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Intensifying intrinsic genetic variation in soybean [*Glycine max* (L.) Merr.] inbred lines

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**Intensifying intrinsic genetic variation in soybean [*Glycine max* (L.) Merr.]
inbred lines**

by

Katherine Espinosa

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Plant Breeding

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Ames, Iowa
2014

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DEDICATION

I dedicate my thesis work to my good friend Paola Tatiana to whom I am forever grateful for the help and encouragement she gave me in making my dreams come true. I would also like to dedicate my work to my forever beloved mother, my siblings, and Scott Carroll. Without the help, support, and encouragement that I received from them, I could not have completed this task.

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ABSTRACT

The soybean [*Glycine max* (L.) Merr.] is an economically important legume crop in the world. It is a source of nutritional vegetable oil and protein that is used for food, feed, and industrial purposes. The United States is the world's leading soybean producer with 32% followed by Brazil with 29%. Despite its economic importance, the genetic base of soybean cultivars has been reported to be narrow thus limiting crop improvement. Even though there is an apparent lack of genetic variation, soybean yields have continuously increased. Inbreeding plant species have shown intrinsic genetic variation; and the sources for this variability have been attributed to seed source, residual heterozygosity, and genetic mechanisms driven by the *de novo* genetic variation. In order to exploit intracultivar variation in established soybean lines, our studies were conducted with the intent of exploring various factors that may be contributing to this genetic variation. The objectives were to evaluate the effect of ultra-low plant densities, the accelerated aging test, simulated-hail, and sexual hybridization as seed- and plant-stress techniques. Single plants from inbred lines were maintained and evaluated during this study. During the growing season, single plants and their progeny were evaluated on the basis of flower color, pubescence color, and phenotypic variation within plots. Further evaluation through laboratory analysis included the examination of segregation patterns for aconitase-2 and aconitase-4 isozymes as well as segregation for DNA-based molecular markers. Phenotypic and genetic variation was observed within cultivars. During field evaluations, several mutants were observed which included lethal-yellow, viable-yellow, semi-sterile, and late maturity phenotypes. A large frequency of unexpected variation was detected in the progeny of plants grown in an ultra-

low planting design and treated with an accelerated aging test. Although there is not a single factor contributing to this genetic variation, it is likely that plants grown in ultra-low densities might have an effect resulting in the generation of new genetic variants.

CHAPTER 1: GENERAL INTRODUCTION

Soybean [*Glycine max* (L.) Merr.] is recognized as the most important legume crop in the world. It is a source of nutritional vegetable oil and protein that can be used for human food and for industrial purposes, and also for livestock feed (Singh and Hymowitz 1999; Boerma and Specht 2004; Wilcox 2004). The United States is the world's leading soybean producer, which accounts for 32%, followed by Brazil with 29%, Argentina with 19%, China with 6%, and India with 5 % (FAO Statistics 2013).

Morphological and molecular data have indicated that the cultivated soybean was domesticated from its annual wild relative [*Glycine soja* (Sieb. and Zucc.)] in China (Broich and Palmer 1980; Kollipara et al. 1997; Carter et al. 2004; Hymowitz 2004; Doebley et al. 2006; Guo et al. 2010). According to Hymowitz (2004), the first introduction of soybean to America from China was by Samuel Bowen in 1765. Beginning in the 1920's soybean plant introductions were brought from Asia to be grown for seed in North America (Hymowitz 2004).

The adaptation and performance of established soybean cultivars, like many of the most important crop species, have undergone several genetic bottleneck events and many years of intense selection, resulting in a narrow genetic base population, which is strengthened by its predominantly autogamous condition (Gizlice et al. 1993; Tanksley and McCouch 1997; Ray et al. 2003; Hyten et al. 2006). Although there are an estimated 45,000 accessions preserved in germplasm collections in the world, only 80 ancestors account for 99% of the parentage of U.S. soybean cultivars (Carter et al. 2004). Only a few Asian landraces, introduced to North America, became the genetic base of North American

cultivars, which has been described as the bottleneck with the greatest impact reducing the number of rare alleles and thus the genetic diversity of modern cultivars (Gizlice et al. 1994, 1996; Carter et al. 2004; Hyten et al. 2006; Guo et al. 2010).

Crop improvement in soybean is focused on the development of inbred lines, which represents the totality of cultivars in the market. These highly homozygous inbred lines are what is grown by the farmer. Each cycle of improvement begins with the selection of parents to be used to create segregating populations; these parents can be either exotic germplasm or elite material (Fehr 1991). Those populations are advanced toward homozygosity through selection for the best phenotypes to produce relatively homozygous lines. These newly developed homozygous lines are evaluated for yield and other agronomic traits in performance trials. In most soybean breeding programs, selecting parents from elite material is preferred, and then crosses are made to create segregating populations. A cross between high yielding parents is more likely to produce desirable progeny than a cross between high and low yielding parents (Schoener and Fehr 1979; Wilcox 2004; Fasoula and Boerma 2005). As a consequence, it is believed that breeding practices may contribute to a pronounced reduction of genetic variation in newly generated cultivars (Gizlice et al. 1994, 1996; Hyten et al. 2006).

Despite the apparent lack of genetic variation, soybean yield in the United States continues to increase. Specht (2012) reported that yields have increased at a linear rate of 23.3 kg ha⁻¹ from 1924 to 2010. The consistent yield gain has been attributed to continued genetic improvement and the adoption of improved agronomic practices by producers (Specht and Williams 1984; Specht 2012). Consequently, yield improvement may be achieved by breeding directly for this trait; for example, crossing high yielding parents, even

though each selection cycle is expected to lead to reduced genetic variation. Yield improvement also results from improved agronomic practices, for example, changes in planting date, row spacing, soil fertility, etc. This continuous genetic gain indicates that breeding progress within an apparently narrow genetic base in soybean is possible.

In recent years, there has been an increased interest to explore additional factors that may be contributing to the increase in genetic gain within apparently narrow genetic pools. For example, Roth et al. (1989) suggested that soybean plants were able to generate their own genetic diversity as a consequence of diverse genetic hybridization in response to genetic stress, which could be useful for breeding programs. Although not published, changes in the enzyme mobility for *Aco-4* locus were found in progeny of sexual crosses of soybean cultivars (Dr. Reid G. Palmer, personal communication, April, 2007). Exceptional *Aco-4* patterns were noticed in F₂ seed examined from crosses between ‘Minsoy’ (PI 27890) and ‘Noir 1’ (PI 290136).

In commercial cultivars, genetic variation within highly inbred cultivars has been exploited using single-plant progenies selected under ultra-low densities in a honeycomb planting design (Fasoulas and Fasoula 1995; Fasoula and Boerma 2005, 2007). According to Fasoulas (1990), single-plant selection in the absence of competition has been effective, because it reduces the masking effects of the negative correlation between a plant’s yield and competitive ability, soil heterogeneity, and maximization of the range of genotypic expression across different entries.

The objective of this study was to enhance intrinsic variation in soybean inbred lines by manipulating plant growing conditions, i.e. honeycomb design pattern, and seed or plant stresses. Enhancing intrinsic genetic variation in already adapted cultivars could provide

additional strategies that help increase genetic gains in soybean and possibly other self-pollinated crop species. Since the narrow genetic base of elite cultivars is perceived as a concern to long-term food security (Tanksley and McCouch 1997), new sources of variation is of interest for the scientific community. Additionally, the role of plant breeding is to provide strategies that can help mitigate effects of global climate change; for example, frequency and intensity of pest attacks, diseases, and radical changes in the rainfall patterns. Finally, although there is not a clear understanding of the genetic mechanisms responsible for enhancing genetic variation, the evaluation of growing environments concurrent with the technical advances made in genomics could improve our understanding of the genetic mechanisms behind these unusual events.

This research was divided into two main research topics: the study of progeny of sexual hybridization and the study of progeny from non-sexual or self-pollinations. The effect of stress treatments, such as accelerated aging tests on seeds, and simulated hail on plants were evaluated. For the evaluation of new variants, we followed some phenotypic variants in the field, as well as the segregation of isozymes *Aco-2* and *Aco-4*, and molecular markers.

Dissertation Organization

This dissertation is organized into five chapters. Chapter one is the general introduction. Chapter two describes characterization of yellow-foliage mutants found in progeny of pure-line experiments. Chapter three describes results of progenies from sexual hybridization and pure-lines experiments. Chapter four describes results of progenies of pure-lines from honeycomb planting design and conventional tractor planting with non-

simulated and simulated hail experiments. Chapter five is the general conclusion of each experiment.

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CHAPTER 2: GENETIC AND MOLECULAR ANALYSIS OF A LETHAL YELLOW AND A VIABLE-YELLOW MUTANT IN SOYBEAN

A paper submitted to *Plant Science*

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Abstract

Genetic variants with altered phenotypes are extremely important for genetic studies.

Chlorophyll-deficient mutants have been extensively used to understand genetic mechanisms regulating complex metabolic pathways. Two chlorophyll-deficient mutants were observed in self-pollinated progeny of ‘IAR2001BSR’ and ‘BSR 101’ inbred lines grown in a honeycomb planting design and that had suffered natural hail-storm damage. During progeny evaluation, one lethal-yellow mutant was observed in progeny of each line.

Segregation patterns suggested single-gene recessive inheritance for the lethal-yellow mutants. F_{2:3} progeny evaluations from a cross between ‘Manchu’ and green plants from the segregating mutant lines showed the expected 1:2 ratio for non-segregating: segregating lethal-yellow. Among these F_{2:3} lines, one showed segregation for green, viable-yellow, and lethal-yellow plants. The objectives of this investigation were to determine the inheritance of lethal- and viable-yellow genes, to study epistatic interaction between them, to molecular

map the viable-yellow gene, and to identify candidate genes that might play a role in photosynthesis. Segregation in the F₂ and F_{2:3} populations between ‘Manchu’ and viable-yellow plants showed single-gene recessive inheritance. Our analysis revealed recessive epistatic interaction between two genes (viable-yellow and lethal-yellow) and might indicate involvement of these two genes in the same chlorophyll biosynthesis pathway. The viable-yellow gene was mapped to ~168 kb region on chromosome Gm02. We located 28 predicted genes between the flanking markers. Of these, *Glyma02g39990* is of particular interest, as it shows homology to a translocon at the inner envelope membrane of chloroplasts (Tic) 110 in pea and Arabidopsis. Tic110 is known to play critical role in plastid biogenesis and heterozygous mutants for *tic110* in Arabidopsis exhibited a pale phenotype.

Introduction

A number of methods have been used to create genetic variation, in an attempt to produce heritable and stable genetic variation, which is required for functional genetic studies and genetic crop improvement. Genetic and molecular analyses of variants can enhance the identification of important agronomic traits and help determine their role in metabolic pathways. Phenotypic and genotypic variation also can be enhanced as a consequence of a particular environment, under which plants are allowed to develop. For example, tissue culture imposes a stress on cultured cells and tissues [1-3]. Due to the dynamic nature of the genomes, stresses can trigger mechanisms that can cause modifications and genome reorganization under a variety of stress conditions leading to modifications [1]. Similar to the stress of plant cells grown under tissue culture conditions, environmental stress in plants can be triggered under natural or controlled conditions, i.e. fertilizer levels, plant

density, biotic and abiotic factors, etc. Some of these changes have been associated with changes at the DNA level, and have created significant variation for agronomic traits [4-6].

Genetic variants with altered phenotype and genotype contribute to a better understanding of metabolisms, genetic mechanisms, and characterization of genomic regions that could be associated with agronomic traits. For example, chlorophyll-deficient mutants have been extensively used to explore chlorophyll metabolism, its regulation, gene identification, and genetic control [7-13]. Chlorophyll metabolism is essential for plant development and yield production in relation to photosynthesis [14]. Chlorophyll has an important role in photosynthetic light-harvesting in antennae systems and energy transfer in the reaction centers of photosynthesis [15]. Identification of genes encoding for biochemical analyses is essential for a better understanding of enzymatic processes. Mutations underlying chlorophyll-deficiency phenotype have been found in genes encoding enzymes that function within the metabolic pathway identified [16-18]. Thus, mutants with chlorophyll-deficiency are essential for identification of gene function.

In soybean, 57 chlorophyll-deficiency mutants have been reported [19]. Some are classified as lethal or non-lethal and maybe nuclearly inherited, or cytoplasmically inherited [19]. At least 25, nuclear genes affecting chlorophyll-deficiency have been reported. Some of these mutant genes have been mapped to different linkage groups [8, 9, 12, 13, 19, 20]. Chlorophyll-deficiency mutants have been recovered among the progeny of independent germinal revertants of the *w4-m* (mutable) line [21], from the progeny of *Y18-m* mutable line [8], from instability of the *y20 Mdh1-n k2* [chlorophyll-deficient foliage (*y20*), mitochondrial malate dehydrogenase (*Mdh1-n*), and tan saddle seed coat (*k2*)] chromosomal region [22], from tissue-cultured derived chimeric plants [10, 23], and from spontaneous

mutations [23]. In a gene-tagging study, Palmer et al. 1989 recovered chlorophyll-deficient mutants among the progeny of independent germinal revertants of *w4-m*, which was an unstable mutation for anthocyanin pigmentation in soybean [21]. These authors suggested that each of the mutants was the result of a deletion which produced the silencing of a mitochondrial malate dehydrogenase (*MDH*) locus, atypical chloroplast development, and an altered chlorophyll composition. Chen et al. 1999 proposed that instability at the *y20 Mdh1-n k2* chromosomal region might be due to transposon activity that could generate chromosomal rearrangements [22].

In an attempt to study genetic variation within inbred lines, single plants of germplasm ‘IAR2001BSR’, and plant introduction ‘BSR 101’ (PI 548519) inbred lines were planted in a honeycomb design. Honeycomb design is a planting pattern consisting of a wide interplant spacing [24]. This planting pattern minimizes interplant competition and allows effective evaluation of the yield potential of individual plants. Field evaluation of seedlings, of progeny of single plants harvested from a honeycomb design affected by natural hail-storm, identified two soybean lines segregating for lethal-yellow mutants. One was found in ‘IAR2001BSR’ and the other one in ‘BSR 101’. Cross-pollinations were made between plant introduction ‘Manchu’ (PI 30593) as female parent with green plants from the segregating mutant lines. During $F_{2:3}$ progeny-line evaluation; a viable-yellow mutant was detected in a progeny row from the cross-pollination between ‘Manchu’ and ‘IAR2001BSR’. The objectives of this investigation were to determine the inheritance of lethal- and viable-yellow genes, to study epistatic interaction between them, to molecular map the viable-yellow gene, and to identify candidate genes that might play a role in photosynthesis.

Materials and Methods

Plant material

Soybean germplasm ‘IAR2001BSR’ and plant introduction ‘BSR 101’ (PI 548519) were used in this study. Bulk seed of ‘BSR 101’ was obtained from Dr. R.G. Palmer, Iowa State University at Ames, IA, and bulk seed of ‘IAR2001BSR’ from Dr. Silvia Ciazio, Iowa State University at Ames, IA.

In 2008, 500 seed of ‘BSR 101’, and ‘IAR2001BSR’ were planted at the Bruner Farm in a honeycomb design [24], using an equidistant spacing of 2 m between each individual plant to minimize the unfavorable effect of competition in response to selection (Figure 1). A code, termed “entry number,” was assigned to each individual plant. Field plots were damaged by natural hail in July reducing yield in most entries. For this reason, the number of harvested plants was reduced to 315 plants for ‘BSR 101’ and 171 for ‘IAR2001BSR’. At maturity, each plant was harvested and threshed by hand.

In 2009, progeny of single plants from 2008 honeycomb experiment were planted at the Bruner Farm near Ames, IA. Fifty seed per entry were sown in a 15-meter long row with a push-planter. Three weeks after planting, each plot was thinned to approximately 35 plants. During the growing season, plots were evaluated on the basis of plant color, flower color, pubescence color, and phenotypic variation within plots. At maturity, each plot was combine-harvested.

Identification of mutants in the field and strategies for genetic analysis

Early identification of lethal-yellow mutants in the field before flowering gave us time to make cross-pollinations for genetic analysis. Green plants from ‘IAR2001BSR’ entry 243, and ‘BSR 101’ entry 345 were tagged and used as male parents in cross-pollinations

with ‘Manchu’ (PI 30593). F_1 seed were planted at the University of Puerto Rico – Iowa State University station near Isabela, Puerto Rico in October 2009. The F_1 plants were single-plant threshed and F_2 seed from each F_1 plant from the cross combinations were planted at the Bruner Farm near Ames, IA, in May 2010. The F_2 plants were classified for plant color at the seedling stage. Green foliage F_2 plants of the segregating families were single-plant threshed and evaluated as F_2 -plant progeny rows. $F_{2:3}$ progenies were evaluated for seedling traits in the USDA greenhouse at Iowa State University in October 2010 – June 2011.

Identification of viable-yellow mutant and development of genetic material

One $F_{2:3}$ progeny line from the cross-pollination between ‘Manchu’ and ‘IAR2001BSR’ entry 243, segregated 20 green, 3 viable-yellow and 9 lethal-yellow plants in the USDA greenhouse sandbench (Figure 2). Three viable-yellow mutant plants were tagged and transplanted to pots in the USDA greenhouse in June 2011. Cross-pollinations were made between ‘Manchu’ as female plant with the viable-yellow plants (KE120-1, KE120-2, and KE120-3) as male parents. F_1 seed were planted at the University of Puerto Rico – Iowa State University station near Isabela, Puerto Rico in October 2011. The F_1 plants were single-plant threshed and 50 F_2 seed from each F_1 plant from the cross combinations were planted in the USDA greenhouse. For genetic and molecular analyses, remnant seed of selected F_2 populations was planted at the Bruner Farm near Ames, IA, in June 2012. The $F_{2:3}$ progenies were grown and evaluated in the USDA greenhouse in November 2012 to determine the genotype of each F_2 plant by checking leaf color phenotype.

DNA isolation and bulked segregant analysis (BSA)

Plant DNA was extracted using genomic DNA isolation technique described earlier [25]. Bulked segregant analysis (BSA) was used to identify molecular markers closely linked to the viable-yellow gene [26]. Two DNA bulks were established based on $F_{2:3}$ phenotypic data. One bulk was created by pooling DNA from 10 F_2 individuals identified as homozygous dominant (green phenotype), and the second was created by pooling DNA from 10 F_2 individuals identified as homozygous recessive (viable-yellow phenotype). Each bulk was diluted to a final concentration of 50 ng/ μ l.

Molecular analysis

Simple Sequence Repeat (SSR) markers were developed using information from Soybase (<http://soybase.org/resources/ssr/php>) [27, 28]. SSR markers were amplified by the polymerase chain reaction (PCR) in a 10 μ l reaction mix that contained 1 \times PCR buffer (10mM Tris-HCl, 50 mM KCl, pH8.3), 2.0 mM $MgCl_2$, 0.25 μ M primer, 200 μ M of each dNTP, 50 ng of genomic DNA, and 0.25 units of Biolase DNA polymerase (Bioline USA Inc, Taunton, MA). The PCR program employed was 2 minutes at 94 °C, 35 cycles of 30 seconds at 94 °C, 30 seconds at 58 °C, followed by 1 minute at 72 °C. The PCR products were separated on a 4 % agarose gel at 150 V for 2 hours in 0.5 \times TBE buffer.

The putative closely linked SSR markers identified in the BSA analysis were used to screen the whole F_2 mapping population. Map positions of the locus and the SSR markers in the final map were calculated with the program Mapmaker 2.0 [29], using a minimum LOD of 3.0 and a maximum recombination value of 0.4 as thresholds. Linkage calculations were done using the Kosambi mapping function [30]).

Results

Summer 2009, 2010, Bruner Farm; lethal-yellow mutants

In summer 2009, 315 entries of 'BSR 101', and 171 entries of 'IAR2001BSR' were evaluated for flower color, plant color, pubescence color, and phenotypic variation within plots. During evaluation at early stage of development, two entries, 'IAR2001BSR' entry 243 and 'BSR 101' entry 345 were segregating for lethal-yellow phenotype. These segregation pattern suggested clear single-gene recessive inheritance for 'IAR2001BSR' entry 243, however 'BSR 101' entry 345 did not conform to single-gene model (Table 1).

Green plants (*AA* or *Aa*) from the entries segregating for green and lethal-yellow plants were used as male parents and crossed with 'Manchu' as female parents. The F_2 Segregation ratios deviated significantly from the expected 3:1 ratio. Data are shown for one F_2 population from the cross between 'Manchu' and 'IAR2001BSR' (Table 2). $F_{2:3}$ progeny-line evaluation showed the expected 1:2 ratio for non-segregating: segregating lethal-yellow (Table 2). Among $F_{2:3}$ progeny lines, one segregated 20 green plants, 3 viable-yellow, and 9 lethal-yellow plants. The viable-yellow was characterized in this study.

Summer 2011, 2012; viable-yellow mutant

'Manchu' (female parent) was crossed with identified viable-yellow mutants (KE120-1, KE120-2, and KE 120-3). All F_1 plants were green. F_2 populations segregated into two phenotypic classes (green: viable-yellow: lethal-yellow plants and green: viable-yellow plants) (Table 3). For genetic and molecular analysis of the viable-yellow mutant, 167 F_2 individuals from the cross between 'Manchu' and KE120-1-2 were analyzed. F_2 and $F_{2:3}$ segregation indicated single-gene recessive inheritance (Tables 4).

Genetic mapping of the viable-yellow gene

To determine the genetic location of the viable-yellow gene, we used 800 SSR markers covering all 20 soybean molecular linkage groups (MLG) on the viable-yellow and green bulks. BARCSOYSSR_02_1477 showed polymorphism between the bulks, which suggested that the viable-yellow mutant was located on chromosome Gm02 (MLG D1b). Markers that were close to BARCSOYSSR_02_1477 were analyzed for polymorphism between the parents. Of these, 10 markers showed polymorphism and were tested on the F₂ generation. The polymorphic markers were Satt537, Satt282, BARCSOYSSR_02_1450, BARCSOYSSR_02_1454, BARCSOYSSR_02_1468, BARCSOYSSR_02_1469, BARCSOYSSR_02_1477, BARCSOYSSR_02_1486, BARCSOYSSR_02_1521, and BARCSOYSSR_02_1539. Analysis of the marker data showed that the viable-yellow gene was flanked by BARCSOYSSR_02_1454 and BARCSOYSSR_02_1468 at a distance of 1.1 cM and 1.6 cM, respectively (Figure 3). SSR markers of the viable-yellow region were physically mapped using the soybean genome sequence (<http://www.phytozome.net/>) [31]. The viable-yellow region between BARCSOYSSR_02_1454 and BARCSOYSSR_02_1468 markers was about 168 kb (Figure 3).

Discussion

Lethal-yellow

During field evaluation at early stage of development, we found mutant lines segregating for lethal-yellow phenotype in ‘IAR2001BSR’ entry 243, and ‘BSR 101’ entry 345. In its original background of ‘IAR2001BSR’, the lethal-yellow was inherited as a single-recessive gene. In the F₂ generation from the cross-pollination between ‘Manchu’ and

‘IAR2001BSR’, the expected ratio deviated significantly from 3 green plants: 1 lethal-yellow plant. However, $F_{2:3}$ segregations confirmed the expected single-recessive gene inheritance of the mutant phenotype. Deviation in the F_2 population might have occurred due to lower percent germination of the lethal-yellow genotypes. Fewer than expected lethal-yellow plants would give a distorted ratio. Although, segregation pattern of selfed progenies of ‘BSR 101’ showed deviation from expected, F_2 and $F_{2:3}$ generations from the cross-pollination between ‘Manchu’ and ‘BSR 101’ entry 345 showed expected segregation ratios for a monogenic inheritance (data not shown). Distorted segregation pattern in selfed progenies of ‘BSR 101’ may also be attributed to low germination percentage of lethal-yellow plants. The lethal-yellow mutants were not characterized further.

Viable-yellow

Viable-yellow plants observed in the $F_{2:3}$ progeny row from the cross between ‘Manchu’ and ‘IAR2001BSR’ were crossed with ‘Manchu’ as female parents. No variegated (chimeric) plants were observed in the F_1 or F_2 generations. All F_1 plants had green foliage, which suggested nuclear gene inheritance. F_2 populations gave two phenotypic classes, green: viable-yellow: lethal-yellow plants and green: viable-yellow plants (Table 3). At least two cross combinations (‘Manchu’ x KE120-1-2 and ‘Manchu’ x KE120-2) showed single-gene inheritance (Table 3). These results were validated when remnant seed of the selected F_2 population (‘Manchu’ x KE120-1-2) were planted in the field for genetic and molecular analyses (Table 4). The F_2 population segregated 129 green plants: 38 viable-yellow plants which fit the expected 3:1 ratio. The $F_{2:3}$ family segregation was also consistent with single-gene inheritance (Table 4).

Two of the cross combinations ('Manchu' x KE120-1-1 and 'Manchu' x KE120-3) showed three phenotypic classes (green: viable-yellow: lethal-yellow) in F_2 that suggested epistatic interaction involving two genes (Table 2). Based on the segregation patterns of the lethal-yellow and viable-yellow phenotypes, we were able to decipher relationship between two genes (Yl and Yv). Homozygous recessive mutation in one gene ($ylyl$) results in lethal-yellow phenotype irrespective of the second gene (Figure 4). Homozygous recessive mutation in the second gene, $yvyv$, in the presence of normal Yl , results in viable-yellow phenotype (Figure 4). Based on this model, homozygous recessive mutation in the Yl gene ($YvYvylyl$) in the 'IAR2001BSR' and 'BSR101' lines resulted in lethal-yellow phenotype. When lethal-yellow (in heterozygous form) was crossed with Manchu ($YvYvYlYl$), it showed monogenic inheritance (Figure 4). In one of the F_2 plants ($YvYvYlyl$) a second mutation occurred leading to a heterozygous genotype for the both genes ($YvyvYlyl$). The $F_{2:3}$ family from this plant segregated 20 green ($Yv_Yl_$): 3 viable-yellow ($yvyvYl_$): 9 lethal-yellow (Yv_ylyl or $yvyvylyl$) (Figure 4). When three viable-yellow plants [KE120-1 ($yvyvYlyl$), KE120-2 ($yvyvYlYl$), and KE120-3 ($yvyvYlyl$)] were crossed with Manchu ($YvYvYlYl$), 3 green: 1 viable-yellow or 9 green: 3 viable yellow: 4 lethal-yellow ratios were observed (Figure 4). Low P values for the chi-square test for 9:3:4 ratios can be explained by poor germination of the lethal-yellow plants (Table 3). For all the crosses shown in table 3, 50 F_2 seeds were germinated (Table 3). For two crosses that segregate into green, viable-yellow and lethal yellow germination percentage is significantly lower as compared to other two crosses that segregate for green and viable-yellow (Table 3). This further endorses low germination rate of yellow lethal plants.

Although we do not know the genetic mechanism leading to the appearance of chlorophyll-deficient mutants, our results showing two mutations for different traits (viable-yellow and lethal-yellow) might indicate involvement of these two genes in the same chlorophyll biosynthesis pathway (Figure 5). Homozygous recessive mutation in the *Yl* gene may result in plants that are yellow in color but are unable to survive. Homozygous recessive mutation in the *Yv* gene may result in plants that are yellow but are viable. Both normal genes will result in normal green plants (Figure 5).

Availability of the soybean genome sequence allowed us to fine map the viable-yellow mutant gene. Using the sequencing information for SSR markers, we were able to place them physically on the chromosome. The results showed that the viable-yellow locus was located on chromosome Gm02 (MLG D1b). An interval of 168 kb region was flanked by BARCSOYSSR_02_1454 and BARCSOYSSR_02_1468 markers on chromosome 2 (MLG D1b). Using this information, we located 28 predicted genes in this region (Table 5; <http://www.phytozome.net/>). Of these, one gene (*Glyma02g39990*) was of particular interest as this may play role in photosynthesis [31]. The locus *Glyma02g39990* shows homology to a translocon at the innner envelope membrane of chloroplasts (Tic) 110 in pea and Arabidopsis [32, 33]. Tic complexes are implicated in several import processes, including the import of thousands of nucleus-encoded proteins synthesized in the cytosol [34]. Tic 110 forms a prominent protein channel and plays critical role in plastid biogenesis and plant viability [33, 35]. Heterozygous mutants for *tic110* in Arabidopsis exhibited pale phenotype, however, homozygous mutants were not viable [33]. In soybean, viable-yellow mutants were viable in homozygous recessive condition. The reason for the soybean mutant to be viable may be the paleopolyploid nature of the soybean genome. Soybean has another gene

(*Glyma14g38140*) on chromosome 14 that is 96% identical to *Glyma02g39990*. Further characterization of *Glyma02g39990* may confirm its role in green/viable-yellow phenotype and help in decoding the nature of the mutation.

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Table 1. Number of green and lethal-yellow soybean plants found in self-pollinated plants of ‘IAR2001BSR’ entry 243 and ‘BSR 101’ entry 345 after honeycomb planting and natural hail-storm damage.

Population	No. of plants			
	Green	Yellow	χ^2 (3:1)	<i>P</i>
‘IAR2001BSR’ entry 243	33	14	0.57	0.45
‘BSR 101’ entry 345	34	4	4.25	0.04

Chi-square values calculated to test goodness of fit to a 3:1 ratio.

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

Table 2. Number of green and lethal-yellow F₂ and F_{2:3} soybean plants from the cross-pollination between ‘Manchu’ and heterozygous lethal-yellow plants (‘IAR2001BSR’) based on single-gene hypothesis.

Population	No. F ₂ plants				No. F _{2:3} families				No. plants in segregating F _{2:3} families			
	Green	Yellow	χ^2 (3:1)	<i>P</i>	All Green	Segregating	χ^2 (1:2)	<i>P</i>	Green	Yellow	χ^2 (3:1)	<i>P</i>
‘Manchu’ × Lethal-yellow (heterozygote)	133	8	28.09	<0.01	37	73	0.004	0.84	1982	569	9.88	<0.01

Chi-square values calculated to test goodness of fit to a 3:1 or 1:2 ratio.

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

Table 3. Number of green, viable-yellow and lethal-yellow F₂ plants from the cross-pollination between ‘Manchu’ and KE120-1, KE120-2, and KE120-3 (viable-yellow mutants).

Cross-combination	No. of F ₂ plants			χ^2 (3:1)	<i>P</i>	χ^2 (9:3:4)	<i>P</i>
	Green	Viable-yellow	Lethal-yellow				
‘Manchu’ × KE120-1-1	27	8	4			4.63	0.1
‘Manchu’ × KE120-1-2	40	8	0	1.77	0.18		
‘Manchu’ × KE120-2	41	9	0	1.31	0.25		
‘Manchu’ × KE120-3	30	7	1			11.09	0.004

Manchu was used as female parent. KE120-1 through 3 are the three viable-yellow plants. There were two cross-pollinations with KE120-1.

Chi-square values calculated to test goodness of fit to a 3:1 or 9:3:4 ratio.

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

Table 4. Segregation pattern, chi-square, and *P* value for the population line of soybean viable-yellow.

Cross-combination	No. of F ₂ plants				No. of F _{2:3} families				No. of plants in segregating F _{2:3} families			
	Green	Yellow	χ^2 (3:1)	<i>P</i>	All Green	Segregating	χ^2 (1:2)	<i>P</i>	Green	Yellow	χ^2 (3:1)	<i>P</i>
'Manchu' × viable-yellow (KE120-1-2)	129	38	0.45	1	45	82	0.25	0.6	1129	346	1.87	0.17

Chi-square values calculated to test goodness of fit to a 3:1 or 1:2 ratio.

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

Table 5. Predicted genes present within the viable-yellow genomic region.

Gene	Start Position	End Position	Predicted Protein/Function
<i>Glyma02g39930</i>	45139091	45140686	O-methyltransferase/O-methyltransferase activity
<i>Glyma02g39950</i>	45147618	45149795	Uncharacterized nodulin-like protein
<i>Glyma02g39960</i>	45163588	45165353	None
<i>Glyma02g39970</i>	45177368	45182655	Pseudouridine synthase activity
<i>Glyma02g39981</i>	45185164	45189541	tRNA pseudouridine synthase/ pseudouridine synthase activity
<i>Glyma02g39990</i>	45189739	45197024	Translocon at the inner envelope membrane of chloroplast (83% homologous with Tca)
<i>Glyma02g40000</i>	45203358	45206518	Peroxidase/ peroxidase activity
<i>Glyma02g40010</i>	45208070	45211347	Peroxidase/ peroxidase activity
<i>Glyma02g40020</i>	45218694	45222348	Peroxidase/ peroxidase activity
<i>Glyma02g40030</i>	45227136	45229954	GCN5-like protein 1/ General control of amino-acid synthesis 5-like 1
<i>Glyma02g40040</i>	45238522	45243014	Peroxidase/ peroxidase activity
<i>Glyma02g40051</i>	45247669	45254494	Armadillo/beta-catenin-like repeat
<i>Glyma02g40060</i>	45259446	45264505	tRNA synthetases class 1 (W and Y)/ tyrosine-tRNA ligase activity
<i>Glyma02g40080</i>	45273260	45275361	Unknown protein
<i>Glyma02g40100</i>	45294882	45295981	None
<i>Glyma02g40110</i>	45303718	45306548	Protein kinase/ signal transduction
<i>Glyma02g40121</i>	45301348	45308471	None
<i>Glyma02g40130</i>	45330334	45332607	Protein kinase/ signal transduction
<i>Glyma02g40140</i>	45342635	45346020	None
<i>Glyma02g40150</i>	45358188	45360601	Cytochrome P450/ electron carrier activity
<i>Glyma02g40170</i>	45366502	45369181	Plant protein of unknown function
<i>Glyma02g40180</i>	45369571	45370463	None
<i>Glyma02g40190</i>	45375210	45376823	Protein of unknown function
<i>Glyma02g40200</i>	45380086	45385677	Protein kinase domain/ ATP binding
<i>Glyma02g40220</i>	45396549	45406180	Topoisomerase-related protein
<i>Glyma02g40230</i>	45412654	45415794	Rare lipoprotein A (RlpA)-like double-psi beta-barrel / Pollen allergen

Figure Legends

Figure 1. Honeycomb design pattern used to study genetic variation within inbred lines ‘IAR2001BSR’ and ‘BSR 101’ (Fasoulas and Fasoula 1995). An equidistant spacing of 2 meters was used between each individual plant.

Figure 2. Viable-yellow mutant detected in a $F_{2:3}$ progeny line from the cross-pollination between ‘Manchu and ‘IAR2001BSR’ entry 243. Progeny row segregated green, viable-yellow, and lethal-yellow plants.

Figure 3. Genetic linkage map and sequence-based physical map of soybean chromosome 2 (MLG D1b) showing locations of SSR markers close to the viable-yellow (YV) gene. Genetic distances are shown in centiMorgans (cM) and physical distances are shown in base pairs (bp).

Figure 4. Inheritance of lethal-yellow and viable-yellow mutant genes in soybean. Genes show recessive epistatic interaction (9 green: 3 viable-yellow: 4 lethal yellow).

Figure 5. Predicted model showing interaction between the viable-yellow and lethal-yellow genes in development of chlorophyll.

Figure 1

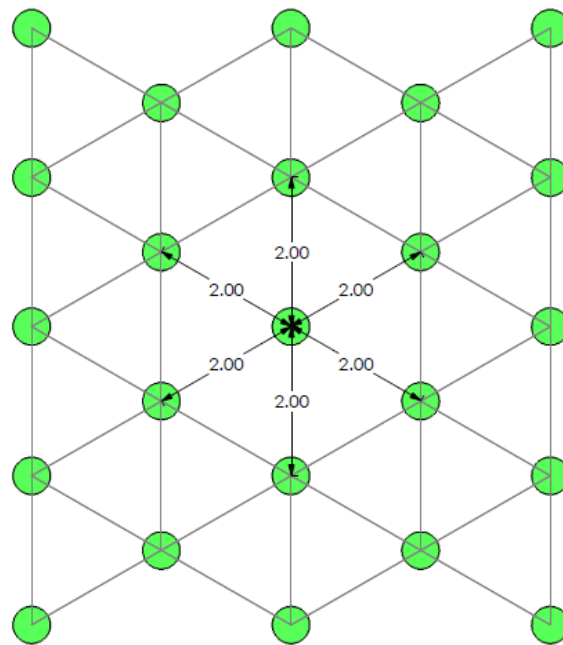


Figure 2

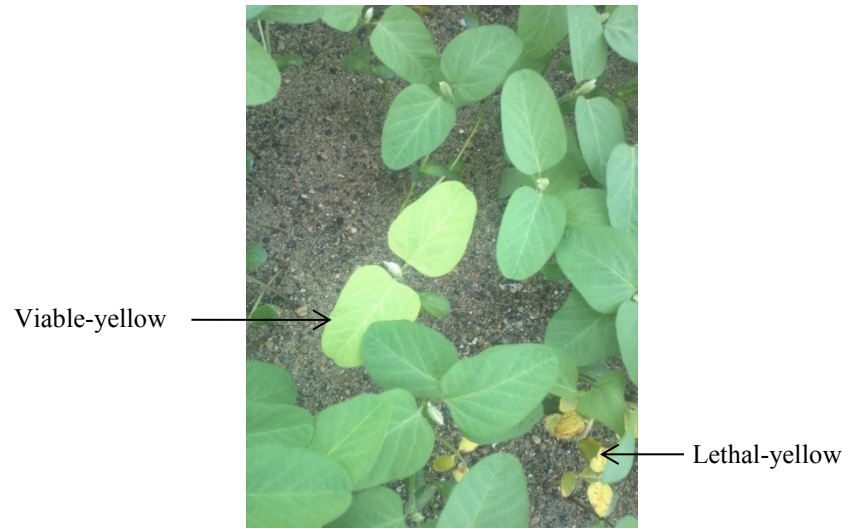
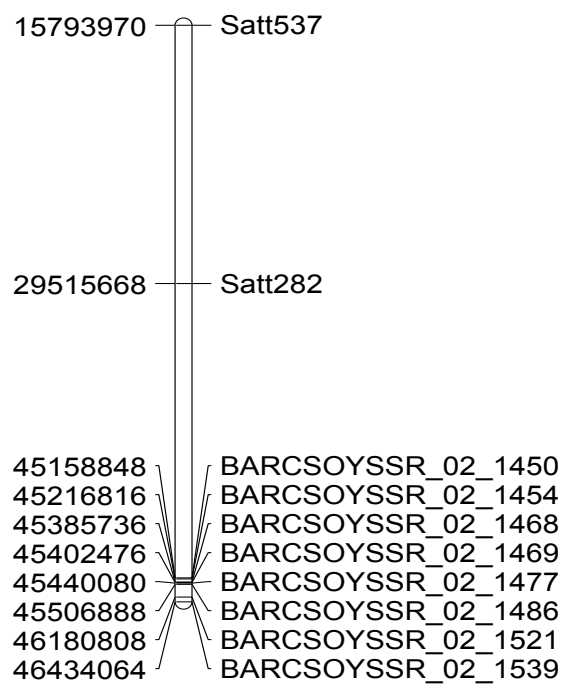
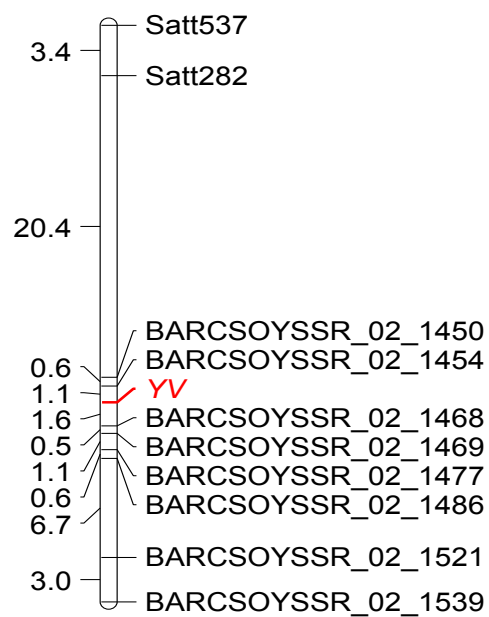


Figure 3



Physical map
Chromosome 2



Linkage map
Chromosome 2

Figure 4

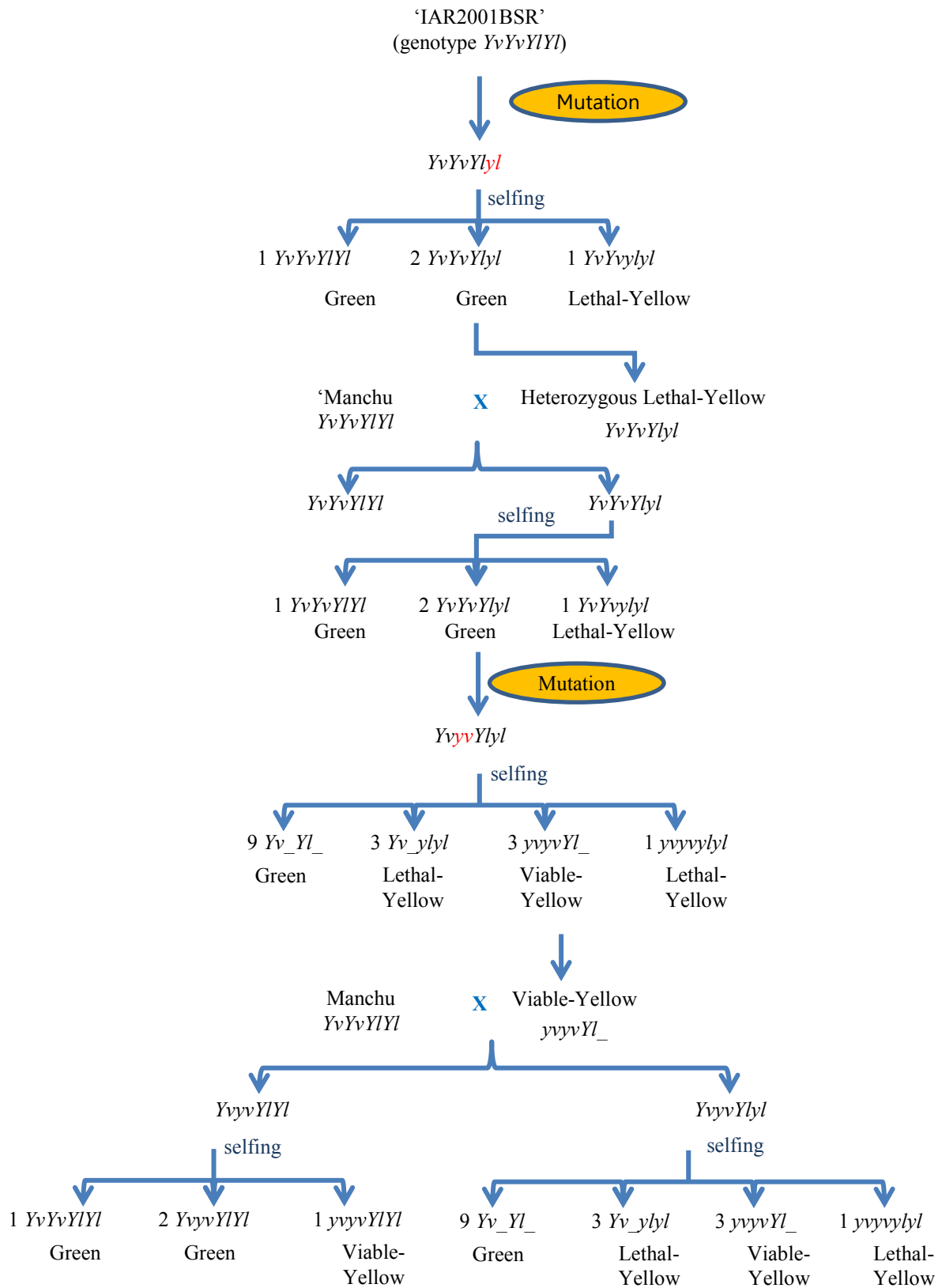
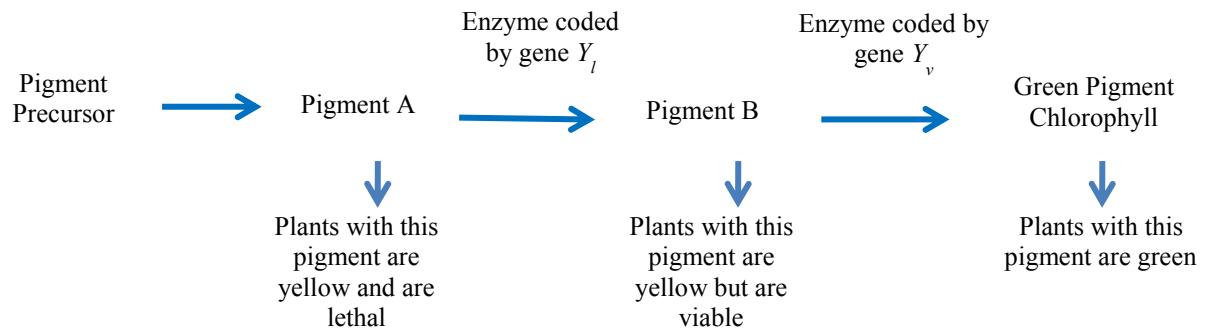


Figure 5



CHAPTER 3: GENETIC VARIABILITY IN SOYBEAN IN RESPONSE TO SEXUAL HYBRIDIZATION AND STRESS IN PURE LINES

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Abstract

Intracultivar variation has been reported in commercial cultivars and in pure lines of soybean [*Glycine max* (L.) Merr.]. This variation has been attributed to residual heterozygosity, traced to seed source, and hypothesized as the result of genetic mechanisms contributing to the de novo genetic variation. In our study, we have identified segregation of allelic variants at the aconitase-4 loci in sexual crosses of Minsoy (PI 27890) x 'BSR 101' (PI 548519). In the pure line cultivars 'BSR 101' (PI 548519) and 'Jack' (PI 540556), we have documented multiple cases of cryptic allelic variation or 'allele switching' that are stable and heritable. For both cultivars, 'BSR 101' and 'Jack', we had 64 entries with two replications and sampled three pods, each three seeded, for a total of 1152 seed per cultivar. We have observed both single and double switches within individual 3-seeded pods. The most unusual pod originated from a homozygous aconitase-4 *aa* 'BSR 101' plant. This plant produced a pod that had the unusual aconitase-4- *bb* for one seed and *aa* for two seeds, i.e. a double switch. In 'BSR 101' we had 13 seed, representing 10 plants, with allele switches. In

‘Jack’ we had five seed, representing four plants, with allele switches. The unusual ‘allele switch’ plants were allowed to self-pollinate and the resulting homozygous genotypes were used in allelism tests with the appropriate testers. All new alleles were allelic to known aconitase-4 alleles. In addition, the seeds from self-pollination of the new homozygous genotypes were true breeding for the new allele. Characterization of molecular variants with InDel markers revealed unexpected variation in sexual hybridization and accelerated aging test experiments. Although it has been difficult to rule out selection of preexisting changes which appear to be spread through the genome, these findings indicate that there was an effect on the generation of endogenous variation in both cultivars for both experiments.

Introduction

Intracultivar variation refers to the genetic or phenotypic variation present from plant-to-plant within a cultivar. Although intracultivar variation has been recognized for several decades (Byth and Weber 1968), it is often ignored due to the belief that elite cultivars, especially cultivars from self-pollinated species, inherently represent a fairly homogeneous gene pool that is transmitted unchanged during reproduction (Orf et al. 2006; Fasoula and Boerma 2007). Nevertheless, increasing evidence of intracultivar 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)

Evidence of intracultivar variation in maize (*Zea mays* L.) was reported in doubled monohaploids lines and long-term inbred lines (Sprague et al. 1960; Russell et al. 1963; Bogenschutz and Russell 1986). According to the authors, these inbred lines accumulated

significant differences among means for several quantitative traits that exceeded commonly reported rates of spontaneous mutation. In flax (*Linum usitatissimum* L.), Durrant (1962, 1971) found heritable changes in stable flax lines, termed genotrophs, grown in specific environments, which persisted for a large number of generations. Genotrophs differed from one another and from the original plants in characteristics such as plant height and weight, total amount of nuclear DNA (Evans et al. 1966), and isozyme band mobility for peroxidase and acid phosphatase (Cullis and Kolodynska 1975). Further studies in flax, demonstrated that these changes were associated with non-random changes in the DNA sequences, and chromosome rearrangements (Cullis 1973; Schneeberger and Cullis 1991; Chen et al. 2005, 2009).

In barley (*Hordeum vulgare* L.), Rasmusson and Phillips (1997) noted that enough variation has remained to sustain breeding progress within elite gene pools of six-row Midwestern cultivars. Genetic gains from selection within barley populations derived from these closely related lines were attributed to enhanced genetic variance, from inherent mechanisms, which provided a continuing source of new genetic variation. Evidence of intracultivar variation also has been reported within lines of advanced generations. For example, in cotton (*Gossypium hirsutum* L.), Fasoulas (2000) reported within cultivar variation for yield and tolerance to Verticillium wilt, caused by *Verticillium dahliae* Kleb, after single-plant and progeny selection in an ultra-low planting, or Honeycomb design (Fasoula and Fasoula 1997). Similarly, for tomato (*Lycopersicon esculentum* L.), bread wheat (*Triticum aestivum* L.), and maize, single-plant selection at ultra-low planting densities was effective at revealing intracultivar 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).

Several genetic bottleneck events were experienced during domestication in soybean [*Glycine max* (L.) Merr.] and are proposed to have contributed to the reduction of genetic diversity and the loss of rare alleles (Hyten et al. 2006). Therefore, 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, Specht (2012) reported that soybean yields have increased from 1924 to 2010 at a linear rate of 23.3 kg ha⁻¹. This suggests that, although breeding processes normally use the parents of those with improved agronomic traits, it is possible to continue achieving agronomic improvement through breeding.

Intracultivar variation due to seed sources differences, which have been known to plant breeders for many years, also have been shown to be significant in the evaluation of cultivars (Fehr and Probst 1971). In soybean, Byth and Weber (1968) recognized significant phenotypic variability within F₅-derived lines for several agronomic traits, which was related to the degree of genetic heterogeneity within the evaluated derived populations.

Advances in DNA-based techniques, have allowed for the detection of genomic changes and has aided in understanding the possible ways genetic variation of individuals can be revealed. Using restriction fragment length polymorphism (RFLP), Roth et al. (1989), identified *de novo* variation in soybean inbred lines. This variation was identified in soybean tissue culture lines derived from the root tissue of single plants of ‘Minsoy’ (PI 27890) and ‘Noir 1’ (PI 290136), and from the root tissue of a F₁ hybrid plant between ‘Minsoy’ and ‘Noir 1’ (Roth et al. 1989). These lines generated novel RFLP alleles, which according to the

authors, already were found and characterized in other soybean cultivars, but not the ‘Minsoy’ or ‘Noir 1’ cultivars from which the explants were obtained. For example, new RFLP variants were observed in ‘Noir 1’, whose size corresponded to RFLP alleles already characterized in ‘Minsoy’ and vice versa. It seems that in response to stress, alleles can be switched to other known alleles.

Following single-plant selection from fields planted in a Honeycomb design, Fasoula and Boerma (2005) reported significant intracultivar variation in commercial soybean cultivars for seed protein, seed oil, and fatty acids within F₄- and F₅-derived cultivars, ‘Benning’, ‘Haskell’, and ‘Cook’. Intracultivar variation existed also for seed weight and other agronomic traits. 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, 2007b, 2007c). Using simple sequence repeat (SSR) markers, Yates et al. (2012) reported that between 82 and 93% of the variation detected by SSR markers in the ‘Benning’, ‘Haskell’, and ‘Cook’ foundation seed, could be traced to residual heterozygosity in the initial plant selections. 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. Similarly, within the soybean reference cultivar ‘Williams 82’, residual heterozygosity also has been documented (Haun et al. 2011). Individuals from different seed stocks of ‘Williams 82’ had genomic structural heterogeneity, differences in single nucleotide polymorphisms (SNPs), and variation in gene content (Haun et al. 2011).

The genetic mechanisms driving *de novo* variation remain unexplained. Researchers have proposed that processes such as spontaneous mutations, DNA transposition, DNA

methylation, gene duplication, unequal crossing over, and genome restoration might be contributing to the *de novo* variation (Sprague et al. 1960; Fukui 1983; Rasmusson and Phillips 1997; Lolle et al. 2005; Morgante et al. 2005; Hopkins et al. 2013; Kempinski et al. 2013).

The main 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 using genetic lines that have had documented instances of marker variation (Roth et al. 1989). Progeny of sexual crosses between soybean plant introductions ‘BSR 101’ (PI 548519), ‘Minsoy’ (PI 27890), and ‘Noir 1’ (PI 290136) were evaluated through the examination of segregation patterns for aconitase-2 and aconitase-4 enzyme variants, and segregation for DNA-based molecular makers. Since both proteins and DNA markers are inherited in Mendelian fashion and expressed co-dominantly, they can be used to provide individual profiles and be understood in genetic terms.

Materials and Methods

Plant material and seed source

Soybean plant introductions ‘BSR 101’ (PI 548519) (Tachibana et al. 1987), ‘Jack’ (PI 540556) (Nickell et al. 1990), ‘Minsoy’ (PI 27890) (introduced from France), and ‘Noir 1’ (PI 290136) (introduced from Hungary) were used in this study. Seed source for the sexual hybridization experiment was obtained from Dr. R.L. Nelson, USDA ARS at Urbana, IL. Seed source for the stress treatment experiment in pure lines was obtained from bulked seed of ‘BSR 101’, obtained from Dr. R.G. Palmer, Iowa State University at Ames, IA, and ‘Jack’ obtained from Dr. R. Shoemaker, USDA ARS at Ames, IA.

Sexual hybridization

CROSS POLLINATION AND GENERATION ADVANCE. Soybean plant introductions 'BSR 101', 'Minsoy', and 'Noir 1' were used to make the following cross-pollinations in 2007 at the Bruner Farm near Ames, Iowa: 'Noir 1' x 'BSR 101', 'Minsoy' x 'Noir 1', and 'Minsoy' x 'BSR 101'.

The F_1 seed were 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 seed 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.

SUMMER 2008; BRUNER FARM. 500 seed of 'BSR 101', 'Minsoy' and 'Noir 1' were analyzed for aconitase-2 and aconitase-4 and the seedlings transplanted to the Bruner Farm. Fifty seed 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 2007 October Puerto Rico planting were analyzed for aconitase isozyme variation and seedlings were transplanted to the Bruner Farm. The seeds used for this experiment included 50 F_2 seed from 18 F_1 plants representing 10 different 'Noir 1' female parent plants crossed to 'BSR 101'; 50 F_2 seed from 18 F_1 plants representing 9 different 'Minsoy' female parent plants crossed to 'BSR 101' and 50 F_2 seed from 19 F_1 plants representing 8 different 'Minsoy' female parent plants crossed to 'Noir 1'.

$F_{2:3}$ seed from self-pollinated F_2 plants of the three cross-combinations from the 2008 February Puerto Rico planting were analyzed for aconitase isozyme variation and seedlings were transplanted to the Bruner Farm. Two seed from each $F_{2:3}$ plant from all three cross-combinations were selected. The seeds used for this experiment included 352 $F_{2:3}$ seed from 176 F_2 plants from the 2008 February plots 6, 16, 17, 24, 25 and 27, representing ‘Noir 1’ as female parent plants crossed to ‘BSR 101’. Also included were 942 $F_{2:3}$ seed from 471 F_2 plants from the 2008 February 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 plants crossed to ‘BSR 101’. Additionally, 308 $F_{2:3}$ seed were included from 154 F_2 plants from the 2008 February plots 63, 64, 65, 66, 69, 70, 75, and 76 representing ‘Minsoy’ as female parent plants crossed to ‘Noir 1’.

Pure lines; stress treatment

SEED SOURCE AND STRESS TREATMENT. Soybean cultivars ‘BSR 101’ and ‘Jack’ were used to study the effect of seed stress in pure lines. In 2008, 500 plants of ‘BSR 101’ and ‘Jack’ were planted in a honeycomb design (Fasoula and Fasoula 1995), using an equidistant spacing of 2.0 m between each individual plant to eliminate the unfavorable effect of competition on response to selection (Fasoula and Boerma 2005). A code, termed “entry number,” was assigned to each individual plant. Field plots were damaged by natural hail in July 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-2 and aconitase-4.

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 (AOSA, 2002).

In the 2009 growing season, 50 seeds of each entry were treated at 41°C for 48 hours and were 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 aconitase isozyme.

Aconitase isozyme analysis

Starch gel electrophoresis techniques described by Cardy and Beversdorf (1984) were used to evaluate isozyme patterns at the aconitase-2 (*Aco-2*) and aconitase-4 (*Aco-4*) loci for the four plant introductions, the F₂ and F_{2:3} progenies from the three different cross combinations, and for the self-pollinated progeny of seed treated with an accelerated aging test.

Seeds were germinated on germination paper for 72 h at 30 °C in the dark. The three day-old seedlings were sampled by punching out three pieces of the cotyledon using a 200- μ L glass-bore pipettor. The samples were placed in 1.5 mL polypropylene microcentrifuge tubes to which 120 μ L cold extraction buffer [0.1 M tris-HCl, pH 7.2, 4% (wt/v) PVP-40 [polyvinylpyrrolidone, molecular weight 40,000], 400 mM sucrose, 1 mM dithiothreitol] were added. Samples were ground for 30 s by using a laboratory stirring motor (TRI-R STIR-R, Model S63C, Chicago, USA) 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 $10,000 \times g$ 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 of Cardy and Beversdorf (1984a). 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 MgCl₂, 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.

Aconitase-4, molecular mapping; DNA isolation and BSA

For the genetic linkage mapping, the F₂ population 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 ten homozygous *aa* or ten homozygous *bb* F₂ plants (Michelmore et al. 1991). Both bulks were diluted to 50 ng/µl final DNA concentration. Seven hundred simple sequence repeat (SSR) markers were tested on both bulks to detect polymorphisms between the bulks.

Aconitase-4, molecular mapping; molecular marker analysis

For the SSR analysis, 50 ng of DNA was used for a 10 µl reaction with 1x reaction buffer (10mM Tris-HCl, 50mM KCl, pH 8.3), 2.0 mM MgCl₂, 0.25µM 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 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 run on a 4 % agarose gel at 150 V for 2-4 h for separation. 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://www.phytozome.net/> (Song et al. 2004, 2010).

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 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 genotype (homozygous or heterozygous) and to estimate the segregation ratio. Chi-square tests of linkage and heterogeneity were calculated according to Mather (1951).

To estimate stable inheritance of the new alleles, homozygous *Aco4* 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.

Leaf collection and DNA analysis

One trifoliolate sample was collected from each plant, placed on ice and stored long-term at -80 °C. DNA was extracted using a modified extraction protocol (Edwards et al. 1991). DNA quantity and quality was assessed with the ND-1000 spectrophotometer (NanoDrop, Fisher Thermo, Wilmington, DE, USA). Molecular profiles were determined by *in vitro* DNA amplification using polymerase chain reaction (PCR). For the unusual variants detected in the sexual hybridization experiment, DNA of the F₁, F₂ and F_{2:3} plants, was analyzed using 11 insertion-deletion (InDel) markers (Table 1). For the progeny of unusual variants detected in the pure lines and stress treatment experiments nine additional InDel markers were used (Table 2). InDel markers were developed by Choi et al. (2003). The InDel markers are distributed across eight soybean chromosomes, and contain unique sequences when compared with the reference genome. These molecular markers consist of genomic DNA sequences between 12 and 48 nucleotides in length that are either present (insertion) or absent (deletion) in the genomic background. For InDel analyses, 10 ng of each DNA sample were used for 25 µl reaction with 2X reaction *Taq* buffer (500 mM KCl, 100 mM Tris-HCl, 15 mM MgCl₂, Triton x-100), 1 µl of each primer, 2.5 mM of each dNTP, and 0.2 units of *Taq* DNA polymerase (GenScript) per reaction. Amplifications were performed using a PTC-100 Programmable Thermal Controller (MJ Research, Inc., Waltham, MA, USA) programmed with the following conditions: temperature of 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 52 °C to 57 °C for 30 s, and 72 °C for 1 min; with a final extension at 72 °C for 7 min. The resulting PCR products were resolved on a 3% agarose/TBE gel stained with ethidium bromide (1 µg mL⁻¹) in 0.5X TBE at 100-120 V for 1 to 2 h. The band patterns were visualized and gels were photographed under UV light.

Images were saved using a Fotodyne Archiver Imaging System (Fotodyne, Inc., Harland, WI), and analyzed with an open source ImageJ analysis software (U.S. National Institutes of Health, Bethesda, MD). The fragment size of each PCR product was approximated by referencing the bands to a low molecular weight DNA ladder (New England BioLabs, Inc., Ipswich, USA).

Genotyping of aconitase-4 variants, sexual hybridization

DNA from F₁, F₂, and F_{2:3} samples of aconitase-4 variant A08-AS-2932 were analyzed with 11 InDel markers. Seventeen F_{2:3} lines from self-pollination of A08-AS-2932 were evaluated and compared with the DNA profile from the F₁ and F₂ samples. Also, progeny of five sibling plants to A08-AS-2932 (A08-AS-2905, A08-AS-2931, A08-AS-2933, A08-AS-2943, and A08-AS-2949) were evaluated and compared to the F₁ DNA profile.

Genotyping of aconitase-4 variants, pure lines

DNA from progeny resulting from the self-pollination of ‘BSR 101’ and ‘Jack’ aconitase-4 variants were evaluated for InDel markers. As a control, progeny of single plants grown in the honeycomb 2008 without accelerated aging treatments were analyzed. Additionally, a sample reference of ‘BSR 101’ and ‘Jack’ from the untreated seed source used for honeycomb experiments was examined.

For ‘BSR 101’, five randomly selected samples from self-pollinated progeny of the aconitase-4 variant *Aco4-bc* (BSR-301A) were evaluated. Additionally, five DNA samples from progeny of four self-pollinated *Aco4-cc* genotypes (A11-AS-156, A11-AS-156, A11-AS-156, and A11-AS-156) were analyzed for InDel markers.

For ‘Jack’, 16 randomly selected samples from self-pollinated progeny of the aconitase-4 variant *Aco4-bc* (Jack 127) were evaluated. Also, DNA samples from progeny of each of the three self-pollinated *Aco4-cc* genotypes (A12-AS-9, A12-AS-15, and A12-AS-19) were evaluated. The number of seedlings analyzed per plant varied between 14 and 19.

Results

Effect of sexual hybridization

SUMMER 2008; BRUNER FARM. 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 to the Bruner Farm. At the aconitase-4 locus ‘BSR 101’ was *Aco4-aa*, ‘Minsoy’ was *Aco4-cc*, and ‘Noir 1’ was *Aco4-bb*, as expected. For aconitase-2, all three plant introductions were *Aco2-bb*, as expected.

A total of 2204 F₂ progeny from the three different sexual crosses were assayed for aconitase-4 (Table 3). This includes a total of 789 F₂ plants derived from ‘Noir 1’ × ‘BSR 101’, 650 F₂ plants from ‘Minsoy’ × ‘Noir 1’ and 765 F₂ plants from ‘Minsoy’ × ‘BSR 101’. The F₂ genotypes fit the expected 1:2:1 genotypic ratio (Table 3), however there was one exception. This F₂ plant, from ‘Minsoy’ × ‘BSR 101’, A08-AS-2932, was *Aco4-ab*, an unexpected genotype (Figure 1). The F₂ homozygous genotypes were true breeding and the F₂ heterozygous genotypes segregated 1:2:1, as expected; (data not presented).

ACONITASE-4 VARIANT. F₂ 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-pollination seed of

the female parent ‘Minsoy’ plant 32 (A07-61-32) were analyzed for aconitase isozyme variation. All seed were *Aco4-cc* as expected. The male parent, ‘BSR 101’ was not identified by individual plant number in the cross-pollinations and therefore could not be tested.

The 49 sibling F₂ plants (A08-AS-2901 to A08-AS-2950; minus A08-AS-2932) from the 2007 October Puerto Rico planting did not show the aconitase-4 allele variant. A sibling F₁ plant that produced 50 F₂ plants (A08-AS-2951 to A08-AS-3000) did not show the aconitase-4 allele variant (Figure 1).

SUMMER 2009 AND 2010: BRUNER FARM; STABILITY TEST. As shown in Table 4, self-pollination of the novel *Aco4-ab* variant (A08-AS-2932) 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, A11-AS-185, A11-AS-191, and A11-AS-196 were used for additional aconitase determinations. Approximately 100 *Aco4-bb* plants were allowed to self-pollinate and a total of 302 seed from each of the three *Aco4-bb* plants analyzed for aconitase isozyme variation. These 302 F₃ plants were *Aco4-bb* as expected (Table 5). Thirty-nine of the 302 F₃ plants were used for an allelism test. A total of 221 seed were produced by manual cross-pollination.

SUMMER 2011: BRUNER FARM; ALLELISM TESTS. The 221 testcross seed from *Aco4-bb* (‘Noir 1’) × *Aco4-bb* genotypes were analyzed for aconitase isozyme variation and transplanted to the Bruner Farm. All 221 seed were *Aco4-bb*, the expected genotype (Table 6).

SUMMER 2008: BRUNER FARM; ACONITASE-2. The F₂ progeny from ‘Noir 1’ × ‘BSR 101’, ‘Minsoy’ × ‘Noir 1’, and ‘Minsoy’ × ‘BSR 101’ sexual crosses all scored *Aco2-bb* as expected.

Effect of seed stress in pure lines

FALL 2008. Samples of single plants of ‘BSR 101’ and ‘Jack’ harvested from the honeycomb 2008 experiment were analyzed for aconitase isozyme variation. At the aconitase-4 locus ‘BSR 101’ was *Aco4-aa* and *Aco4-bb*, and for ‘Jack’ was *Aco4-bb* and *Aco4-cc*. For aconitase-2, all three plant introductions were *Aco2-bb* as expected.

SUMMER 2009: BRUNER FARM; ACONITASE-2 AND ACONITASE-4. Single-three seeded pods from progeny of treated ‘BSR 101’ and ‘Jack’ were collected from three separate plants per entry per replication. A total of 1152 seed were analyzed separately per cultivar for aconitase-2 and aconitase-4. Progeny of both plant introductions were homozygous as expected for aconitase-2 isozyme (*Aco2-bb*). For aconitase-4, however, ‘BSR 101’ and ‘Jack’ showed *Aco4* variants identified in seeds from single pods collected from individual plants. Ten entries of ‘BSR 101’ and four entries of ‘Jack’ were characterized either by single allele switches, the change of one allele to another, or double allele switches, the change of both alleles to another for the *Aco4* isozyme (Table 7). In ‘BSR 101’, the expected isozyme pattern had single allele switches from *Aco4-aa* to *Aco4-ab* and from *Aco4-bb* to *Aco4-ab* or *Aco4-bc*. Single allele switches in ‘Jack’ were from *Aco4-bb* to *Aco4-bc* and 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 7).

‘BSR 101’ ENTRY 301; ACONITASE-4 VARIANT. A single seed from a three-seeded pod of ‘BSR-101’ entry 301 that scored heterozygous for aconitase-4 (*Aco4-bc*), originated from self-pollination of a ‘BSR-101’ entry 301 plant homozygous for aconitase-4 (*Aco4-bb*). This individual was expected to be an *Aco4-bb* homozygous, but instead was an *Aco4-bc* genotype, where the ‘c’ allele was unexpected.

SUMMER 2010 AND 2011: BRUNER FARM; STABILITY TEST. As shown in Table 8, self-pollination of the novel *Aco4-bc* variant (‘BSR-101’ entry 301) gave rise to progeny that segregated a good fit to the expected ratio (16 *Aco4-bb*: 52 *Aco4-bc*: 17 *Aco4-cc* plants). To confirm inheritance of the new allele ‘c’, approximately 60 seed from five plants (A11-AS-156, A11-AS-159, A11-AS-160, A11-AS-172, and A11-AS-175) of the 17 *Aco4-cc* plants were self-pollinated and the progeny were analyzed to determine the genotype (Table 9). All 298 plants were *Aco4-cc* as expected (Table 9).

SUMMER 2011: BRUNER FARM; ALLELISM TESTS. Four ‘BSR 101’ entry 301 *Aco4-cc* plants were crossed to ‘Minsoy’ (*Aco4-cc*); the standard *Aco4-cc* genotype. The number of testcross seed using four variant *Aco4-cc* plants varied from 8-17 seed, for a total of 50 testcross seed. All 50 seed were the expected *Aco4-cc* genotype (Table 10).

‘Jack’ ENTRY 127; ACONITASE-4 VARIANT. A single seed from a three-seeded pod of ‘Jack’ entry 127 that scored heterozygous for aconitase-4 (*Aco4-bc*), originated from

self-pollination of a ‘Jack’ entry 127 plant homozygous for aconitase-4 (*Aco4-bb*). This individual was expected to be an *Aco4-bb* homozygous, but instead was an *Aco4-bc* genotype, where the ‘c’ allele was unexpected (Table 7).

SUMMER 2011 AND 2012: BRUNER FARM; STABILITY TEST. As shown in Table 11, 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).

SUMMER 2012: BRUNER FARM; ALLELISM TESTS. Six ‘Jack’ entry 127 *Aco4-cc* plants were crossed to ‘Minsoy’ (*Aco4-cc*); the standard *Aco4-cc* genotype. The number of testcross seed using four variant *Aco4-cc* plants varied from 3-7 seed, for a total of 33 testcross seed.

Genetic linkage mapping; *Aconitase-4* gene

To find the location of the *Aconitase-4* gene, 700 SSR markers covering the entire soybean genome were tested on the bulks. Satt_509 showed polymorphism between the bulks, indicating the gene was on chromosome 11, MLG B1 (Song et al. 2004). Eighty-nine SSR markers on MLG B1 near Satt_509 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 Satt_509. The 12 SSR markers were tested on the entire population. It was found that *Aconitase-4* is flanked by

BARCSOYSSR_11_323 and BARCSOYSSR_11_336. BARCSOYSSR_11_336 was found to be 1.4 cM away from the gene (Figure 2). Using the soybean genome sequence (www.phytozome.net/) (Schmutz et al. 2010), a physical map of the markers in the vicinity of the *Aconitase-4* gene was created. The region between BARCSOYSSR_11_323 and BARCSOYSSR_11_336 is about 292 kb. We were able to use the soybean genome sequence flanked by these markers to locate putative genes present in the region. There are 40 predicted genes in this region (Table 12). One of the candidate genes, *Glyma11g08550*, codes for C-terminal domain of aconitase protein (Table 12). This analysis strongly suggests that most likely *Glyma11g08550* codes for *Aconitase-4* gene in soybean. Future studies focusing on characterization of the candidate gene may result in cloning of the *Aconitase-4* gene.

InDel genotyping, aconitase-4 variant; sexual hybridization

DNA from F₁, F₂, and F_{2:3} plants originating from the cross-pollination between ‘Minsoy’ and ‘BSR 101’ were analyzed with 11 InDel markers (Table 13). There were 17 F_{2:3} lines evaluated with each showing expected segregation in all of the InDel markers except for InDel marker BARC-065401-19428. This marker was discordant between the F₁-47-1 and the aconitase-4 variant (F₂-A08-AS-2932). According to these results, the genetic marker went from homozygous insertion in the F₁ to heterozygous in the F₂. The F_{2:3} lines were segregating as expected from a heterozygous parent F₂ plant.

Due to the discordance with InDel marker BARC-065401-19428, progeny of five sibling plants (A08-AS-2905, A08-AS-2931, A08-AS-2933, A08-AS-2943, and A08-AS-2949) also were analyzed. Although we did not analyze DNA from the F₂ plants, F_{2:3} lines

showed homozygous insertion as expected from a self-pollination of homozygous insertion F_1 parent (Table 13).

InDel genotyping, aconitase-4 variant; pure lines

DNA from progeny of both ‘BSR 101’ and ‘Jack’ variants for aconitase-4 (*Aco4-bc*) were analyzed using 20 InDel markers (Table 2). Selected homozygous *Aco4-cc* plants were allowed to self-pollinate and the resulting progeny were analyzed using the same InDel markers. As a control, additional analysis was performed on DNA samples from progeny of single plants grown in the honeycomb 2008 experiment. Finally, as a reference, ‘BSR 101’ and ‘Jack’ samples that were used as a seed source for honeycomb experiments also were analyzed. Both the control and reference DNA samples for ‘BSR 101’ and ‘Jack’ showed the expected profiles with all InDel markers. Progeny of aconitase-4 variants, however, showed unexpected DNA profiles (Table 14, 17).

For ‘BSR 101’, several InDel markers (BARC-055739-13673, BARC-028361-05843, BARC-014413-01360, BARC-001499-00119, BARC-019345-03881, BARC-007970-00182, BARC-028339-05837, and BARC-015905-02012) only showed unexpected DNA profiles for the control (‘BSR 101’ entry 301) when compared with the reference sample (DNA from seed source used for honeycomb experiments). Among the polymorphic InDel markers, four showed the expected DNA profile (BARC-025705-05003, BARC-065401-19428, BARC-017059-02188, and BARC-047166-12877).

Progeny of the aconitase-4 variant (‘BSR 101’ entry 301A) gave unexpected DNA profiles when compared with the reference sample (DNA from seed source used for honeycomb experiments) and with the control samples (DNA from progeny of plants grown in the honeycomb 2008 experiment with no accelerated aging treatment). Twelve out of 18

InDel markers gave unexpected DNA profiles when compared with the reference sample (BARC-055739-13673, BARC-028361-05843, BARC-014413-01360, BARC-065401-19428, BARC-001499-00119, BARC-047681-10375, BARC-019345-03881, BARC-017059-02188, BARC-047166-12877, BARC-007970-00182, BARC-028339-05837, and BARC-015905-02012) because they went from homozygous insertion to homozygous deletion or vice versa, indicating double allele switching (Table 14). Six out of 18, on the other hand, were unexpected when compared with the control (BARC-055739-13673, BARC-065401-19428, BARC-047681-10375, BARC-017059-02188, BARC-047166-12877, and BARC-015905-02012), because they went from homozygous insertion or homozygous deletion to heterozygous genotypes (Table 14). DNA samples from self-pollinated progeny for selected homozygous *Aco4-cc* gave the expected DNA profile when compared with the aconitase-4 variant samples.

For ‘Jack’, the control (HC-J-127) gave the expected DNA profile when compared with the reference sample with all of the InDel markers (Table 15). However, unexpected profiles were observed with six InDel markers (BARC-025705-05003, BARC-015905-02012, BARC-028361-05843, BARC-065341-19358, BARC-047166-12877, and BARC-007970-00182) when the progeny of aconitase-4 variant (Jack 127) were compared with the control samples. Some of these InDel markers went from homozygous insertion or deletion to heterozygous (BARC-025705-05003, BARC-015905-02012, BARC-028361-05843, and BARC-007970-00182), while the others went from homozygous insertion to homozygous deletion and or vice versa (BARC-065341-19358 and BARC-047166-12877). Progeny of selected homozygous *Aco4-cc* gave the expected DNA profile when compared with the aconitase-4 variant samples.

Discussion

Soybean cultivars are maintained as inbred lines that contain highly homozygous individuals. A variety of phenotypic and genotypic variants, however, have been identified and characterized agronomically and molecularly (Fasoula and Boerma 2005, 2007; Haun et al. 2011; Roth et al. 1989; Yates et al. 2012). Soybean allele-switching was noticed using restriction fragment length polymorphism (RFLP) markers in suspension cultures obtained from leaf, cotyledon, and stem tissues of ‘Minsoy’ and ‘Noir 1’ (Roth et al. 1989). In this study, the authors found that the cultures prepared from root tissue showed changes in a significant number of RFLP markers. Interestingly, they found that most of the newly generated RFLP alleles were the same as ones previously characterized in other cultivars. Although not published, unexpected variation for aconitase-2 and aconitase-4 isozymes also were identified in the progeny of sexual crosses using the same genetic material by Dr. R. G. Palmer (unpublished data). Roth et al. (1989) hypothesized that inbreeding organisms such as soybean have evolved internal generators of genetic variation in response to stress.

In our study, evidence of allele switching was obtained by following segregation patterns of the aconitase-4 isozyme in both the sexual hybridization and pure line experiments. Among the 765 F₂ plants from the cross-pollination between ‘Minsoy’ and ‘BSR 101’, one individual had switched from the expected heterozygous genotype *Aco4-ac* to *Aco4-ab* with the unexpected ‘b’ allele. In the pure-line experiment, progeny of plants that were grown in a honeycomb planting design, and treated with an accelerated aging test, resulted in genetic variation within ‘BSR 101’ and ‘Jack’ cultivars. The frequency of *Aco4* variants was 13 out of 1152 seeds for ‘BSR 101’ and 5 out of 1152 for ‘Jack’. Similar evidence of new isozyme variants has been reported as a consequence of genomic stress

(Amberger et al. (1992). In their study, the authors reported isozyme variants for *Aco2* via somatic embryogenesis. The frequency of somaclonal isozyme mutants in their study was 2 out of 185 regenerated plants. The genetic test for the evaluated *Aco4* variants, in both the sexual hybridization and pure-line experiments, indicated that these variants were inherited as a single-gene recessive. Similar to our study, Amberger et al. (1992) demonstrated that somatic embryogenesis in soybean generates isozyme variants that were heritable. Our method for isozyme analysis differs from methods used in previous studies because it was performed using tissue from the cotyledons of seedlings grown from individual seeds within single pods from each entry. This strategy not only indicated that the genomic variation was within plants in a cultivar, but also that variation existed within seed from individual pods collected from the same individual plant.

If outcrossing was not the source of observed variation in our case, then our findings were similar to the ones reported by Roth et al. (1989). In our study, we observed a low frequency of changes in the sexual hybridization experiment (1 out of 765 F₂ plants) when compared with the pure line experiment (13/1152 and 5/1152 for ‘BSR’ and ‘Jack’ respectively). According to Roth et al. (1989), the low frequency observed in cell cultures from the roots of hybrid plants, when compared with the frequency of change observed in cells from homozygous plants, suggests that heterozygosity may inhibit the process of variation.

Characterization of variants, sexual hybridization

Molecular analyses of the evaluated *Aco4* variants showed unexpected InDel marker variation. For the sexual hybridization experiment, the InDel marker BARC-065401-19428 was discordant between the F₁-47-1 and the F₂ aconitase-4 variant (F₂-A08-AS-2932). The

F₁ InDel marker went from a homozygous insertion to a heterozygous genotype in the F₂. F_{2:3} lines, which are progeny lines of the F₂ aconitase variant, gave the expected genotype when compared only with the F₂ parental genotype. To further investigate this event, we analyzed progeny of sibling plants of F₂-A08-AS-2932. Although we did not analyze DNA from F₂ plants, we verified that these F_{2:3} lines, the progeny of sibling plants, had the expected genotype which followed the F₁ segregation pattern. Unexpected InDel marker segregation between the F₁-47-1 and the F₂ aconitase-4 variant (F₂-A08-AS-2932) could be attributed to pollen contamination or to the *de novo* genetic variation induced by sexual hybridization.

Characterization of variants, pure line

For the pure-line experiments, there was a large number of InDel markers that were discordant between progeny of the aconitase-4 variant when compared with the reference sample and the control. For ‘BSR 101’, segregation patterns of eight InDel markers were discordant between the reference sample and the control and six were discordant between the control and the progeny of the aconitase-4 variant.

However, InDel marker segregation was as expected for selected homozygous *Aco4-cc* progeny from the aconitase-4 variant. Discordant markers were only detected in the progeny of the aconitase-4 variant. InDel markers went from homozygous insertion or deletion to InDel segregation in progeny of the aconitase-4 variant and outcrosses lead to heterozygous genotypes and the presence of non-parental alleles, as shown in our results.

Changes that occurred in the control (progeny of the single plants that were grown in honeycomb 2008 and affected by a natural-hail storm), when compared with the reference sample, are difficult to explain as a result of outcrossing. InDel markers went from

homozygous insertion to homozygous deletion or vice versa; in any case there was segregation in the individuals evaluated as a control. Although not characterized in this study, identified *Aco4-bb* variant plants could not be the result of an outcrossing event. In this case, the mother plant was *Aco4-aa* and both alleles switched to '*bb*'. If outcrossing was the source of this variation, at least one of the parental alleles should be present.

For 'Jack', the control gave the expected InDel marker genotypes when compared with the reference sample. However, discordant InDel markers were identified between the control and progeny of the aconitase-4 variant. Contrary to the findings for 'BSR 101', discordant InDel markers for 'Jack' went from homozygous insertion or deletion to heterozygous and from homozygous deletion to homozygous insertion or vice versa. Again, outcrossing could be explained by the segregation of InDels in progeny of the aconitase-4 variant, but in the case of homozygous InDel genotypes, outcrossing is the less likely explanation for these events.

In this study, there was only one aconitase-4 variant detected in the sexual hybridization experiment while 18 variants were detected in the pure-line experiments, 13 from 'BSR 101' and 5 from 'Jack' inbred lines. In the pure-line experiment, each of these variants was detected in single seed within three-seeded pods. Genetic and molecular analysis was only conducted on two variants, one from each cultivar. The novel alleles identified in these variants were not present in the parental lines and, in some cases, progeny plants appeared to have inherited insertion alleles from parental lines that had scored as homozygous for the deletion or vice versa. Since progeny from the sexual hybridization experiment originated from plants with known genotypes, marker discordance cannot be explained by residual heterozygosity.

In the pure-line experiment, aconitase-4 variants were detected in single seed within a three-seeded pod. These results indicate self-pollination and possibly even cross-pollination events within the same pod. To our knowledge, there are no reports of these two events within the same pod. Although pollen contamination has not been completely ruled out as the source of these genetic variants, outcrossing rates in the mainly autogamous domesticated soybean plant have been shown to be below 1% (Anderson and Vicente 2010). Weber and Fehr (1967) stated that natural cross-pollination in soybean is dependent upon the distance between plants, the environment, the genotypes, and the abundance of pollinators. In a study comparing emasculation and non-emasculation, as procedures for testing hybridization of soybeans, Walker et al. (1979) reported that emasculation did not result in the decrease of number of selfed seed. The percentage of selfed seed observed was 5.8% and 2.7% for emasculation and non-emasculation respectively. From 577 pods obtained, 22 were selfed seed and 2 pods contained both selfed and hybrid seeds. Natural hybridization between plants is known to occur in a range from 0.004 to 2.5% (Ahrent and Caviness 1994; Weber and Fehr 1967) and is reported to decrease rapidly with longer distances, dropping to less than 1.5% beyond 1 m and less than 0.1% beyond 2 m (Anderson and Vicente 2010). Ray et al. (2003) reported that cross-pollination rates ranged from 0.41% at 0.9 m from the pollen source to 0.003% at 5.4 m from the pollen source in soybean cultivars.

Plants grown in the honeycomb planting design were planted with an equidistant spacing of approximately 2 m between individual plants. The accelerated aging test experiment, however, used a row design with a row spacing of approximately 1 m and a 0.3 m gap between 1.5 m long entry plantings within each row. ‘BSR 101’ and ‘Jack’ entries were not mixed together, but rather planted in adjacent blocks. In the design, the only plants

from a given cultivar that were planted directly adjacent to plants from the other cultivar were minimized to those located in the border rows between blocks. This might suggest, if outcrossing has occurred in all of the identified variants including the ones not characterized in the pure-line experiment, that outcrossing rates could be exceeding the values of what has been previously reported. These higher rates of outcrossing would work out to be 1.13% and 0.43% for ‘BSR 101’ and ‘Jack’ respectively. Furthermore, these rates have been calculated using only the three randomly selected three-seeded pods per entry number.

Treatment effect

The accelerated aging test, through the use of high temperatures and high relative humidity, has been reported to cause seed stress (Parrish and Leopold 1978; Hsu et al. 2003). Treatments have been reported to lower germination rates, emergence and promote 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 that was affected by a natural-hail storm. Aconitase isozyme essays and InDel markers showed genetic variation within each cultivar, with the most predominance being within the ‘BSR 101’ cultivar. Rolling (2012) evaluated the effect of seed stress on agronomic traits such as plant height, plant maturity, and yield. In this study the author reported intracultivar variation for some traits, for example ‘Jack’ showed a decrease of 8% in yield for plants grown from the stressed seeds. These yield values ranged from 920 to 280 g. Although some single entries from within both cultivars consistently performed in the top 25% or top 10% of agronomic performance trait values, none of this was associated with changes observed for detected aconitase isozyme and InDel marker variants.

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

Undoubtedly, the combination of classical genetic analyses and molecular approaches has impacted the process of following genetic changes considerably and may even constitute contributing mechanisms to newly developed genetic diversity. Our results indicate there is intra-cultivar variation that could be further characterized. Although it is difficult to rule out selection of preexisting changes, it seems unlikely since we have observed a large number of specific changes which appear to be spread through the genome after stress treatments, i.e. sexual hybridization, accelerated aging test, etc. It is important to separate the effect of the honeycomb design from the accelerated aging test, as well as from the effect of the natural-hail that occurred in 2008 experiments.

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Table 1. InDel marker distribution, pair of primers used for InDel genotyping, amplicon product size, InDel size and expected polymorphism in progenies from cross-pollination between ‘Minsoy’ and ‘BSR 101’ inbred lines.

InDel marker	Chr ^a	LG ^b	Primer	Size ^c	InDel ^d	‘Minsoy’ ^e	‘BSR 101’ ^f
BARC-025705-05003	6	C2	F: TCTAACTCAGTTGATTGATG R: TTCGGTCAATCAGAACTGAAG	188	26	I	D
BARC-017117-02205	7	M	F: CATGTTTCAGCAGAAGGAGAG R: GTGAATTCGTAAGTAGTCATTTTC	177	27	I	D
BARC-028361-05843	8/5	A2/A1	F: CTTGCTGCAGTTGAAGAACCAAC R: CTAGTTATGTCATCATTGTCATG	163	26	D	D
BARC-014413-01360	9	K	F: TAGAGCCACCCTTTATGTCATGTTAC R: GGTTTCCACATTACATGCATAG	170	48	I	I
BARC-065401-19428	10	O	F: CAAAGGTGAATTCTATCTC R: TGATTATCCTTGTGCAAGTAC	164	26	I	I
BARC-030173-06820	12	H	F: GAACAAGAATCATAGACATG R: CGTTACTCCTAATAATTTAGC	153	30	I	D
BARC-007975-00194	13	F	F: CTCAAACATTAATATTTCCATCC R: TTGAGCATCAGGTGTGTTTG	161	27	I	D
BARC-014619-01598	17	D2	F: GATATCACATAATCATAACACG R: ATACTCCAGGGTACGAATATC	268	30	I	I
BARC-019505-03652	17	D2	F: AGAAGCTTCCGTTGGTGTC R: ACATACTTTAATTTGATTTAG	158	27	I	I
BARC-012245-01768	18	G	F: CATTACAAGACTTTCTCTATTACTTG R: TGAAAATCAATTTATCAAGAATTG	328	21	I	I
BARC-055739-13673	19	L	F: GCAAATCCTTCCGTGATAG R: TTGCTAACCAAGTAGAGTCAC	143	33	I	D

^a Chr: Chromosome number where the InDel is located. In some cases an alignment pair (query to hit) has high bit score and expect value (E-value) in two chromosomes. Example, BARC-017059-2188.

^b LG: Denotes linkage group

^c PCR product size (bp)

^d Insert size (bp)

^e Expected InDel polymorphism for ‘Minsoy’; “I” denotes insertion, “D” denotes deletion.

^f Expected InDel polymorphism for ‘BSR 101’; “I” denotes insertion, “D” denotes deletion.

Table 2. InDel marker distribution, pair of primers used for InDel genotyping, amplicon product size, InDel size and expected polymorphism in 'BSR 101 and 'Jack' inbred lines.

InDel marker	Chr ^a	LG ^b	Primer	Size ^c	InDel ^d	'BSR 101' ^e	'Jack' ^f
BARC-015905-02012	5	A1	F: ATTTACTAAACTATGCTAGCTTTG R: TGTGGGACCTGCTATACTG	143	14	D	I
BARC-047166-12877	5	A1	F: TTGATTAAGGTTGTGTTGTG R: AAGTAGCAATTTTACCTCTACTCC	120	12	I	D
BARC-017059-02188	5/17	A1/D2	F: GAGCACTATCAATGTCAGAC R: AGCATCACTAATTGTTGCTG	111	19	D	I
BARC-025705-05003	6	C2	F: TCTAACTCAGTTGATTGATG R: TTCGGTCAATCAGAACTGAAG	188	26	D	I
BARC-017117-02205	7	M	F: CATGTTTCAGCAGAAGGAGAG R: GTGAATTCGTAAGTAGTCATTTTC	177	27	D	D
BARC-028361-05843	8/5	A2/A1	F: CTTGCTGCAGTTGAAGAACCAAC R: CTAGTTATGTCATCATTGTCATG	163	26	D	I
BARC-014413-01360	9	K	F: TAGAGCCACCCTTTATGTCATGTTAC R: GGTTTCCACATTCACATGCATAG	170	48	I	D
BARC-065401-19428	10	O	F: CAAAGGTGAATTCTATCTC R: TGATTATCCTTGTGCAAGTAC	164	26	I	D
BARC-030173-06820	12	H	F: GAACAAGAATCATAGACATG R: CGTTACTCCTAATAATTTAGC	153	30	D	D
BARC-007975-00194	13	F	F: CTCAAACATTAATATTTCCATCC R: TTGAGCATCAGGTGTGTTTG	161	27	D	D
BARC-028339-05837	16	J	F: CACTCATTCTGGTCTTTAGGAC R: AAAGTCACCTAGCCTTCATTG	163	18	D	I
BARC-065341-19358	17	D2	F: AGCTTAAAATTAAGGAAATTG R: TTATAATGGTGCTGACTG	126	19	D	I
BARC-014619-01598	17	D2	F: GATATCACATAATCATAACACG R: ATACTCCAGGGTACGAATATC	268	30	I	I
BARC-019505-03652	17	D2	F: AGAAGCTTCCGTTGGTGTC R: ACATACTTTAATTGATTTTAG	158	27	I	I

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Table 2. Continued

InDel marker	Chr ^a	LG ^b	Primer	Size ^c	InDel ^d	'BSR 101' ^e	'Jack' ^f
BARC-047681-10375	18	G	F: ATGAGCATGGATTGCAAC R: CAAAGGGTTAGAGAAGACTGAG	159	15	D	I
BARC-019345-03881	18	G	F: GAATAATGAGAATGAAAGTTCTCC R: CGTTATTTTCGTACTTATTTTG	115	15	D	I
BARC-012245-01768	18	G	F: CATTACAAGACTTTCTCTATTACTTG R: TGAAAATCAATTTATCAAGAATTG	328	21	I	I
BARC-055739-13673	19	L	F: GCAAATCCTTCCGTGATAG R: TTGCTAACCAAGTAGAGTCAC	143	33	D	D
BARC-001499-00119	20/10	I/O	F: GGATTGGTAAGTATCATCCAAC R: CATGTTTTAGTTAAATACATG	132	21	I	D
BARC-007970-00182	20/10	I/O	F: GACCCATATGAATTTTATCCAAC R: TTATCTATTGGACACAACCTCTCGC	132	21	I	D

^a Chr: Chromosome number where the InDel is located. In some cases an alignment pair (query to hit) has high bit score and expect value (E-value) in two chromosomes. Example, BARC-017059-2188.

^b LG: Denotes linkage group

^c PCR product size (bp)

^d Insert size (bp)

^e Expected InDel polymorphism for 'BSR 101'; "I" denotes insertion, "D" denotes deletion.

^f Expected InDel polymorphism for 'Jack'; "I" denotes insertion, "D" denotes deletion.

Table 3. Genotypic frequency of progeny of self-pollination of F₁ 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 ^z			No. F ₂ plants	χ^2 (1:2:1) ^y	P ^x
<i>Aco4-bb</i> × <i>Aco4-aa</i> 'Noir 1' × 'BSR 101'	<i>Aco4-ab</i>	18	<i>Aco4-aa</i> 215	<i>Aco4-ab</i> 373	<i>Aco4-bb</i> 201	789	2.83	0.24
<i>Aco4-cc</i> × <i>Aco4-bb</i> 'Minsoy' × 'Noir 1'	<i>Aco4-bc</i>	19	<i>Aco4-bb</i> 154	<i>Aco4bc</i> 328	<i>Aco4-cc</i> 168	650	0.66	0.72
<i>Aco4-cc</i> × <i>Aco4-aa</i> 'Minsoy' × 'BSR 101'	<i>Aco4-ac</i>	18	<i>Aco4-aa</i> 201	<i>Aco4-ac</i> 378	<i>Aco4-cc</i> 186	765	0.68	0.71

^zGenotype determined by progeny test from self-pollination of *Aco4-ab*, *Aco4-cb*, and *Aco4-ac* plants.

^yChi-square values calculated to test goodness of fit to a 1:2:1 ratio.

^xP = probability of a greater value of chi-square.

Table 4. Genotypic frequency of progeny from self-pollination of aconitase F₂ variant plant A08-AS-2932 (*Aco4-ab*) derived from a cross between ‘Minsoy’ (*Aco4-cc*) × ‘BSR 101’ (*Aco4-aa*)

Aconitase-4 profiles ^z	No. plants
<i>aa</i>	17
<i>ab</i>	45
<i>bb</i>	16
χ^2 (1:2:1) ^y	1.87
<i>P</i> ^x	0.4

^zGenotype determined by progeny test from self-pollination of *Aco4-ab* plant (A08-AS-2932).

^yChi-square values calculated to test goodness of fit to a 1:2:1 ratio.

^x*P* = probability of a greater value of chi-square.

Table 5. Genotypic frequency of progeny from self-pollination of three homozygous *Aco4-bb* plants (A11-AS-185, A11-AS-191, and A11-AS-196) derived from self-pollination of A08-AS-2932 (*Aco4-ab*)

Parent A11-AS-	<i>Aconitase-4</i> profiles ^z	No. plants
185	<i>bb</i>	100
191	<i>bb</i>	103
196	<i>bb</i>	99

^zGenotype determined by progeny test from self-pollination of *Aco4-bb* plants (A11-AS-185, A11-AS-191, and A11-AS-196).

Table 6. Allelism test (testcrosses) of progeny from three homozygous *Aco4-bb* plants (A11-AS-185, A11-AS-191, and A11-AS-196), derived from self-pollination of A08-AS-2932 (*Aco4-ab*), and crossed to ‘Noir 1’ (*Aco4-bb*) as female parent.

Parent A11-AS-	No. plants used in testcrosses ^z	Range of the no. of hybrid seed from each male parent used in testcrosses	No. plants	Aconitase-4 profiles
185	10	3-10	50	<i>bb</i>
191	17	1-9	80	<i>bb</i>
196	12	2-12	91	<i>bb</i>

^zFor example; 10 of 100 *Aco4-bb* plants derived from self-pollination of A11-AS-185 were used as male parents crossed with ‘Noir 1’ as female parent. See Table 3 for number of plants of A11-AS-185, A11-AS-191, and A11-AS-196.

Table 7. Summary of aconitase-4 (*Aco4*) variants found in individual seeds from single three-seeded pods harvested from ‘BSR 101’ and ‘Jack’ plants.

Entries with <i>Aco4</i> variant ^z	Repetition number ^y	Expected isozyme pattern ^x	<i>Aco4</i> variant ^w
BSR 101-4-1	2	<i>aa</i>	<i>ab</i>
BSR 101-6-1	2	<i>bb</i>	<i>ab</i>
BSR 101-34-3	1	<i>aa</i>	<i>ab</i>
BSR 101-34-3	1	<i>aa</i>	<i>bb</i>
BSR 101-46-1	2	<i>aa</i>	<i>bb</i>
BSR 101-78-1	2	<i>aa</i>	<i>ab</i>
BSR 101-160-3	1	<i>aa</i>	<i>ab</i>
BSR 101-160-3	1	<i>aa</i>	<i>ab</i>
BSR 101-160-3	1	<i>aa</i>	<i>ab</i>
BSR 101-190-3	1	<i>aa</i>	<i>ab</i>
BSR 101-213-1	2	<i>aa</i>	<i>ab</i>
BSR 101-301-1	2	<i>bb</i>	<i>bc</i>
BSR 101- 335-1	1	<i>bb</i>	<i>ab</i>
Jack – 26-1	2	<i>bb</i>	<i>bc</i>
Jack – 28-2	2	<i>cc</i>	<i>bc</i>
Jack – 28-2	2	<i>cc</i>	<i>bc</i>
Jack – 77-2	2	<i>cc</i>	<i>bc</i>
Jack – 127-1	1	<i>cc</i>	<i>bc</i>

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Table 7. Continued

^zEntries with *Aco4* variant labeled in the following format: cultivar name

- entry number - pod number. Three, three seeded pods were collected from each entry.

^yTwo repetitions of each entry number were planted.

^xExpected isozyme 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.

^w*Aco4* variant represents the new isozyme pattern for each seed with single or double-allele switching.

Table 8. Genotypic frequency of progeny of self-pollination of aconitase variant plant 'BSR 101' entry 301 (*Aco4-bc*)

Genotype ^z	Number of plants
<i>bb</i>	16
<i>bc</i>	52
<i>cc</i>	17
χ^2 ^y	4.27
<i>P</i> ^x	0.12

^zGenotype determined by progeny test from self-pollination of *Aco4-bc* plant ('BSR 101' entry 301)

^yChi-square values calculated to test goodness of fit to a 1:2:1 ratio.

^x*P* = probability of a greater value of chi-square.

Table 9. Genotypic frequency of progeny from self-pollination of five homozygous *Aco4-cc* plants (A11-AS-156, A11-AS-159, A11-AS-160, A11-AS-172, and A11-AS-175) derived from self-pollination of 'BSR 101' entry 301 (*Aco4-bc*)

Parent A11-AS-	<i>Aconitase-4</i> profiles ^z	No. plants
156	<i>cc</i>	60
159	<i>cc</i>	60
160	<i>cc</i>	60
172	<i>cc</i>	59
175	<i>cc</i>	59

^zGenotype determined by progeny test from self-pollination of *Aco4-cc* plants (A11-AS-156, A11-AS-159, A11-AS-160, A11-AS-172, and A11-AS-175).

Table 10. Allelism test (testcrosses) of progeny of three homozygous *Aco4-cc* plants (A11-AS-156, A11-AS-159, A11-AS-160, and A11-AS-175) derived from self-pollination of ‘BSR 301’ entry 301 (*Aco4-bc*) crossed to ‘Minsoy’ (*Aco4-cc*) as female parent.

Parent A11-AS-	No. plants used in testcrosses ²	No. plants	Aconitase-4 profiles
156	14	3	<i>cc</i>
159	17	3	<i>cc</i>
160	8	2	<i>cc</i>
175	11	3	<i>cc</i>

Table 11. Genotypic frequency of progeny from self-pollination of aconitase variant plant 'Jack' entry 127 (*Aco4-bc*).

Genotype ^z	Number of plants
<i>bb</i>	12
<i>bc</i>	24
<i>cc</i>	14
χ^2 ^y	0.08
<i>P</i> ^x	1

^zGenotype determined by progeny test from self-pollination of *Aco4-bc* plant ('Jack' entry 127)

^yChi-square values calculated to test goodness of fit to a 1:2:1 ratio.

^x*P* = probability of a greater value of chi-square.

Table 12. Genes present in the *Aconitase-4* region. Name and predicted functions of the putative proteins encoded by 40 genes that are flanked by BARCSOYSSR_11_323 and BARCSOYSSR_11_336 on Gm11 (MLG B1) are shown. Gene of interest is shown in bold font.

Gene	Start Position	End Position	Predicted Protein/ Function
Glyma11g08190	5799683	5800802	DnaJ Domain; heat shock protein building
Glyma11g08210	5814338	5816899	Armadillo/beta-catenin-like repeat
Glyma11g08230	5828902	5832369	Oxidoreductase NAD-binding domain; Oxidoreductase FAD-binding domain
Glyma11g08240	5836830	5838915	Thaumatococcus family
Glyma11g08250	5849969	5852241	Thaumatococcus family
Glyma11g08260	5858172	5860046	Rhodanese-related sulfurtransferase
Glyma11g08275	5860216	5865580	Mlo family; cell death; integral to membrane
Glyma11g08290	5867998	5874448	Ubiquitin interaction motif; 26S proteasome non-ATPase regulatory subunit 4
Glyma11g08300	5876589	5882182	Senescence-associated protein

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Table 12. Continued

Glyma11g08310	5890482	5898263	Histidine kinase-, DNA gyrase B, and HSP90-like ATPase; Response regulator receiver domain; regulation of transcription, DNA-dependent
Glyma11g08320	5900559	5903101	Peroxidase; oxidation reduction; heme binding
Glyma11g08330	5904208	5907846	Protein of unknown function
Glyma11g08340	5910216	5913305	None
Glyma11g08350	5915036	5918910	3-dehydroquinate synthase
Glyma11g08360	5921704	5923940	PPR repeat
Glyma11g08365	5923506	5926312	None
Glyma11g08370	5928196	5932492	Isocitrate/isopropylmalate dehydrogenase
Glyma11g08380	5933800	5936481	Ras family; Rho type; GTP binding
Glyma11g08390	5937829	5946945	Vacuolar protein sorting-associated protein 35; Membrane coat complex Retromer
Glyma11g08400	5951533	5951533	Copper chaperone; metal ion transport. Metal ion binding
Glyma11g08420	5959575	5962576	GDSL-like Lipase/Acylhydrolase; zinc finger fyve domain containing protein

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Table 12. Continued

Glyma11g08430	5970175	5976464	PLAC8 family
Glyma11g08440	5978465	5980679	tetramerisation domain; SCF ubiquitin ligase
Glyma11g08450	5981810	5983729	PPR repeat
Glyma11g08470	5986226	5987561	Trm112p-like protein
Glyma11g08480	5990036	5991373	Zinc finger, C3HC4 type (ring finger)
Glyma11g08500	6008627	6013845	Lupus la ribonucleoprotein; RNA-binding protein LARP/SRO9
Glyma11g08510	6018136	6019954	Putative methyltransferase
Glyma11g08520	6020442	6022324	Peroxidase; heme binding
Glyma11g08530	6026622	6030797	Eukaryotic aspartyl protease; proteolysis and peptidolysis
Glyma11g08540	6035792	6038920	Ring finger protein 11
Glyma11g08550	6048905	6056598	Aconitase C-terminal domain; 3-isopropylmalate dehydrase subunit; RNA-binding translational regulator IRP
Glyma11g08560	6058479	6061791	Prolyl 4-hydroxylase alpha subunit; oxidoreductase activity

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Table 12. Continued

Glyma11g08570	6069900	6072242	None
Glyma11g08580	6074093	6077300	Peroxidase; heme binding
Glyma11g08590	6078276	6079157	None
Glyma11g08601	6080626	6081150	NADH-Ubiquinone/plastoquinone (complex 1); NADH Dehydrogenase; ATP synthesis coupled electron transport
Glyma11g08610	6084908	6093534	RNA polymerase Rpb1
Glyma11g08621	6094773	6095625	None
Glyma11g08630	6098360	6101239	PPR repeat

Table 13. Summary of InDel markers profiles for aconitase-4 variant in the sexual hybridization experiment

	InDel	00194	01598	01768	02205	03652	05003	13673	05843	01360	06820	19428
Plant sample		M=I B=D	MB=I	MB=I	M=I B=D	MB=I	M=I B=D	M=I B=D	MB=D	MB=I	M=I B=D	MB=I
F1 47-1	<i>Aco-4</i>	H	I	I	H	I	H	H	D	I	H	I
F2-A08-AS-2932		H	I	I	H	I	D	H	D	I	D	H?
F2:3 UW_AS_2932_26	<i>aa-ab-bb?</i>	H	I	I	D	I	D	H	D	I	D	I
F2:3 UW_AS_2932_27	<i>aa-ab-bb?</i>	H	I	I	H	I	D	I	D	I	D	D
F2:3 UW_AS_2932_30	<i>aa-ab-bb?</i>	H	I	I	H	I	D	D	D	I	D	H
F2:3 UW_AS_2932_31	<i>aa-ab-bb?</i>	D	I	I	H	I	D	I	D	I	D	H
F2:3 UW_AS_2932_32	<i>aa-ab-bb?</i>	D	I	I	D	I	D	H	D	I	D	I
F2:3 UW_AS_2932_33	<i>aa-ab-bb?</i>	D	I	I	I	I	D	D	D	I	D	H
F2:3 UW_AS_2932_34	<i>aa-ab-bb?</i>	I	I	I	D	I	D	I	D	I	D	H
F2:3 UW_AS_2932_35	<i>aa-ab-bb?</i>	H	I	I	H	I	D	D	D	I	D	H
F2:3 UW_AS_2932_36	<i>aa-ab-bb?</i>	H	I	I	D	I	D	H	D	I	D	I
F2:3 UW_AS_2932_37	<i>aa-ab-bb?</i>	I	I	I	D	I	D	H	D	I	D	H
F2:3 UW_AS_2932_38	<i>aa-ab-bb?</i>	I	I	I	H	I	D	I	D	I	D	H
F2:3 UW_AS_2932_39	<i>aa-ab-bb?</i>	H	I	I	I	I	D	I	D	I	D	H
F2:3 UW_AS_2932_40	<i>aa-ab-bb?</i>	H	I	I	H	I	D	D	D	I	D	I
F2:3 UW_AS_2932_41	<i>aa-ab-bb?</i>	H	I	I	H	I	D	D	D	I	D	D
F2:3 UW_AS_2932_42	<i>aa-ab-bb?</i>	H	I	I	I	I	D	H	D	I	D	D
F2:3 UW_AS_2932_43	<i>aa-ab-bb?</i>	D	I	I	H	I	D	H	D	I	D	H
F2:3 UW_AS_2932_44	<i>aa-ab-bb?</i>	D	I	I	D	I	D	I	D	I	D	I

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Table 13. Continued

	<i>Aco4</i>	00194	01598	01768	02205	03652	05003	13673	05843	01360	06820	19428
F2:3 A08-AS-2905-1	<i>aa-ac-cc?</i>	I	I	I	I	I	I	D	D	I	I	I
F2:3 A08-AS-2905-2	<i>aa-ac-cc?</i>	I	I	I	D	I	I	D	D	I	D	I
F2:3 A08-AS-2905-3	<i>aa-ac-cc?</i>	na	I	I	I	I	I	D	D	I	D	I
F2:3 A08-AS-2905-4	<i>aa-ac-cc?</i>	I	I	I	na	I	I	D	D	I	D	I
F2:3 A08-AS-2905-5	<i>aa-ac-cc?</i>	I	I	I	D	I	I	D	D	I	I	I
F2:3 A08-AS-2931-1	<i>aa-ac-cc?</i>	D	I	I	I	I	D	H	D	I	I	I
F2:3 A08-AS-2931-2	<i>aa-ac-cc?</i>	D	I	I	D	I	D	D	D	I	I	I
F2:3 A08-AS-2931-3	<i>aa-ac-cc?</i>	D	I	I	D	I	D	D	D	I	I	I
F2:3 A08-AS-2931-4	<i>aa-ac-cc?</i>	na	I	I	D	I	D	H	D	I	I	I
F2:3 A08-AS-2931-5	<i>aa-ac-cc?</i>	na	I	I	I	I	D	I	D	I	I	I
F2:3 A08-AS-2931-6	<i>aa-ac-cc?</i>	na	I	I	I	I	D	D	D	I	I	I
F2:3 A08-AS-2933-1	<i>aa-ac-cc?</i>	na	I	I	I	I	I	D	D	I	D	I
F2:3 A08-AS-2933-2	<i>aa-ac-cc?</i>	na	I	I	I	I	I	H	D	I	D	I
F2:3 A08-AS-2943-1	<i>aa-ac-cc?</i>	I	I	I	I	I	I	D	D	I	H	I
F2:3 A08-AS-2943-2	<i>aa-ac-cc?</i>	I	I	I	I	I	I	H	D	I	H	I
F2:3 A08-AS-2943-3	<i>aa-ac-cc?</i>	na	I	I	D	I	I	I	D	I	H	I
F2:3 A08-AS-2943-4	<i>aa-ac-cc?</i>	na	I	I	I	I	I	D	D	I	D	I
F2:3 A08-AS-2949-1	<i>aa-ac-cc?</i>	H	I	I	na	I	I	D	D	I	D	I
F2:3 A08-AS-2949-2	<i>aa-ac-cc?</i>	na	I	I	I	I	I	D	D	I	D	I
F2:3 A08-AS-2949-3	<i>aa-ac-cc?</i>	na	I	I	I	I	I	D	D	I	D	I
F2:3 A08-AS-2949-4	<i>aa-ac-cc?</i>	H	I	I	I	I	I	H	D	I	D	I
F2:3 A08-AS-2949-5	<i>aa-ac-cc?</i>	H	I	I	I	I	D	H	D	I	D	I
F2:3 A08-AS-2949-6	<i>aa-ac-cc?</i>	D	I	I	I	I	D	H	D	I	D	I
F2:3 A08-AS-2949-7	<i>aa-ac-cc?</i>	H	I	I	I	I	D	D	D	I	D	I

M = 'Minsoy', B = 'BSR 101', I = insertion, D = deletion, H = heterozygous, NA = not available

Table 14. Summary of InDel markers profiles for aconitase-4 variant in ‘BSR 101’ entry 301 in the pure-line experiment

	Indel markers	01598	01768	02205	03652	05003	13673	06820	05843	01360	19428	00119	10375	03881	02188	12877	00182	05837	02012
Plant sample	<i>Aco-4</i>																		
BSR-101 ref	<i>bb</i>	I	I	D	I	D	D	D	D	I	I	I	D	D	D	I	I	D	D
Jack ref	<i>bb</i>	I	I	D	I	I	D	D	I	D	D	D	D	I	I	D	D	I	I
HC-BSR-2	<i>bb</i>	I	I	D	I	D	I	D	I	D	I	D	D	I	D	I	D	I	I
HC-BSR-7	<i>bb</i>	I	I	D	I	D	I	D	I	D	I	D	D	I	D	I	D	I	I
HC-BSR-11	<i>bb</i>	I	I	D	I	D	I	D	I	D	I	D	D	I	D	I	D	I	I
HC-BSR-18	<i>bb</i>	I	I	D	I	D	I	D	I	D	I	D	D	I	D	I	D	I	-
HC-BSR-15	<i>bb</i>	I	I	D	I	D	I	D	I	D	I	D	D	I	D	I	D	I	I
BSR-301A-2	<i>bb,bc, cc?</i>	I	I	D	I	D	H	D	I	D	I	D	D	I	I	I	D	I	-
BSR-301A-5	<i>bb,bc, cc?</i>	I	I	D	I	D	D	D	I	D	H	D	H	I	D	I	D	I	-
BSR-301A-10	<i>bb,bc, cc?</i>	I	I	D	I	D	H	D	I	D	D	D	H	I	H	D	D	I	H
BSR-301A-13	<i>bb,bc, cc?</i>	I	I	D	I	D	H	D	I	D	D	D	H	I	I	I	D	I	D
BSR-301A-17	<i>bb,bc, cc?</i>	I	I	D	I	D	H	D	I	D	D	D	D	I	I	D	D	I	H
AS-156-4	<i>cc</i>	I	I	D	I	D	D	D	I	D	D	D	D	I	I	D	D	I	H
AS-156-10	<i>cc</i>	I	I	D	I	D	H	D	I	D	H	D	H	I	I	-	D	I	H
AS-156-14	<i>cc</i>	I	I	D	I	D	H	D	I	D	H	D	D	I	I	H	D	I	D
AS-156-19	<i>cc</i>	I	I	D	I	D	H	D	I	D	H	D	H	I	I	I	D	I	I
AS-156-20	<i>cc</i>	I	I	D	I	D	I	D	I	D	H	D	H	I	I	D	D	I	D

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Table 14. Continued

	Indel markers	01598	01768	02205	03652	05003	13673	06820	05843	01360	19428	00119	10375	03881	02188	12877	00182	05837	02012
AS-159-4	cc	I	I	D	I	D	I	D	I	D	-	D	D	I	H	D	D	I	H
AS-159-8	cc	I	I	D	I	D	I	D	I	D	H	D	D	I	H	D	D	I	-
AS-159-12	cc	I	I	D	I	D	I	D	I	D	D	D	D	I	H	D	D	I	-
AS-159-15	cc	I	I	D	I	D	I	D	I	D	D	D	D	I	I	D	D	I	D
AS-159-20	cc	I	I	D	I	D	I	D	I	D	I	D	D	I	I	D	D	I	H
AS-160-4	cc	I	I	D	I	D	I	D	I	D	H	D	I	I	H	D	D	I	I
AS-160-9	cc	I	I	D	I	D	I	D	I	D	D	D	I	I	H	D	D	I	I
AS-160-11	cc	I	I	D	I	D	I	D	I	D	I	D	I	I	D	D	D	I	I
AS-160-13	cc	I	I	D	I	D	I	D	I	D	H	D	I	I	H	D	D	I	I
AS-160-15	cc	I	I	D	I	D	I	D	I	D	-	D	I	I	H	D	D	I	I
AS-175-3	cc	I	I	D	I	D	I	D	I	D	-	D	D	I	I	I	D	I	D
AS-175-9	cc	I	I	D	I	D	I	D	I	D	I	D	D	I	I	I	D	I	H
AS-175-13	cc	I	I	D	I	D	I	D	I	D	H	D	D	I	I	I	D	I	D
AS-175-16	cc	I	I	D	I	D	I	D	I	D	-	D	D	I	I	I	D	I	I
AS-175-19	cc	I	I	D	I	D	I	D	I	D	H	D	D	I	I	I	D	I	H

HC = Honeycomb, B = 'BSR 101' variant, AS = homozygous *Aco4*, I = insertion, D = deletion, H = heterozygous, - = not available

Table 15. Summary of InDel markers profiles for aconitase-4 variant in ‘Jack’ entry 127 in the pure-line experiment

Indel markers		00194	01598	01768	02205	03652	05003	13673	06820	19428	02012	05843	19358	02188	12877	00182	05837	01360
Plant sample	<i>Aco-4</i>	LAB	LAB	LAB	LAB	LAB	LAB	LAB	LAB	LAB	DNA F	DNA F	DNA F	DNA F	DNA F	DNA F	DNA F	DNA F
BSR-101 ref	<i>bb</i>	D	I	I	D	I	D	D	D	I	D	D	D	D	I	I	D	I
Jack ref	<i>bb</i>	D	I	I	D	I	I	D	D	D	I	I	I	I	D	D	I	D
HC-J-127-1	<i>bb</i>	D	I	I	D	I	I	D	D	D	I	I	I	I	D	D	I	D
HC-J-127-2	<i>bb</i>	D	I	I	D	I	I	D	D	D	I	I	I	I	D	D	I	D
HC-J-127-3	<i>bb</i>	D	I	I	D	I	I	D	D	D	I	I	I	I	D	D	I	D
HC-J-127-4	<i>bb</i>	D	I	I	D	I	I	D	D	D	I	I	I	I	D	D	I	D
HC-J-127-5	<i>bb</i>	D	I	I	D	I	I	D	D	D	I	I	I	I	D	D	I	D
HC-J-127-6	<i>bb</i>	D	I	I	D	I	I	D	D	D	I	I	I	I	D	D	I	D
HC-J-127-7	<i>bb</i>	D	I	I	D	I	I	D	D	D	I	I	I	I	D	D	I	D
HC-J-127-8	<i>bb</i>	D	I	I	D	I	I	D	D	D	I	I	I	I	D	D	I	D
HC-J-127-9	<i>bb</i>	D	I	I	D	I	I	D	D	D	I	I	I	I	D	D	I	D
HC-J-127-10	<i>bb</i>	D	I	I	D	I	I	D	D	D	I	I	I	I	D	D	I	D
HC-J-127-11	<i>bb</i>	D	I	I	D	I	I	D	D	D	I	I	I	I	D	D	I	D
HC-J-127-12	<i>bb</i>	D	I	I	D	I	I	D	D	D	I	I	I	I	D	D	I	D
HC-J-127-13	<i>bb</i>	D	I	I	D	I	I	D	D	D	I	I	I	I	D	D	I	D
HC-J-127-14	<i>bb</i>	D	I	I	D	I	I	D	D	D	I	I	I	I	D	D	I	D
HC-J-127-15	<i>bb</i>	D	I	I	D	I	I	D	D	D	I	I	I	I	D	D	I	D
HC-J-127-17	<i>bb</i>	D	I	I	D	I	I	D	D	D	I	I	I	I	D	D	I	D
HC-J-127-18	<i>bb</i>	D	I	I	D	I	I	D	D	D	I	I	I	I	D	D	I	D
HC-J-127-19	<i>bb</i>	D	I	I	D	I	I	D	D	D	I	I	I	I	D	D	I	D

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Table 15. Continued

	Indel markers	00194	01598	01768	02205	03652	05003	13673	06820	19428	02012	05843	19358	02188	12877	00182	05837	01360
HC-J-127-20	<i>bb</i>	D	I	I	D	I	I	D	D	D	I	I	I	I	D	D	I	D
Jack-127-1-1	<i>bb - bc - cc?</i>	D	I	I	D	I	Seg	D	D	D	H	H	D	I	I	D	I	D
Jack-127-1-2	<i>bb - bc - cc?</i>	D	I	I	D	I	Seg	D	D	D	H	D	D	I	I	H	I	D
Jack-127-1-3	<i>bb - bc - cc?</i>	D	I	I	D	I	Seg	D	D	D	H	H	D	I	I	H	I	D
Jack-127-1-4	<i>bb - bc - cc?</i>	D	I	I	D	I	Seg	D	D	D	I	H	D	I	I	D	I	D
Jack-127-1-5	<i>bb - bc - cc?</i>	D	I	I	D	I	Seg	D	D	D	D	D	D	I	I	D	I	D
Jack-127-1-6	<i>bb - bc - cc?</i>	D	I	I	D	I	Seg	D	D	D	I	H	D	I	I	D	I	D
Jack-127-1-7	<i>bb - bc - cc?</i>	D	I	I	D	I	Seg	D	D	D	H	D	D	I	I	I	I	D
Jack-127-1-8	<i>bb - bc - cc?</i>	D	I	I	D	I	Seg	D	D	D	H	H	D	I	I	I	I	D
Jack-127-1-9	<i>bb - bc - cc?</i>	D	I	I	D	I	Seg	D	D	D	I	H	D	I	I	H	I	D
Jack-127-1-10	<i>bb - bc - cc?</i>	D	I	I	D	I	Seg	D	D	D	I	H	D	I	I	D	I	D
Jack-127-1-11	<i>bb - bc - cc?</i>	D	I	I	D	I	Seg	D	D	D	D	H	D	I	I	D	I	D
Jack-127-1-12	<i>bb - bc - cc?</i>	D	I	I	D	I	Seg	D	D	D	I	D	D	I	I	I	I	D
Jack-127-1-13	<i>bb - bc - cc?</i>	D	I	I	D	I	Seg	D	D	D	I	H	D	I	I	I	I	D
Jack-127-1-14	<i>bb - bc - cc?</i>	D	I	I	D	I	Seg	D	D	D	D	H	D	I	I	H	I	D
Jack-127-1-15	<i>bb - bc - cc?</i>	D	I	I	D	I	Seg	D	D	D	I	D	D	I	I	D	I	D
Jack-127-1-16	<i>bb - bc - cc?</i>	D	I	I	D	I	Seg	D	D	D	H	I	D	I	I	H	I	D
A12-AS-9-1	<i>cc</i>	D	I	I	D	I	I	D	D	D	D	I	D	I	I	H	I	D
A12-AS-9-2	<i>cc</i>	D	I	I	D	I	I	D	D	D	D	I	D	I	I	D	I	D
A12-AS-9-3	<i>cc</i>	D	I	I	D	I	I	D	D	D	D	I	D	I	I	Seg	I	D
A12-AS-9-4	<i>cc</i>	D	I	I	D	I	I	D	D	D	D	I	D	I	I	Seg	I	D
A12-AS-9-5	<i>cc</i>	D	I	I	D	I	I	D	D	D	D	I	D	I	I	Seg	I	D
A12-AS-9-6	<i>cc</i>	D	I	I	D	I	I	D	D	D	D	I	D	I	I	Seg	I	D

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Table 15. Continued

	Indel markers	00194	01598	01768	02205	03652	05003	13673	06820	19428	02012	05843	19358	02188	12877	00182	05837	01360
A12-AS-9-7	cc	D	I	I	D	I	I	D	D	D	D	I	D	I	I	Seg	I	D
A12-AS-9-8	cc	D	I	I	D	I	I	D	D	D	D	I	D	I	I	Seg	I	D
A12-AS-9-9	cc	D	I	I	D	I	I	D	D	D	D	I	D	I	I	Seg	I	D
A12-AS-9-10	cc	D	I	I	D	I	I	D	D	D	D	I	D	I	I	Seg	I	D
A12-AS-9-11	cc	D	I	I	D	I	I	D	D	D	D	I	D	I	I	Seg	I	D
A12-AS-9-12	cc	D	I	I	D	I	I	D	D	D	D	I	D	I	I	Seg	I	D
A12-AS-9-13	cc	D	I	I	D	I	I	D	D	D	D	I	D	I	I	Seg	I	D
A12-AS-9-14	cc	D	I	I	D	I	I	D	D	D	D	I	D	I	I	Seg	I	D
A12-AS-9-15	cc	D	I	I	D	I	I	D	D	D	D	I	D	I	I	Seg	I	D
A12-AS-9-16	cc	D	I	I	D	I	I	D	D	D	D	I	D	I	I	Seg	I	D
A12-AS-9-17	cc	D	I	I	D	I	I	D	D	D	D	I	D	I	I	Seg	I	D
A12-AS-9-18	cc	D	I	I	D	I	I	D	D	D	D	I	D	I	I	Seg	I	D
A12-AS-9-19	cc	D	I	I	D	I	I	D	D	D	D	I	D	I	I	Seg	I	D
A12-AS-15-1	cc	D	I	I	D	I	D	D	D	D	D	D	D	I	I	I	I	D
A12-AS-15-2	cc	D	I	I	D	I	D	D	D	D	H	D	D	I	I	I	I	D
A12-AS-15-3	cc	D	I	I	D	I	D	D	D	D	H	D	D	I	I	I	I	D
A12-AS-15-4	cc	D	I	I	D	I	D	D	D	D	I	D	D	I	I	I	I	D
A12-AS-15-5	cc	D	I	I	D	I	D	D	D	D	H	D	D	I	I	I	I	D
A12-AS-15-6	cc	D	I	I	D	I	D	D	D	D	H	D	D	I	I	I	I	D
A12-AS-15-7	cc	D	I	I	D	I	D	D	D	D	D	D	D	I	I	I	I	D
A12-AS-15-8	cc	D	I	I	D	I	D	D	D	D	D	D	D	I	I	I	I	D
A12-AS-15-9	cc	D	I	I	D	I	D	D	D	D	-	D	D	I	I	I	I	D
A12-AS-15-10	cc	D	I	I	D	I	D	D	D	D	-	D	D	I	I	I	I	D

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Table 15. Continued

	Indel markers	00194	01598	01768	02205	03652	05003	13673	06820	19428	02012	05843	19358	02188	12877	00182	05837	01360
A12-AS-15-11	cc	D	I	I	D	I	D	D	D	D	-	D	D	I	I	I	I	D
A12-AS-15-12	cc	D	I	I	D	I	D	D	D	D	-	D	D	I	I	I	I	D
A12-AS-15-13	cc	D	I	I	D	I	D	D	D	D	-	D	D	I	I	I	I	D
A12-AS-15-14	cc	D	I	I	D	I	D	D	D	D	-	D	D	I	I	I	I	D
AS-A12-19-1	cc	D	I	I	D	I	D	D	D	D	I	H	D	I	I	D	I	D
AS-A12-19-2	cc	D	I	I	D	I	D	D	D	D	I	I	D	I	I	D	I	D
AS-A12-19-3	cc	D	I	I	D	I	D	D	D	D	I	H	D	I	I	D	I	D
AS-A12-19-4	cc	D	I	I	D	I	D	D	D	D	I	I	D	I	I	D	I	D
AS-A12-19-5	cc	D	I	I	D	I	D	D	D	D	I	H	D	I	I	D	I	D
AS-A12-19-6	cc	D	I	I	D	I	D	D	D	D	I	H	D	I	I	D	I	D
AS-A12-19-7	cc	D	I	I	D	I	D	D	D	D	I	I	D	I	I	D	I	D
AS-A12-19-8	cc	D	I	I	D	I	D	D	D	D	I	D	D	I	I	D	I	D
AS-A12-19-9	cc	D	I	I	D	I	D	D	D	D	I	D	D	I	I	D	I	D
AS-A12-19-10	cc	D	I	I	D	I	D	D	D	D	I	D	D	I	I	D	I	D
AS-A12-19-11	cc	D	I	I	D	I	D	D	D	D	I	H	D	I	I	D	I	D
AS-A12-19-12	cc	D	I	I	D	I	D	D	D	D	I	I	D	I	I	D	I	D
AS-A12-19-13	cc	D	I	I	D	I	D	D	D	D	I	D	D	I	I	D	I	D
AS-A12-19-14	cc	D	I	I	D	I	D	D	D	D	I	H	D	I	I	D	I	D
AS-A12-19-15	cc	D	I	I	D	I	D	D	D	D	I	H	D	I	I	D	I	D
AS-A12-19-16	cc	D	I	I	D	I	D	D	D	D	I	I	D	I	I	D	I	D

HC = Honeycomb, J = 'Jack' variant, AS = homozygous *Aco4*, I = insertion, D = deletion, H = heterozygous, - = not available, Seg = segregating

Figure 1. Graphic representation of the history of the F₂ aconitase variant plant A08-AS-2932 (*Aco4-ab*) from a cross between ‘Minsoy’ (*Aco4-cc*) x ‘BSR 101’ (*Aco4-aa*)

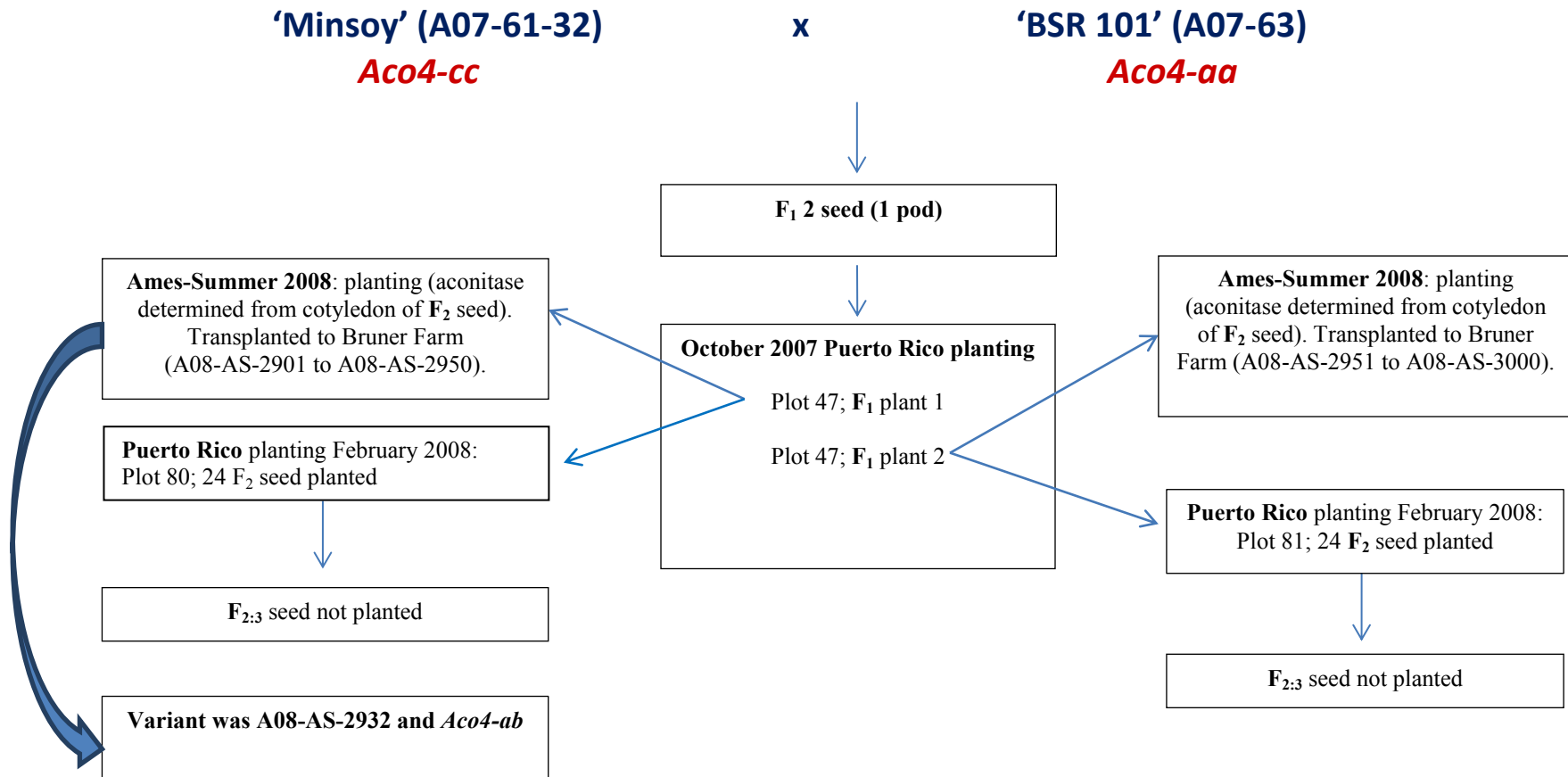
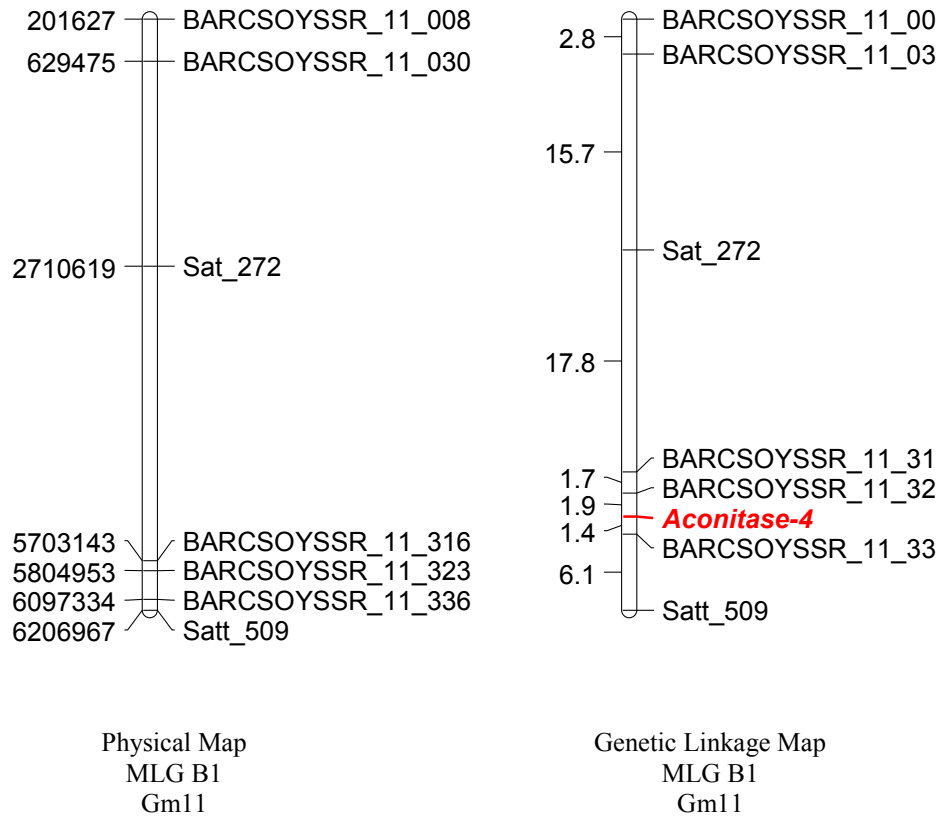


Figure 2. Genetic linkage mapping of the *Aconitase-4* gene from the cross 'BSR 101' \times 'Noir 1'. Genetic and physical maps of soybean chromosome Gm11 (MLG B1) showing location of the *Aconitase-4* gene. Genetic distances are shown in centiMorgans (cM) and physical distances are shown in base pairs (bp).



**CHAPTER 4: EFFECT OF SIMULATED-HAIL AND CONVENTIONAL TRACTOR
PLANTING ON SINGLE PLANTS OF SOYBEAN [*Glycine max* (L.) Merr.] GROWN
IN A HONEYCOMB PLANTING DESIGN**

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Abstract

Environmental stress factors such as elevated temperatures, fertilizer levels, and high salinity have been demonstrated to affect the growth, development, and genetic stability of plants. Detailed evaluation of cultivars for their response to plant stresses is important because it might be possible to select cultivars tolerant to biotic and abiotic stresses, and to identify sources that enhance genetic variation. Furthermore, there is value in establishing whether treatments that enhance genetic variation have broad application and can be used for different cultivars. In 2008 experiments, we used honeycomb planting designs in order to produce a large amount of seed to use in the stress experiments and to evaluate agronomic performance of ‘BSR 101’, ‘Jack’, and ‘IAR2001BSR’ cultivars. Phenotypic and genotypic variability observed in progeny of single plants grown in a honeycomb design could not be attributed to the effect of the planting pattern or due to severe damage caused by a natural-hail storm in 2008. The objective of this study was to investigate the effect of simulated-hail

on single plants grown in a honeycomb design, and as control for the possible effect of the honeycomb design, we evaluated the effect of simulated hail on plants grown in a conventional tractor-planting design. The simulated-hail procedure was performed approximately at the V4-V5 vegetative stage. Leaf samples were collected at three different dates for DNA evaluation and twelve InDel molecular markers were used to evaluate genomic changes. There was no detected variation in the simulated hail experiments for both honeycomb and conventional tractor-planting patterns. However, we found unusual DNA variation in the last date of collection (date-2) for 'BSR 101' cultivar in the honeycomb control experiment.

Introduction

Plants are influenced by abiotic and biotic environmental stresses such as drought, suboptimal temperatures, nutrient availability, diseases, and insects. As a consequence, the growth, development, and productivity of crop species can be significantly affected (Madlung and Comai 2004). Due to their sessile nature, plants must develop strategies to succeed in adverse environments. Mechanisms of adaptation include changes in plant physiology, changes in plant morphology, and through the generation of genomic and epigenetic alterations (Madlung and Comai 2004; Bruce et al. 2007). In plants, for example, germline cells emerge late in development, thus allowing acquired genomic changes to be inherited (Cullis 1987; Walbot 1996; Gorbunova and Levy 1999). For example, Raval et al. (2013) identified a male-sterile and female-sterile mutant from germinal revertant of the *w4-m* mutant allele. The mutable allele resulted from the insertion of *Tmg9*, a CACTA-like transposable element that excises at high frequencies from both somatic and germinal tissues;

in this case, late excision resulted in a novel mutable line, which was useful to identify candidate genes for the male-fertile, female-fertile genotype.

Immediate short-term responses to environmental stresses occur in the form of regulation of physiological processes. For example, under drought stress, rapid induction of response seems to be associated with drought-responsive genes, which are controlled at the molecular level by changes in gene expression and are dependent on developmental stages or stress effect (Le et al. 2012). Changes in the expression of stress-related genes may occur through epigenetic modifications. These mechanisms regulate genetic functions such as transcription, replication, DNA repair, gene transposition, and cell differentiation (Angers et al. 2010; Sahu et al. 2013). Stress can alter patterns in DNA methylation, histone modification, and chromatin remodeling. Under stress conditions, some genes are selectively demethylated and subsequently translated resulting in alteration of gene expression (Choi and Sano 2007). In their study, they proposed that environmental responses of plants might be mediated through active alteration of DNA methylation patterns.

Stress not only results in the modification of gene expression but it also results in genomic alterations. Under extremely stressful conditions, plants may adapt or create variation in order to develop a long-term stress response (Walbot and Cullis 1985). The plant genome is particularly fluid, which allows large differences in genome size and organization to occur in closely related species as a strategy of adaptability to changing environments (McClintock 1984; Walbot and Cullis 1985; Casacuberta and Puigdomenech 2000). According to McClintock (1984), genomic changes in response to stress are attributed to a rearrangement or reorganization of the genome facilitated by transposon activation, transposition of mobile elements, and chromosome breakage.

Unusual patterns of genetic variation induced by the growing environment or stress treatments have been reported in several studies. These changes have furthermore been shown to be stably inherited for a large number of generations. For example, in flax (*Linum usitatissimum* L.), several studies have demonstrated that the genome is responsive to a number of external stimuli and is extremely fluid (Cullis 1987). After the plants were grown for one generation under different fertilizer combinations and temperature treatments, stable and genetically altered plants were obtained, which were referred to as genotrophs (Cullis 2005). Phenotypic alterations such as plant height at maturity were observed (Durrant 1962), and the extreme phenotypes were characterized by a significant difference in nuclear DNA content (Evans et al. 1966). Other observed alterations include changes in the number of genes coding for the 25S, 18S, and 5S ribosomal RNAs as well as in other repetitive sequence families (Schneeberger and Cullis 1991), and a novel 5.8 kb element designated as *Linum Insertion Sequence 1* (*LIS-1*) (Cullis 2005). In *Arabidopsis* (*Arabidopsis thaliana* L.), when plants were grown under different temperature regimes and stress conditioned by salicylic acid for five generations, they displayed differences in gene copy number variation when compared with non-stressed plants (DeBolt 2010). Puchta et al. (1995) reported that *Arabidopsis* plants grown under high salt environments had an increase in recombination frequency compared to the control. Recombination frequencies also were enhanced several fold by plants exposed to ultraviolet-B radiation (Ries et al. 2000).

In soybean [*Glycine max* (L.) Merr.], Roth et al. (1989) have proposed that, in response to stress, inbred line have evolve a system for generating genetic diversity. In their study, when DNA extracted from tissue cultures prepared from leaf, cotyledon, stem, or root tissue from single ‘Minsoy’ or ‘Noir 1’ plants were analyzed for RFLPs, only the root tissue

showed differences at various loci. Additionally, they reported variation in the root tissue of F_1 plants from the cross between ‘Minsoy’ (PI 29890) and ‘Noir 1’ (PI 290136). Yates et al. (2012), observed *de novo* variation within three soybean cultivars. These cultivars were selected by performing single-plant selection at ultra-low plant density within plants that were grown in a honeycomb pattern design (Fasoula and Boerma 2005, 2007). Although the source of this *de novo* variation is unknown and suggested to be the result of mutation or some other genetic mechanisms, the honeycomb planting pattern could be an approach for enhancing *de novo* variation. Ultra-low plant spacing is believed to minimize stress, allowing maximum grain production per plant (Duvick 1997; Fasoula and Fasoula 1997). Reducing interplant competition has been demonstrated to avoid yield deterioration and to exploit the outcome of favorable genetic modifications that might have occurred within the cultivar over time (Fasoula and Fasoula 2000).

In summer 2008 experiments, we used a honeycomb planting design to produce a large amount of seed per single-plant that was later used in stress experiments and for evaluating agronomic performance within ‘BSR 101’, ‘Jack’, and ‘IAR2001BSR’ cultivars. However, during the growing season, these single plants were severely injured by a natural-hail storm, which affected the number of single plants that could be harvested. During summer 2009, progeny from these plants, from honeycomb design and natural-hail storm were planted in a 15-meter long row and remnant seed from some entries was used for an aging test and grafting stress treatments. Plant treatments resulted in phenotypic and genotypic variation for plant and seed traits as well as aconitase isozymes. Despite this variability within cultivars, we could not attribute these differences to the effect caused by the honeycomb planting, or to the effect of stress treatments due to the confounding variable

of natural-hail storm damage. As mentioned above, studies have revealed genetic changes caused by specific growing environments. The severe damage caused by the hail storm was therefore considered as an additional stress that could have an effect on single plants.

Although there is no evidence that genetic changes can be attributed to the effect of the honeycomb planting design on growing plants or to the effect on the first generations, studies have demonstrated the success of single-plant selection for several agronomic traits in soybean (Fasoula and Boerma 2005, 2007).

The purpose of this study was to separate the possible effects of hail from that of a honeycomb design. In this study, we evaluated the effect of simulated hail on single plants grown in a honeycomb design; and as control for the possible effect of the honeycomb design, we evaluated the effect of simulated hail on plants grown in a conventional tractor-planting design.

Materials and Methods

Plant material and seed source

Soybean plant introductions ‘BSR 101’ (PI 548519) (Tachibana et al. 1987), and ‘Jack’ (PI 540556) (Nickell et al., 1990) were used in this study. Seed source of both inbred lines were hand-harvested single-plants. Each single plant was given a number from 1-200 called an entry number. For example, seeds of entry number one of ‘BSR 101’ were used for the honeycomb and conventional-tractor design with both control and simulated hail treatments. Single plants of ‘BSR 101’ were obtained from Dr. R.G. Palmer, Iowa State University at Ames, IA, and ‘Jack’ from Dr. R. Shoemaker, USDA ARS at Ames, IA.

Honeycomb planting design; 2010

In 2010, 150 entries of 'BSR 101' and 'Jack' were grown in a honeycomb design (Fasoulas and Fasoula 1995), using a plant-to-plant spacing of 2.0 m. Four seeds per hill were planted, and approximately three weeks after planting, each hill plot was thinned to 1 plant per plot. Two replicates from each entry number were planted. The first replicate was used for the control (non-simulated hail experiment) and the second one was for the simulated hail experiment.

Honeycomb planting design and conventional planting; 2011

In 2011, 50 entries of 'BSR 101' and 'Jack' were planted in a honeycomb design as described previously. Two replicates also were planted for the control and simulated hail experiments. Additionally, seed of the same 50 entries of both cultivars were planted in a conventional-planting design. Two replicates of approximately 50 seed/entry from selected single plants were planted in one-row plots with a row spacing of 0.76 m and a row length of 3.5 m. One replicate was used for the control (non-simulated) hail experiment and the other one for the simulated hail experiment.

Leaf tissue collection, tagging, and harvest

Leaf tissue from young trifoliolate from the main stem was collected three times per plant at different developmental stages (Fehr et al. 1977). The first collection (date-0) was at the V3-V4 vegetative stage, the second collection (date-1) was at the R1-R2 reproductive stage, and the last collection (date-2) was at the R3-R4 reproductive stage (Figure 1). Every single plant in the honeycomb 2010 and in the honeycomb and conventional planting 2011 experiments was sampled. For the conventional planting in 2011, a single plant/plot/entry was selected at random and sampled three times as explained previously. Plants were tagged

at every point of leaf collection as date-0, date-1 and date-2. At maturity, approximately four pods at each point of leaf collection were harvested in separated envelopes. Remnant seed from each plant were single-plant hand shelled and saved in a different envelope.

Simulated hail treatment

Single plants in the honeycomb experiment and all the plants in the conventional planting plots were treated with a simulated hail treatment (simulated hail experiments). The procedure was done approximately two weeks after the first leaf collection (date-0) at the V4-V5 stage. Two thirds of every trifoliolate were removed manually with scissors. The procedure was done the same day for all the experiments.

Honeycomb 2010 progeny; 2012 experiment

Since 11 entries of 'BSR 101' showed unusual DNA patterns, we decided to analyze progeny of these 11 entries in 'BSR 101' and another 11 entries at random for a total of progeny of 22 entries analyzed. Additionally, we analyzed the same number of progeny of entries for 'Jack' cultivar. Then, progeny of 22 entries of both, 'BSR 101' and 'Jack', from honeycomb 2010 plants were evaluated for DNA variation. Approximately five seeds per date of collection were evaluated, for a total of 15 seeds per plant. Seeds were surface sterilized with chlorine gas for 16 hours. Five seeds per date of collection were placed in disposable 60 × 15 mm Petri dishes and sterilized with chlorine gas into a tightly sealed desiccator for 16 h by mixing 100 ml of commercial bleach (12% sodium hypochlorite) with 3.5 ml of 12 N HCl. After overnight exposure, Petri dishes were allowed to air out for about 30 min. Disinfected seeds were placed on germination paper 72 to 100 h at 30 °C in a growth chamber. Seedlings were transplanted to peat pots in the USDA greenhouse. Leaf tissue was collected from the first trifoliolate and stored at -80 °C until DNA was extracted. After leaf

collection for DNA analysis, plants were transplanted to the field in Brunner Farm, near Ames, IA. At harvest, each plant was hand harvested.

InDel marker development

The InDels (insertion/deletion) were identified by Dr. Perry Cregan (USDA ARS, Beltsville, MD) (Choi et al. 2007). The identified polymorphisms were sequenced from EST libraries from cultivars ‘Archer’ (PI 546487), ‘Minsoy’ (PI 27890), ‘Noir 1’ (PI 290136), ‘Evans’, ‘PI 209332’, and ‘Peking’ (PI 438496). Sequence information from the database was used to design the flanking primers for InDel markers. The selected InDel markers were distributed across eight soybean chromosomes, and contained unique sequences when compared with the reference genome (Table 1). These markers consisted of genomic DNA sequences between 12 and 48 nucleotides in length that are either present (insertion) or absent (deletion) in the ‘BSR 101’ and ‘Jack’ genomic backgrounds.

InDel genotyping; honeycomb 2010

Leaf samples were collected at different developmental stages and freeze-dried using a vacuum freeze dryer (Labconco, USA) prior to DNA extraction. Approximately 30 mg of freeze dried tissues were used for DNA extraction. DNA extraction was performed using the DNA facility’s Autogen Autogenprep 740 DNA extraction robot. DNA quantity and quality was assessed with the ND-1000 spectrophotometer (Fisher Thermo, USA). DNA was analyzed using 12 InDel markers fluorescently labeled with Dye Set G5 (6-FAM, VIC, NED, PET, and LIZ-size standard) (Applied Biosystems, USA). Four targets were amplified in a single reaction tube. For each reaction, 10 ng of each DNA sample were used for 25 µl reaction with 2X reaction *Taq* buffer (500 mM KCl, 100 mM Tris-HCl, 15 mM MgCl₂, Triton x-100), 0.4 µl of each primer, 2.5 mM of each dNTP, and 0.2 units of *Taq* DNA

polymerase (GenScript, USA) per reaction. Multiplex PCR reactions were performed using a PTC-100 Programmable Thermal Controller (MJ Research, USA) programmed with the following conditions: temperature of 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, annealing (the optimal temperature varies between primer sets) for 30 s, and 72 °C for 1 min; with a final extension at 72 °C for 7 min. PCR reactions were sent to the Iowa State University DNA facility for genotyping service. Amplified products were separated by a capillary electrophoresis in an ABI-3730 Genetic Analyzer (Applied Biosystems, USA) and results were analyzed by GeneMapper software version 4.0 (Applied Biosystems, USA).

Unusual DNA patterns

Unusual DNA patterns were detected using the GeneMapper software version 4.0 (Applied Biosystems). PCR amplifications were repeated in the laboratory using non-labeled InDel primers. The resulting PCR products were resolved on a 3% agarose/TBE gel stained with ethidium bromide ($1 \mu\text{g mL}^{-1}$) in 0.5X TBE at 100-120 V for 1 to 2 h. The band patterns were visualized and gels were photographed under UV light. Images were saved using a Fotodyne Archiver Imaging System (Fotodyne, Inc., Harland, WI). The fragment size of each PCR product was approximated by referencing the bands to a low molecular weight DNA ladder (New England BioLabs, Inc., Ipswich, USA).

Additional InDel genotyping

External and internal primers were used for testing individual plants with unusual DNA patterns. External primers flanking the InDel markers were designed to obtain larger amplicon sizes. To test if the unusual amplicon was indeed the same or different from the expected DNA pattern, internal primers were designed. Target sequences were amplified

using one external primer paired with a primer homologous to sequences within the insertion. Internal and external primers are listed in Table 2.

Sequencing of PCR products

Genomic DNA was PCR amplified and sequenced directly. PCR products were cleaned with ExoSAP-IT® (Affymetrix, CA, USA) according to the manufacturer's instructions. Sequencing was performed using the Iowa State University DNA facility with a 3730xl DNA Analyzer (Applied Biosystems, CA, USA). Sequence alignments were generated using CLC Main Workbench 6 software (CLC Bio, MA, USA).

Results

Honeycomb planting design 2010

The 150 entries of 'BSR 101' and 'Jack' inbred lines from control (non-simulated hail) and simulated hail were analyzed molecularly using 12 InDel markers. Six hundred samples per experiment were analyzed. This includes DNA analysis of date-0 and date-2 collections per entry number. Date-1 collection was analyzed only if an unusual pattern was detected in date-0 or date-2.

'Jack' inbred lines

DNA variation was detected in 'Jack' inbred lines. 'Jack' entry number 36 was polymorphic using 5 out of 12 InDel markers (BARC-017059-02188, BARC-028361-05843, BARC-065401-19428, BARC-047681-10375, and BARC-007970-00182). 'Jack' entry 36 had a different genetic profile when compared with the other entry numbers and with the expected DNA profile. This pre-existing variation was detected in all dates of collection and in the control and simulated hail experiments. Additional to this pre-existing variation, a

deletion event was found in 64 entries of ‘Jack’ using the InDel marker BARC-047681-10375. This variation also was found in all dates of collection and in all experiments.

De novo or unexpected genetic variation was not observed in the control or the simulated hail experiments for ‘Jack’ inbred lines at date-0 and date-2 collections. DNA patterns were identical to the mother plant.

‘BSR 101’ inbred lines

Unusual DNA patterns were detected in the control experiment of ‘BSR 101’. Eleven entries showed unusual DNA variation when date-0 and date-2 collections were analyzed with 9 out of 12 InDel markers. These results were verified using non-labeled primers, the same PCR machines, and DNA fragments were visualized in agarose gels. When these unusual results were verified, date-1 collection was included (Table 3). Results were consistent when DNA fragments were visualized using the capillary system and agarose gels. The vast majority of the variation was found in date-2 collection, which was approximately done at R3-R4 reproductive stage. There were two cases where variation was found in date-0 collection, which was approximately done at V4-V5 vegetative stage (Table 3). For some locations, for example BARC-047166-12877, ‘BSR 101’ entries 113 and 139 went from homozygous insertion to heterozygous or from homozygous insertion to a deletion (Table 4). The entry number with the largest number of changes was ‘BSR 101’ entry 113. There were changes at nine different InDel locations, followed by entries 68, 139 and 148, which were different at 6, 5 and 5 different InDel locations, respectively.

From the 12 InDel markers analyzed, 9 showed unusual differences compared with the expected DNA pattern (Table 4). InDel marker BARC-014413-01360 showed the largest number of unusual DNA patterns, with nine entries showing differences, followed by InDel

markers BARC-028339-05837 and BARC-028361-05843 in eight and seven entries, respectively.

De novo or unexpected genetic variation was not observed in the simulated hail experiments for ‘BSR 101’ inbred lines at date-0 and date-2 of collection. DNA patterns were identical to the mother plant using 12 InDel markers.

Replication of unusual DNA variants; strategies for replication of results

Although results obtained from the DNA facility using the capillary system for DNA fragment visualization were verified by repeating the PCR reaction and visualizing DNA fragments in agarose gels; we were unable to reproduce the same results approximately three months later. Several strategies for replication of results were used, among them, preparing new DNA dilutions, using different PCR machines, incorporating new PCR reactive solutions (dNTPs, different suppliers of *Taq* DNA polymerase, and buffer), and generating a new order of the same InDel primers.

DNA extraction from remnant lyophilized leaf tissue was performed and external and internal primers were designed. These external and internal primers were designed for the InDel markers BARC-047166-12877, BARC-017059-02188, BARC-014413-01360, and BARC-028339-05837 (Table 2) in order to improve the chances of obtaining a robust PCR product and a reliable score from DNA samples containing insertion sequences. Figure 2 shows results from agarose gels obtained with the original set of primers (Table 1). Five external primers with a PCR product size ranging from 187 to 248 bp were evaluated in the 11 unusual entries from ‘BSR 101’ control experiment at all dates of collection (Table 2). There were no differences between samples collected on different dates and the amplicon sizes were as expected.

With regard to the internal primers, positioned on either side of the target sequence (Table 2), a total of nine sets of primers were used with a PCR product size ranging from 108 to 250 bp. On average, two sets of primers were designed and evaluated for each InDel marker; BARC-047166-12877, BARC-017059-02188, BARC-014413-01360, and BARC-028339-05837. There were no differences between dates of collection of the control experiment, except when set 2 of primers designed for InDel marker BARC-017059-02188 was used (Forward primer: ACAATCATTGTACATGACAAAAG and reverse primer: TTGACTGCATGTCTTCACGA) (Table 2). With this set of primers, there was no PCR product detected for date-0 and date-1 collection but there was a PCR product detected for date-2 collection (Figure 3). The PCR product was approximately 239 bp as expected. There was no detected PCR fragment when samples of the same entry numbers were evaluated for the simulated hail experiment for all dates of collection.

DNA sequencing

Amplicon samples using internal primer set 2 designed for InDel marker BARC-017059-02188 from ‘BSR 101’ entries 68 and 119 date-2 collection were subjected to DNA sequence analysis. There was no PCR product detected in samples from date-0 and date-1 collections for these two entries. In order to get information for date-0 and date-1 collections, amplicon samples from all three collection dates of ‘BSR 101’ entry 119 using external primer set 1 designed for InDel marker BARC-017059-02188 were also subjected to DNA sequence analysis.

Sequence analyses of DNA from ‘BSR 101’ entries 68 and 119 date-2 collection were identical and both samples showed that these plants had acquired a 19-nucleotide insertion fragment (Figure 4). In the ‘BSR 101’ genetic background, the InDel marker BARC-

017059-02188 corresponded to a deletion marker. However, this same insertion was absent in sequence analyses of DNA from ‘BSR 101’ entry 119 at all dates of collection; they all were identical (Figure 5).

Honeycomb planting design and conventional tractor planting; 2011

The 50 entries of ‘BSR 101’ and ‘Jack’ inbred lines from honeycomb and conventional planting both with control (non-simulated hail) and simulated hail treatments were analyzed molecularly using 12 InDel markers. There were not genetic changes among samples evaluated. Two hundred samples per experiment were analyzed, which included DNA analysis of date-0 and date-2 collections per entry number.

Discussion

Highly inbred lines in soybean are not permanent genetic pools but genotypes which contain phenotypic and genotypic variation (Roth et al. 1989; Fasoula and Boerma 2005, 2007; Haun et al. 2011; Yates et al. 2012). Environmental stress is considered a trigger for enhancing naturally occurring phenotypic and genotypic variation in plants (Durrant et al. 1962; McClintock 1984; Cullis 2005; Chen et al. 2005; Madlung and Comai 2004). The honeycomb planting design has been shown to enhance the expression of genetic variation in several crop species (Fasoulas 1998; Christakis and Fasoulas 2002; Fasoula and Boerma 2005, 2007; Tokatlidis et al. 2010). In summer 2008, we used a honeycomb design in order to produce large amount of seed from single plants to use in seed-stress and plant-stress experiments. However, during this growing season, a severe hail storm affected the plants reducing seed production on most plants. The damage sustained by the soybean plants was severe and was considered an additional stress. Phenotypic and genotypic variation was

found in progeny of 2008 honeycomb plants; for example yellow-lethal mutants, semi-sterile mutants, etc. The honeycomb experiments were repeated during summers of 2010 and 2011. The main purpose of this simulated hail experiment was to separate the possible effects of hail from that of a honeycomb design and to evaluate the combined effect of simulated hail on single-plants grown in a honeycomb design. As a control for the possible effect of the honeycomb design, we evaluated the effect of simulated hail on plants grown in a conventional tractor-planting design. ‘BSR 101’ and ‘Jack’ genotypes were each represented by single plants grown in a honeycomb design or progeny from single plants grown in a conventional tractor planting. DNA variation detected in ‘Jack’ cultivar was pre-existing in the original cultivar seed source. For ‘BSR 101’, however, DNA variation was unusual and limited to the last date of collection (date-2) for single plants evaluated in the honeycomb design control experiment.

InDel variation detected in the original seed source

We employed molecular InDel markers to assess the extent and pattern of DNA variation in the control and in response to simulated hail treatments, within a honeycomb and conventional tractor planting designs. The InDel markers that were used in the genotyping analysis were polymorphic between ‘BSR 101’ and ‘Jack’ (Table 1). The use of a multiplex PCR reaction allowed us to evaluate a large number of samples at low cost due to the reduced amount of reagents and DNA needed for PCR amplification. InDel markers were developed based on sequence comparisons between species or genetically similar plant introductions, and were useful for studies of genetically related genotypes (Choi et al. 2007; Wu et al. 2013). Furthermore, InDel markers have become a valuable tool in many plant

genetic studies due to the simplicity of the technique and accuracy of the results (Garcia et al. 2012; Kempinski et al. 2012; Hopkins et al. 2013; Wu et al. 2013).

InDel markers in ‘Jack’ showed polymorphism, which also was present in the original foundation seed source, or mother plant. The results found in the ‘Jack’ inbred line could be attributed to residual heterozygosity or heterogeneity in the parental inbreds. ‘Jack’ is an F₃-derived line from the cross between ‘Fayette’ × ‘Hardin’ that was released in 1989 (Nickell et al. 1990). The F₂ generation was advanced by single-seed descent. Ten sublines, selected from the F₃-derived line, were composited in the F₁₀ generation to produce ‘Jack’. As an F₃-derived line, ‘Jack’ is expected to maintain residual heterozygosity at approximately 25% of the polymorphic loci from its parents. This suggests that it is likely the result of segregation and fixation of residual heterozygosity in the initial F₃ generation of ‘Fayette’ × ‘Hardin’. Therefore, the polymorphic region observed within ‘Jack’ single plants might be a consequence of heterogeneity between the 10 sublines originally selected and composited at the F₃ generation. In contrast, pre-existing DNA variation was not found in ‘BSR 101’ inbred lines in the control and simulated hail experiments. Although ‘BSR 101’ was derived from a F₄ plant selection from the cross between L69U40-16-4 × A76-304020, and the population was advanced by single-seed descent to the F₄ generation (Tachibana et al. 1987). Thus no sublines were composited to produce the breeder seed. This could explain why we did not find pre-existing DNA variation for the 12 InDel makers tested.

Intracultivar variation due to seed source differences, which have been shown to be significant in the evaluation of cultivars, was reported by Fehr and Probst (1971). More recently, Haun et al. 2011 reported that ‘Williams 82’ contained intracultivar variation primarily derived from the segregation of residual heterozygosity of parental loci of

‘Williams’ and ‘Kingwa’. Although, it is predicted that the level of heterozygosity is reduced by half every generation with inbreeding, maintenance of a selectable trait could allow the retention of heterozygosity in the early cycles of selection (Haun et al. 2011). Yates et al. (2012) reported that most of the Simple Sequence Repeats (SSR) variation detected within three commercial soybean cultivars was the result of residual heterozygosity in the initial plant selected to become a cultivar. The levels of residual heterozygosity reported were 82, 93, and 82 percent for ‘Benning’, ‘Haskell’ and ‘Cook’ seed source respectively. Similar to the inbred lines used in this study, one cultivar was F_4 -derived (‘Benning’), and two cultivars (‘Cook’ and ‘Haskell’) were F_5 -derived lines. Although both ‘Cook’ and ‘Haskell’ are F_5 -derived lines, ‘Cook’ showed a lower level of residual heterozygosity when compared with ‘Haskell’.

Control (non-simulated) and simulated hail treatments

A natural-hail storm occurred in the 2008 growing season. A few plants died; most plants were damaged, had few seed, and were hand harvested. A total of 315 out of 500 single plants for ‘BSR 101’ and a total of 305 out of 500 single plants of ‘Jack’ were used in 2009 experiments. Results from these experiments showed genetic and phenotypic variation in some entry numbers within cultivars. However, it was unclear if this variation could not be attributed to the effect of the honeycomb design or to the treatments evaluated, due to the confounding effect of the natural hail.

To discriminate variation originating from these experiments, we decided to use a seed source that was not progeny of single plants affected by natural hail for 2010 experiments. Seed source for 2010 was 2007 seed planted and reproduced in 2009. We evaluated the effect of plants grown in a honeycomb design and a conventional tractor

planting with control (non-simulated) and with simulated hail treatment. Simulated hail treatment was performed at approximately the same developmental stage as the natural hail that occurred in 2008. Molecular results did not show genetic variation using 12 InDel markers in any simulated hail treatment. Simulated hail treatment was done approximately at V3-V4 vegetative stage in every single plant in the honeycomb experiment and in all the plants in the conventional tractor planting experiment. Since it is impossible to completely duplicate actual hail damage, damage was limited and only 2/3 of each trifoliolate removed. The treated plants produced enough seed for future experiments and replication procedures.

Several studies suggest that the absence of DNA variation seen in this study might be due to insufficient damage or damage rendered at the wrong developmental stage. Plant stress caused by defoliation affects soybean yield differently at specific developmental stages (Teigen and Vorst 1975; Fehr et al. 1981, 1983; Petersen 2004; Conley et al. 2009; Board et al. 2010). Less impact on yield losses occurs during vegetative stages, which have been related to leaf regrowth and delayed leaf senescence (Peterson and Higley 1996). Other authors also have reported that the response of plants to injury vary among different cultivars, depending on the developmental stages of the plants, and the severity of damage. For example, yield reductions were greater during reproductive stages than during vegetative stages, and for determinate versus indeterminate cultivars (Fehr et al. 1977; Haile et al. 1998a; Board 2004; Conley et al. 2009; Moscardi et al. 2012). Mechanisms for plant compensation, such as compensatory growth and delayed leaf senescence, have been reported as likely plant response mechanisms to defoliation (Haile et al. 1998b). These authors commented that yield recovery is directly related to the light interception capacity of the soybean canopy, which suggested that cultivars with narrow-leaf morphology were able

to have equal or greater yields when compared with wide-leaf cultivars under defoliation treatments (Haile et al. 1998b).

Another reasonable explanation for the lack of plant response to simulated hail could be that the recovery of plants from leaf defoliation might be associated with the genes involved in the plant's defense mechanisms. For example, in response to stress, plants, including soybean, activate a wide range of defense mechanisms that increase tolerance to these limited conditions (Nanjo et al. 2011; Le et al. 2012). During drought stress, many photosynthesis-related genes were downregulated, which contribute to growth retardation, an adaptive mechanisms associated with plant survival (Le et al. 2012). Because we did not target specific regions for any agronomic or physiological trait, we cannot discard that there could have been variation in the expression of genes related, for example, with photosynthetic activity or cell wall synthesis for the simulated hail treatments within cultivars.

There is evidence that plants have a “memory” of encountered stress situations that allow them to better adapt to adverse conditions (Bruce et al. 2007; Slaughter et al. 2012). According to Bruce et al. 2007, when the plant's defense state is triggered, it can result in acquired resistance in the case of biotic or abiotic stresses when a stress factor occurs after the priming (against biotic stresses) or hardening (against abiotic stresses) event. Studies have demonstrated that primed or hardened plants display either faster and, or stronger, activation of the various defense responses that are induced following attack by biotic or abiotic factors. For example, Slaughter et al. (2012) demonstrated in *Arabidopsis* that the primed state of plants was transferred to their progeny, conferring improved protection from pathogen attack as compared to the descendants of unprimed plants. In this study,

Arabidopsis plants were primed with β -amino-butyric acid (BABA) or with an avirulent isolate of the bacteria *Pseudomonas syringae* pv *tomato* (*PstacrRpt2*). The descendants of primed plants showed a faster and higher accumulation of transcript of defense-related genes in the salicylic acid signaling pathway and enhanced disease resistance upon challenge inoculation with a virulent isolate of *P. syringae*. According to these findings, it would be interesting to evaluate the progeny of simulated hail treated plants under an additional stress treatment.

Alterations of the genome, correlated with changes in gene expression or with specific plant traits, have been observed during plant development and under stress conditions (Madlung and Comai 2004; Cullis 2005). Although our data suggested there were no effects of the simulated hail treatments in both, honeycomb and conventional planting, we could not infer that stress-induced variation for specific agronomic traits should not be found in subsequent generations. Future experiments will evaluate the variation present in selfed progeny from controls and treatments for agronomic performance, such as plant height, maturity, lodging, seed oil and protein content, and grain yield. If phenotypic or genotypic variation is found for some of these traits, variations could be associated with the treatment or with the environmental response.

Rolling (2012) demonstrated the presence of variation in agronomic performance for agronomic traits such as plant height, maturity, lodging, seed oil and protein content, and grain yield in plants grown from seeds produced during 2008 honeycomb and affected by natural-hail storm, and additionally stressed under accelerated aging conditions. Progeny from these plants showed a higher rate of variation for many agronomic traits than untreated seeds. The variation present in selfed progeny from control and simulated hail treatments for

agronomic performance should be evaluated to assess if phenotypic of genotypic variation is expressed in subsequent generations of stressed plants.

Genetic and phenotypic variation detected in progeny of treated plants

Genetic and phenotypic variation within inbred lines has been detected in progeny of plants grown under stressful environments (Durrant 1971; Fasoula and Boerma 2005, 2007; Hopkins et al. 2012; Roth et al. 1989; and Yates et al. 2012). For example, in soybean, genomic changes have been demonstrated when soybean cells have been taken through a cycle of tissue culture (Roth et al. 1989). In their study, tissue cultures prepared from roots of F₁ hybrid plants and from inbred plants showed RFLP allelic differences at various loci. According to the authors, this generated variation may be a genetic response to physiological stress inducted by hormones, or genetic stress caused by hybridization between diverse genetic stocks. In highly inbred commercial soybean cultivars ‘Benning’, ‘Haskell’, and ‘Cook’, honeycomb design was effective for selecting variants within each cultivar. The single-plant selected lines were superior for seed oil and protein content, seed weight, plant height, and maturity (Fasoula and Boerma 2005, 2007). Using progeny of each selected line and from each source cultivar, Yates et al. (2012) were able to find *de novo* genetic variation that could be associated to some of the traits. In flax (*Linum usitatissimum*), genomic changes associated with the environment in which the plant was grown have been found in the progeny of treated inbred flax variety ‘Stormont Cirruss’ plants (Durrant 1971; Cullis 1977; Schneeber and Cullis 1991; Cullis 2005). The term “plastic” was used to describe the ability of the plants to respond to changes in the environmental conditions, of soil fertility or temperature which resulted in progenies with phenotypic and genotypic differences. These differences were revealed when first selfed generation progenies were compared under

uniform growing conditions. Progenies varied in plant height depending on treatments applied to their parents (Durrant 1971). Other morphological, biochemical, and genetic differences have been associated with environmental induction which were stably inherited while others were unstably inherited to subsequent generations (Durrant 1971; Schneeber and Cullis 1991; Chen et al. 2005).

Unusual InDel variation detected at different stages of single plants

In the honeycomb design, single plants were sampled three times at different developmental stages (Figure 1). As shown in Table 2, we found unusual DNA patterns in 11 entries of ‘BSR 101’ single plants grown in a honeycomb design with non-simulated hail treatment, i.e. the control. This variation was mostly found at the last date of collection (date-2) at approximately at R3-R4 reproductive stage. There were two cases where we found unusual patterns at date-0 collection (Table 2). Although this variation was detected using the same forward and reverse primer sequences with labeled and non-labeled sets of primers, subsequent replications failed using conventional PCR amplification. Consequently, sequencing of unusual fragments was not possible. From several strategies used to replicate these results, only the use of an internal primer paired with an external primer gave similar results to the ones obtained with original primers (Figure 3). ‘BSR 101’ genotype has the deletion at InDel marker BARC-017059-02188. At this position, using primers flanking the InDel, PCR reactions produced unexpected DNA patterns for ‘BSR 101’ entries 68, 88, 113, 139, and 148 at date-2 collection (Figure 3). For this location, when we used an internal primer paired with an external primer, an amplicon was detected at date-2 collection in ‘BSR 101’ entries 68, 88, 95, 119, 120, and 148 for the control. Amplicon fragments were not

detected at date-0 and date-1 collections. When we tested this primer set in the ‘BSR 101’ simulated hail experiment, there was no amplicon product in any of the collection dates.

Using the internal and external primers to the target sequence, we found that ‘BSR 101’ entries 95, 119, and 120 showed an amplicon in date-2 that previously was not found using the original set of primers. Additionally, there was no amplicon product for entries numbers 113 and 139 date-2, which was shown initially using the original set of primers.

Sequencing analysis of DNA products from ‘BSR 101’ entries 68 and 119 at date-2 collection, using the internal and external set of primers, showed that these DNA samples had acquired a 19-nucleotide insertion, which is unexpected for the ‘BSR 101’ genetic background (Figure 4). However, sequencing analysis of DNA products from ‘BSR 101’ entry 119 at all dates of collection, using the original set of primers, did not show the insertion fragment at any collection date. Although the 19-nucleotide insertion was not detected using the original set of primers, our results suggest there is not a random event for the detected unusual DNA amplifications. This pattern is detected in the DNA extracted from date-2 collection, R3-R4 reproductive stages, in plants evaluated in the honeycomb design control experiment.

Using highly inbred lines and evaluating single plants at different developmental stages, we consider pollen contamination and seed mixture very unlikely explanations for these experimental results. Based on the fact that some InDel markers might have similar sequences in two chromosomes, for example, BARC-001499-00119 and BARC-017059-02188, we could have amplification of similar sequences in two separate genomic regions, which could be the reason for this *de novo* variation. However, the pattern of unusual events is consistently identified in the majority of cases in date-2 samples. Expression of unusual

DNA patterns in the control experiment with single plants of ‘BSR 101’ grown in a honeycomb design might be associated with the planting design rather than with the stress treatment (simulated hail) evaluated in the growing season. As we mentioned above, ultra-low planting density is believed to reduce stress, due to the abundance of resources; for example, water, nutrients, and light interception (Duvick 1997; Fasoula and Fasoula 1997).

Ultra-low spacing effect

In our study, results from ‘BSR 101’ grown in a honeycomb design with non-simulated hail treatment (control), suggested that abundant availability of resources per single-plant might have a contribution for the genetic changes observed at date-2 of collection. Interplant competition, which is defined as a decrease in the amount of light interception per plant due to proximity to adjacent plants, has been long recognized to affect selection for yield and yield components (Fasoulas 1973; Duvick 1997; Fasoula and Fasoula 1997). Management factors adopted to reduce this effect, for example, plant spacing, population density, and timing of loss in plant stand, have been developed. Fasoulas (1973) identified interplant competition and soil heterogeneity as factors which make single-plant selection for yield ineffective. He proposed the honeycomb selection method, which is based on the theory that single-plant selection can be effective, if interplant competition is eliminated. Single plants grown in a honeycomb design are space-planted and arranged in a hexagonal pattern of plant positions, like in a honeycomb, such that every plant is in the center of a hexagon. This arrangement of single plants allows reliable sampling for environmental diversity and high selection pressures.

Ultra-low plant spacing is believed to minimize stress and allows maximum grain production per plant, by increasing the amount of light, water, and nutrients to which an

individual plant has access (Duvick 1997; Fasoula and Fasoula 1997). According to their theory, in the absence of competition, phenotypic expression and differentiation are maximized, which allow optimization of plant heritability. In several studies, honeycomb selection was effective for improving the potential yield per plant and yield components (Tokatlidis et al. 1998, 2000; Christakis and Fasoulas 2002; Fasoula and Boerma 2005, 2007; Tokatlidis et al. 2010). These authors showed that selection within cultivars could be an effective way to either improve or maintain the cultivar. Although genetic variation was reduced in every generation of self-pollination, exploitation of intracultivar variation using honeycomb selection was effective in advanced generations (Lungu et al. 1987; Christakis and Fasoulas 2002; Fasoula and Boerma 2005, 2007).

Recently, Yates et al. (2012) reported that single-plant soybean lines, developed at ultra-low plant density from honeycomb selection by Fasoulas et al. (2007a, 2007b, 2007c), had a genotypic component identified by SSR markers that could not be explained as residual heterozygosity. This *de novo* genetic variation within commercial cultivars, was estimated to be 18% for ‘Benning’, 7% for ‘Haskell’ and 18% for ‘Cook’ cultivars, which Yates et al. 2013 attributed to spontaneous mutation or other genetic mechanisms.

Genetic variation in progeny of single plants grown in a honeycomb design has been reported for many plant species. However, no reported studies have investigated the presence of *de novo* genetic variation within a single plant at different developmental stages following stress. We evaluated DNA from leaf tissue collected at different developmental stages from single plants. Due to unusual variation, detected in the control of ‘BSR 101’ honeycomb treatment, we evaluated DNA from progeny of 22 out of 150 entries of ‘BSR 101’ and ‘Jack’ control treatment. Although variation was not observed in the next

generation, at least in the 15 seedlings evaluated for each entry number, de *nov*o variation might be revealed by increasing the number of seedlings evaluated per progeny or in further selfing generations.

Genetic changes in single plants

InDel markers evaluated at different developmental stages in single plants within each cultivar resulted in unusual DNA patterns at the last date of collection (date-2) approximately at R3-R4 reproductive stage (Figure 1, Table 3). Changes during the inducing treatment or plant development have been reported in soybean, flax and Arabidopsis (Roth et al. 1989; Cullis 2005; Hopkins et al. 2013). In soybean, cell cultures prepared from roots, stems, cotyledons or leaves taken from the same plant showed RFLP allelic differences at various loci (Roth et al. 1989). Cultured root tissue generated difference while cotyledon, stem or leaf derived culture tissue did not. In flax, data collected from measurements of the nuclear DNA, determination of the ribosomal RNA gene number, and the identification of an insertion event (termed LIS-1, for *Linum* Insertion Sequence 1), during growth of plants, indicated that DNA changes occurred during the vegetative growth of the plants before flowering (Cullis 2005). The author also reported that DNA changes occurred in the apical meristem during vegetative growth and not in differentiated tissue. It is possible that at flowering time the plants were chimeric. Identification of multiple sectors in single plants of Arabidopsis also was reported by Hopkins et al. (2013). Self-fertilization of homozygous F₂ mutants (*hth*) for HOTHEAD (*HTH*) organ fusion gene was reported to produce F₃ progeny that was phenotypically wild type for *HTH*. Genotypic changes also were verified when InDel makers profiles were compared between F₂ parental lines and F₃ progenies (Hopkins et al. 2013). InDel marker evaluation of individual *hth* mutant plants at multiple locations

allowed them to confirm that genetic discordance between F₂ and F₃ individuals was due to sectoring. This event also was found in individual wild-type hybrid lines when roots and shoots from the same seedling were compared (Hopkins et al. 2013).

Occurrences of genetic variation in single plants of different backgrounds and species have been associated with the differences in the growing environment and developmental stages (Roth et al. 1989; Cullis 2005; Hopkins et al. 2013). Roth et al. (1989) suggested these differences arise as a result of differences between cell types which might have a roll in the genetic stability. Following these findings, we hypothesize that the variation which we observed in single plants of ‘BSR 101’ non-simulated hail occurs in the plant in a non-random manner. Honeycomb design might have contributed to unusual DNA patterns observed in date-2 collection. This variation in the last date of collection might suggest that the plants were able to express the effect of an ultra-spacing planting at a later developmental stage.

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Table 1. InDel marker distribution, pair of primers used for InDel genotyping, amplicon product size, InDel size and expected polymorphism in ‘BSR 101’ and ‘Jack’ inbred lines.

InDel marker	Chr ^a	LG ^b	Primer	Size ^c	InDel ^d	‘BSR 101’ ^e	‘Jack’ ^f
BARC-015905-02012	5	A1	F: ATTTACTAAACTATGCTAGCTTTG R: TGTGGGACCTGCTATACTG	143	14	D	I
BARC-047166-12877	5	A1	F: TTGATTAAGGTTGTGTTGTG R: AAGTAGCAATTTTACCTCTACTCC	120	12	I	D
BARC-017059-02188	5/17	A1/D2	F: GAGCACTATCAATGTCAGAC R: AGCATCACTAATTGTTGCTG	111	19	D	I
BARC-028361-05843	8/5	A2/A1	F: CTTGCTGCAGTTGAAGAACCAAC R: CTAGTTATGTCATCATTGTGTCATG	163	26	D	I
BARC-014413-01360	9	K	F: TAGAGCCACCCCTTTATGTCATGTTAC R: GGTTTCCACATTCACATGCATAG	170	48	I	D
BARC-065401-19428	10	O	F: CAAAGGTGAATTCTATCTC R: TGATTATCCTTGTGCAAGTAC	164	26	I	D
BARC-028339-05837	16	J	F: CACTCATTCTGGTCTTTAGGAC R: AAAGTCACCTAGCCTTCATTTG	163	18	D	I
BARC-065341-19358	17	D2	F: AGCTTAAAATTAAGGAAATTG R: TTATAATGGTGCTGACTG	126	19	D	I
BARC-047681-10375	18	G	F: ATGAGCATGGATTGCAAC R: CAAAGGGTTAGAGAAGACTGAG	159	15	D	I
BARC-019345-03881	18	G	F: GAATAATGAGAATGAAAGTTCTCC R: CGTTATTTTCGTA CT TATTTTG	115	15	D	I
BARC-001499-00119	20/1 0	I/O	F: GGATTGGTAAGTATCATCCAAC R: CATGTTTTAGTTAAATACATG	132	21	I	D
BARC-007970-00182	20/1 0	I/O	F: GACCCATATGAATTTTATCCAAC R: TTATCTATTGGACACA ACTCTCGC	132	21	I	D

^a Chr: Chromosome number where the InDel is located. In some cases an alignment pair (query to hit) has high bit score and expect value (E-value) in two chromosomes. Example, BARC-017059-2188, ^b LG: Denotes linkage group,

^c PCR product size (bp), ^d Insert size (bp), ^e Expected InDel polymorphism for ‘BSR 101’; “I” denotes insertion, “D” denotes deletion, ^f Expected InDel polymorphism for ‘Jack’; “I” denotes insertion, “D” denotes deletion.

Table 2. Pair of external and internal primers used for testing unusual DNA patterns. Primers set per each InDel maker, forward and reverse primer sequences, relative position to the target sequence and amplicon size.

InDel	Primer set ^a	Forward primer sequence	Position ^b	Reverse primer sequence	Position ^c	Size ^d
BARC-047166-12877	1	GGATGGAAGGTGGTGAAGAA	External	CATCACCCATCCACCATGTA	External	187
BARC-017059-02188	1	CGGCTTCTTTCTTCAGTTGG	External	TTTGTTTTTCGTTTTGGAGCA	External	228
BARC-014413-01360	1	TGGCATTTCGAATCCACAA	External	ACCCACAACCTCAGAGCAAT	External	222
BARC-028339-05837	1	GAGCTGAACTGCAATGGTGA	External	TTGCAGCTGATGGTCCAATA	External	226
BARC-028339-05837	2	CTGCAATGGTGATGAGTGCT	External	ATTTCCATGCCAAAGTCACC	External	248
BARC-047166-12877	1	TGGGGTCTTTCTTTGATTGG	External	ATCAATATCCACAAGCACATGAC	Internal	185
BARC-047166-12877	2	TGGGGTCTTTCTTTGATTGG	External	CACAAGCACATGACACAGAGG	Internal	176
BARC-017059-02188	1	CGGCTTCTTTCTTCAGTTGG	External	GAAGAGATAATTAAAAAGTCACAT	Internal	193
BARC-017059-02188	2	ACAATCATTGTACATGACAAAAG	Internal	TTGACTGCATGTCTTCACGA	External	239
BARC-014413-01360	1	TCATGTTACATCCAAATGAACCTAT	External	GAGTACTCTTTATGATTGTGGATT	Internal	130
BARC-014413-01360	2	GAGCCAACCTTTATGTCATGTT	External	CATAGGAGTACTCTTTATGATTGT	Internal	150
BARC-028339-05837	1	CACTCATTCTGGTCTTTAGGAC	External	CCGTTTCAGTTACATCAGTTGTTT	Internal	108
BARC-028339-05837	2	CACTCATTCTGGTCTTTAGGAC	External	GACACAGCCGTTTCAGTTACATC	Internal	115
BARC-028339-05837	3	GGTGCTACAACTGATGTAAGTAAAC	Internal	CCCCTAGCCGAAATAGGTCT	External	250

^a Primer set: In some cases there were two primer sets used per InDel marker.

^b Forward primer position relative to the target sequence

^c Left primer position relative to the target sequence

^d PCR product size (bp)

Table 3. Genetic variation detected in 'BSR 101' non-simulated hail experiment

'BSR 101' - entry number	Date-0	Date-1	Date-2	InDel
68	False	False	True	BARC-017059-2188, BARC-007970-00182, BARC-047681-10375, BARC-014413-1360, BARC-028339-05837, BARC-028361-05843
88	False	False	True	BARC-017059-2188, BARC-014413-1360, BARC-028339-05837, BARC-028361-05843
95	False	False	True	BARC-028339-05837, BARC-028361-05843
97	False	False	True	BARC-014413-1360, BARC-028339-05837
103	False	False	True	BARC-014413-1360
113	False	False	True	BARC-017059-2188, BARC-007970-00182, BARC-047166-12877, BARC-047681-10375, BARC-014413-1360, BARC-028339-05837, BARC-065401-19428, BARC-065341-19358, BARC-028361-05843
119	False	False	True	BARC-014413-1360, BARC-028339-05837, BARC-028361-05843
120	False	False	True	BARC-014413-1360, BARC-028339-05837, BARC-028361-05843
133	True	False	True	BARC-047166-12877, BARC-065401-19428
139	True	False	True	BARC-017059-2188, BARC-047166-12877, BARC-014413-1360, BARC-065401-19428, BARC-028361-05843
148	False	False	True	BARC-017059-2188, BARC-047166-12877, BARC-014413-1360, BARC-028339-05837, BARC-065401-19428

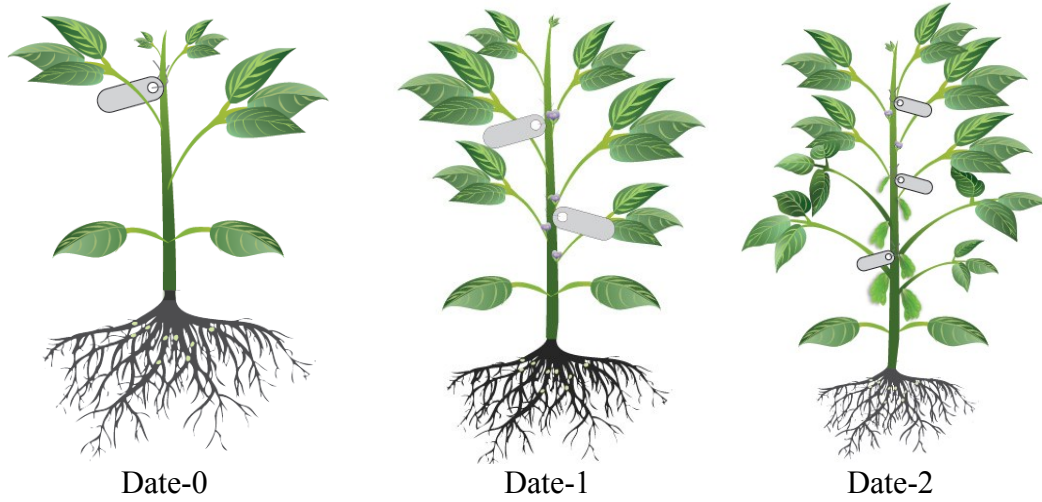
Table 4. Unusual DNA patterns detected in 11 entries of ‘BSR 101’ non-simulated hail experiment.

Genotype/InDel	02012	12877	02188	05843	01360	19428	05837	19358	10375	03881	00119	00182
‘BSR 101’	D	I	D	D	I	I	D	D	D	D	I	I
‘Jack’	I	D	I	I	D	D	I	I	I	I	D	D
‘BSR 101’- 68-0	D	I	D	D	I	I	D	D	D	D	I	I
‘BSR 101’- 68-1	D	I	D	D	I	I	D	D	D	D	I	I
‘BSR 101’- 68-2	D	I	H	H	H	I	H	D	H	D	I	D
‘BSR 101’- 88-0	D	I	D	D	I	I	D	D	D	D	I	I
‘BSR 101’- 88-1	D	I	D	D	I	I	D	D	D	D	I	I
‘BSR 101’- 88-2	D	I	H	H	H	I	H	D	D	D	I	I
‘BSR 101’- 95-0	D	I	D	D	I	I	D	D	D	D	I	I
‘BSR 101’- 95-1	D	I	D	D	I	I	D	D	D	D	I	I
‘BSR 101’- 95-2	D	I	D	H	I	I	H	D	D	D	I	I
‘BSR 101’- 97-0	D	I	D	D	I	I	D	D	D	D	I	I
‘BSR 101’- 97-1	D	I	D	D	I	I	D	D	D	D	I	I
‘BSR 101’- 97-2	D	I	D	D	H	I	H	D	D	D	I	I
‘BSR 101’- 103-0	D	I	D	D	I	I	D	D	D	D	I	I
‘BSR 101’- 103-1	D	I	D	D	I	I	D	D	D	D	I	I
‘BSR 101’- 103-2	D	I	D	D	H	I	D	D	D	D	I	I
‘BSR 101’- 113-0	D	I	D	D	I	I	D	D	D	D	I	I
‘BSR 101’- 113-1	D	I	D	D	I	I	D	D	D	D	I	I
‘BSR 101’- 113-2	D	D	I	I	H	H	I	D	H	D	I	D
‘BSR 101’- 119-0	D	I	D	D	I	I	D	D	D	D	I	I
‘BSR 101’- 119-1	D	I	D	D	I	I	D	D	D	D	I	I
‘BSR 101’- 119-2	D	I	D	H	H	I	I	D	D	D	I	I
‘BSR 101’- 120-0	D	I	D	D	I	I	D	D	D	D	I	I
‘BSR 101’- 120-1	D	I	D	D	I	I	D	D	D	D	I	I
‘BSR 101’- 120-2	D	I	D	H	H	I	H	D	D	D	I	I
‘BSR 101’- 133-0	D	I	D	D	H	I	D	D	D	D	I	I
‘BSR 101’- 133-1	D	I	D	D	I	I	D	D	D	D	I	I
‘BSR 101’- 133-2	D	H	D	D	I	H	D	D	D	D	I	I
‘BSR 101’- 139-0	D	I	D	D	H	I	D	D	D	D	I	I
‘BSR 101’- 139-1	D	I	D	D	I	I	D	D	D	D	I	I
‘BSR 101’- 139-2	D	H	H	H	H	H	D	D	D	D	I	I
‘BSR 101’- 148-0	D	I	D	D	I	I	D	D	D	D	I	I
‘BSR 101’- 148-1	D	I	D	D	I	I	D	D	D	D	I	I
‘BSR 101’- 148-2	D	H	H	D	H	H	H	H	D	D	I	I

D: Denotes deletion, I: Denotes insertion, H: Denotes heterozygote

Figure 1. Leaf tissue collection and tagging at different developmental stages. A. Non-simulated hail experiment B. Simulated-hail experiment

A.



B.

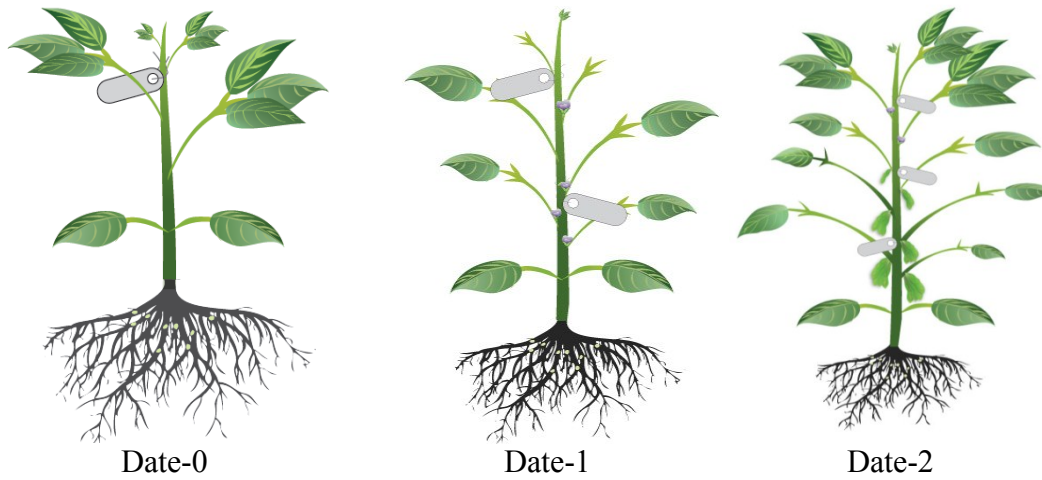


Figure 2. PCR product obtained with original set of primers BARC-017059-02188. Products are only shown for 7 out 11 entries of 'BSR 101' control (non-simulated hail)

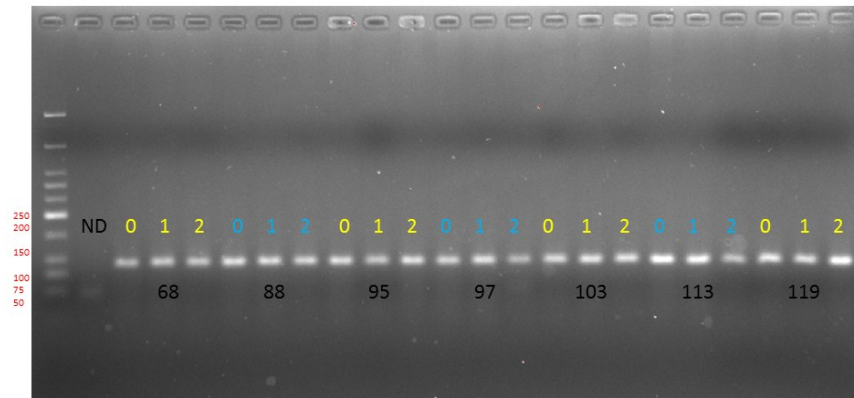


Figure 3. PCR product obtained with internal set of primers designed for InDel BARC-017059-02188. Products are shown for all 11 unusual entries of 'BSR 101' control (non-simulated hail)

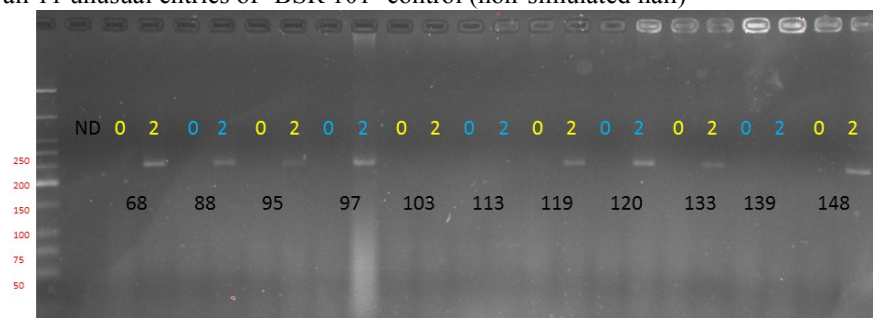


Figure 4. DNA sequence alignment obtained from 'BSR 101' control (non-simulated hail) entries 68 and 119 at date-2 of collection, using internal set of primers for InDel BARC-017059-02188

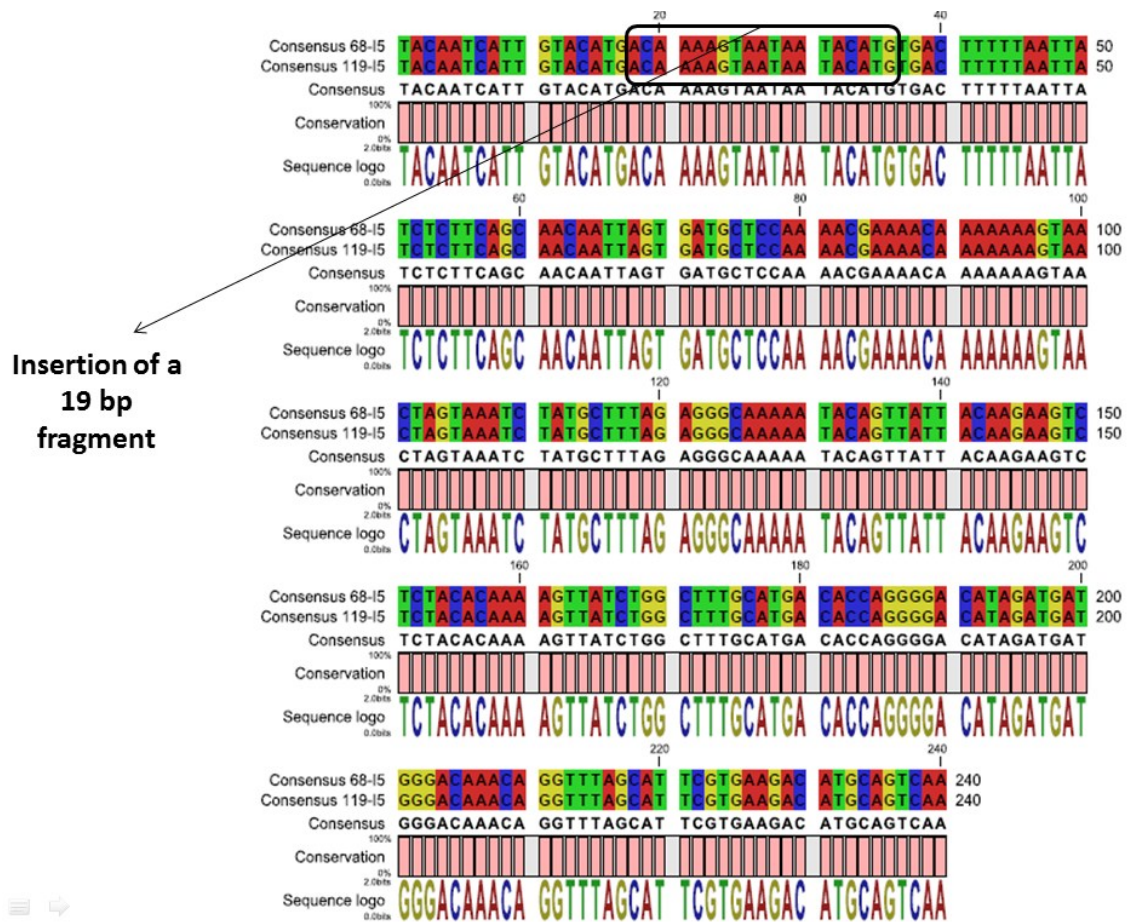
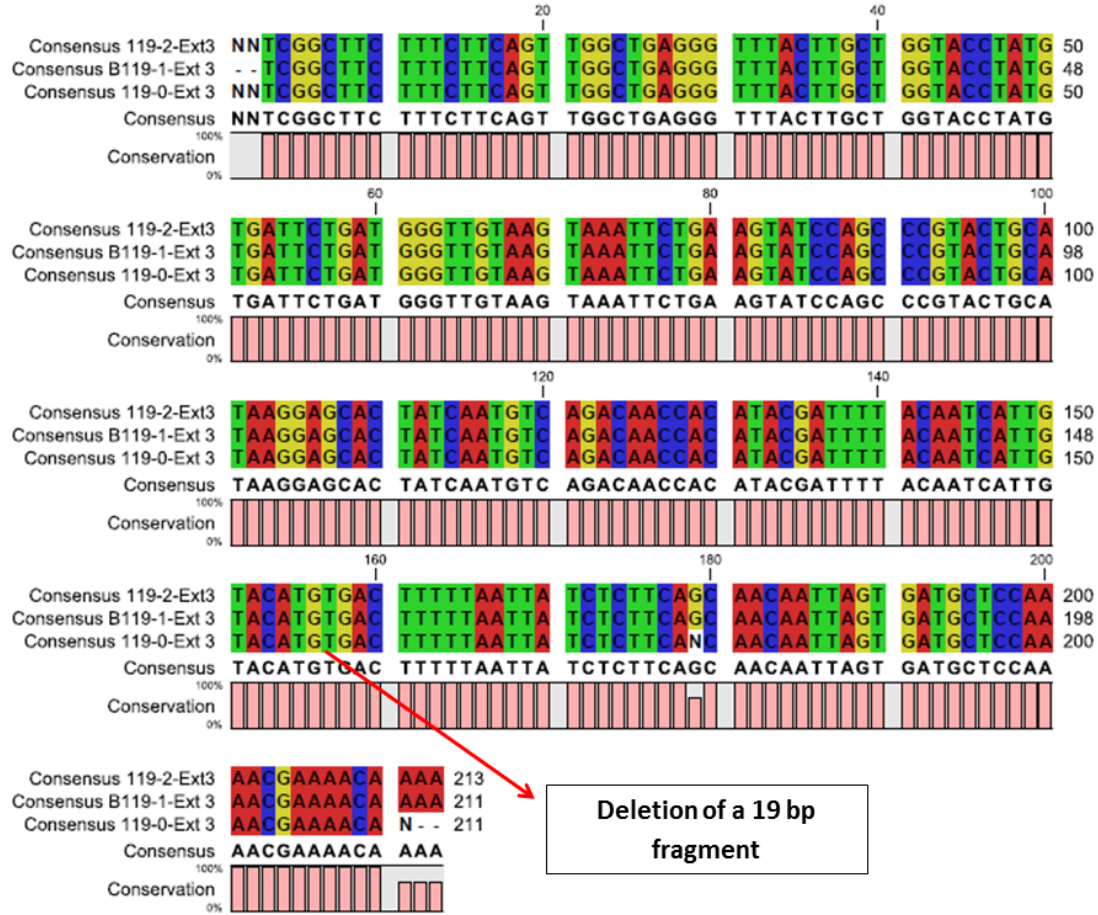


Figure 5. DNA sequence alignments obtained from 'BSR 101' control (non-simulated hail) entry 119 at all dates of collection, using external set of primers for InDel BARC-017059-02188



CHAPTER 5: GENERAL CONCLUSIONS

The main objective of this research project was to enhance intrinsic genetic variation in soybean inbred lines. Strategies included a honeycomb planting design (ultra-low plant density), and an accelerated aging test as seed stress to evaluate their effect in progeny of treated plants. These methods have been demonstrated to result in phenotypic and genetic changes in many plant species.

A honeycomb design was used to produce large amounts of seed for additional experiments, and to evaluate the effect on the agronomic performance within ‘IAR2001BSR’, ‘BSR 101’, and ‘Jack’ soybean inbred lines. In summer 2008, the honeycomb experiment was performed, however, field plots were damaged by natural-hail storm in July reducing yield in most entries. In 2009, progeny of single plants from the honeycomb 2008 experiment were planted for seed increase. During field evaluation lethal-yellow and semi-sterile mutants were identified in three entries, one in each cultivar. Using genetic and molecular analysis, the viable-yellow mutant was shown to behave as a recessive single-gene inheritance located on chromosome Gm02 (MLG D1b), flanked by BARC_02_1454 and BARC_02_1468. Using information between flanking markers, we located 28 predicted genes, for which *Glyma02g39990* and *Glyma02g40150* are of particular interest as they might play role in photosynthesis. Although we have a confounding effect due to the injuries caused by the natural-hail storm, these results could be indicative that stress caused by ultra-low plant densities by the natural-hail damage or their interaction might result in enhanced genetic variation within already established cultivars, and/or highly homozygous inbred lines.

In the second study we evaluated the effect of genomic stress caused by sexual hybridization and an accelerated aging test on pure lines. For the sexual hybridization experiment, cross-combinations were made between ‘BSR 101’, ‘Minsoy’, and ‘Noir 1’. For the pure-line experiment, ‘BSR 101’ and ‘Jack’ lines were used. Evaluation of changes was performed through the examination of segregation patterns for aconitase-2 and aconitase-4 enzyme variants, and the segregation for DNA-based molecular makers. We found a large frequency of unexpected variation in the pure-line experiment, which were plants from seed treated with an accelerated aging test. This variation was found among seed evaluated within a pod. Although it is difficult to rule out outcrossing events, these results are intriguing since both self-pollination and outcrossing could occur within a single pod. If different mechanisms are enhancing this genetic variation within single pods and within cultivars, these results are evidence that accelerated aging treatment can be used to induce stress which results in enhanced genetic variation that could be useful for improvement of cultivars. Again, the confounding effect of the natural-hail needs to be separated from the effect of the honeycomb, and the accelerated aging test.

In the last study, we investigated the effect of simulated-hail on single plants grown in a honeycomb design, and as control for the possible effect of the honeycomb design, we evaluated the effect of simulated hail on plants grown in a conventional tractor-planting design. The simulated-hail procedure was performed approximately at the V4-V5 vegetative stage. Leaf samples were collected at three different dates. For DNA evaluation, 12 InDel molecular markers were used. There was no detected variation in the simulated hail experiments for both honeycomb and conventional tractor planting patterns in cultivars ‘BSR 101’ or ‘Jack’. However, we did find unusual DNA variation at the last date of collection

(date-2) for 'BSR 101' cultivar, but not in 'Jack' cultivar. According to these results, genetic changes might occur at specific developmental stages of the plant, or after treatment delayed in time due to the difference in days between date-0 and date-2 of collection; which might suggest that genomic changes are non-randomly distributed.