

Genetic Analysis of 4 New Mutants at the Unstable *k2 Mdb1-n y20* Chromosomal Region in Soybean

MIN XU AND REID G. PALMER

From the Department of Agronomy and Interdepartmental Genetics Graduate Program, Iowa State University, Ames, IA 50011 (Xu); and US Department of Agriculture–Agricultural Research Service, Corn Insects and Crop Genetics Research Unit, Department of Agronomy, and Interdepartmental Genetics Graduate Program, Iowa State University, Ames, IA 50011 (Palmer).

Address correspondence to R. G. Palmer at the address above, or e-mail: rpalmer@iastate.edu.

Abstract

In soybean (*Glycine max* (L.) Merr.), a chromosomal region defined by 3 closely linked loci, *k2* (tan-saddle seed coat), *Mdb1-n* (malate dehydrogenase 1 null), and *y20* (yellow foliage), is highly mutable. A total of 31 mutants have been reported from this region. In this study, a mutation with tan-saddle seed coat was found from bulk-harvested seed of cultivar Kenwood. Genetic analysis established that this tan-saddle seed coat mutation is allelic to the *k2* locus and inherited as a recessive gene. Simple sequence repeat analysis showed that this mutant is not a contaminant from other existing *k2* mutants. The mutant was named Kenwood-*k2*. To test for genetic instability at the *k2 Mdb1-n y20* chromosomal region, Kenwood-*k2* was crossed reciprocally with cultivars Harosoy and Williams. No new mutants were found in F₂ families. In the genetic instability tests of T239 (*k2*) with cultivar Williams, 3 new mutants with yellow foliage (*y20*) and malate dehydrogenase 1 null (*Mdb1-n*) were identified. In the genetic instability tests of T261 (*k2 Mdb1-n*) with cultivar Williams, no new mutants were found. The Kenwood-*k2* and the 3 yellow-foliage, malate dehydrogenase 1–null mutants provide additional genetic materials to study chromosomal aberrations in this mutable/unstable chromosomal region.

Several mutable or unstable loci have been described in soybean (*Glycine max* (L.) Merr.). They include *Y18-m*, *cyt-Y3* (T284M), *w4-m*, *wp-m*, and *r-m* (for review, see Palmer and others 2004). These unstable loci conditioned variegated phenotypes. Different from these unstable loci, the unstable *k2 Mdb1-n y20* chromosomal region does not condition mutable or variegated phenotypes. It is mutable because it is a “hotspot” for generating mutations (Chen and Palmer 1998).

The unstable *k2 Mdb1-n y20* chromosomal region was defined by 3 closely linked loci, *k2*, *Mdb1-n*, and *y20*. The *k2* locus conditions a tan-saddle pattern on the seed coat. Plants homozygous for a null allele, *Mdb1-n*, do not show malate dehydrogenase 1 activity on starch-gel electrophoresis (Hedges and Palmer 1992). The *y20* locus conditions yellowish-green leaves.

So far, 31 mutants have been reported from the *k2 Mdb1-n y20* chromosomal region (Xu and Palmer 2005a). A total of 25 *y20* mutants have been described; 18 are associated with *k2*, but all 25 are associated with *Mdb1-n*. No *k2k2*

Mdb1Mdb1 y20y20 plants have been identified. For example, tan-saddle mutant seed were identified in a bulk harvest of cultivar Harosoy, precluding the identity of the original plants. The self-pollinated progeny of some tan-saddle seed were true breeding and were green plants (T239), and some were true breeding and were yellowish-green foliage at seedling stage. The latter plants also were identified as malate dehydrogenase null (*Mdb1-n*) and added to the US Department of Agriculture–Agricultural Research Service Soybean Genetic Type Collection as T253 (*k2 Mdb1-n y20*). It was suspected that a transposon was responsible for the mutant phenotypes (Palmer 1984; Palmer and others 1989). Genetic Type T261 (*k2 Mdb1-n*) was found as a spontaneous mutation in cultivar Mandarin (Ottawa). The Clark-*k2* (L67-3843) tan saddle was found after seed X-irradiation of cultivar Clark.

Imсанде and others (2001), using Southern blot analysis, reported that the *Mdb1-n* mutants examined (T253, T317, T323, and T324) were the result of deletions. The null phenotype correlated with the deletion of specific genomic restriction fragments that encoded the *Mdb1* gene. The *k2*,

Table 1. Soybean lines used in the instability studies

Parents	Genotype	Phenotype
Harosoy	<i>K2K2 Mdb1Mdb1 Y20Y20</i>	Wild type, yellow seed coat, MDH 1 present, green foliage
Williams	<i>K2K2 Mdb1Mdb1 Y20Y20</i>	Wild type, yellow seed coat, MDH 1 present, green foliage
Kenwood- <i>k2</i>	<i>k2k2 Mdb1Mdb1 Y20Y20</i>	Tan-saddle seed coat, MDH 1 present, green foliage
Clark- <i>k2</i>	<i>k2k2 Mdb1Mdb1 Y20Y20</i>	Tan-saddle seed coat, MDH 1 present, green foliage
T239	<i>k2k2 Mdb1Mdb1 Y20Y20</i>	Tan-saddle seed coat, MDH 1 present, green foliage
T261	<i>k2k2 Mdb1-nMdb1-n Y20Y20</i>	Tan-saddle seed coat, MDH 1 null, green foliage

MDH, malate dehydrogenase 1.

Mdb1-n, and *y20* loci were mapped on molecular linkage group H by using 5 mapping populations (Xu and Palmer 2005b). Three simple sequence repeat (SSR) markers that were closely linked to the *k2 Mdb1-n y20* chromosomal region were identified that corresponded to deleted chromosome segments in T261 (*Mdb1-n y20*).

Chen and Palmer (1998) reported that tan-saddle mutant T239 was effective in generating *Mdb1-n y20* mutants in certain cross-combinations. This “instability” experiment gave about 2.6% *Mdb1-n* mutants (10 out of 383 F₂ families) from crosses of T239 with parents of *w4-m* and *Y18-m*, which were proposed to contain an active transposable element in their genomes. The control population of 833 F₂ families from crosses of T239 with cultivar Harosoy gave no mutants. Genetic Type T261 (*k2 Mdb1-n*) crossed with parents of *w4-m* and *Y18-m* gave about 0.3% *y20* mutants (1 mutant out of 323 F₂ families). Tan-saddle mutant Clark-*k2* (L67-3483) crossed with parents of *w4-m* and *Y18-m* gave no mutants in 273 F₂ families. Chen and Palmer (1998) have proposed that the instability at the *k2 Mdb1-n y20* chromosomal region was due to transposon activity or special chromosome structures that could generate chromosomal rearrangements such as deletions.

In 1993, tan-saddle mutant seed were found in a bulk harvest of cultivar Kenwood. Our objectives were 1) to determine the inheritance and allelism of this new tan-saddle mutant in Kenwood and 2) to test for instability of Kenwood-*k2*, T239, and T261 in crosses with cultivars Harosoy and Williams.

Materials and Methods

The soybean lines, genotypes, and phenotypes used in the inheritance, allelism, and instability studies are given in Table 1.

Origin and Inheritance of Kenwood-*k2*

Tan-saddle seed were found in a bulk harvest of cultivar Kenwood (Cianzio and others 1990) in 1993 at the Bruner Farm near Ames, IA. The plant with the most intense tan

saddle was single-plant threshed in 1994. Planting, selecting for intense tan saddle, and harvesting self-pollinated seed were continued for the next 3 years.

The Kenwood-*k2* plant with the most intense tan saddle was crossed as male parent with cultivar Harosoy (Weiss and Stevenson 1955). The F₁ seed were advanced to the F₂ at the University of Puerto Rico/Iowa State University soybean nursery near Isabela, Puerto Rico. F₂ and F₃ seed were planted at the Bruner Farm for seed coat color evaluation.

Allelism and Genetic Instability Studies with Kenwood-*k2*

For the allelism test, the Kenwood-*k2* plant with the most intense tan saddle was crossed as male parent to tan-saddle Clark-*k2* (L67-3483), which arose from X-irradiation of cultivar Clark (Johnson 1958).

To test for instability at the *k2 Mdb1-n y20* chromosomal region, cultivars Harosoy and Williams (Bernard and Lindahl 1972) were crossed reciprocally with Kenwood-*k2*. The F₁ seed from both tests were advanced to the F₂ at Isabela, Puerto Rico. F₂ and F₃ seed were planted at the Bruner Farm for evaluation of the presence/absence of tan-saddle seed and to look for seedling/adult plant traits in the genetic instability study. The seedling traits would include chimeric-foliage, yellow-foliage, multileaflet plants, etc. The adult plant traits would include partial male and/or female sterility, complete male and female sterility, dwarfs, etc. Four male-sterile, female-fertile mutants were identified by Chen and Palmer (1996) in a test for instability and genetically characterized by Palmer (2000).

Origin Test of Kenwood-*k2* with SSR Analysis

SSR analysis was conducted as previously described (Xu and Palmer 2005a). Six soybean lines, including Kenwood, Kenwood-*k2*, Harosoy, T239, Clark, and Clark-*k2*, were evaluated with 100 SSR markers randomly selected from the 20 molecular linkage groups constructed by Song and others (2004), which represented the 20 chromosomes of the soybean genome, 5 from each molecular linkage group, respectively.

Test of Instability from Reciprocal Crosses of T239 and T261 with cultivar Williams

To test for instability at the *k2 Mdb1-n y20* chromosomal region, Genetic Types T239 and T261 were crossed reciprocally with cultivar Williams. The F₁ seed were advanced to the F₂ at Isabela, Puerto Rico. F₂ and F₃ seed were planted at the Bruner Farm for evaluation of the presence/absence of tan-saddle seed and to look for seedling/adult plant traits as mentioned in the allelism and genetic instability studies with Kenwood-*k2*.

Genetic Evaluation of Mutants

From the test for instability, 3 F₂ families from crosses involving T239 and Williams were identified that segregated

Table 2. Inheritance of tan-saddle seed coat from the cross of Harosoy with Kenwood-*k2* (F₂ data)

Entry	Seed coat patterns		$\chi^2_{(3:1)}$	P
	Yellow	Tan saddle		
A02-353-1	282	84	0.82	0.37
A02-353-2	319	100	0.29	0.59
A02-353-3	213	67	0.17	0.68
Total	814	251	1.16	0.28

about 3 green:1 yellow viable plant. Green and yellow F₂ plants were threshed individually and evaluated as plant-progeny rows.

Self-pollinated seed from the original yellow plants within each entry were evaluated for malate dehydrogenase according to the procedure of Cardy and Beversdorf (1984).

For inheritance studies, yellow plants from each of the 3 entries were crossed reciprocally with cultivar Harosoy. Seed generation advance and data evaluation were similar to the instability study with T239 and T261.

For allelism tests, yellow plants from each of the 3 entries were crossed with T325 (*Mdb1-n y20*). A small piece of cotyledon was taken from each F₁ and F₂ seed. The samples were evaluated for malate dehydrogenase. The F₁ and F₂ plants were classified for plant color at the Bruner Farm.

Results and Discussion

The Mutation of Tan-Saddle Seed Coat Found in Kenwood Was Conditioned by a New *k2* Allele

During the inheritance experiment of Kenwood-*k2*, 3 hybrid seeds were obtained from the cross of Harosoy with Kenwood-*k2*. The 3 F₂ progenies each segregated about 3 yellow seed coats:1 tan-saddle seed coat (Table 2), which suggested that the mutation with tan-saddle seed coat found in cultivar Kenwood was conditioned by a single gene and that it was recessive to wild-type yellow seed coat.

In soybean, existing tan-saddle seed coat mutations are conditioned by a recessive *k2* locus (for review, see Palmer and others 2004). To test if this mutant found in Kenwood was conditioned by the *k2* locus, tan-saddle Clark-*k2* was crossed with Kenwood-*k2*. The F₁ seed were advanced to the F₂, and all F₂ plants were tan-saddle seed. Twenty F₂ plants were evaluated as F₃ families the following summer at the Bruner Farm. Ten F₃ plants from each of the 20 progeny rows were evaluated for seed coat color pattern. All plants were tan-saddle seed. This result established that tan-saddle seed coat mutations of these 2 lines were allelic. They both were conditioned by the *k2* allele.

One hundred SSR markers representing the 20 chromosomes (20 linkage groups) of the soybean genome were used to determine if Kenwood-*k2* was a mutation in cultivar Kenwood. Results showed that no polymorphisms were detected between Kenwood-*k2* and Kenwood by

Table 3. Test for instability of Kenwood-*k2* with cultivars Harosoy and Williams

Entry	No. F ₂ families	No. F ₂ families segregating green and yellow plants
Harosoy × Kenwood- <i>k2</i>	322	0
Kenwood- <i>k2</i> × Harosoy	118	0
Williams × Kenwood- <i>k2</i>	601	0
Kenwood- <i>k2</i> × Williams	664	0
Total	1705	0

these 100 markers, but about 25% polymorphisms were found between Kenwood-*k2* and T239 and between Kenwood-*k2* and Clark-*k2*. This suggested that the Kenwood-*k2* was not a contamination from other lines with *k2* alleles but a new mutation derived from cultivar Kenwood.

Compared with T239, Kenwood-*k2* Was Stable

Harosoy and Williams were crossed reciprocally with Kenwood-*k2* to test for instability at the *k2 Mdb1-n y20* chromosomal region. A total of 1705 F₂ progeny rows were evaluated 6 times from emergence to maturity, among which 440 progeny rows were from reciprocal crosses of Harosoy and Kenwood-*k2* and 1265 progeny rows were from reciprocal crosses of Williams and Kenwood-*k2* (Table 3). The only variant observed was one foliage chimeric plant from the Kenwood-*k2* with Williams combination. Progenies of this plant were wild type, and no variation was observed.

Williams was crossed reciprocally with T239 and T261 to produce 216 F₂ families (Table 4). Three of 119 F₂ progenies from the reciprocal crosses with Williams and T239 were observed to segregate about 3 green:1 yellow plant (Table 5), which is about 2.6% frequency. This was consistent with the previous results from crossing T239 with the parents of *w4-m* and *Y18-m* (Chen and Palmer 1998). Green and yellow plants were threshed individually, and progeny was tested. The F₂ green plants gave about 1 all-green:2 segregating (green and yellow) progenies (Table 5). Within segregating progenies, the ratio was about 3 green:1 yellow plant (Table 5). The yellow F₂ plants were true breeding.

Table 4. Test for instability of T239 and T261 with cultivar Williams

Cross	No. F ₂ families	No. F ₂ families segregating green and yellow plants ^a
Williams × T239	15	1
T239 × Williams	104	2
Williams × T261	6	0
T261 × Williams	91	0
Total	216	3

^a A00-R-78, A00-R-147, and A00-M-41 were the F₂ families segregating green and yellow plants.

Table 5. Inheritance of the 3 yellow mutants found in the instability test from reciprocal crosses of T239 with cultivar Williams

Family number ^a	No. F ₂ plants				No. F _{2,3} families				No. plants within segregating F _{2,3} families			
	Green	Yellow	$\chi^2_{(3:1)}$	P	Green	Segregating	$\chi^2_{(1:2)}$	P	Green	Yellow	$\chi^2_{(3:1)}$	P
A00-R-78	127	43	0.01	0.93	39	85	0.20	0.66	2797	913	0.30	0.58
A00-R-147	51	18	0.04	0.83	18	30	0.37	0.54	1418	465	0.09	0.76
A00-M-41	72	26	0.12	0.73	23	42	0.12	0.73	1703	574	0.05	0.82

^a A00-R-78 and A00-R-147 were identified in different F₂ families from the cross of T239 × Williams. A00-M-41 was identified in an F₂ family from the cross of Williams × T239.

No mutations were found from the reciprocal crosses of Williams and T261. The reason could be that too few F₂ families were evaluated. According to previous results, the mutation rate of T261 in crosses with parents of *w4-m* and *Y18-m* was about 0.3% (Chen and Palmer 1998), but only 97 F₂ families were evaluated in this study (Table 4).

The *k2* alleles in T239, T261, and Kenwood-*k2* arose spontaneously and independently. Compared with T239, the *k2* allele in Kenwood-*k2* in crosses with cultivars Harosoy and Williams did not induce mutations. The mutant-generating abilities of the *k2* alleles in Kenwood-*k2*, T239, and T261 are different even though they condition similar tan-saddle phenotypes.

Genetic Characterization of the Yellow Plants Found in Reciprocal Crosses of T239 with cultivar Williams

Yellow plants were crossed reciprocally with cultivar Harosoy. The F₁ plant color from all crosses was green, which suggested that the yellow phenotype was the result of a nuclear mutation. The F₂ data and F_{2,3} family data were in agreement with a single recessive gene inheritance pattern (Table 6). The yellow F₂ plants were true breeding.

For allelism tests, the yellow plants were crossed with T325, a *Mdb1-n y20* mutant identified in a gene-tagging study with *w4-m* (Hedges and Palmer 1992). The parents, F₁, and a random sample of F₂ seed were tested for malate dehydrogenase and evaluated for plant color. The parents, F₁, and F₂ plants were all yellow foliage (*y20*), and the seeds were malate dehydrogenase null (*Mdb1-n*) (Table 7). The 3 independently derived mutants are alleles of *Mdb1-n y20*.

In soybean, the *k2* locus was tightly linked to the *Mdb1-n* and *y20* loci and resides in a chromosomal segment that is a hotspot for mutation (Chen and Palmer 1998). The mutations of *Mdb1-n* and *y20* are suspected to correspond to a chromosomal segment deletion. The instability of the *k2 Mdb1-n y20* chromosomal region could be due to an inactive transposable element or chromosomal rearrangements such as duplications or inversions residing in or nearby this region that could lead to deletions in this region (Chen and Palmer 1998).

As we know, the insertion and excision of transposable elements would generate rearrangements of the sequences flanking their insertion sites, including deletions, inversions, and duplications. Sometimes the deletion size could be very large. For example, the *Tam3* transposable element has induced a deletion with size more than 20 kbp at the *niv* locus in *Antirrhinum majus* (for review, see Martin and Lister 1989).

This study identified a new tan-saddle mutant that occurred spontaneously in cultivar Kenwood. This is the first *k2* mutant identified in cultivar Kenwood. Two *Mdb1-n y20* mutants were identified in the cultivar Williams with T239 (*k2*) cross, and one *Mdb1-n y20* mutant was identified in the T239 with cultivar Williams cross. The results of the instability study with T239 and cultivar Williams gave about 2.5% *Mdb1-n y20* mutants (3 out of 119 F₂ families). This is similar to the T239 crosses (10 out of 383 F₂ families) reported by Chen and Palmer (1998). Thus, 35 mutants are known for the *k2 Mdb1-n y20* chromosomal region in soybean. No mutants were found in reciprocal crosses of cultivar Williams with Kenwood-*k2* or in reciprocal crosses of Williams with T261 (*k2 Mdb1-n*).

Table 6. Inheritance of the 3 yellow mutants found in the instability test from reciprocal crosses of T239 with cultivar Williams

Parent 1 ^a	Parent 2 ^a	No. F ₂ plants				No. F _{2,3} families				No. plants within segregating F _{2,3} families			
		Green	Yellow	$\chi^2_{(3:1)}$	P	Green	Segregating	$\chi^2_{(1:2)}$	P	Green	Yellow	$\chi^2_{(3:1)}$	P
A00-R-78	Harosoy	609	199	0.06	0.81	41	86	0.06	0.80	3093	1001	0.66	0.42
A00-R-147	Harosoy	735	238	0.15	0.70	44	95	0.18	0.67	5097	1686	0.07	0.78
A00-M-41	Harosoy	485	157	0.10	0.75	37	69	0.12	0.73	3052	1029	0.10	0.75

^a Data from reciprocal crosses bulked, and homogeneity tests indicated that all samples were homogeneous.

Table 7. Allelism tests of the 3 yellow-foilage and malate dehydrogenase 1-null F₂ plants

Female	Male	No. F ₁ plants		No. F ₂ plants	
		Foliage color	MDH pattern	Foliage color	MDH pattern ^a
A00-R-78	T325 (<i>Mdb1-n y20</i>)	10, yellow	10, null	369, yellow	30, null
A00-R-147	T325 (<i>Mdb1-n y20</i>)	12, yellow	12, null	381, yellow	36, null
A00-M-41	T325 (<i>Mdb1-n y20</i>)	14, yellow	14, null	411, yellow	42, null

MDH, malate dehydrogenase 1.

^a Three F₂ seeds descended from each of the F₁ plants were evaluated for malate dehydrogenase.

Molecular studies are necessary to elucidate the basic of this chromosomal instability. The new mutants found or generated in this study would provide additional genetic materials for studying the chromosomal aberrations in this chromosomal region.

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