Molecular Genetic Resources for Development of 1% Linolenic Acid Soybeans

Kristin Bilyeu,* Lavanya Palavalli, David A. Sleper, and Paul Beuselinck

ABSTRACT

Advanced plant breeding will incorporate the most efficient methods available to introgress new traits and develop improved crops. Molecular markers that are specifically targeted to desirable alleles are important molecular genetic resources for selection of traits. Reducing the amount of linolenic acid in soybeans [Glycine max (L.) Merr.] is a desired breeding objective so that oxidatively stable soybean oil can be produced without the production of trans fatty acids. The objective of this work was to determine the molecular genetic basis for soybeans containing 1% (10 g kg\(^{-1}\)) linolenic acid in the seed oil fraction and to develop molecular markers specific for identified alleles. Utilizing the soybean homologs of Arabidopsis FAD3 as candidate genes, mutations were discovered in all three GmFAD3 genes in the soybean line A29. The mutations were associated with the linolenic acid phenotype in segregating populations. Molecular markers specific for the mutant alleles enabled capture of the phenotype. Novel combinations of mutant alleles at the three GmFAD3 loci allowed the development of new germplasm containing 1% linolenic acid in the seed oil along with SNP-based molecular markers that can be used in a backcross breeding strategy.

The impetus for developing soybean cultivars with lower linolenic acid concentration in the oil fraction of the seed is the need to rapidly respond to emerging needs related to production of healthful foods. For human nutrition, the fatty acid profile of foods has received considerable attention in recent years with generalized warnings concerning the amount of saturated fats and trans fatty acids in the diet. As part of an effort to reduce consumption of ingredients that increase the risk of coronary heart disease, the addition of trans fatty acid content to food labels begins in 2006. The need to improve oxidative and flavor stability of typical soybean oil results in the production of trans fatty acids as a consequence of chemical hydrogenation of polyunsaturated fatty acids. Linolenic acid content of the oil is responsible for the oxidative instability and flavor problems in soybean oil (Dutton et al., 1951; Lui and White, 1992), and development of soybean containing reduced linolenic acid concentrations is a current breeding goal.

Breeding based on chemical phenotype for single seed composition traits has been successful in lowering linolenic acid from wild-type levels of 60 to 100 g kg\(^{-1}\) oil to as low as 10 g kg\(^{-1}\) oil for lines containing three independent mutations (Fehr et al., 1992; Rahman et al., 1998; Ross et al., 2000). Because linolenic acid content in soybean is controlled by multiple genes, a more efficient breeding strategy would be to use molecular markers for rapid introgression of the trait in a backcross breeding strategy to develop elite lines (Bilyeu et al., 2005). Molecular markers for the lowered linolenic acid trait would also allow the combination of multiple traits in one line where a backcrossing strategy could be utilized to capture mutant alleles of a low linolenic acid donor parent with a recurrent parent that has other desirable seed composition traits with or without associated molecular markers.

Genetics of the lowered linolenic acid trait have received considerable attention. After the original fan mutation corresponding to lower linolenic acid levels was detected in line C1640 (Wilcox and Cavins, 1985; Wilcox and Cavins, 1987), other independent loci (fan2, fan3, and fanx) were also identified in lines with further reductions in linolenic acid content (Fehr et al., 1992; Rahman et al., 1998; Ross et al., 2000). In an effort to provide a molecular understanding of the low linolenic acid trait, we identified and characterized the soybean homologs of the Arabidopsis omega-three fatty acid desaturase gene, FAD3 (Bilyeu et al., 2003; Yadav et al., 1993). At least three versions of this gene exist in the soybean genome, GmFAD3A, GmFAD3B, and GmFAD3C, and GmFAD3A was shown to be Fan (Bilyeu et al., 2003; Anai et al., 2005). Recently, we determined the molecular genetic basis for the low linolenic acid soybean line CX1512–44, which had mutant alleles of both GmFAD3A and GmFAD3C (Bilyeu et al., 2005). The GmFAD3A gene was shown to have a greater impact on seed linolenic acid levels than GmFAD3C, consistent with higher expression of GmFAD3A in developing seeds (Bilyeu et al., 2005; Bilyeu et al., 2003).

The lowest level of linolenic acid reported for a soybean line is 1% of the oil fraction (10 g kg\(^{-1}\) oil) described for line A29 (Ross et al., 2000). A29, which is not related to other low linolenic acid lines with reported molecular information (CX1512–44, J18, M5, and M24 [Anai et al., 2005; Bilyeu et al., 2005]), was developed by combining three independent mutations: fan from line A5 (Hammond and Fehr, 1983), fan2 from A23, and fan3 from a mutagenized derivative of line A89–144003 (Ross et al., 2000). The fan mutation in line A5 is caused by a GmFAD3A deletion (Bilyeu et al., 2003; Byrum et al., 1997). The very low linolenic acid lines A29, IA3017, and IA3018 are related by pedigree (W. Fehr, personal communication, 2005). The objective of this research was to investigate the FAD3 genes in soybean line A29 to

Abbreviations: PCR, polymerase chain reaction; SNP, single nucleotide polymorphism.
identify possible mutations and determine if the mutant alleles were associated with the 1% linolenic acid trait.

MATERIALS AND METHODS

Cloning and Sequence Analysis

Primers were designed based on the sequences deposited in GenBank to amplify soybean *GmFAD3A* (AY204710), *GmFAD3B* (AY204711), and *GmFAD3C* (AY204712) cDNAs or genomic DNA from line A29. Reverse transcriptase reactions, PCR (polymerase chain reaction) amplification, isolation of products from agarose gels, and cloning were as previously described (Bilyeu et al., 2003).

Plant Growth

The low linolenic acid soybean lines A29 (10 g kg$^{-1}$ oil), IA3017 (10 g kg$^{-1}$ oil), and IA3018 (26 g kg$^{-1}$ oil) were chosen as candidates for potential FAD3 gene mutations. Low linolenic soybean lines A29, IA3017, and IA3018 were provided by Dr. Walter Fehr, Iowa State University (Ross et al., 2000). The low linolenic acid line 2721 (30 g kg$^{-1}$ oil) was an F$_2$, line produced from a cross between ‘Pana’ and CX1512–44. Line 2721 was homozygous for the CX1512–44 mutant alleles of *GmFAD3A* and *GmFAD3C* (Bilyeu et al., 2003).

SS97–6946 is an experimental breeding line with normal soybean cyst nematode HG types, and appropriate maturity (D. Sleper, unpublished data, 2003). A cross of SS97–6946 × IA3017 produced F$_1$ seeds in the summer of 2003 at the Bradford Research and Extension Center (BREC), located near Columbia, MO. F$_1$ plants were grown in Guacima, Costa Rica, and F$_2$ seed was chipped for fatty acid analysis with the remainder of the seed planted and grown in Nazareth, Costa Rica, during the fall of 2003 and spring of 2004. Chips were shipped to BREC and analyzed for linolenic acid concentration. Single-plant thresher F$_2$;F$_3$ seed of lines containing the lowest linolenic acid content (18 lines containing up to linolenic acid concentrations of 15 g kg$^{-1}$ oil) was returned to BREC for planting progeny rows in summer 2004. Because no lines were recovered which were homozygous for all three mutations, five individual aabbCc F$_2$;F$_3$ plants derived from an aabbCc F$_2$ parent were used as pollen donors for a SS97–6946 × F$_2$;F$_3$ backcross (SS97–6946 × SS97–6946 × IA3017). The resulting three confirmed BC$_1$F$_1$ F$_2$;F$_3$ seed were germinated in moist germination packets containing the lowest linolenic acid content (18 lines containing up to linolenic acid concentrations of 15 g kg$^{-1}$ oil) was returned to BREC for planting progeny rows in summer 2004. Because no lines were recovered which were homozygous for all three mutations, five individual aabbCc F$_2$;F$_3$ plants derived from an aabbCc F$_2$ parent were used as pollen donors for a SS97–6946 × F$_2$;F$_3$ backcross (SS97–6946 × SS97–6946 × IA3017). The resulting three confirmed BC$_1$F$_1$ F$_2$;F$_3$ seed were germinated in moist germination packets and transferred to soil for growth in controlled chambers set at 27.5/23°C day/night with 14.5 h daylength at a light intensity of 750 μmol m$^{-2}$ s$^{-1}$. Eighty seeds were produced, and fatty acid gel chromatography of fatty acid methyl esters. Genotype and phenotype data were collected for 61 individuals. Seeds of a A29 × 2721 cross were produced in Costa Rica in 2003. F$_1$ (1 seed), F$_2$ (62 seeds), and F$_3$;F$_4$ seeds were germinated in moist germination packets and transferred to soil for growth in controlled chambers set at 27.5/23°C day/night with 14.5 h daylength at a light intensity of 750 μmol m$^{-2}$ s$^{-1}$. Twelve lines were identified in the F$_2$ generation to have homozygous mutant alleles for *GmFAD3B* and *GmFAD3C*, and a subset (8) of those lines were followed to the F$_3$ generation to test for segregation of *GmFAD3A* alleles.

Genotype Analysis

Detection of mutant alleles followed the procedure as described by Bilyeu et al. (2005) with PCR amplification of the genomic region encompassing the single nucleotide polymorphism (SNP) followed by restriction enzyme digestion of products. When primer sequences are indicated, the forward primer is listed first, and all primers are listed in the 5’ to 3’ orientation.

For the A29 *GmFAD3B* allele assay, amplification primers were B932: AGCCACAGAACTCATCCATCAA and IABrev: TGGGACAGTTAGAACATTAGT. Following amplification conditions as described for the CX1512–44 alleles (Bilyeu et al., 2005), products were digested with 2.5 U *HpaI* (New England Biolabs, Beverly, MA) per reaction and resolved on agarose gels. Wild-type alleles produced fragments of 196 and 162 bp while A29-derived mutant alleles produced a single 388-bp fragment.

For the A29 *GmFAD3C* allele assay, amplification primers were IACleft: TTGGATCAACAACATTCCA and IAC-right: CATCACATGTGTTGTTGGCTTGA. Following amplification, products were digested with 5 U *BecI* (New England Biolabs) per reaction and resolved by MsCPN analysis (Ye et al., 2002) or on agarose gels. Wild-type alleles produced fragments of 199 and 56 bp while A29-derived mutant alleles produced a single 250-bp fragment.

For the *GmFAD3A* deletion assay, a Taqman assay was used in a reaction with primers that detected both the *GmFAD3A* gene (3AD1/3AIX amplification primers [Bilyeu et al., 2005]; dual-labeled probe [FMABHQ1] WTprobe: TGCACGGAC-GGTAAACAAAGGTTTGAATGTT and Lprobe: CTGCAGATGTTGCGCTAATG. Reverse transcriptase reaction, products were digested with 2.5 U *HpaI* (New England Biolabs, Beverly, MA) per reaction and resolved on agarose gels. Wild-type alleles were identified by bands of approximate size 200 bp while A29-derived mutant alleles were reduced to approximately 150 bp.

Genotype Analysis

For the A29 *GmFAD3B* allele assay, amplification primers were B932: AGCCACAGAACTCATCCATCAA and IABrev: TGGGACAGTTAGAACATTAGT. Following amplification conditions as described for the CX1512–44 alleles (Bilyeu et al., 2005), products were digested with 2.5 U *HpaI* (New England Biolabs, Beverly, MA) per reaction and resolved on agarose gels. Wild-type alleles produced fragments of 196 and 162 bp while A29-derived mutant alleles produced a single 388-bp fragment.

For the A29 *GmFAD3C* allele assay, amplification primers were IACleft: TTGGATCAACAACATTCCA and IAC-right: CATCACATGTGTTGTTGGCTTGA. Following amplification, products were digested with 5 U *BecI* (New England Biolabs) per reaction and resolved by MsCPN analysis (Ye et al., 2002) or on agarose gels. Wild-type alleles produced fragments of 199 and 56 bp while A29-derived mutant alleles produced a single 250-bp fragment.

For the *GmFAD3A* deletion assay, a Taqman assay was used in a reaction with primers that detected both the *GmFAD3A* gene (3AD1/3AIX amplification primers [Bilyeu et al., 2005]; dual-labeled probe [FM/ABHQ1] WTprobe: TGCACGGAC-GGTAAACAAAGGTTTGAATGTT and Lprobe: CTGCAGATGTTGCGCTAATG. Reverse transcriptase reaction, products were digested with 2.5 U *HpaI* (New England Biolabs, Beverly, MA) per reaction and resolved on agarose gels. Wild-type alleles were identified by bands of approximate size 200 bp while A29-derived mutant alleles were reduced to approximately 150 bp.

RESULTS AND DISCUSSION

Identification of Mutant FAD3 Alleles and Molecular Marker Assays

We characterized the soybean FAD3 genes as candidates for potential mutations from the low linolenic acid soybean lines A29 (10 g kg$^{-1}$ oil), IA3017 (10 g kg$^{-1}$ oil), and IA3018 (26 g kg$^{-1}$ oil). Our previous work identified the $\text{fan}$ mutation in line A5 as a deletion of *GmFAD3A* gene sequences, which presumably is a null allele (Bilyeu et al., 2005; Bilyeu et al., 2005). We determined that the 1 and 2% linolenic acid lines A29, IA3017, and IA3018 also have deletions of the *GmFAD3A* gene (data not shown). For genotyping assays, either simulta-
neous amplification of both the *GmFAD3A* and *GmFAD3B* genes or an allele-specific Taqman assay can be used to detect the deletion of *GmFAD3A*, as previously described (Bilyeu et al., 2005; Bilyeu et al., 2003; see materials and methods). Although unambiguous identification of homozygous mutant individuals is robust, neither detection method distinguishes heterozygous individuals from homozygous wild-type lines.

When cDNA from line A29 was initially used as template for RT-PCR, the *GmFAD3B* gene reactions failed to produce a product. Therefore, genomic DNA from line A29 was used to amplify the *GmFAD3B* gene region, and the product was cloned, sequenced, and compared to the partial *GmFAD3B* genomic sequence present in GenBank (accession AX088031). Among several minor differences in intron sequences, a SNP was identified in the 3’ splice site consensus (Brown et al., 1996) sequence preceding exon 4 (Fig. 1). Improper splicing was subsequently detected in mRNA from this region that would lead to a frameshift and premature termination of the protein product (data not shown). An assay was designed to distinguish wild-type and mutant alleles of *GmFAD3B* based on the presence or absence of a *HpaI* restriction enzyme site for wild-type and A29-derived mutant alleles, respectively (Fig. 1C).

The *GmFAD3C* gene was amplified by RT-PCR from A29 cDNA. After cloning and sequencing of *GmFAD3C*, a SNP was discovered (C910T) that resulted in a H304Y mutation (Fig. 2) in the histidine-rich region II of the protein sequence (Shanklin et al., 1994). The altered histidine residue corresponds to one shown to be essential for desaturase activity (Shanklin et al., 1994). An assay was designed to distinguish between wild-type and A29-derived mutant alleles at *GmFAD3C* based on the presence or absence of a *BccI* restriction enzyme site for wild-type and A29-derived mutant alleles, respectively (Fig. 2B).

Both 1% linolenic acid lines A29 and IA3017 were found to have the same set of *GmFAD3A*, *GmFAD3B*, and *GmFAD3C* mutant alleles. The related line IA3018, which contains 2.6% linolenic acid grown under field conditions, was genotyped and found to contain the *GmFAD3A* and *GmFAD3C* mutations, but not the A29 allele of *GmFAD3B*. Thus, soybean lines selected for the lowest known linolenic acid concentration contain mutant alleles of three *GmFAD3* genes.

### Using Molecular Markers in a Backcross Breeding Strategy

Part of our interest in developing molecular markers for the low linolenic acid trait is to provide the tools to accelerate introgression of traits into elite soybean cultivars. The use of molecular markers supercedes the need for extensive chemical analysis and facilitates the capture of the trait in a backcrossing program that can rely on selection of unrelated traits. Toward this end, we initiated a cross between SSS7–6946 (D. Sleper, unpublished data, 2003) and IA3017. Following the production of F1 seed, a typical breeding strategy was used in which F1 plants were grown, allowed to self-fertilize, and the F2 seed was chipped for fatty acid analysis. Agronomic selections and selections based on fatty acid analysis were made on F2 plants. As an initial genotyping screen, two individuals from each F2:F3 family were genotyped. No F2:F3 families were identified that contained all three homozygous mutant alleles of the *FAD3* genes. However, individual plants were identified by genotype to be homozygous mutants, and these individual homozygous mutant F2:F3 plants were used as pollen donors in backcrosses to SSS7–6946.
The association of genotype and phenotype was assessed in field grown plants derived from a single cross (SS97–6946 × [SS97–6946 × IA3017]). F2:F4 seeds produced on field grown selfed F2:F3 plants of selected plants with known genotypes were tested for fatty acid composition. While the recurrent parent germplasm contained an average of 7.7% linolenic acid (77.0 g kg\(^{-1}\) oil), the five F2:F3 lines identified with the homozygous mutant genotype for all three genes produced F2:F4 seed with an average of 1.3% linolenic acid (13.0 g kg\(^{-1}\) oil). Grown in the same field environment, IA3017 produced seeds with an average of 1.2% linolenic acid (12.0 g kg\(^{-1}\) oil).

Over the subsequent cycle of backcross breeding (SS97–6946 × [SS97–6946 × IA3017]), plants were grown in controlled environment chambers. BC1F1 plants (derived from SS97–6946 × F2:F3) were confirmed using molecular markers. The BC1F2 seeds were chipped for fatty acid analysis and germinated for further growth and genotyping (Fig. 3). One BC1F2 line was recovered with the homozygous mutant genotype for all three GmFAD3 genes, and it contained the lowest concentration of linolenic acid (12.0 g kg\(^{-1}\) oil). Because 27 genotypes are possible from the segregation of the three GmFAD3 loci, and the assay for the GmFAD3A deletion does not distinguish wild-type from heterozygous individuals, the association of genotype and phenotype is not entirely clear. In general, samples homozygous for the GmFAD3A deletion produce the lowest linolenic acid phenotype, regardless of the genotype at GmFAD3B and GmFAD3C. In addition, mutations in GmFAD3B and GmFAD3C also appear to additively contribute to lowered linolenic acid levels as demonstrated by the relatively low linolenic acid phenotype for samples with at least one copy of a wild-type GmFAD3A allele in combination with homozygous mutations in GmFAD3B and GmFAD3C (Fig. 3, samples 8, 12, 15, 17, 19, and 36, gray circles). Conversely, samples with at least one copy of a wild-type GmFAD3A allele in combination with homozygous wild-type GmFAD3B and GmFAD3C contained some of the highest linolenic acid levels.

When BC1F2:F3 seeds from a BC1F2 plant with the GmFAD3A deletion but still segregating for both the GmFAD3B and GmFAD3C mutant alleles were ana-
Table 1. Listing of available mutant alleles at the three soybean GmFAD3 loci with perfect molecular markers developed from four low linolenic acid soybean lines.

<table>
<thead>
<tr>
<th>Mutant line</th>
<th>Linolenic acid g kg⁻¹ oil</th>
<th>GmFAD3A</th>
<th>GmFAD3B</th>
<th>GmFAD3C</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A5</td>
<td>40</td>
<td>aa²⁹ (deletion)†</td>
<td>BB</td>
<td>CC</td>
<td>Bilyeu et al., 2003</td>
</tr>
<tr>
<td>CX1512–44</td>
<td>28</td>
<td>aa²⁸ (SNP)</td>
<td>BB</td>
<td>cc²⁹ (SNP)</td>
<td>Bilyeu et al., 2005</td>
</tr>
<tr>
<td>IA3017</td>
<td>10</td>
<td>aa²⁹ (deletion)</td>
<td>BB</td>
<td>cc²⁹ (SNP)</td>
<td>Bilyeu et al., 2005</td>
</tr>
<tr>
<td>C1640</td>
<td>40</td>
<td>aaC1640(SNP)</td>
<td>BB</td>
<td>CC</td>
<td>Chappell and Bilyeu, 2006</td>
</tr>
</tbody>
</table>

† Mutant genotype for three GmFAD3 genes. Superscript designates source of mutant allele derived from a mutant soybean line; 29 = A29 and cx = CX1512–44. Uppercase designates wild-type alleles.

lyzed for fatty acid content, additional evidence for an association between mutant alleles and low linolenic acid phenotype was observed (Fig. 4). The individual contribution of wild-type alleles of GmFAD3B and GmFAD3C to linolenic acid phenotype cannot be determined with confidence from this limited dataset, but additional experiments support nearly equivalent contributions for GmFAD3B and GmFAD3C that are smaller than the GmFAD3A contribution to linolenic acid levels when substituting wild-type and mutant alleles (data not shown).

Five BC1F2:F3 seeds derived from the single BC1F2 plant containing all three mutations were analyzed for fatty acid profile. The phenotype of the BC1F2:F3 seeds, which theoretically contain 75% of the recurrent parent genome, was a linolenic acid average of 12% (12.0 g kg⁻¹ oil). A discrepancy was noted in the linolenic acid phenotype of seed chips when compared to the phenotype of the remainder of the same crushed seed; the linolenic acid level of the seed chips averaged 12 g kg⁻¹ higher than the crushed seed. Whole crushed seeds provide a complete sample to more precisely determine the true phenotype, but eliminate the possibility to regenerate the individual genotype (i.e., the seeding).

The association data presented here supports a model in which all three GmFAD3 genes contribute to the total enzymatic capacity to produce linolenic acid in the seed oil. Consistent with our earlier work, the mutation in the GmFAD3A gene described here caused a greater reduction in linolenic acid concentration than mutations in GmFAD3B or GmFAD3C (Bilyeu et al., 2005; Bilyeu et al., 2003).

Recombination of Mutant Alleles

Together with the previously identified mutations in GmFAD3A and GmFAD3C present in the low linolenic acid lines C1640 and CX1512–44 (Bilyeu et al., 2005; Chappell and Bilyeu, 2006), there are now a total of six available mutant alleles with perfect (i.e., specific to the causative mutation) molecular markers at three soybean GmFAD3 loci (Table 1). The mutant GmFAD3 genotype in A29 can be designated aa²⁹bb²⁹cc²⁹ and distinguished from the CX1512–44 derived mutant genotype, designated aa²⁸bb²⁹cc²⁹. No mutations were identified in the CX1512–44 GmFAD3B gene (Bilyeu et al., 2005).

For crosses with A29 or IA3017 as a parent, the inability to distinguish lines heterozygous for the GmFAD3A mutant allele from lines with homozygous wild-type GmFAD3A alleles prevented the use of molecular markers for the most efficient backcrossing strategy. Because the CX1512–44 line contains a mutant allele of GmFAD3A that is the result of a SNP that can be assayed to easily distinguish the two homozygotes and the heterozygotes, it could be used as an alternative source of the GmFAD3A mutant allele (Bilyeu et al., 2005).

We initiated a cross between A29 (aa²⁹bb²⁹cc²⁹) and 2721 (aa²⁸bb²⁹cc²⁹, Bilyeu et al., 2005), and analyzed progeny for genotype and phenotype in the F2 and F2:F3 generations to select lines homozygous for different combinations of the three SNP-containing mutant alleles (aa²⁸bb²⁹cc²⁹ or aa²⁹bb²⁹cc²⁹). As expected for segregation of only mutant alleles at GmFAD3A and GmFAD3C and mutant and wild-type alleles of GmFAD3B, the range of linolenic acid concentrations in F2 seeds was narrow (12 to 51 g kg⁻¹ oil). Two F2 individuals (lines designated B1–52 and B1–62) were confirmed in the F3 generation to be homozygous for both the CX1512–44-derived mutant alleles and homozygous for the A29-derived GmFAD3B allele, aa²⁸bb²⁹cc²⁹. One F3 individual (designated line B1–15) had the alternate genotype, aa²⁹bb²⁸cc²⁹. The linolenic acid contents for these lines are listed in Table 2, along with a line with the aa²⁸bb²⁹cc²⁹ genotype. These results indicate that the available mutant alleles can be substituted for each other and in combinations that enable SNP detection for all three FAD genes as well as stable inheritance of the 1% linolenic acid phenotype.

CONCLUSIONS

Introgression of soybean seed composition traits into elite cultivars can be accomplished by utilizing chemical

Table 2. Linolenic acid phenotype in Fₙ generations of lines derived from the cross A29 (aa²⁹bb²⁹cc²⁹) × 2721 (aa²⁸bb²⁹cc²⁹) with different genotype combinations of mutant FAD3 alleles.

<table>
<thead>
<tr>
<th>Generation</th>
<th>Linolenic acid g kg⁻¹ oil</th>
<th>Linolenic acid g kg⁻¹ oil</th>
<th>Linolenic acid g kg⁻¹ oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1–40 (aa²⁸bb²⁹cc²⁹)†</td>
<td>14</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>B1–15 (aa²⁸bb²⁹cc²⁹)†</td>
<td>15</td>
<td>13</td>
<td>16</td>
</tr>
<tr>
<td>B1–52 (aa²⁸bb²⁹cc²⁹)†</td>
<td>Not determined</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>B1–62 (aa²⁸bb²⁹cc²⁹)†</td>
<td>15</td>
<td>13</td>
<td>15</td>
</tr>
</tbody>
</table>

† Homozygous mutant genotype for three GmFAD3 genes. Superscript designates source of mutant allele derived from a mutant soybean line; cx = CX1512–44.

† Chipped seed was used for fatty acid analysis in the F2 generation otherwise analysis was performed on whole seed.
phenotyping, using molecular markers, or combinations of the two screening strategies. The lowered linolenic acid trait is controlled by multiple genes, with three of those genes shown to be the three fatty acid desaturase candidate genes described in this work, GmFAD3A, GmFAD3B, and GmFAD3C. For the lines A29 and IA3017, three soybean FAD3 genes had deleterious mutations. Relatively straightforward molecular marker assays were designed to distinguish these mutant alleles from their wild-type counterparts. Selection for the three mutations in early generations can achieve reductions in linolenic acid concentration from approximately 8% (80.0 g kg\(^{-1}\) oil) to below 2% (20.0 g kg\(^{-1}\) oil) with stable inheritance of the trait. The most efficient use of time and resources would need to be determined for each breeding program, but a combination of chemical phenotype screening followed by genotype selection in the F\(_2\) generation using molecular markers would keep costs low while allowing complete capture of the trait. Either a backcrossing strategy could be used or screening in advanced generations could emphasize other traits.

The mutant alleles identified as part of this project were readily substituted with other mutant alleles of GmFAD3A and GmFAD3C that had been previously identified (Bilyeu et al. 2005). Breeding programs that have already incorporated a fan mutation (an allele of GmFAD3A) to lower linolenic acid concentration now have the option of accomplishing further reductions in linolenic acid by incorporating mutant alleles of GmFAD3B and GmFAD3C. In addition, the novel combinations of mutant GmFAD3 genes that impart a 1% (10.0 g kg\(^{-1}\) oil) linolenic acid phenotype developed as part of this project provide new resources to the soybean breeding community.

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