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Abstract
Intra-cultivar variation reported in pure lines of soybean has been hypothesized to result from genetic mechanisms contributing to de novo genetic variation. In this study we have detected allele switching by following segregation pattern of Aconitase-4 isozyme in sexual crosses and pure lines. In sexual crosses, one F2 plant showed switch at the Aconitase- 4 (Aco4) locus from the expected heterozygous genotype Aco4-ac to Aco4-ab. In the pure lines grown in a honeycomb planting design and treated with an accelerated aging test, multiple cases of allele switching were detected at the Aco4 locus. Both single and double switches were detected that were stable and heritable. These findings indicate that the generation of endogenous variation continues in pure lines as a result of intrinsic genetic mechanisms. With a long-term goal of understanding the genetic nature of the changes, we genetically mapped the Aco4 gene to a 3.3 cM region on Chromosome 11. The corresponding physical region is ~293 kb with 39 predicated genes. Of these, Glyma.11g080600 is of particular interest, as it shows 93% and 88% identity to Medicago truncatula and Arabidopsis aconitase genes, respectively. Further characterization of the soybean Aco4 gene may shed light on genetic mechanisms responsible for allele switching.

Keywords
Allele switching, soybean, aconitase, linkage mapping, isozyme

Disciplines
Agricultural Science | Agronomy and Crop Sciences | Plant Breeding and Genetics

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

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ABSTRACT

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

Running Title: Allele switching in soybean

Keywords: Allele switching, soybean, aconitase, linkage mapping, isozyme

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Intra-cultivar variation has been reported in most commercial cultivars for decades, and yet it is often ignored or attributed to outcrossing or contamination (Byth and Weber 1968). Nevertheless, increasing evidence of intra-cultivar variation within already established cultivars has been revealed when subjected to a wide range of conditions and factors (Byth and Weber 1968; Durrant 1962; Fasoula and Boerma 2005; Fasoula and Boerma 2007; Gordon and Byth 1972; Haun et al. 2011; Rasmusson and Phillips 1997; Roth et al. 1989). In maize (*Zea mays* L.) evidence of intra-cultivar variation was reported in doubled haploid lines and long-term inbred lines (Bogenschutz and Russell 1986; Russell et al. 1963). These inbred lines were shown to accumulate significant differences for several quantitative traits that exceeded commonly reported rates of spontaneous mutation. In flax, heritable changes were found in stable lines for plant height, plant weight, total amount of nuclear DNA (Evans et al. 1966), and isozyme band mobility for peroxidase and acid phosphatase (Cullis and Kolodynska 1975). Further studies demonstrated that in flax these changes were associated with non-random changes in the DNA sequence, and chromosomal rearrangements (Chen et al. 2009; Chen et al. 2005; Schneeberger and Cullis 1991).

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

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carbon isotope discrimination, and ash content (Christakis and Fasoulas 2002; Tokatlidis 2000; Tokatlidis et al. 2004; Tokatlidis et al. 2005).

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

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

Significant intra-cultivar variation in commercial soybean cultivars, 'Benning', 'Haskell', and 'Cook' for seed protein, seed oil, fatty acids, seed weight and other agronomic

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

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

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

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

MATERIALS AND METHODS

Plant material and seed source

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

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University at Ames, IA, and 'Jack' seed was obtained from Dr. R.C. Shoemaker, USDA ARS at Ames, IA.

Sexual hybridization

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

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

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

F_2 seed from self-pollinated F_1 plants of the three cross-combinations from the October 2007 Puerto Rico planting were analyzed for Aconitase-4 isozyme variation and seedlings were transplanted at the Bruner Farm. The seeds used for this experiment included 50 F_2 seeds from 18 F_1 plants representing 10 different 'Noir 1' female parent plants crossed to 'BSR 101'; 50 F_2 seeds from 18 F_1 plants representing 9 different 'Minsoy' female parent plants crossed to 'BSR 101'and 50 F_2 seeds from 19 F_1 plants representing 8 different 'Minsoy' female parent plants crossed to 'Noir 1'.

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158 $F_{2:3}$ seeds from self-pollinated F_2 plants of the three cross-combinations from the
159 February 2008 Puerto Rico planting were analyzed for Aconitase-4 isozyme variation and
160 seedlings were transplanted at the Bruner Farm. Two seeds from each $F_{2:3}$ plant from all three
161 cross-combinations were selected. The seeds used for this experiment included 352 $F_{2:3}$ seeds
162 from 176 F_2 plants from the February 2008 plots 6, 16, 17, 24, 25, and 27, representing 'Noir
163 1' as female parent plant crossed to 'BSR 101'. Also included were 942 $F_{2:3}$ seeds from 471
164 F_2 plants from the February 2008 plots 29, 30, 31, 32, 33, 36, 37, 45, 46, 47, 48, 51, 52, 53,
165 54, 55, 56, 59, and 60 representing 'Minsoy' as female parent plant crossed to 'BSR 101'.
166 Additionally, 308 $F_{2:3}$ seeds were included from 154 F_2 plants from the February 2008 plots
167 63, 64, 65, 66, 69, 70, 75, and 76 representing 'Minsoy' as female parent plant crossed to
168 'Noir 1'.
169
170 **Pure lines; accelerated aging seed stress treatment**
171 Soybean cultivars 'BSR 101' and 'Jack' were used to study the effect of seed stress in
172 pure lines. In 2008, 500 seeds of 'BSR 101' and 'Jack' were planted in a honeycomb design
173 (Fasoula and Fasoula 1997; Fasoula and Boerma 2005), using an equidistant spacing of 2.0 m
174 between each individual plant to eliminate the unfavorable effect of competition in response
175 to selection (Fasoula and Boerma 2005). A code, termed "entry number", was assigned to
176 each individual plant. Field plots were damaged by a natural hail in July 2008 reducing yield
177 in most entries. For this reason, the number of harvested plants was reduced to 315 plants for
178 'BSR 101' and 305 plants for 'Jack'. At harvest, plants were single-plant threshed and
179 analyzed for Aconitase-4 isozyme.

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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 exposed to ~100% relative humidity and partially hydrated seeds maintained at 41°C for 48 hours, and hand-planted at the Bruner Farm near Ames, IA, in a completely randomized design with two replications. At harvest, a single three-seeded pod was collected randomly from three separate plants per entry per replication and analyzed for the Aconitase-4 isozyme.

Aconitase isozyme analysis

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

Seeds were germinated on germination paper for 72 h at 30 °C in the dark. The three day-old seedlings were sampled by punching out three pieces of the cotyledon using a 200-µL glass-bore pipettor. The samples were placed in 1.5 mL polypropylene microcentrifuge tubes to which 120 µL cold extraction buffer {0.1 M tris-HCl, pH 7.2, 4% (wt/v) PVP-40 (polyvinylpyrrolidone, molecular weight 40,000), 400 mM sucrose, 1 mM dithiothreitol} was added. Samples were ground for 30 s by using a laboratory stirring motor (TRI-R STIR-R, Model S63C, Chicago, 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 × *g* for 3 min. The supernatant was

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

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

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

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

Genetic analysis of aconitase variants

After isozyme analysis, seedlings of progeny that expressed variants in the isozyme pattern were saved and transplanted into pots containing a standard greenhouse soil mix (2 soil: 1 sand: 1 peat). These seedlings were maintained in the USDA-ARS greenhouse (Ames, Iowa) 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

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electrophoretically to determine the Aconitase-4 genotype (homozygous or heterozygous) and to estimate the segregation ratio.

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

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

***Aco4*, molecular mapping; DNA isolation and Bulked Segregant Analysis (BSA)**

For the genetic linkage mapping, an F_2 population of 94 plants, generated by crossing parent plants 'BSR 101' (*Aco4-aa*) and 'Noir 1' (*Aco4-bb*) was used. Genomic DNA was isolated according to the method described previously (Sandhu et al. 2004). Bulks were created with *aa* or *bb* allele types by taking 1 µg DNA from ten homozygous *Aco-aa* or ten homozygous *Aco-bb* F_2 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.

***Aco4*, molecular mapping; molecular marker analysis**

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247 For the SSR analysis, 50 ng of DNA was used for a 10 µl reaction with 1x reaction
248 buffer (10mM Tris-HCl, 50mM KCl, pH 8.3), 2.0 mM $MgCl_2$, 0.25µM of each primer, 200
249 µM of each dNTP, and 0.25 units of Biolase DNA polymerase (Bioline USA, Inc., Tauton,
250 MA). PCR was completed with one cycle at 94 °C for 3 min, followed by 11 cycles of 94 °C
251 for 30 s, 58 °C for 30 s with an increment of -1 °C per cycle and 72 °C for 1 min, 35 cycles
252 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.
253 The PCR products were resolved on a 4 % agarose gel at 150 V for 2-4 h. The genetic
254 linkages and distances were determined using Mapmaker 2.0 (Kosambi 1944; Lander et al.
255 1987). The order of the markers was determined at LOD threshold of 3.0. Markers were
256 developed using information from http://soybase.org/resources/ssr.php and
257 http://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Gmax (Song et al. 2010; Song
258 et al. 2004).
259
260 **RESULTS**
261 **Effect of sexual hybridization**
262 To establish a baseline in the absence of sexual hybridization, approximately 500
263 transplants of 'BSR 101', 'Minsoy', and 'Noir 1' were analyzed for aconitase isozyme
264 variation and were transplanted at the Bruner Farm. At the *Aco4* locus all individuals of
265 'BSR 101' were *Aco4-aa*, 'Minsoy' were *Aco4-cc*, and 'Noir 1' were *Aco4-bb*, as expected.
266 A total of 2204 F_2 progeny from the three different sexual crosses were assayed for
267 Aconitase-4 (Table 1). This includes a total of 789 F_2 plants derived from 'Noir 1'× 'BSR
268 101', 650 F_2 plants from 'Minsoy' × 'Noir 1' and 765 F_2 plants from 'Minsoy' × 'BSR 101'.
269 The F_2 genotypes fit the expected 1:2:1 genotypic ratio (Table 1), with one exception. One F_2

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plant, from 'Minsoy' × 'BSR 101', A08-AS-2932, was *Aco4-ab*, an unexpected genotype (Figure 1). The F_2 homozygous genotypes were true breeding and the F_2 heterozygous genotypes segregated 1:2:1, as expected (data not shown).

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

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

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

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

Effect of seed stress in pure lines

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

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

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315 ACONITASE-4 VARIANT; STABILITY TEST. As shown in Table 4, self-
316 pollination of the novel *Aco4-bc* variant ('Jack' entry 127) gave rise to progeny that
317 segregated a good fit to the expected ratio (12 *Aco4-bb*: 24 *Aco4-bc*: 14 *Aco4-cc* plants). Four
318 of the 12 plants that scored as homozygous for the Aconitase-4 '*b*' allele (*Aco4-bb*), A12-
319 AS-8, A12-AS-11, A12-AS-13, and A12-AS-41, were used for additional aconitase
320 determinations. Plants A12-AS-8, A12-AS-11, A12-AS-13, and A12-AS-41 were self-
321 fertilized and 21, 25, 22, and 21 seedlings, respectively, were analyzed for aconitase enzyme
322 variations. All 89 F_3 plants were *Aco4-bb* as expected.
323
324 ALLELISM TESTS. Four 'Jack' entry 127 *Aco4-bb* plants were crossed to 'Noir1'
325 (*Aco4-bb*); the standard *Aco4-bb* genotype. The number of testcross seeds using four variant
326 *Aco4-bb* plants varied from 7-15, for a total of 43 testcross seeds. All the seeds showed the
327 expected *Aco4-bb* genotype.
328
329 **Genetic linkage mapping; The *Aco4* gene**
330 With an objective of developing a better understanding of genetic mechanisms
331 leading to allele switching, we mapped the *Aco4* gene. To find the location of the *Aco4* gene,
332 700 SSR markers covering the entire soybean genome were tested on the bulks. Satt509
333 showed polymorphism between the bulks, indicating the candidate gene was on chromosome
334 11, MLG B1 (Song et al. 2004). Eighty-nine SSR markers on MLG B1 near Satt509 were
335 tested for polymorphism between the parents. Of these, 12 showed polymorphism:
336 BARCSOYSSR_11_001, BARCSOYSSR_11_008, BARCSOYSSR_11_030,
337 BARCSOYSSR_11_056, BARCSOYSSR_11_316, BARCSOYSSR_11_323,

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338 BARCSOYSSR_11_336, BARCSOYSSR_11_338, BARCSOYSSR_11_339,
339 BARCSOYSSR_11_345, Sat_272, and Satt509. The 12 polymorphic SSR markers were
340 tested on the entire population. The putative *Aco4* gene is flanked by
341 BARCSOYSSR_11_323 and BARCSOYSSR_11_336. Marker BARCSOYSSR_11_336 was
342 1.4 cM away from the gene (Figure 2). Using the soybean genome sequence
343 (http://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Gmax) (Schmutz et al. 2010),
344 a physical map of the markers in the vicinity of the *Aco4* gene was created. The region
345 between BARCSOYSSR_11_323 and BARCSOYSSR_11_336 is about 293 kb. We were
346 able to use the soybean genome sequence flanked by these markers to locate putative genes
347 present in the region. There are 39 predicted genes in this region (Table 5) with one
348 candidate gene sharing partial sequence similarity with an aconitase (*Glyma.11g080600*).
349

350 **DISCUSSION**

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

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such as soybean may have evolved internal generators of genetic variation in response to stress or other environmental stimuli (Roth et al. 1989).

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

Similar evidence of new soybean isozyme variants has previously been reported and attributed to genomic stress induced by tissue culture manipulations (Amberger et al. 1992a). In these studies the frequency of isozyme variants for Aconitase-2 in somaclonal mutants was 2 out of 185 regenerated plants and the variants were heritable (Amberger et al. 1992b). Unlike the isozyme analyses described in the previous studies, in this study tissue samples were taken from the cotyledons of seedlings grown from individual seeds within single pods from each entry. This approach not only revealed that the genomic variation existed within plants in a cultivar, but also that variation was expressed within seed from individual pods collected from the same individual plant. In the pure-line experiment, Aconitase-4 variants

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were detected in a single seed within a three-seeded pod. Furthermore, the appearance of plants showing double switches (BSR 101-34-3 and BSR 101-46-1) completely rules out pollen contamination or outcrossing as the source of these genetic variants (Table 3).

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

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

The use of high temperatures and high relative humidity has been reported to cause seed stress (Hsu et al. 2003; Parrish and Leopold 1978) resulting in lower germination rates,

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406 emergence and in promoting the formation of free radicals (Hsu et al. 2003). In this study,
407 the accelerated aging test was used on progeny seed of single plants that were grown in a
408 honeycomb design in 2008 but these same plants were also affected by a natural-hail storm.
409 In related study, the effect of seed stress on agronomic traits such as plant height, plant
410 maturity, and yield was studied (Rolling 2012). 'Jack' showed a decrease of 8% in yield for
411 plants grown from the stressed seeds.

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

422 Characterization of the *Aco4* gene may help us understand the genetic basis of the
423 changes happening during allele switching. We genetically mapped the *Aco4* gene using SSR
424 markers (Figure 2). The 293 kb region was flanked by the BARCSOYSSR_11_323 and
425 BARCSOYSSR_11_336 markers on chromosome 11 (MLG B1). Using this information, we
426 located 39 predicted genes in this region (Table 5;
427 http://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Gmax). Of these, one gene
428 (*Glyma.11g080600*) was of particular interest as this showed 93% identity to *Medicago*

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429 *truncatula* aconitase (Young et al. 2011) and 88% identity to the *Arabidopsis* Aconitase 1 at
430 protein level (Peyret et al. 1995). Future studies focusing on characterization of the candidate
431 gene may result in cloning of the *Aco4* gene and may shed light on genetic mechanisms
432 involved in allele switching at the *Aco4* locus.
433

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592 **FIGURE LEGENDS**

593

594 Figure 1. Graphic representation of the history of the F_2 Aconitase-4 variant plant A08-AS-
595 2932 (*Aco4-ab*) from a cross between 'Minsoy' (*Aco4-cc*) x 'BSR 101' (*Aco4-aa*).

596

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

601

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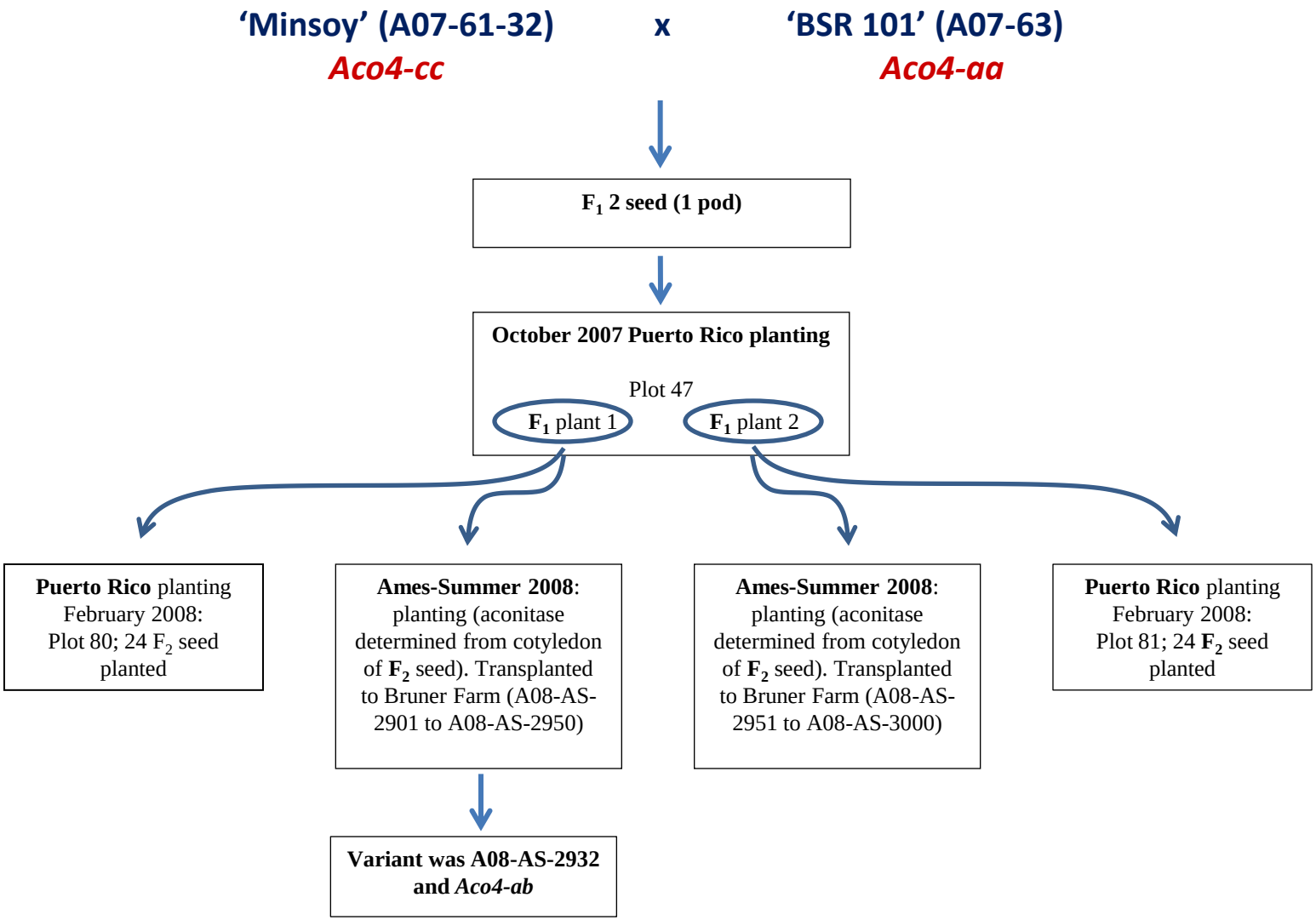

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Figure 2

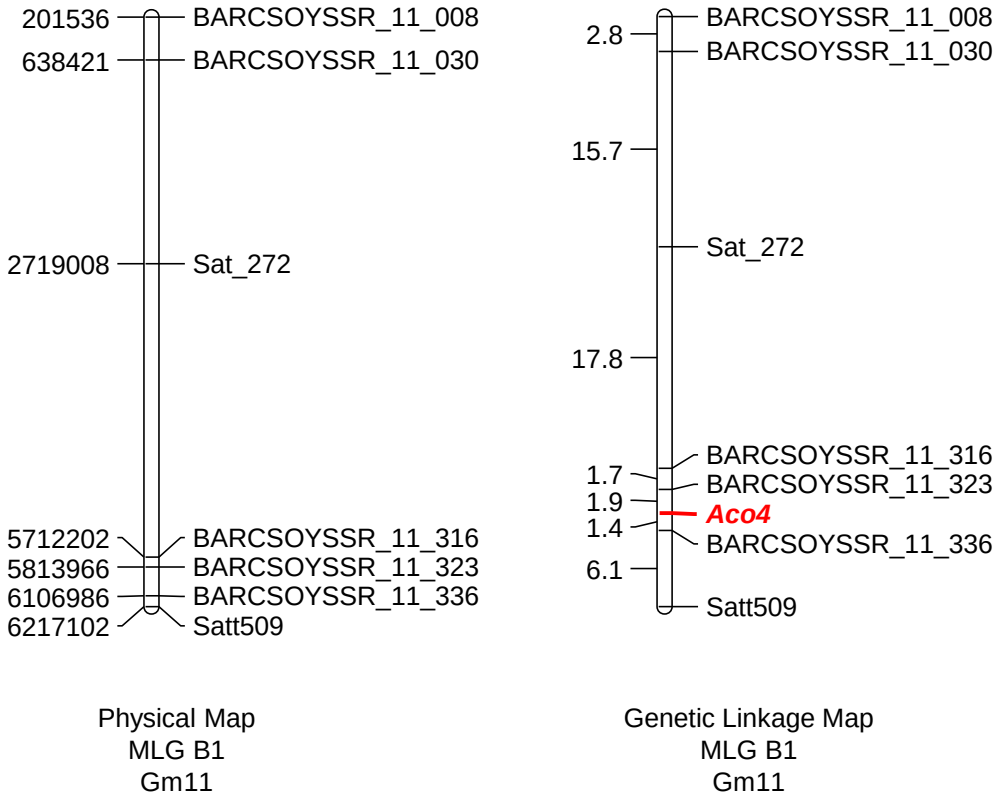

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Table 1. Genotypic frequency of progeny of self-pollination of F_1 plants (*Aco4-ab*, *Aco4-bc*, and *Aco4-ac*) assayed for Aconitase-4.

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

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

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

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

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

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

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

[z]Entries with Aconitase-4 variant labeled in the following format: cultivar name - entry number - pod number. Three, three-seeded pods were collected from each entry.

[y]Expected Aconitase-4 pattern for any of the seeds within the pod that do not have allele switches. This is the same pattern that appears in the original parental seed source.

[x]Aconitase-4 variant represents the new isozyme pattern for each seed with single or double-allele switches.

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

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

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

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

Gene	Start position	End position	Protein Function
Glyma.11g077500	5823379	5825992	Armadillo/beta-catenin-like repeat
Glyma.11g077600	5838011	5841370	Oxidoreductase NAD-binding domain; Oxidoreductase FAD-binding domain
Glyma.11g07700	5845936	5847989	Thaumatin family
Glyma.11g077800	5859113	5861305	Thaumatin family
Glyma.11g077900	5867279	5869126	Rhodanese-related sulfurtransferase
Glyma.11g078000	5869398	5874647	Mlo family; cell death; integral to membrane
Glyma.11g078100	5877078	5883528	Ubiquitin interaction motif; 26S proteasome non-ATPase regulatory subunit 4
Glyma.11g078200	5885675	5891247	Senescence-associated protein
Glyma.11g078300	5897790	5907341	Histidine kinase-, DNA gyrase B, and HSP90-like ATPase; Response regulator receiver domain; regulation of transcription, DNA-dependent
Glyma.11g078400	5909639	5912304	Peroxidase; oxidation reduction; heme binding
Glyma.11g078500	5913292	5917166	Protein of unknown function
Glyma.11g078600	5919282	5922385	None
Glyma.11g078700	5924116	5927990	3-dehydroquinate synthase
Glyma.11g078800	5930818	5933005	PPR repeat

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Glyma.11g078900	5932586	5935392	None
Glyma.11g079000	5937276	5941473	Isocitrate/isopropylmalate dehydrogenase
Glyma.11g079100	5942881	5945544	Ras family; Rho type; GTP binding
Glyma.11g079200	5446944	5955940	Vacuolar protein sorting-associated protein 35; Membrane coat complex Retromer
Glyma.11g079300	5960613	5960978	Copper chaperone; metal ion transport. Metal ion binding
Glyma.11g079400	5968757	5971655	GDSL-like Lipase/Acylhydrolase; zinc finger fyve domain containing protein
Glyma.11g079500	5979267	5985576	PLAC8 family
Glyma.11g079600	5987638	5989759	tetramerisation domain; SCF ubiquitin ligase
Glyma.11g079700	5991289	5992851	PPR repeat
Glyma.11g079800	5995314	5996613	Trm112p-like protein
Glyma.11g079900	5999169	6000451	Zinc finger, C3HC4 type (ring finger)
Glyma.11g080000	6005401	6006947	None
Glyma.11g080100	6018320	6023418	Lupus la ribonucleoprotein; RNA-binding protein LARP/SRO9
Glyma.11g080200	6027758	6029576	Putative methyltransferase
Glyma.11g080300	6030076	6031938	Peroxidase; heme binding
Glyma.11g080400	6036330	6040326	Eukaryotic aspartyl protease; proteolysis and peptidolysis
Glyma.11g080500	6045490	6048516	Ring finger protein 11
Glyma.11g080600	**6058457**	**6066818**	**Aconitase C-terminal domain; 3-isopropylmalate dehydrase subunit; RNA-binding translational regulator IRP**
Glyma.11g080700	6068106	6071401	Prolyl 4-hydroxylase alpha subunit; oxidoreductase activity
Glyma.11g080800	6079536	6081820	None

Glyma.11g080900	6083715	6086922	Peroxidase; heme binding
Glyma.11g081000	6087915	6088796	None
Glyma.11g081100	6090248	6091010	NADH-Ubiquinone/plastoquinone (complex 1); NADH Dehydrogenase; ATP synthesis coupled electron transport
Glyma.11g081200	6094537	6103135	RNA polymerase Rpb1
Glyma.11g081300	6104383	6105246	None

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