). It is still unclear whether this mottling is truly a mutant phenotype or a result from increased gene expression of the *Nc*'s.

Dosage and pleiotropic effects in the putative EMS-induced mutants

In addition to the variable expressivity in kernel color, mottling and spotting observed in the progeny and homozygous alleles, and possible incomplete penetrance, a dosage effect was observed. Dosage effects are present when the progeny receive two copies of the *r1* allele from the female parent and a single copy from the male. Reciprocal crosses were attempted and in all

cases the mottling or kernel color effects were more enhanced when transmitted through the female. Depending on the allele, mottling was observed when transmission occurred from the male, but the effect was not as strong visually. In crosses between the putative EMS-induced mutants and *r-Δ902*, partial dominance was observed when transmitted through the female (Figures 14J and14N). Although the observations are consistent with dosage effects, imprinting cannot be ruled out at this time.

The variable expressivity and mottling were also accompanied by changes in normal development in three putative EMS-induced mutants and potentially indicate pleiotropic effects. Delays were seen in plant height (dwarf plants) and leaf structure (wilty) during development in one of the homozygous putative EMS-induced mutants (*05-032-24*). This suggests that a subset of the genes involved in the canalization of *r1* are also involved or closely involved with developmental genes and were affected by the mutagenesis.

Molecular analyses of the Nc and Sc enhancers

Of the 20 *Sc* and *Nc* enhancers analyzed, 19 showed that the 5' and 3' regions of the *Sc* and *Nc* genes were intact when digested with *Hind*III, a methylation insensitive enzyme (Eggleston *et al*. 1995). Two were dominant *R* contaminants, which also showed hybrid vigor in M2 plants, consistent with pollen contamination origin. One of the mutant alleles, *08-146-2,* revealed a faint fragment, representing the *Nc* genes, at the 5' end when compared to *R-sc:86* (Figure 17).Based on these observations there appear to be no large structural changes in the regions probed of the *Sc* or *Nc*'s (Eggleston *et al*. 1995). However the presence of EMS-induced point mutations is a likely cause for the observed changes in phenotypes in the putative EMS-induced mutants. Point

mutations could create changes in amino-acid and protein structure which could alter seed color pigmentation.

5-meC patterns in Nc and Sc enhancers

Of the 20 *Sc* and *Nc* enhancers analyzed 16 revealed that there is lower methylation at the 5' end of *Sc* and high methylation at the 3' end of the *Nc*'s similar to the control *08-105-11*. Partial methylation of *Sc* was observed in the dominant *Nc* enhancer *05-052-16* and *Sc* enhancer *08-113-8*. Also at the 5' end partial methylation was observed for the *Sc* enhancer *08-178-1* as the *Sc‖nc1*, *Nc2*, and *Nc3* fragment was lower in intensity and in the *Sc* enhancer *08-146-2* a very faint fragment for the *Nc* genes was observed. At the 3' end low methylation was observed in the *Nc* genes of the *Sc* enhancer *08-178-1,* as indicated by the 0.95kb fragment. These four alleles which show a change in methylation from *R-sc:86* and *R-sc:86-17(L*) (Figure 5 and Figure 28) indicate that methylation is most likely involved at the 5' end of the genes in silencing those select alleles. The most unusual *Sc* enhancer, in regards to methylation levels, is *08-178-1* which had methylation at both the 5' and 3' ends of the genes.

Extended the mapping of the genetic basis for R-sc:86-17(L)

Eggleston (pers. comm.) previously found that restoration of full-color in progeny of *R-sc:86-17*(*L*)/*P* resulted from recombination of *Sc‖nc1* with *P*. The current work analyzed an additional six heritable full-color revertants from *R-sc:86-17*(*L*) and *r-r:n142* heterozygotes. Molecular results indicate the presence of the 5' region of *Sc*, the 3' end of *P* and a lack of *Nc genes.* Together these results extend the original conclusion that the lesion responsible for *R-sc:86-17pale* is at the 3' end of the *Sc‖nc1* gene.

Loss of canalization at the r1 locus does not affect Lc expression in cis

Previously *Lc* was combined with *R-sc:86-17(L)* in *trans* and no loss of *Lc* expression was observed suggesting that the silencing mechanism was located at or near *r1* and was not *trans* acting with *R-sc:86-17*(*L*) (W. Eggleston pers. comm.). *Lc* was recombined with the *R-sc:86-17(L), R-sc:124, R-sc:86,* and *R-sc:86-17(D)* and germinated to determine if *R-sc:86-17(L)* would silence *Lc* in *cis*. Loss of *Lc* expression was observed in two out of the six families screened and no restoration of *Lc* was identified, consistent with the loss of the *Lc* gene. The positive *R-sc:86-17*(*L*) *Lc* individual contained *Lc*, *Sc*‖*nc1*, *Nc2,* and *Nc3* (Eggleston *et al*. 1995). The three *Lc* losses tested contained none of the genes present in the *Lc* control. An additional two fragments at 3.3kb and 2.5kb were observed in the three *Lc* losses tested which indicate the presence of *R-g:8 pale* (Dietrich 1998). One possibility is that the samples tested represent homozygous *R-g:8 pale* instead of *R-sc:86-17*(*L*) *Lc*. Also, in an additional three families, *Lc* was placed in *cis* with *R-sc:86-17*(*L*), *R-sc:86-17*(*D*), and *R-sc:86* and no silencing was observed. No molecular analysis was completed on the additional families to determine if *Lc* was actually silenced and present or if the gene was missing. In the future, more plants of *R-sc:86* in *cis* with *Lc* and its' derivatives need to be germinated and analyzed to determine structure and 5-meC patterns. If *Lc* has been lost then the study becomes uninformative to determine if *R-sc:86-17*(*L*) affects *Lc* in *cis*. A study currently in progress has identified 5 out of 1831 *Lc* losses on *R-sc:86-17*(*L*) *Lc* indicating that the loss of *Lc* expression is a rare event (W. Eggleston pers. comm.).

Potential mechanisms in the loss of kernel color at r1 (*R-sc:86-17 to R-sc:86-17pale*)

The results presented here indicate that the lesion(s) responsible for an increase in kernel color and potentially canalization is at or near the *r1* locus and elsewhere in the genome. This data also indicates that the loss of kernel color from *R-sc:86-17* to *R-sc:86-17pale* lies at the 3' end of the *Sc* gene. Additional support is the lack of *trans* silencing when *R-sc:86-17*(*L*) was combined with *R-sc:86-17(D), R-sc:124,* and *R-sc:86* as evidenced by the presence of *r1* expression (W. Eggleston unpubl. obs.). These observations are not consistent with RNA-directed DNA methylation, *trans* acting, pathways such as siRNA or miRNA. It does not however rule out a novel mechanism of the pathway as observed at the *Pl* locus (Erhard *et al*. 2013). There is still the potential for a *cis* acting enhancer element, as observed in Erhard *et al*. (2013), or that an epigenetic effect of RNA polymerase: *POLIV* was responsible for the initial reduction of seed color expression in *R-sc:86-17pale*. As the structure for *R-sc:86-17pale*, *R-sc:86-17*(*L-D*) and 24 out of 25 putative EMS-induced mutants was the same, it is unlikely that the habituation of *R-sc:86-17pale* is due to variable expressivity, but rather to loss of canalization of the genes at *r1* resulting in highly variable and selectable phenotypes.

Are potential mechanisms which restore kernel color also related to the canalization of r1?

The variable kernel phenotypes of dark seed color and mottling observed in the putative EMS-induced mutants offer insight into the mechanisms related to the restoration of seed color. The question that remains is whether the restoration of seed color is directly related to canalization at *r1*. As *trans* acting pathways have been ruled out as causing the original loss of canalization at *r1* and current reduction of color in *R-sc:86-17*(*L*) there is evidence of a second site enhancer as

demonstrated from the complementation tests of the EMS mutants. One example of an enhancement gene or element is *enr* (enhancement of *r1*) a dominant enhancer that produces spots or mottling on several near colorless *r1* haplotypes as suggested by Stinard *et al*. (2009). The dominant enhancers are suggested to act in conjunction with *FCU*, a mutable enhancer of *r1* aleurone expression. The mutability is thought to occur when an autonomous transposon, located at the *enr* locus, excises resulting in mottling at *r1* (Stinard *et al.* 2009). A second possibility is that the original loss of canalization was caused by a change in methylation states at the 3' end of the genes as evidenced here and previously (W. Eggleston upubl obs.) by the high methylation observed in the EMS mutants. Pleiotropic effects were also observed in three of the EMS mutants as evidenced by the developmental delays and changes to height and plant phenotypes. This is comparable to the function of *Hsp 90* in *Drosophila*. When the functions of certain genes are rendered non-functional or reduced, the result is greater selection on other genes and processes (Sangster *et al*. 2007; Sato and Siomi, 2010). The representative data for *R-sc:86-17*(*L*) *Lc* to determine if *Lc* is silenced in *cis* shows that there is a loss of the *Lc* gene indicative of a genetic rather than epigenetic change. These tests need to be repeated and molecular data completed to determine whether there is a true loss of the *Lc* gene or *Lc* expression. The homozygous EMS-induced mutants should be crossed to more null alleles in order to determine penetrance, as well as determine more information about the element outside of *r1* causing an expression change at *r1*. The difficulty arises in that as darker seed color has been selected, an inherent bias exists after several generations of selection or habituation. This was seen originally in previous work by K. McWhirter (unpubl. obs.) where *R-sc:86-17* was habituated over five

generations resulting in the light and dark seed color. This work with *R-sc:86-17*(*L*) supplies evidence for restoration of gene expression, important not just for color, but also offers a more practical solution for breeding strategies that may utilize epigenetics versus current transgenic technology. For example, if a trait like leaf shape could be habituated over several generations, then if a line is grown in a location where wilty is needed, e.g. in order to conserve water, then this could be developed without changing the genetic make-up and in turn, habituated back to typical leaf shape as needed.

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