

# Inheritance of *R* Locus Expressing Brown Hilum on Black Seed Coat in Soybean

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## Abstract

Color is one of the phenotypic markers mostly used to study soybean (*Glycine max* L. Merr.) in the study of genetic, molecular and biochemical processes, due to their easy recognizability. The genetic control of several soybean natural variants has not been studied. The standard phenotype of *R* gene is black hilum on black seed. The genetic type T16 is the only occurrence with brown hilum on black seed coat and its genetic control was not described until now. The aim of this study is to understand the genetic control of seed coat and hilum color in the genetic types T16 and in the natural variants of Bragg, BR6 and BR13. T16 was combined with Bragg P and BR13P (black seed color) and BR6M (brown seed color) and T236 (*r-m*). It was found that the genetic control of the brown hilum trait in black seed coat of the T16 genotype was controlled by two loci segregating independently and controlling the expression of the color of the hilum and the seed coat color. The expression of the brown hilum trait in black seed coat is dependent on locus *T<sub>-</sub>*, which controls pubescence color; therefore it occurs only in genotypes with tawny brown pubescence (*T<sub>-</sub>*), which characterizes the pleiotropic effect of this locus on the trait hilum brown trait in black seed coat. The color of the hilum and the seed coat color belong to the same allelic sequence. No maternal effect was found in the expression of hilum brown trait in black seed coat.

## Keywords

Seed Coat Colour, *Glycine max*, Mutable Allele, Soybean

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## 1. Introduction

Seed color is controlled by a small number of genes and because of that, this trait is frequently used by breeders as a readily assayed visible marker for the presence of “off-types” in soybean seed lots.

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The visual appearance of the soybean seed has been altered as result of domestication during the transition of the wild *Glycine soja* to the current cultivated *Glycine max*. All modern commercial high yielding *Glycine max* germplasms possess yellow seed coats with a range of hilum colors (brown, black, imperfect black, buff, yellow), whereas the soybean *Glycine soja* accessions in the USDA Soybean Germplasm Collections possess black seed coats. The wild type soybean seed coat and hilum color in soybean are secondary metabolites derived from flavonoid pathway such as proanthocyanidins and anthocyanins which play a relevant role in plant pathogen defense and protection from UV light exposure [1].

In soybean (*Glycine max* L. Merrill) the *R* gene is involved in anthocyanin synthesis in seed coat, the standart *R* locus produces black hilum and black seed coat. In additional it is found that the locus *R* is also involved in the variegation phenotype affecting seed coat color. The *r-m* allele of this gene produces striped seed coat superimposed on an brown seed color, and the locus *R* and two additional independent loci *I* anthocyanin inhibitor and *T* tawny, control pigmentation and anthocyanin accumulation [2] [3].

The *I* locus controls the presence or absence and spatial distribution of flavonoid pigments and has four alleles (*I*, *i*<sup>i</sup>, *i*<sup>k</sup>, *i*). In the presence of *I* the total inhibition of pigmentation occurs; the double recessive allele *i*<sup>i</sup> allows full pigmentation of the seed; with *i*<sup>i</sup> allele, pigmentation is restricted to the hilum; with the allele *i*<sup>k</sup> pigmentation in hilum and in part of the seed coat occurs. Plants carrying the *I* or *i*<sup>i</sup> allele show complete inhibition of pigmentation in the seed coat or pigmentation only in the hilum, respectively, resulting in a yellow seed phenotype. However, there are four main seed coat colors (black, brown, yellow and green), and the inheritance of black and brown pigments is genetically independent from the green and yellow ones [4]-[6].

The the dominant allele *T* conferred brown (or tawny) and *t* conferred gray pubescence color. Further, *T* generally darkens hilum and/or seed coat color in combination with genotypes at the *I*, *W*<sub>1</sub>, *R* and *O* loci [7] [8]. The allelic series *T/t-r/t* produces the seed coat or hilum colors of black, red-buff and buff, respectively [9]. The dominant allele *T* was completely shown to suppress the development of abnormal pigmentation around the hilum region and to partially suppress the cracking of seed coat associated with chilling treatments. One recessive gene conditioning the phenotype spread hilum in the T48 genotype was found. The expression of the spread hilum is dependent of locus *T*<sub>-</sub>, which controls pubescence color; therefore it occurs only in genotypes with brown pubescence (*T*<sub>-</sub>), which characterizes pleiotropic effect of this locus on the trait spread hilum [10].

The inheritance black hilum/black seed coat was described by Kovinich *et al.* [4]; Carpentieri-Pipolo *et al.* [9]; Yang *et al.* [6] and Senda *et al.* [5]. More recently Gillman *et al.* [10] identified the specific gene and causative basis behind the phenomenon of brown hilum/seed coat coloration in soybean (*R* locus), and discovered an allelic series of four loss of function mutations affecting our *R* locus gene candidate (*R*<sub>2</sub>*R*<sub>3</sub> *MYB* gene). The presence of each one of these mutations was perfectly correlated with the brown seed coat/hilum phenotype in a broadly distributed survey of soybean cultivars. These findings strongly suggest that loss of function for one particular seed coat-expressed *R*<sub>2</sub>*R*<sub>3</sub> *MYB* gene is responsible for the brown seed coat/hilum phenotype in soybean.

The allelic sequence *R*, *r-m*, and *r* controls the pigment color and the presence of stripes in the seed coat. The *r-m* allele is dominant over *r* and recessive to *R*. The *r-m* allele exhibits instability in somatic and germinal cell layers and the revertants phenotypic forms are not stable with expression forms of the allele at high frequency [11]. The expression of this not typical phenotypic germinal instability was suggested as transposable elements residing in or near the *r-m* allele. Since the revertants of *r-m* alleles may be not be identical to the standard *R* and *r* forms they were designated by Chandlee and Vodkin [11] as designate *R*<sup>\*</sup> and *r*<sup>\*</sup>. There is one phenotypic difference between the standart *R* allele, and the *R*<sup>\*</sup> carryng brown hilum in black seed and *r*<sup>\*</sup> carryng *r-m* (striped seed coat); thus the locus *R* is not stable and could produce all three forms *R*<sup>\*</sup>, *r-m* and *r* [11].

The standard phenotype *R* gene is black hilum on black seed [11]. The genetic type T16 is the only occurrence with brown hilum on black seed coat in the *Glycine max* accession of USDA soybean germplasm and its genetic control until now is not described yet.

The aim of this study is to understand the genetic control of seed coat and hilum color in the genetic types T16 and the natural variants of the commercial cultivars Bragg, BR13 and BR6.

## 2. Materials and Methods

The genetic type T236 is an *Glycine max* accession of USDA Soybean Germplasm Collections with white flower, gray pubescence and a usual red-buff (*Rbf*) seed coat which conditioned by allele at the *T/t* locus. LC 91-26 is a

PI91073 isolate, it is homozygous (*r-m/r-m*) (brown striped) and a source of *r-m* allele in the USDA GRIN germplasm collection, accessed on 30/06/2015 (<http://www.ars-grin.gov/npgs/>). The standard soybean cultivars Bragg and BR13 are yellow seed coat with a black hilum and the standard BR6 is brown seed coat with a brown hilum; the isolines natural variants, BraggP and BR13P with black hilum and black seed coat and BR6M with Brown hilum and brown seed coat respectively were used in the crossing as parental (Brazilian Ministry of Agriculture and Food Supply, MAPA, accessed 30/06/2015, [http://extranet.agricultura.gov.br/php/snpc/cultivarweb/cultivares\\_registradas.php](http://extranet.agricultura.gov.br/php/snpc/cultivarweb/cultivares_registradas.php)). The soybeans seeds cultivars including the isolines natural variants were provided by the Brazilian Agricultural Research Corporation (Embrapa Soybean), Soybean active germplasm Bank of Embrapa.

The phenotypes of the parental inheritance involved in this study, the color of the seed coat and hilum are shown in **Table 1**.

F<sub>1</sub> and F<sub>2</sub> generations resulting from crosses between soybeans genotypes T16 × Bragg, T16 × BraggP, T16 × BR 13P, T16 × BR 6M, T16 × LC 91-26, T16 × T236, were studied. The parental population F<sub>1</sub> and F<sub>2</sub> were handled in a greenhouse, under temperature conditions ranging from 25°C to 30°C, and 16 hours of light at Brazilian Agricultural Research Corporation, Embrapa Soybean, in Londrina, PR, Brazil at 23°22' latitude south.

Crossing were performed from December 2009 to February to 2010. F<sub>1</sub> plants, were obtained in the winter of 2010. Authentication of hybrid origin of F<sub>1</sub> plants was carried out and they were compared with their female parental. Additional phenotypic markers such as flower, pubescence and pod color were used to verify that the seeds produced were hybrid. Having in mind that the age of the seed influences in the expression of the genetic traits of the soybean, renewing of parent seeds was carried out during the winter of 2009 and F<sub>2</sub> generation, were planted in December 2010.

Seed coat colors were assessed by inspecting mature and dried seeds visually. The F<sub>2</sub> segregating generations were separated into classes through comparison with a parental sample. The data obtained was grouped according to the distribution of the frequency of the plants for the characteristic, and the  $\chi^2$  (chi-square) test was applied to test the genetic hypothesis of monogenic, digenic and polygenic inheritance.

### 3. Results and Discussion

No difference between the original and reciprocal crosses was found, suggesting that there was no effect of maternal control on the characteristic hilum color. Hilum tissue is not maternally-derived, in contrast to the seed coat. Phenotypes of F<sub>1</sub> seeds showed that the black hilum was dominant over the brown hilum, a previously expected result as described by Chandlee and Vodkin [11] in their study of the relation between black and brown pigment in soybean hilum and seed coat. Regarding the seed coat, the yellow color was dominant over the black one in breeding T16 × Bragg, and over tanned-red in the crossing T16 × T236, the same dominance relation that was observed by Seo *et al.* [9] in his study of the inheritance of Buff-red seed coat in genotype T236.

F<sub>2</sub> segregation from T16 × Bragg (**Table 2**) indicated that the brown hilum pigmentation in black seed coat is controlled by a single allele *R*. We have shown that the phenotype brown hilum pigmentation in black seed coat in the genetic type T146 (*R*) is controlled by a single allele [11].

In the crosses T16 × BraggP and T16 × BR 13P, the F<sub>2</sub> segregation presented correspondence to the 3:1 proportion (black hilum: brown hilum). This result was confirmed by the low value observed in the chi-square ( $\chi^2 = 0.009$  and  $p = 0.922$  and  $\chi^2 = 0.099$  and  $p = 0.751$ , respectively), which shows that the inheritance of seed color the hilum color were determined by a single gene in these crosses, and the black hilum dominates over brown hilum.

The dominance relation (3:1) was observed in crosses T16 × BR 6M ( $\chi^2 = 0.007$  and  $p = 0.930$ ) and T16 × LCV 91-26 ( $\chi^2 = 0.206$  and  $p = 0.649$ ) with dominance of brown striped seed coat color over brown seed coat. However, there was only segregation of seed coat color, and all the seeds showed brown hilum, confirming that all T16 and BR6 Brown genotypes and LCV91-26 (brown striped) have the same genetic constitution for hilum color. Chandlee and Vodkin [11] have examined the inheritance of *r-m* allele through several generation and found that it exhibits unusual allele instability which changes in gene expression that can convert to the other expression form of the alleles at high frequencies. This behavior has not been seen in other unstable alleles in soybean and suggests that an insertion sequence or transposable elements resid in or near the *r-m* allele.

The F<sub>2</sub> population of T16 × Bragg demonstrated segregation in four different phenotype classes (yellow seed coat with black hilum, yellow seed coat with brown hilum, black seed coat with black hilum and black seed coat with brown hilum). The proportion 244:84:72:24 observed in F<sub>2</sub> showed concordance with the expected one of

**Table 1.** Simplified description of phenotypic effects of four different genetic loci affecting seed coat and hilum colors of the cultivars and the isolines natural variants used as parents plants.

Parents genotype	Phenotypes				Genotype
	Seed coat	Pubescence	Flower	Hilum	
T 16	Black	Tawny	white	Brown	<i>iirrTTw1w1</i>
Bragg	Yellow	Tawny	white	Black	<i>i<sup>i</sup>RRTTw1w1</i>
Bragg P	Black	Tawny	white	Black	<i>iiRRTTw1w1</i>
BR 13 P	Black	Tawny	white	Black	<i>iiRRTTw1w1</i>
BR 6 M	Brown	Tawny	white	Brown	<i>iirrTTw1w1</i>
LC 91-26	Black + striped	Tawny	white	Black	<i>ii-r-m-r-mTTw1w1</i>
T 236	Red-buff	gray	white	Brown	<i>iirrttoow1w1</i>

**Table 2.** Distributions of *R* allele frequencies among progeny F<sub>2</sub> of soybean plants.

Cross	Number of observed F <sub>2</sub> individuals								Theoretical frequencies	$\chi^2$	p
	Seed coat						Red buff	Buff			
	Yellow	Black	Brown	Brown + striped							
	Hilum				Brown	Red buff	Buff				
Black	Brown	Black	Brown								
T16 × Bragg P			237	80					03:01	0.009	0.922
T16 × BR13 P			494	160					03:01	0.099	0.751
T16 × BR6 M					98	291			03:01	0.007	0.930
T16 × LCV 91-26					87	276			03:01	0.206	0.649
T16 × Bragg	244	84	72	24					9:3:3:1	1.324	0.723
T16 × T236			146	45			45	15	9:3:3:1	0.160	0.983

9:3:3:1 ( $\chi^2 = 1.324$  and  $p = 0.723$ ) for two loci segregating independently and controlling the expression of the color of the hilum and the seed coat color.

The *I* allele inhibits the production and accumulation of pigments over the entire seed coat, resulting in uniformly yellow-colored seeds, whereas the *i* allele leads to completely pigmented seeds by allowing the production and accumulation of pigments over the entire seed coat. The remaining two alleles, *i<sup>i</sup>* and *i<sup>k</sup>*, inhibit pigmentation except in the hilum and a saddle-shaped region, respectively. Yellow soybean cultivars carry the *I* allele for a light (no pigmented) hilum or the *i<sup>i</sup>* allele for a dark (pigmented) hilum [4]-[6], as observed T16 and Bragg, that carry *i* and *i<sup>i</sup>*, respectively. The dominance relationships among the four alleles are  $I > i^i > i^k > i$ . Inhibition of seed coat pigmentation by the *I* locus, at least the *I* and *i<sup>i</sup>* alleles, is the result of RNA silencing of chalcone synthase (CHS) genes [5].

In cross T16 × T236, segregation of the F<sub>2</sub> population in four phenotype classes also occurred (black seed coat with black hilum, black seed coat with brown hilum, red-buff seed coat with buff hilum and red-buff seed coat with red-buff hilum). The obtained results for hilum color and seed coat color correspond to the segregation of 9:3:3:1 ( $\chi^2 = 0.160$  and  $p = 0.983$ ), indicating two loci segregating independently. The F<sub>2</sub> population have shown two phenotypes for pubescence color: tawny brown and gray pubescence. Plants with gray pubescence (*tt*) did not produce seeds with tawny brown hilum on black seed coat (*T\_*), it only was observed in the plants with tawny brown pubescence, therefore the expression of the brown hilum trait on black seed coat is dependent on locus *T\_*, which controls pubescence color; therefore it occurs only in genotypes with tawny brown pubescence (*T\_*), which characterize pleiotropic effect of this locus on the trait hilum brown trait on black seed coat.

Seo *et al.* [9] observed that chi-square analysis indicated that the segregation of F<sub>2</sub> population of cross be-

tween T236 and Clark isolate L68-2077 (*r-m*) were not significantly, different from the 3:1 ratio expected for a monogenic inheritance model, with black dominant on red-buff seed coat.

The genetic control of the brown hilum trait in black seed coat in the T16 genotype is controlled by a single recessive. There is no cytoplasmatic effect in the expression of this trait and the genetic control of the brown hilum trait in the black seed coat, although belonging to the same allelic sequence, there is a different control from the hilum and black seed coat trait.

#### 4. Conclusion

The presence of multiple independent alleles suggested that this gene was selected during domestication either directly for brown coloration or indirectly for hilum colors.

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