Evaluation of spontaneous generation of allelic variation in soybean in response to sexual hybridization and stress

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[1]Department of Agronomy, Iowa State University, Ames, IA 50011-1010, USA; [2]Department of Biology, University of Wisconsin-Stevens Point, Stevens Point, WI 54481, USA; and [3]Department of Biology, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1.

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Espinosa, K., Boelter, J., Lolle, S., Hopkins, M., Goggi, S., Palmer, R. G. and Sandhu, D. 2015. **Evaluation of spontaneous generation of allelic variation in soybean in response to sexual hybridization and stress**. Can. J. Plant Sci. **95**: 405–415. Intra-cultivar variation reported in pure lines of soybean has been hypothesized to result from genetic mechanisms contributing to de novo genetic variation. In this study we have detected allele switching by following segregation patterns of Aconitase-4 isozyme in sexual crosses and pure lines. In sexual crosses, one F_2 plant showed a switch at the *Aconitase-4* (*Aco4*) locus from the expected heterozygous genotype *Aco4-ac* to *Aco4-ab*. In the pure lines grown in a honeycomb planting design and treated with an accelerated aging test, multiple cases of allele switching were detected at the *Aco4* locus. Both single and double switches were detected that were stable and heritable. These findings indicate that the generation of endogenous variation continues in pure lines as a result of intrinsic genetic mechanisms. With a long-term goal of understanding the genetic nature of the changes, we genetically mapped the *Aco4* gene to a 3.3 cM region on Chromosome 11. The corresponding physical region is ~293 kb with 39 predicted genes. Of these, *Glyma.11g080600* is of particular interest, as it shows 93% and 88% identity to *Medicago truncatula* and *Arabidopsis* aconitase genes, respectively. Further characterization of the soybean *Aco4* gene may shed light on genetic mechanisms responsible for allele switching.

Key words: Allele switching, soybean, aconitase, linkage mapping, isozyme

Espinosa, K., Boelter, J., Lolle, S., Hopkins, M., Goggi, S., Palmer, R. G. et Sandhu, D. 2015. **Évaluation des variations alléliques spontanées chez le soja consécutivement à l'hybridation et au stress**. Can. J. Plant Sci. **95**: 405–415. On a formulé l'hypothèse que les variations intra-cultivar signalées chez les lignées pures de soja pourraient découler de mécanismes génétiques autorisant une variation génétique *de novo*. Les auteurs ont identifié la permutation d'allèles en suivant la ségrégation de l'isozyme aconitase-4 dans les croisements sexuels et les lignées pures. Dans les croisements sexuels, un plant de la F_2 présentait une permutation au locus *Aconitase-4* (*Aco4*) du génotype hétérozygote prévu *Aco4-ac* au génotype *Aco4-ab*. Dans les lignées pures cultivées en quinconce et soumises à un essai de vieillissement accéléré, les auteurs ont détecté de nombreux cas de permutation d'allèles au locus *Aco4*. Des permutations simples et doubles stables et transmissibles ont ainsi été discernées. Ces résultats indiquent que les variations endogènes se poursuivent dans les lignées pures en raison de mécanismes génétiques intrinsèques. Dans le but ultime d'élucider la nature génétique de tels changements, les auteurs ont situé le gène *Aco4* sur une région de 3,3 cM du chromosome 11. La zone physique correspondante mesure environ 293 kb et comprend 39 gènes explicatifs. Parmi eux, *Glyma.11g080600* revêt un intérêt particulier, car il ressemble à 93 % et à 88 % aux gènes de l'aconitase de *Medicago truncatula* et d'*Arabidopsis*, respectivement. Une caractérisation plus poussée du gène *Aco4* du soja pourrait nous éclairer sur les mécanismes génétiques à l'origine de la permutation des allèles.

Mots clés: Permutation d'allèles, soja, aconitase, cartographie des liaisons, isozyme

Intra-cultivar variation has been reported in most commercial cultivars for decades, and yet it is often ignored or attributed to outcrossing or contamination (Byth and Weber 1968). Nevertheless, increasing evidence of intra-cultivar variation within already established cultivars has been revealed when subjected to a wide range of conditions and factors (Durrant 1962; Byth and Weber 1968; Gordon and Byth 1972; Roth et al. 1989; Rasmusson and Phillips 1997; Fasoula and Boerma 2005, 2007; Haun et al. 2011). In maize (*Zea mays* L.) evidence of intra-cultivar variation was reported in doubled haploid lines and long-term inbred lines (Bogenschutz and Russell 1986; Russell et al. 1963). These inbred lines were shown to accumulate significant differences for several quantitative traits that exceeded commonly reported rates of spontaneous mutation. In flax, heritable changes were found in stable lines for plant height, plant weight, total amount of nuclear DNA (Evans et al. 1966), and isozyme band mobility for peroxidase and acid phosphatase (Cullis and Kolodynska 1975). Further

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Abbreviations: **RFLP**, restriction fragment length polymorphism; **SSR**, simple sequence repeat

studies demonstrated that in flax these changes were associated with non-random changes in the DNA sequence, and chromosomal rearrangements (Schneeberger and Cullis 1991; Chen et al. 2005, 2009).

Evidence of intra-cultivar variation has also been reported among lines of advanced generations following single-plant selection. For example, in cotton (*Gossypium hirsutum* L.), intra-cultivar variation for yield and Verticillium wilt tolerance was reported in an ultra-low planting, or Honeycomb design (Fasoula and Fasoula 1997, 2000). Similarly, for tomato (*Lycopersicon esculentum* L.), bread wheat (*Triticum aestivum* L.), and maize, single-plant selections of plants reared under conditions of ultra-low planting densities was effective at revealing intra-cultivar variation for per plant yield, seed protein content, carbon isotope discrimination, and ash content (Tokatlidis 2000; Christakis and Fasoulas 2002; Tokatlidis et al. 2004, 2005).

In soybean [*Glycine max* (L.) Merr.], the continued generation of intra-cultivar variation is particularly perplexing in light of genetic bottleneck events experienced during domestication that are proposed to have contributed to the reduction of genetic diversity and the loss of rare alleles (Hyten et al. 2006). Because of the loss of diversity during domestication the magnitude of genetic variation within homogeneous soybean gene pools is expected to be very limited. Despite these limitations, analyses of genetic gains in soybean across time for yield shows a tendency for continuous progress. For example, annual soybean yields have increased from 1924 to 2010 at a linear rate of 23.4 kg ha^{-1} (Wilson et al. 2014). This suggests that although soybean breeders normally use parents with improved agronomic traits, it is possible to continue achieving agronomic improvement through breeding.

Advances in DNA-based techniques have aided in understanding the possible ways genetic variation of individuals can be revealed at the genomic level. For example, restriction fragment length polymorphisms (RFLPs) were used to identify de novo variation in soybean-inbred lines subjected to tissue culture manipulations (Roth et al. 1989). In these lines, root tissue of single plants of 'Minsoy' and 'Noir 1' and from the root tissue of a F_1 hybrid plant between 'Minsoy' and 'Noir 1' showed novel RFLP alleles (Roth et al. 1989). These particular alleles were already characterized in other soybean cultivars but not in 'Minsoy' or 'Noir 1' from which the explants were obtained. These authors suggested that alleles could switch to other known alleles in response to stress such as in vitro propagation.

Significant intra-cultivar variation in commercial soybean cultivars, 'Benning', 'Haskell', and 'Cook' for seed protein, seed oil, fatty acids, seed weight and other agronomic traits (Fasoula and Boerma 2005, 2007) has been reported following single-plant selection from cultivars planted in a Honeycomb design. These selections resulted in the release of new true-breeding variants within each cultivar; five lines from cultivar 'Benning', six from cultivar 'Haskell', and seven from cultivar 'Cook' (Fasoula et al. 2007a, b, c). Using simple sequence repeat (SSR) markers it was determined that between 82 and 93% of the variation detected in the 'Benning', 'Haskell', and 'Cook' foundation seed could be traced to residual heterozygosity in the initial plant selections (Yates et al. 2012). However, 7 to 18% of the variation could not be explained by residual heterozygosity and was attributed to de novo variation within the three cultivars.

In addition to DNA-based molecular markers several different isozymes have classically been used as markers in molecular studies in plants. Like DNA-based markers isozyme markers are inherited in Mendelian fashion, expressed co-dominantly and can be used to provide individual profiles and be understood in genetic terms. Aconitase isozymes have been used to study cultivated soybean and related wild species and to elucidate the diversity and genetic structure of soybean populations (Griffin and Palmer 1987; Hirata et al. 1999). Aconitase isozymes catalyze the interconversion of the three tricarboxylic acids: citrate, *cis*-aconitate, and isocitrate in the Krebs cycle and are expressed constitutively at low levels (Peyret et al. 1995). Expression, however, is greatly increased during seed germination and maturation making aconitase isozyme markers a good system to be analyzed in the seed (Peyret et al. 1995). Furthermore, aconitase isozyme assays generally do not compromise seedling viability, are relatively robust and simple to perform.

Five aconitase genes have been identified in soybean: *Aco1*, *Aco2*, *Aco3*, *Aco4*, and *Aco5* (Doong and Kiang 1987; Rennie et al. 1987; Kiang and Bult 1991) that are all known to assort independently (Rennie et al. 1987). The *Aco3* locus has been mapped to chromosome 6 (Griffin and Palmer 1987), while map locations for the other *Aco* loci are not known. In this investigation we have used Aconitase-4 isozyme allele variants to monitor allele switching.

The primary objective of this study was to evaluate the spontaneous generation of de novo allelic variants in soybean sexual crosses and in seeds of inbred lines treated with an accelerating aging test to induce abiotic stress. Progeny of sexual crosses between soybean plant introductions 'BSR 101', 'Minsoy', and 'Noir 1' were evaluated through the examination of segregation patterns for Aconitase-4 isozyme variants while 'BSR 101' and 'Jack' were evaluated for the stable inheritance of Aconitase-4 isozyme alleles following an accelerated aging test. We also mapped the *Aconitase-4* (*Aco4*) locus to a small physical region on soybean chromosome 11 containing 39 predicted genes. Future gene isolation and characterization of the *Aco4* locus will aid in understanding the molecular basis of allele switching.

MATERIALS AND METHODS

Plant Material and Seed Source

Soybean cultivar 'BSR 101' (PI 548519), cultivar 'Jack' (PI 540556), and plant introductions 'Minsoy' (PI 27890) (introduced from France), and 'Noir 1' (PI 290136) (introduced from Hungary) were used in this study. Seed for the sexual hybridization experiment was obtained from Dr. R. L. Nelson, USDA ARS at Urbana, IL. 'BSR 101' seed for the stress treatment experiment was obtained from Dr. R. G. Palmer, Iowa State University at Ames, IA, and 'Jack' seed was obtained from Dr. R. C. Shoemaker, USDA ARS at Ames, IA.

Sexual Hybridization

Soybean 'Minsoy', 'Noir 1' and 'BSR 101' were used to make the following cross-pollinations in 2007 at the Bruner Farm near Ames, Iowa: 'Noir 1' × 'BSR 101', 'Minsoy' × 'Noir 1', and 'Minsoy' × 'BSR 101'.

The F_1 seed was planted at the University of Puerto Rico – Iowa State University Station near Isabela, Puerto Rico, in October 2007. The F_1 plants were single-plant threshed and 24–32 F_2 seeds from each F_1 plant from the three cross combinations were planted in Puerto Rico in February 2008. All F_2 plants were single-plant threshed. The remnant F_2 seed and the $F_{2:3}$ seed were sent to Iowa State University in May 2008.

Five hundred seeds of 'BSR 101', 'Minsoy' and 'Noir 1' were analyzed for Aconitase-4 and the seedlings transplanted at the Bruner Farm. Fifty seeds from each of 10 plants from each of the three soybean parental genotypes were selected.

F_2 seed from self-pollinated F_1 plants of the three cross-combinations from the October 2007 Puerto Rico planting were analyzed for Aconitase-4 isozyme variation and seedlings were transplanted at the Bruner Farm. The seeds used for this experiment included 50 F_2 seeds from 18 F_1 plants representing 10 different 'Noir 1' female parent plants crossed to 'BSR 101'; 50 F_2 seeds from 18 F_1 plants representing 9 different 'Minsoy' female parent plants crossed to 'BSR 101' and 50 F_2 seeds from 19 F_1 plants representing eight different 'Minsoy' female parent plants crossed to 'Noir 1'.

$F_{2:3}$ seeds from self-pollinated F_2 plants of the three cross-combinations from the February 2008 Puerto Rico planting were analyzed for Aconitase-4 isozyme variation and seedlings were transplanted at the Bruner Farm. Two seeds from each $F_{2:3}$ plant from all three cross-combinations were selected. The seeds used for this experiment included 352 $F_{2:3}$ seeds from 176 F_2 plants from the February 2008 plots 6, 16, 17, 24, 25, and 27, representing 'Noir 1' as female parent plant crossed to 'BSR 101'. Also included were 942 $F_{2:3}$ seeds from 471 F_2 plants from the February 2008 plots 29, 30, 31, 32, 33, 36, 37, 45, 46, 47, 48, 51, 52, 53, 54, 55, 56, 59, and 60 representing 'Minsoy' as female parent plant crossed to 'BSR 101'. Additionally, 308 $F_{2:3}$ seeds were included from 154 F_2 plants from the February 2008 plots 63, 64, 65, 66, 69, 70, 75, and 76 representing 'Minsoy' as female parent plant crossed to 'Noir 1'.

Pure Lines; Accelerated Aging Seed Stress Treatment

Soybean cultivars 'BSR 101' and 'Jack' were used to study the effect of seed stress in pure lines. In 2008, 500 seeds of 'BSR 101' and 'Jack' were planted in a honeycomb design (Fasoula and Fasoula 1997, 2005), using an equidistant spacing of 2.0 m between each individual plant to eliminate the unfavorable effect of competition in response to selection (Fasoula and Boerma 2005). A code, termed "entry number", was assigned to each individual plant. Field plots were damaged by a natural hail storm in July 2008 reducing yield in most entries. For this reason, the number of harvested plants was reduced to 315 plants for 'BSR 101' and 305 plants for 'Jack'. At harvest, plants were single-plant threshed and analyzed for Aconitase-4 isozyme.

Seeds from 64 selected entries of 'BSR 101' and 'Jack', from the 2008 honeycomb harvest were stressed using a modified version of the accelerated aging test (Association of Official Seed Analysts 2002). In the 2009 growing season, 50 seeds of each entry were exposed to $\sim$100% relative humidity and partially hydrated seeds maintained at 41°C for 48 h, and hand-planted at the Bruner Farm near Ames, IA, in a completely randomized design with two replications. At harvest, a single three-seeded pod was collected randomly from three separate plants per entry per replication and analyzed for the Aconitase-4 isozyme.

Aconitase Isozyme Analysis

Starch gel electrophoresis was used to evaluate isozyme patterns at the *Aco4* locus for the two plant introductions and the two cultivars (Cardy and Beversdorf 1984). The F_2 and $F_{2:3}$ progenies from the three different cross-combinations, and the self-pollinated progenies of seed treated with an accelerated aging test were used for Aconitase-4 assays.

Seeds were germinated on germination paper for 72 h at 30°C in the dark. The 3-d-old seedlings were sampled by punching out three pieces of the cotyledon using a 200-μL glass-bore pipettor. The samples were placed in 1.5 mL polypropylene microcentrifuge tubes to which 120 μL cold extraction buffer [0.1 M tris-HCl, pH 7.2, 4% (wt/vopl) PVP-40 (polyvinylpyrrolidone, molecular weight 40 000), 400 mM sucrose, 1 mM dithiothreitol] was added. Samples were ground for 30 s by using a laboratory stirring motor (TRI-R STIR-R, Model S63C, Chicago, IL) fitted with a pointed acrylic rod that fit loosely in the microcentrifuge tubes. The samples were placed in a refrigerated microcentrifuge (Eppendorf 5417C, Hamburg, Germany) and centrifuged at 10 000 × *g* for 3 min. The supernatant was loaded directly onto

starch gels by first absorbing the supernatant onto 2.4 × 10 mm wicks punched from Whatman no. 2 filter paper.

Aconitase isozymes were resolved on 13% starch gels with the "D" buffer system (Cardy and Beversdorf 1984). Electrophoresis was carried out at 9.5 W 500 mL^{-1} gel for 5.5 h, or until a bromophenol-blue dye marking the front had migrated 100 mm. After electrophoresis, gels were sliced horizontally into pieces 1.5 mm thick to allow analysis of several isozymes from one gel.

Aconitase activity [aconitate hydratase, enzyme commission (EC) 4.2.1.3] was visualized by incubating gel slices at 37°C in a solution of 100 mL 0.2 M tris-HCl (pH 8.0), 200 mg cis-aconitic acid, 40 units isocitrate dehydrogenase, 100 mg $MgCl_2$, 20 mg β-nicotinamide adenine dinucleotide phosphate, 20 mg methyl thiazolyl tetrazolium bromide, and 4 mg phenazine methosulfate.

Gel slices were incubated at 38°C for 60 to 90 min in the stain solution at room temperature. Each gel was screened to determine if there were any deviations from the expected isozyme patterns.

Genetic Analysis of Aconitase Variants

After isozyme analysis, seedlings of progeny that expressed variants in the isozyme pattern were saved and transplanted into pots containing a standard greenhouse soil mix (2 soil: 1 sand: 1 peat). These seedlings were maintained in the USDA–ARS greenhouse (Ames, IA) where they were allowed to self-pollinate. At harvest, each plant was hand-threshed.

The mode of inheritance of the aconitase variants was determined by the genotype segregation of self-pollination of the variant plants. Cotyledon samples were analyzed electrophoretically to determine the Aconitase-4 genotype (homozygous or heterozygous) and to estimate the segregation ratio.

Two Aconitase-4 variants were observed. The F_2 plant (A08-AS-2932) from the cross of 'Minsoy' (*Aco4-cc*) × 'BSR101' (*Aco4-aa*) was *Aco4-ab*, not the expected *Aco4-ac* genotype. The second variant was 'Jack' entry 127, grown in a honeycomb planting design and treated with an accelerated aging test. The 'Jack' 127 variant was *Aco4-bc*, not expected *Aco4-cc* genotype.

To estimate stable inheritance of the new alleles, homozygous Aconitase-4 variants were allowed to self-pollinate, and progeny seed from each plant was analyzed for isozyme pattern. In the allelism test, crosses were made between homozygous plants for Aconitase-4 variant and a standard Aconitase-4 genotype.

Aco4, Molecular Mapping; DNA Isolation and Bulked Segregant Analysis

For the genetic linkage mapping, an F_2 population of 94 plants, generated by crossing parent plants 'BSR 101' (*Aco4-aa*) and 'Noir 1' (*Aco4-bb*) was used. Genomic DNA was isolated according to the method described previously (Sandhu et al. 2004). Bulks were created with *aa* or *bb* allele types by taking 1 μg DNA from 10 homozygous *Aco-aa* or 10 homozygous *Aco-bb* F_2 plants (Michelmore et al. 1991). Both bulks were diluted to 50 ng μL^{-1} final DNA concentration. Seven hundred SSR markers were tested on both bulks to detect polymorphisms between the bulks.

Aco4, Molecular Mapping; Molecular Marker Analysis

For the SSR analysis, 50 ng of DNA was used for a 10-μL reaction with 1 × reaction buffer (10 mM tris-HCl, 50 mM KCl, pH 8.3), 2.0 mM $MgCl_2$, 0.25 μM of each primer, 200 μM of each dNTP, and 0.25 units of Biolase DNA polymerase (Bioline USA, Inc., Tauton, MA). PCR was completed with one cycle at 94°C for 3 min, followed by 11 cycles of 94°C for 30 s, 58°C for 30 s with an increment of −1°C per cycle and 72°C for 1 min, 35 cycles of 94°C for 30 s, 46°C for 30 s, and 72°C for 1 min, with a final cycle of 72°C for 10 min. The PCR products were resolved on a 4% agarose gel at 150 V for 2-4 h. The genetic linkages and distances were determined using Mapmaker 2.0 (Kosambi 1944; Lander et al. 1987). The order of the markers was determined at LOD threshold of 3.0. Markers were developed using information from http://soybase.org/resources/ssr.php and http://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Gmax (Song et al. 2004, 2010).

RESULTS

Effect of Sexual Hybridization

To establish a baseline in the absence of sexual hybridization, approximately 500 transplants of 'BSR 101', 'Minsoy', and 'Noir 1' were analyzed for aconitase isozyme variation and were transplanted at the Bruner Farm. At the *Aco4* locus all individuals of 'BSR 101' were *Aco4-aa*, 'Minsoy' were *Aco4-cc*, and 'Noir 1' were *Aco4-bb*, as expected.

A total of 2204 F_2 progeny from the three different sexual crosses were assayed for Aconitase-4 (Table 1). This includes a total of 789 F_2 plants derived from 'Noir 1' × 'BSR 101', 650 F_2 plants from 'Minsoy' × 'Noir 1' and 765 F_2 plants from 'Minsoy' × 'BSR 101'. The F_2 genotypes fit the expected 1:2:1 genotypic ratio (Table 1), with one exception. One F_2 plant, from 'Minsoy' × 'BSR 101', A08-AS-2932, was *Aco4-ab*, an unexpected genotype (Fig. 1). The F_2 homozygous genotypes were true breeding and the F_2 heterozygous genotypes segregated 1:2:1, as expected (data not shown).

Aconitase-4 Variant

The F_2 plant A08-AS-2932 that scored heterozygous for Aconitase-4 (*Aco4-ab*) originated from the cross 'Minsoy' *Aco4-cc* (A07-61-32) × 'BSR 101' *Aco4-aa* (A07-63). The *Aco4-ac* heterozygous genotype was expected, but an *Aco4-ab* genotype was observed where the '*b*' allele was unexpected. Fifty self-pollinated seeds of the female parent 'Minsoy' plant 32 (A07-61-32) were analyzed for aconitase isozyme variation. All seeds were *Aco4-cc,* as expected.

Table 1. Genotypic frequency of progeny of self-pollination of F_1 plants (*Aco4-ab*, *Aco4-bc*, and *Aco4-ac*) assayed for Aconitase-4

Cross	F_1 genotype	No. F_1 plants	No. plants and aconitase-4 profiles			No. F_2 plants	$\chi^2_{(1:2:1)}$	*P*
Aco4-bb × *Aco4-aa* 'Noir 1' × 'BSR 101'	*Aco4-ab*	18	*Aco4-aa* 215	*Aco4-ab* 373	*Aco4-bb* 201	789	2.83	0.24
Aco4-cc × *Aco4-bb* 'Minsoy' × 'Noir 1'	*Aco4-bc*	19	*Aco4-bb* 154	*Aco4bc* 328	*Aco4-cc* 168	650	0.66	0.72
Aco4-cc × *Aco4-aa* 'Minsoy' × 'BSR 101'	*Aco4-ac*	18	*Aco4-aa* 201	*Aco4-ac* 378	*Aco4-cc* 186	765	0.68	0.71

P = probability of a greater value of chi-square.

The 49 sibling F_2 plants (A08-AS-2901 to A08-AS-2950; minus A08-AS-2932) from the October 2007 Puerto Rico planting did not show the Aconitase-4 *'b'* allele variant. A sibling F_1 plant that produced 50 F_2 plants (A08-AS-2951 to A08-AS-3000) similarly did not show the Aconitase-4 *'b'* allele variant (Fig. 1).

Stability Test

As shown in Table 2, self-pollination of A08-AS-2932, the plant with the novel *Aco4-ab* variant gave rise to progeny that segregated a good fit to the expected 1:2:1 ratio (17 *Aco4-aa*: 45 *Aco4-ab*: 16 *Aco4-bb* plants). Three of the 16 plants that scored as homozygous for the Aconitase-4 '*b*' allele (*Aco4-bb*), A11-AS-185, A11-AS-191, and A11-AS-196 were used for additional aconitase determinations. Plants A11-AS-185, A11-AS-191, and A11-AS-196 were self-fertilized and 100, 103, and 99 seedlings, respectively, were analyzed for aconitase enzyme variations. These 302 F_3 plants were *Aco4-bb,* as expected.

Allelism Tests

Thirty-nine of the 302 F_3 plants (*Aco4-bb*) were used for allelism tests. A total of 221 testcross seeds from *Aco4-bb* ('Noir 1') × *Aco4-bb* genotypes were analyzed for aconitase isozyme variations and transplanted at the Bruner Farm. All 221 seed were *Aco4-bb*, the expected genotype.

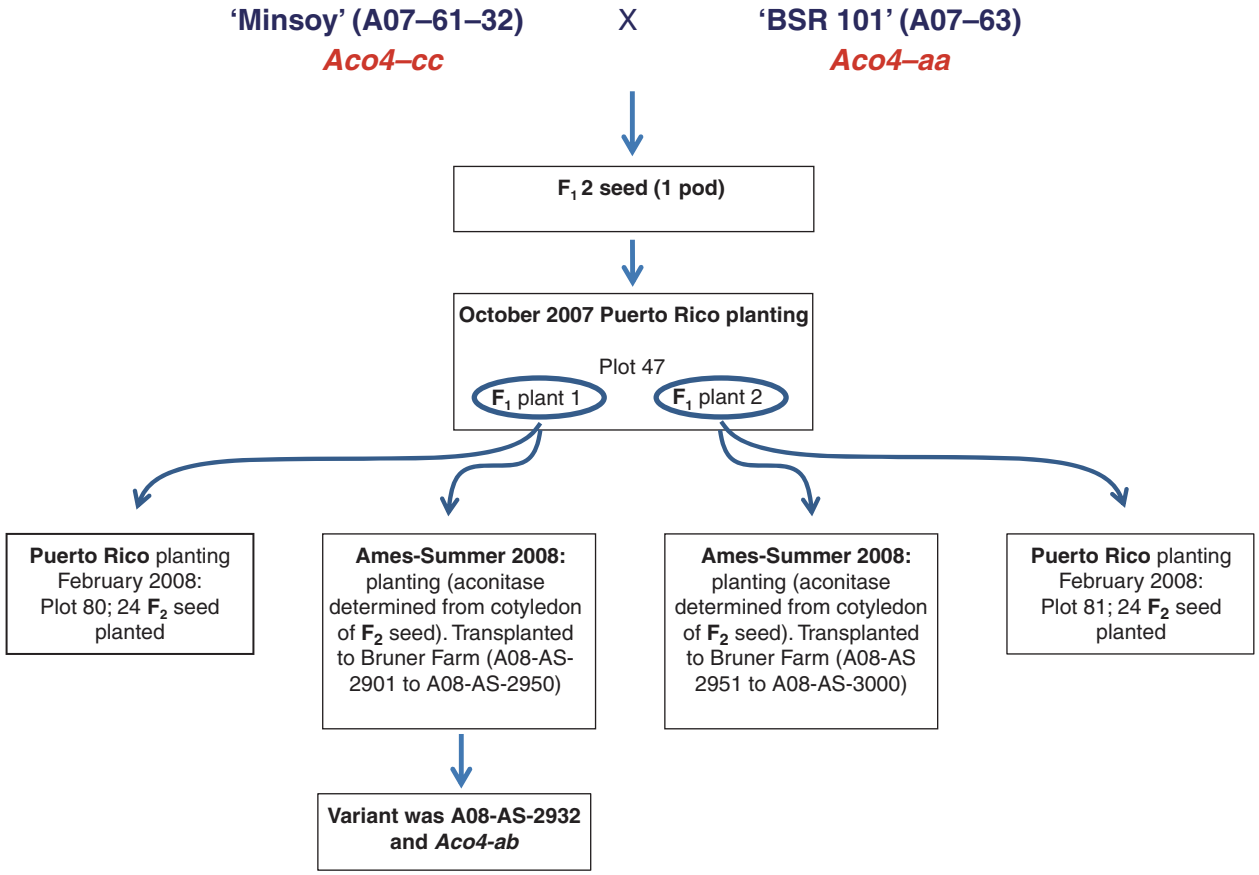

Fig. 1. Graphic representation of the history of the F_2 Aconitase-4 variant plant A08-AS-2932 (*Aco4-ab*) from a cross between 'Minsoy' (*Aco4-cc*) x 'BSR 101' (*Aco4-aa*).

Table 2. Genotypic frequency of progeny from self-pollination of Aconitase-4 F_2 variant plant A08-AS-2932 (*Aco4-ab*) derived from a cross between 'Minsoy' (*Aco4-cc*) × 'BSR 101' (*Aco4-aa*)

Aconitase-4 profiles	No. plants
aa	17
ab	45
bb	16
$\chi^2_{(1:2:1)}$	1.87
P	0.4

P = probability of a greater value of chi-square.

Effect of Seed Stress in Pure Lines

Samples of single plants of 'BSR 101' and 'Jack' harvested from the honeycomb 2008 experiment were analyzed for aconitase isozyme variations. At the *Aco4* locus 'BSR 101' was *Aco4-aa*, and 'Jack' was *Aco4-cc*.

Single three-seeded pods from progeny of 'BSR 101' and 'Jack', grown in a honeycomb planting design and treated with an accelerated aging test, were collected from three separate plants per entry per replication. A total of 1152 seeds were analyzed separately per cultivar for Aconitase-4. Acontiase-4 variants were identified in seeds from single pods collected from individual plants for ten entries of 'BSR 101' and four entries of 'Jack' and were characterized either by single allele switches, the change of one allele, or double allele switches, the change of both alleles for the Aconitase-4 isozyme (Table 3). In 'BSR 101', the expected isozyme pattern had single allele switches from *Aco4-aa* to *Aco4-ab*, however in 'Jack' single allele switches were from *Aco4-cc* to *Aco4-bc*. Double allele switches from the expected isozyme pattern only occurred in two 'BSR 101' entries. Both of these double switches were from *Aco4-aa* to *Aco4-bb* (Table 3).

Aconitase-4 Variant; Stability Test

As shown in Table 4, self-pollination of the novel *Aco4-bc* variant ('Jack' entry 127) gave rise to progeny that segregated a good fit to the expected ratio (12 *Aco4-bb*: 24 *Aco4-bc*: 14 *Aco4-cc* plants). Four of the 12 plants that scored as homozygous for the Aconitase-4 '*b*' allele (*Aco4-bb*), A12-AS-8, A12-AS-11, A12-AS-13, and A12-AS-41, were used for additional aconitase determinations. Plants A12-AS-8, A12-AS-11, A12-AS-13, and A12-AS-41 were self-fertilized and 21, 25, 22, and 21 seedlings, respectively, were analyzed for aconitase enzyme variations. All 89 F_3 plants were *Aco4-bb* as expected.

Allelism Tests

Four 'Jack' entry 127 *Aco4-bb* plants were crossed to 'Noir1' (*Aco4-bb*); the standard *Aco4-bb* genotype. The number of testcross seeds using four variant *Aco4-bb* plants varied from 7 to 15, for a total of 43 testcross seeds. All the seeds showed the expected *Aco4-bb* genotype.

Table 3. Summary of Aconitase-4 variants found in individual seeds from single three-seeded pods harvested from 'BSR 101' and 'Jack' plants

Entries with Aconitase-4 variant[z]	Expected Aconitase-4 pattern[y]	Aconitase-4 Variant[x]
BSR 101-4-1	*aa*	*ab*
BSR 101-34-3	*aa*	*ab*
BSR 101-34-3	***aa***	***bb***
BSR 101-46-1	***aa***	***bb***
BSR 101-78-1	*aa*	*ab*
BSR 101-160-3	*aa*	*ab*
BSR 101-160-3	*aa*	*ab*
BSR 101-160-3	*aa*	*ab*
BSR 101-190-3	*aa*	*ab*
BSR 101-213-1	*aa*	*ab*
Jack – 28-2	*cc*	*bc*
Jack – 28-2	*cc*	*bc*
Jack – 77-2	*cc*	*bc*
Jack – 127-1	*cc*	*bc*

[z]Entries with Aconitase-4 variant labeled in the following format: cultivar name – entry number – pod number. Three, three-seeded pods were collected from each entry.
[y]Expected Aconitase-4 pattern for any of the seeds within the pod that do not have allele switches. This is the same pattern that appears in the original parental seed source.
[x]Aconitase-4 variant represents the new isozyme pattern for each seed with single or double-allele switches.

Genetic Linkage Mapping; The *Aco4* Gene

With an objective of developing a better understanding of genetic mechanisms leading to allele switching, we mapped the *Aco4* gene. To find the location of the *Aco4* gene, 700 SSR markers covering the entire soybean genome were tested on the bulks. Satt509 showed polymorphism between the bulks, indicating the candidate gene was on chromosome 11, MLG B1 (Song et al. 2004). Eighty-nine SSR markers on MLG B1 near Satt509 were tested for polymorphism between the parents. Of these, 12 showed polymorphism: BARCSOYSSR_11_001, BARCSOYSSR_11_008, BARCSOYSSR_11_030, BARCSOYSSR_11_056, BARCSOYSSR_11_316, BARCSOYSSR_11_323, BARCSOYSSR_11_336, BARCSOYSSR_11_338, BARCSOYSSR_11_339, BARCSOYSSR_11_345, Sat_272, and Satt509. The 12 polymorphic SSR markers were tested on the entire population. The putative *Aco4* gene is flanked by BARCSOYSSR_11_323 and BARCSOYSSR_11_336. Marker BARCSOYSSR_11_336 was 1.4 cM away from the gene

Table 4. Genotypic frequency of progeny from self-pollination of Aconitase-4 variant plant 'Jack' entry 127 (*Aco4-bc*)

Aconitase-4 profile	No. plants
bb	12
bc	24
cc	14
$\chi^2_{(1:2:1)}$	0.08
P	1

P = probability of a greater value of chi-square.

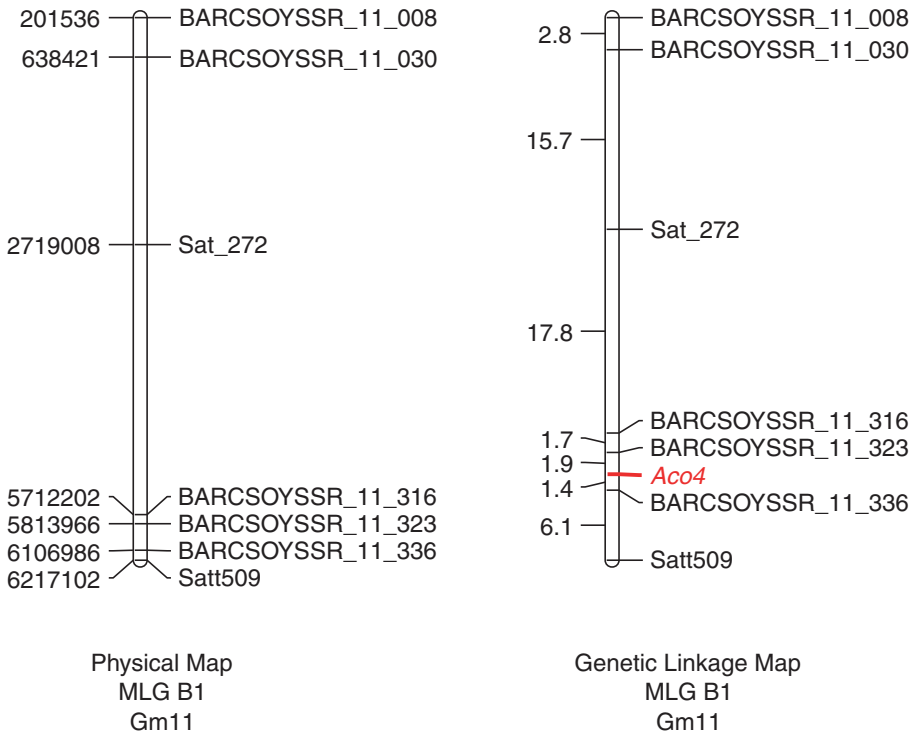

Fig. 2. Genetic linkage map of the *Aco4* gene from the cross 'BSR 101' × 'Noir 1'. Genetic and physical maps of soybean chromosome Gm11 (MLG B1) showing location of the *Aco4* gene. Genetic distances are shown in centiMorgans (cM) and physical distances are shown in base pairs (bp).

(Fig. 2). Using the soybean genome sequence (http://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Gmax) (Schmutz et al. 2010), a physical map of the markers in the vicinity of the *Aco4* gene was created. The region between BARCSOYSSR_11_323 and BARCSOYSSR_11_336 is about 293 kb. We were able to use the soybean genome sequence flanked by these markers to locate putative genes present in the region. There are 39 predicted genes in this region (Table 5) with one candidate gene sharing partial sequence similarity with an aconitase (*Glyma.11g080600*).

DISCUSSION

Soybean cultivars are maintained as inbred lines that theoretically contain highly homozygous individuals. Nevertheless, phenotypic and genotypic variants have been identified and characterized agronomically and molecularly (Roth et al. 1989; Fasoula and Boerma 2005, 2007; Haun et al. 2011; Yates et al. 2012). Early molecular evidence for genetic variation, or allele switching, was shown in soybean where a significant number of RFLP markers in suspension cultures prepared from root tissue of 'Minsoy' and 'Noir 1' deviated from the expected profiles (Roth et al. 1989). Interestingly, most of the newly generated RFLP alleles were the same as ones previously characterized in other soybean cultivars or plant introductions. The study suggested that inbreeding organisms such as soybean may have evolved internal generators of genetic variation in response to stress or other environmental stimuli (Roth et al. 1989).

In this study, evidence of allele switching was obtained by following segregation patterns of the Aconitase-4 isozyme in both sexual hybridization and pure line experiments. Among the 765 F_2 plants from the cross-pollination between 'Minsoy' (*Aco4-cc*) × 'BSR 101' (*Aco4-aa*), one individual had switched from the expected heterozygous genotype *Aco4-ac* to *Aco4-ab* with the unexpected '*b*' allele (Table 2). This switch cannot be explained based on cross-pollination or contamination as the switch happened in the gamete from the female parent 'Minsoy'. In the pure-line experiment, where progenitors were grown in a honeycomb planting design and treated with an accelerated aging test progeny plants showed genetic variation within 'BSR 101' and 'Jack' cultivars. Aconitase variants were identified in 10 out of 1152 seeds for 'BSR 101' and 4 out of 1152 for 'Jack'. The genetic test for these Aconitase-4 variants, in both the sexual hybridization and pure-line experiments, indicated that these variants were inherited as a single-gene.

Similar evidence of new soybean isozyme variants has previously been reported and attributed to genomic stress induced by tissue culture manipulations (Amberger et al. 1992a). In these studies the frequency of isozyme

Table 5. Genes present in the *Aco4* region. Name and predicted functions of the putative proteins encoded by 39 genes that are flanked by BARCSOYSSR_11_323 and BARCSOYSSR_11_336 on Gm11 (MLG B1) are shown. The gene of interest is shown in bold

Gene	Start position	End position	Protein function
Glyma.11g077500	5823379	5825992	Armadillo/beta-catenin-like repeat
Glyma.11g077600	5838011	5841370	Oxidoreductase NAD-binding domain; Oxidoreductase FAD-binding domain
Glyma.11g07700	5845936	5847989	Thaumatin family
Glyma.11g077800	5859113	5861305	Thaumatin family
Glyma.11g077900	5867279	5869126	Rhodanese-related sulfurtransferase
Glyma.11g078000	5869398	5874647	Mlo family; cell death; integral to membrane
Glyma.11g078100	5877078	5883528	Ubiquitin interaction motif; 26S proteasome non-ATPase regulatory subunit 4
Glyma.11g078200	5885675	5891247	Senescence-associated protein
Glyma.11g078300	5897790	5907341	Histidine kinase-, DNA gyrase B, and HSP90-like ATPase; Response regulator receiver domain; regulation of transcription, DNA-dependent
Glyma.11g078400	5909639	5912304	Peroxidase; oxidation reduction; heme binding
Glyma.11g078500	5913292	5917166	Protein of unknown function
Glyma.11g078600	5919282	5922385	None
Glyma.11g078700	5924116	5927990	3-dehydroquinate synthase
Glyma.11g078800	5930818	5933005	PPR repeat
Glyma.11g078900	5932586	5935392	None
Glyma.11g079000	5937276	5941473	Isocitrate/isopropylmalate dehydrogenase
Glyma.11g079100	5942881	5945544	Ras family; Rho type; GTP binding
Glyma.11g079200	5446944	5955940	Vacuolar protein sorting-associated protein 35; Membrane coat complex Retromer
Glyma.11g079300	5960613	5960978	Copper chaperone; metal ion transport. Metal ion binding
Glyma.11g079400	5968757	5971655	GDSL-like Lipase/Acylhydrolase; zinc finger fyve domain containing protein
Glyma.11g079500	5979267	5985576	PLAC8 family
Glyma.11g079600	5987638	5989759	tetramerisation domain; SCF ubiquitin ligase
Glyma.11g079700	5991289	5992851	PPR repeat
Glyma.11g079800	5995314	5996613	Trm112p-like protein
Glyma.11g079900	5999169	6000451	Zinc finger, C3HC4 type (ring finger)
Glyma.11g080000	6005401	6006947	None
Glyma.11g080100	6018320	6023418	Lupus la ribonucleoprotein; RNA-binding protein LARP/SRO9
Glyma.11g080200	6027758	6029576	Putative methyltransferase
Glyma.11g080300	6030076	6031938	Peroxidase; heme binding
Glyma.11g080400	6036330	6040326	Eukaryotic aspartyl protease; proteolysis and peptidolysis
Glyma.11g080500	6045490	6048516	Ring finger protein 11
Glyma.11g080600	**6058457**	**6066818**	**Aconitase C-terminal domain; 3-isopropylmalate dehydrase subunit; RNA-binding translational regulator IRP**
Glyma.11g080700	6068106	6071401	Prolyl 4-hydroxylase alpha subunit; oxidoreductase activity
Glyma.11g080800	6079536	6081820	None
Glyma.11g080900	6083715	6086922	Peroxidase; heme binding
Glyma.11g081000	6087915	6088796	None
Glyma.11g081100	6090248	6091010	NADH-Ubiquinone/plastoquinone (complex 1); NADH Dehydrogenase; ATP synthesis coupled electron transport
Glyma.11g081200	6094537	6103135	RNA polymerase Rpb1
Glyma.11g081300	6104383	6105246	None

variants for Aconitase-2 in somaclonal mutants was 2 out of 185 regenerated plants and the variants were heritable (Amberger et al. 1992b). Unlike the isozyme analyses described in the previous studies, in this study, tissue samples were taken from the cotyledons of seedlings grown from individual seeds within single pods from each entry. This approach not only revealed that the genomic variation existed within plants in a cultivar, but also that variation was expressed within seed from individual pods collected from the same individual plant. In the pure-line experiment, Aconitase-4 variants were detected in a single seed within a three-seeded pod. Furthermore, the appearance of plants showing double switches (BSR 101-34-3 and BSR 101-46-1) completely rules out pollen contamination or outcrossing as the source of these genetic variants (Table 3).

In our study, the frequency with which changes were observed differs between the sexual hybridization experiment (1 out of 765 F_2 plants) and the pure line experiment (10/1152 and 4/1152 for 'BSR 101' and 'Jack', respectively) with fewer detectable events occurring in hybrid lines (Table 2 and Table 3). A similarly low frequency of changes was observed in cell cultures from the roots of hybrid soybean plants as compared with cells from homozygous soybean plants. Although a number of factors could account for these differences, it is also possible that heterozygosity may inhibit the mechanisms driving this variation (Roth et al. 1989).

Although the results presented here indicate that in the pure-line experiment there was an effect on the generation of endogenous variation in both cultivars, the confounding effect of the natural hail that occurred in July 2008 needs to be separated from the effect of the accelerated aging test as well as the honeycomb planting design before a direct causal link can be made. The initial seed source used in this experiment was harvested from plants grown in a wide-spaced or honeycomb design. This design is thought to maximize phenotypic expression by minimizing competition between plants and thus allow even limited genetic variability to be identified (Fasoula and Fasoula 1997). This hypothesis has been supported by further studies in which variation in agronomic traits enabled the selection for new cultivars (Fasoula et al. 2007a, b, c).

The use of high temperatures and high relative humidity has been reported to cause seed stress (Parrish and Leopold 1978; Hsu et al. 2003) resulting in lower germination rates, emergence and in promoting the formation of free radicals (Hsu et al. 2003). In this study, the accelerated aging test was used on progeny seed of single plants that were grown in a honeycomb design in 2008, but these same plants were also affected by a natural-hail storm. In a related study, the effect of seed stress on agronomic traits such as plant height, plant maturity, and yield was studied (Rolling 2012). 'Jack' showed a decrease of 8% in yield for plants grown from the stressed seeds.

The combination of classical genetic analyses and molecular approaches has considerably impacted the process of tracking genetic changes and may even help to decode contributing mechanisms for newly developed genetic diversity. Our results indicate intra-cultivar variation is continuously generated and is heritable. Although the genetic mechanisms driving de novo variation remain unclear, in some previous studies researchers have proposed that processes such as spontaneous mutations, DNA transposition, DNA methylation, gene duplication, unequal crossing over, gene conversion, and genome restoration might be contributing to this de novo variation (Sprague et al. 1960; Fukui 1983; Rasmusson and Phillips 1997; Lolle et al. 2005; Morgante et al. 2005; Haun et al. 2011; Hopkins et al. 2013; Kempinski et al. 2013).

Characterization of the *Aco4* gene may help us understand the genetic basis of the changes happening during allele switching. We genetically mapped the *Aco4* gene using SSR markers (Fig. 2). The 293 kb region was flanked by the BARCSOYSSR_11_323 and BARC SOYSSR_11_336 markers on chromosome 11 (MLG B1). Using this information, we located 39 predicted genes in this region (Table 5; http://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Gmax). Of these, one gene (*Glyma.11g080600*) was of particular interest as this showed 93% identity to *Medicago truncatula* aconitase (Young et al. 2011) and 88% identity to the *Arabidopsis* Aconitase 1 at protein level (Peyret et al. 1995). Future studies focusing on characterization of the candidate gene may result in cloning of the *Aco4* gene and may shed light on genetic mechanisms involved in allele switching at the *Aco4* locus.

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Amberger, L. A., Palmer, R. G. and Shoemaker, R. C. 1992a. Analysis of culture-induced variation in soybean. Crop Sci. **32**: 1103–1108.

Amberger, L. A., Shoemaker, R. C. and Palmer, R. G. 1992b. Inheritance of 2 independent isozyme variants in soybean plants derived from tissue-culture. Theor. Appl. Genet. **84**: 600–607.

Association of Official Seed Analysts. 2002. Seed vigor testing handbook. Association of Official Seed Analysts, Lincoln, NE (Contribution 32).

Bogenschutz, T. G. and Russell, W. A. 1986. An evaluation for genetic variation within maize inbred lines maintained by sib-mating and self-pollination. Euphytica **35**: 403–412.

Byth, D. and Weber, C. 1968. Effects of genetic heterogeneity within two soybean populations I. Variability within environments and stability across environments. Crop Sci. **8**: 44–47.

Cardy, B. J. and Beversdorf, W. D. 1984. Identification of soybean cultivars using isoenzyme electrophoresis. Seed Sci. Technol. **12**: 943–954.

Chen, Y., Lowenfeld, R. and Cullis, C. A. 2009. An environmentally induced adaptive (?) insertion event in flax. Int. J. Genet. Mol. Biol. **3**: 38–47.

Chen, Y., Schneeberger, R. G. and Cullis, C. A. 2005. A site-specific insertion sequence in flax genotrophs induced by environment. New Phytol. **167**: 171–80.

Christakis, P. A. and Fasoulas, A. C. 2002. The effects of the genotype by environmental interaction on the fixation of heterosis in tomato. J. Agric. Sci. **139**: 55–60.

Cullis, C. A. and Kolodynska, K. 1975. Variation in the isozymes of flax (*Linum usitatissimum*) genotrophs. Biochem. Genet. **13**: 687–697.

Doong, J. Y. H. and Kiang, Y. T. 1987. Inheritance of aconitase isozymes in soybean. Genome **29**: 713–717.

Durrant, A. 1962. The environmental induction of heritable change in *Linum*. Heredity **17**: 27–61.

Evans, G. M., Durrant, A. and Rees, H. 1966. Associated nuclear changes in the induction of flax genotrophs. Nature **212**: 697–699.

Fasoula, V. A. and Boerma, H. R. 2005. Divergent selection at ultra-low plant density for seed protein and oil content within soybean cultivars. Field Crops Res. **91**: 217–229.

Fasoula, V. A. and Boerma, H. R. 2007. Intra-cultivar variation for seed weight and other agronomic traits within three elite soybean cultivars. Crop Sci. **47**: 367–373.

Fasoula, D. A. and Fasoula, V. A. 1997. Competitive ability and plant breeding. Plant Breed. Rev. **14**: 89–138.

Fasoula, V. A. and Fasoula, D. A. 2000. Honeycomb breeding: principles and applications. Plant Breed. Rev. **18**: 177–250.

Fasoula, V. A., Boerma, H. R., Yates, J. L., Walker, D. R., Finnerty, S. L., Rowan, G. B. and Wood, E. D. 2007a. Registration of five soybean germplasm lines selected within the cultivar 'Benning' differing in seed and agronomic traits. J. Plant Reg. **1**: 156–157.

Fasoula, V. A., Boerma, H. R., Yates, J. L., Walker, D. R., Finnerty, S. L., Rowan, G. B. and Wood, E. D. 2007b. Registration of seven soybean germplasm lines selected within the cultivar 'Cook' differing in seed and agronomic traits. J. Plant Reg. **1**: 158–159.

Fasoula, V. A., Boerma, H. R., Yates, J. L., Walker, D. R., Finnerty, S. L., Rowan, G. B. and Wood, E. D. 2007c. Registration of six soybean germplasm lines selected within the cultivar 'Haskell' differing in seed and agronomic traits. J. Plant Reg. **1**: 160–161.

Fukui, K. 1983. Sequential occurrence of mutations in a growing rice callus. Theor. Appl. Genet. **65**: 225–230.

Gordon, I. L. and Byth, D. E. 1972. Comparisons among strains of the tobacco cultivar Hicks illustrating variability within a single cultivar. Queensl. J. Agric. Anim. Sci. **29**: 255–264.

Griffin, J. D. and Palmer, R. G. 1987. Inheritance and linkage studies with five isozyme loci in soybean. Crop Sci. **27**: 885–892.

Haun, W. J., Hyten, D. L., Xu, W. W., Gerhardt, D. J., Albert, T. J., Richmond, T., Jeddeloh, J. A., Jia, G., Springer, N. M., Vance, C. P. and others 2011. The composition and origins of genomic variation among individuals of the soybean reference cultivar Williams 82. Plant Physiol. **155**: 645–655.

Hirata, T., Abe, J. and Shimamoto, Y. 1999. Genetic structure of the Japanese soybean population. Genet. Resour. Crop Evol. **46**: 441–453.

Hopkins, M., Khalid, A., Chang, P.-C., Vanderhoek, K., Lai, D., Doerr, M. and Lolle, S. 2013. *De novo* genetic variation revealed in somatic sectors of single *Arabidopsis* plants. F1000Res. 2:5.

Hsu, C. C., Chen, C. L., Chen, J. J. and Sung, J. M. 2003. Accelerated aging-enhanced lipid peroxidation in bitter gourd seeds and effects of priming and hot water soaking treatments. Sci. Hortic. **98**: 201–212.

Hyten, D. L., Song, Q., Zhu, Y., Choi, I.-Y., Nelson, R. L., Costa, J. M., Specht, J. E., Shoemaker, R. C. and Cregan, P. B. 2006. Impacts of genetic bottlenecks on soybean genome diversity. Proc. Natl. Acad. Sci. USA **103**: 16666–16671.

Kempinski, C. F., Crowell, S. V., Smeeth, C. and Barth, C. 2013. The novel *Arabidopsis thaliana svt2* suppressor of the ascorbic acid-deficient mutant *vtc1–1* exhibits phenotypic and genotypic instability. F1000Res. 2:6.

Kiang, Y. T. and Bult, C. J. 1991. Genetic and linkage analysis of aconitate hydratase variants in soybean. Crop Sci. **31**: 322–325.

Kosambi, D. D. 1944. The estimation of map distance from recombination values. Ann. Eugen. **12**: 172–175.

Lander, E. S., Green, P., Abrahamson, J., Barlow, A., Daly, M. J., Lincoln, S. E. and Newburg, L. 1987. MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics **1**: 174–181.

Lolle, S. J., Victor, J. L., Young, J. M. and Pruitt, R. E. 2005. Genome-wide non-mendelian inheritance of extra-genomic information in *Arabidopsis*. Nature **434**: 505–509.

Michelmore, R. W., Paran, I. and Kesseli, R. V. 1991. Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc. Natl. Acad. Sci. USA **88**: 828–832.

Morgante, M., Brunner, S., Pea, G., Fengler, K., Zuccolo, A. and Rafalski, A. 2005. Gene duplication and exon shuffling by helitron-like transposons generate intraspecies diversity in maize. Nat. Genet. **37**: 997–1002.

Parrish, D. J. and Leopold, A. C. 1978. On the mechanism of aging in soybean seeds. Plant Physiol **61**: 365–368.

Peyret, P., Perez, P. and Alric, M. 1995. Structure, genomic organization, and expression of the *Arabidopsis thaliana* aconitase gene. Plant aconitase show significant homology with mammalian iron-responsive element-binding protein. J. Biol. Chem. **270**: 8131–8137.

Rasmusson, D. C. and Phillips, R. L. 1997. Plant breeding progress and genetic diversity from *de novo* variation and elevated epistasis. Crop Sci. **37**: 303–310.

Rennie, B. D., Beversdorf, W. D. and Buzzell, R. I. 1987. Genetic and linkage analysis of an aconitate hydratase variant in the soybean. J. Hered. **78**: 323–326.

Rolling, R. A. 2012. Variability in soybean agronomic performance traits in response to 41°C heat and high relative humidity seed stress Ph.D. Master's. Iowa State Univeristy, Ames, IA.

Roth, E. J., Frazier, B. L., Apuya, N. R. and Lark, K. G. 1989. Genetic variation in an inbred plant: variation in tissue cultures of soybean [*Glycine max* (L.) Merrill]. Genetics **121**: 359–368.

Russell, W., Sprague, G. and Penny, L. 1963. Mutations affecting quantitative characters in long-time inbred lines of maize. Crop Sci. **3**: 175–178.

Sandhu, D., Gao, H., Cianzio, S. and Bhattacharyya, M. K. 2004. Deletion of a disease resistance nucleotide-binding-site leucine-rich- repeat-like sequence is associated with the loss of the *Phytophthora* resistance ene *Rps4* in oybean. Genetics **168**: 2157–2167.

Schmutz, J., Cannon, S. B., Schlueter, J., Ma, J., Mitros, T., Nelson, W., Hyten, D. L., Song, Q., Thelen, J. J., Cheng, J. and others 2010. Genome sequence of the palaeopolyploid soybean. Nature **463**: 178–183.

Schneeberger, R. G. and Cullis, C. A. 1991. Specific DNA alterations associated with the environmental induction of heritable changes in flax. Genetics **128**: 619–630.

Song, Q., Jia, G., Zhu, Y., Grant, D., Nelson, R. T., Hwang, E.-Y., Hyten, D. L. and Cregan, P. B. 2010. Abundance of SSR motifs and development of candidate polymorphic SSR markers (BARCSOYSSR_1.0) in soybean. Crop Sci. **50**: 1950–1960.

Song, Q. J., Marek, L. F., Shoemaker, R. C., Lark, K. G., Concibido, V. C., Delannay, X., Specht, J. E. and Cregan, P. B. 2004. A new integrated genetic linkage map of the soybean. Theor. Appl. Genet. **109**: 122–128.

Sprague, G. F., Russell, W. A. and Penny, L. H. 1960. Mutations affecting quantitative traits in the selfed progeny of doubled monoploid maize stocks. Genetics **45**: 855–866.

Tokatlidis, I. S. 2000. Variation within maize lines and hybrids in the absence of competition and relation between hybrid potential yield per plant with line traits. J. Agric. Sci. **134**: 391–398.

Tokatlidis, I. S., Tsialtas, J. T., Xynias, I. N., Tamoutsidis, E. and Irakli, M. 2004. Variation within a bread wheat cultivar for grain yield, protein content, carbon isotope discrimination and ash content. Field Crop Res. **86**: 33–42.

Tokatlidis, I. S., Xynias, I. N., Tsialtas, J. T. and Papadopoulos, I. I. 2005. Single-plant selection at ultra-low density to improve stability of a bread wheat cultivar. Crop Sci. **46**: 90–97.

Wilson, E. W., Rowntree, S. C., Suhre, J. J., Weidenbenner, N. H., Conley, S. P., Davis, V. M., Diers, B. W., Esker, P. D., Naeve, S. L., Specht, J. E. and others 2014. Genetic gain × management interactions in soybean: II. Nitrogen utilization. Crop Sci. **54**: 340–348.

Yates, J. L., Boerma, H. R. and Fasoula, V. A. 2012. SSR-marker analysis of the intracultivar phenotypic variation discovered within 3 soybean cultivars. J. Hered. **103**: 570–578.

Young, N. D., Debelle, F., Oldroyd, G. E., Geurts, R., Cannon, S. B., Udvardi, M. K., Benedito, V. A., Mayer, K. F., Gouzy, J., Schoof, H. and others 2011. The *Medicago* genome provides insight into the evolution of rhizobial symbioses. Nature **480**: 520–524.