Combination of Novel Mutation in \textit{FAD3C} and \textit{FAD3A} for Low Linolenic Acid Soybean

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ABSTRACT

Conventional commodity soybean oil can typically have levels of linolenic acid up to 8% of the total oil fraction. As linolenic acid is unsaturated, unstable, and contributes to off-flavors, and the hydrogenation process can produce harmful \textit{trans} fats, genetic alternatives for soybean \textit{[Glycine max (L.) Merr.]} low in linolenic acid are desirable. We identified a novel mutant allele of the gene encoding the omega-3 desaturase \textit{FAD3C} in a screen of a N-nitroso-N-methylurea (NMU)-mutagenized population. This allele resulted in a 2 to 3% reduction in linolenic acid levels as a fraction of the total fatty acids. We measured the effect of combining this mutation with mutations in the homeologous \textit{FAD3A} desaturase gene on the soybean fatty acid profile. This \textit{FAD3C} polymorphism may be useful to breeders seeking conventional approaches to reduce linolenic acid levels. In particular, combinations of mutant alleles of \textit{FAD3A} and \textit{FAD3C} result in soybean with linolenic acid comprising only 2 to 3% of the total fatty acids.

Soybean oil accounts for almost 30% of the vegetable oil consumed globally, and more than half of the vegetable oil consumed in the United States at 9.5 million Mg in 2017 (soystats.com, American Soybean Association, 2019). Although a natural source of omega fatty acid, the linolenic acid content of soybean seeds affects the utility of soybean oil in several ways. Oxidative stability is negatively influenced by content of polyunsaturated fats in soybean oil, which contributes to reduced shelf life and undesirable flavors. Historically most soybean oil has been subjected to partial hydrogenation; however, this process results in the creation of \textit{trans} fats, and the use of partial hydrogenation in foods has been recently banned (Federal Register, 2015). Therefore, the genetic reduction of linolenic acid content and increases in oleic acid levels are important goals of soybean breeding and biotechnology.

There are at least three characterized genes that, when mutated, result in reduced levels of linolenic acid in soybean \textit{[Glycine max (L.) Merr.]} seed. Originally named the \textit{fan} loci, it is now known that the genes underlying all three are orthologs of the microsomal \textit{\omega-3} fatty-acid desaturase, also known as \textit{FATTY ACID DESATURASE3} (\textit{FAD3}) (Anai et al., 2005; Bilyeu et al., 2003). A number of induced and naturally occurring mutations in the gene encoding the seed-expressed desaturase \textit{FAD3A} (Glyma.14g194300) have been isolated that demonstrate that mutation in this gene alone can result in a 40 to 50% reduction in linolenic acid with respect to wild-type levels (Anai et al., 2005, Bilyeu et al., 2005; Chappell and Bilyeu, 2006, 2007; Kim et al., 2015; Thapa et al., 2018; Silva et al., 2018). These mutations include deletions, splicing errors, missense, and nonsense mutations. Fewer mutations have been described in \textit{FAD3B} and \textit{FAD3C}. Mutations identified to date in the \textit{FAD3B} gene include a 19-bp deletion that results in a frameshift, as well as a point mutation that results in a splicing error (Anai et al., 2005; Reinprecht et al., 2009). A missense mutation (G128E) in \textit{FAD3C} alone resulted in a 30% reduction in linolenic acid levels with respect to a wild-type control (Bilyeu et al., 2005). In a reverse genetic approach, a single nucleotide polymorphism in the eighth and final exon of the \textit{FAD3C} gene that resulted in both an amino acid substitution in the predicted protein as well as the production of an alternative shorter transcript was also
associated with a 35% reduction of linolenic acid levels with respect to the unmutagenized control (Hoshino et al., 2014). In general mutation in either the FAD3B (Glyma.02g227200) or FAD3C (Glyma.18g06200) genes have a less significant impact on linolenic acid levels, resulting in a reduction in the range of 30% of normal levels of linolenic acid (Bilyeu et al., 2005). However, combinations of *fad3a* mutations with mutations in the other FAD3 genes enhanced the low linolenic acid phenotype, indicating some extent of functional redundancy among the FAD3 genes (Anai et al., 2005; Bilyeu et al., 2005; Hoshino et al., 2014). It was further determined that linolenic acid content could be reduced to <2% of total oil when soybean lines contain all three *fad3* mutant loci (Bilyeu et al., 2011; Ross et al., 2000; Pham et al., 2014) or when all three FAD3 genes were silenced using RNA interference (Flores et al., 2008).

Here we describe the discovery of a new mutant allele of the FAD3C gene and evaluate its effect in combination with other mutations affecting linolenic acid levels.

### MATERIALS AND METHODS

#### Plant Growth and Fatty Acid Phenotyping

Plants were grown in the field or in the greenhouse in West Lafayette, IN. To determine the statistical significance of the *fad3c* mutation on seed composition in Table 1, two-tailed type II *t*-tests were performed on fatty acid values from mutant and wild-type five-seed bulks from individual plants grown in the field over multiple seasons (2013–2017). For the data presented in Fig. 1, homozygous mutant lines were used as females in a backcross to Williams-82 in the field in 2015, F1 plants were advanced in the greenhouse and genotyped for the mutation in FAD3C during the winter, and F2 individuals were planted in the field in 2016. Fatty acid profiling on 71 samples of five-seed F2 bulks was performed by gas chromatography from 73 F2 plants.

For tests of additivity with *fad3a* mutations, the new *fad3c* mutant line was crossed with three different *fad3a* mutants originally isolated in the Williams-82 background (described in Thapa et al., 2016b). These mutants are the nonsense mutant FAD3A<sub>W81STOP</sub> isolated in the Williams-82 background (described in Thapa et al., 2016b), and two missense mutants FAD3A<sub>W81ARG</sub> and FAD3A<sub>W81GLU</sub>. FAD3A<sub>W81STOP</sub> was used as the male parent in a cross to FAD3C<sub>P266</sub> in the field in 2017. In both cases the F1 plants were propagated and genotyped for both alleles in the greenhouse, and 44 and 48 F2 seeds, respectively, were analyzed by gas chromatograph and genotyped. Genotypic and phenotypic analysis of seed chips and FAD3A genotyping markers were described previously (Thapa et al., 2018).

### Plant DNA Extraction, Amplification, and Genotyping

Plant DNA extraction and sequencing were described previously (Carrero-Colón et al., 2014). For sequencing, the coding region of FAD3C was amplified with primers KJH37 (5'-GCATTGTAACAGAGAAAAG), KJH38 (5'-CACATCCTCTCAGATACC) (Exons 1 and 2), KJH39 (5'-CAGTTGTTATATTGGTGT) KJH40 (5'-ACTTGTGAGGACAAATTGGT) (Exons 3–6), and KJH41 (5'-TGCACTGGTTTGCATCTTCA) KJH42 (5'-CAACTAAATGTAGCATTGGG) (Exons 7 and 8). Primers for other FAD3 orthologs are described elsewhere (Thapa et al., 2018). The FAD3C<sub>P266</sub> mutation abolishes a restriction site for the StyI enzyme (New England Biolabs, Ipswich, MA) that enabled the

#### Table 1. Linolenic acid levels are reduced in FAD3C<sub>P266</sub>.

<table>
<thead>
<tr>
<th>Year</th>
<th>Line</th>
<th>Palmitic acid</th>
<th>Stearic acid</th>
<th>Oleic acid</th>
<th>Linoleic acid</th>
<th>Linolenic acid</th>
<th>No. of plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>2013</td>
<td>W82</td>
<td>11.4 ± 1.4</td>
<td>3.3 ± 0.7</td>
<td>23.7 ± 3.9</td>
<td>53.6 ± 2.4</td>
<td>8.1 ± 1.4</td>
<td>5</td>
</tr>
<tr>
<td>2014</td>
<td>W82</td>
<td>9.9 ± 0.5</td>
<td>4.7 ± 0.5</td>
<td>30.1 ± 3.9</td>
<td>50.2 ± 3.5</td>
<td>5.1 ± 0.6</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>FAD3A&lt;sub&gt;P266&lt;/sub&gt;</td>
<td>10.4 ± 0.8</td>
<td>4.0 ± 0.3</td>
<td>21.7 ± 0.6</td>
<td>56.0 ± 1.0</td>
<td>7.8 ± 0.1</td>
<td>5</td>
</tr>
<tr>
<td>2015</td>
<td>W82</td>
<td>10.3 ± 0.4</td>
<td>4.3 ± 0.6</td>
<td>26.1 ± 3.1</td>
<td>54.1 ± 2.4</td>
<td>5.2 ± 0.6</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>FAD3A&lt;sub&gt;P266&lt;/sub&gt;</td>
<td>10.7 ± 0.8</td>
<td>4.0 ± 0.7</td>
<td>22.1 ± 1.2</td>
<td>54.4 ± 1.7</td>
<td>8.8 ± 1.8</td>
<td>5</td>
</tr>
<tr>
<td>2016</td>
<td>W82</td>
<td>10.4 ± 0.4</td>
<td>4.5 ± 0.5</td>
<td>25.8 ± 4.1</td>
<td>54.1 ± 3.4</td>
<td>5.2 ± 0.5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>FAD3A&lt;sub&gt;P266&lt;/sub&gt;</td>
<td>10.8 ± 0.3</td>
<td>3.7 ± 0.2</td>
<td>24.1 ± 1.7</td>
<td>54.5 ± 1.6</td>
<td>6.9 ± 0.2</td>
<td>5</td>
</tr>
<tr>
<td>2017</td>
<td>W82</td>
<td>10.2 ± 0.3</td>
<td>4.1 ± 0.4</td>
<td>27.4 ± 2.7</td>
<td>53.3 ± 2.2</td>
<td>5.0 ± 0.2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>FAD3A&lt;sub&gt;P266&lt;/sub&gt;</td>
<td>10.3 ± 0.3</td>
<td>4.3 ± 0.2</td>
<td>23.0 ± 1.8</td>
<td>54.4 ± 1.4</td>
<td>7.9 ± 0.4</td>
<td>5</td>
</tr>
</tbody>
</table>

** Significant at the 0.001 probability level.
*** Significant at the 0.01 probability level.
*** Significant at the 0.001 probability level.

![Fig. 1. Cos segregation of the FAD3C<sub>P266</sub> allele and low linolenic acid levels.](image-url)
creation of a codominant PCR marker for genotyping the FAD3C<sub>P266S</sub> mutation. Amplification with primers KJH39 and KJH40 generated an 881-bp fragment that was subsequently digested with StyI overnight and analyzed on a 1% TBE agarose gel. The amplified from the wild-type allele is cleaved.

RESULTS

A low linolenic acid mutant was identified in the course of screening the NMU-mutagenized soybean mutant population for variants in soybean seed fatty acid composition using gas chromatography (Hudson, 2012). Fatty acid composition was determined from this line grown annually in the field from 2013 to 2017. Multiple mutant individuals from the line were harvested as single plants, and seeds were assayed by gas chromatograph over multiple growing seasons to determine that the line showed consistent and significantly reduced levels of linolenic acid, with a reduction of 25 to 40% compared with the Williams-82 wild-type (Table 1). Although linolenic acid content varied somewhat from year to year, we observed limited variability between individuals in the mutant line each season, and therefore presumed that this line was likely to be homozygous for the mutation, causing the reduction in linolenic acid.

To determine the molecular identity of the causative gene, the coding regions of the FAD3C gene were amplified and the sequence of PCR amplicons was compared to the Williams-82 reference sequence to identify mutations. The low linolenic line carried a cytosine to thymine polymorphism in the FAD3C gene that converts the conserved proline at position 266 to serine (Fig. 2). Interestingly, we have previously identified mutations at this conserved proline position within both the FAD2-1A (Thapa et al., 2016a) and FAD2-1B (Sweeney et al., 2017) genes.

To test for co-segregation of the low linolenic phenotype and the FAD3C<sub>P266S</sub> polymorphism, we backcrossed the mutant to the wild-type Williams 82 parent. DNA and seeds were individually collected from each F<sub>2</sub> plant. A PCR-based genotyping marker was developed that took advantage of the restriction site that is lost in the mutant (Fig. 1A). We observed co-segregation of the polymorphism and low linolenic acid ranging from 4 to 6% of the total fatty acids (Fig. 1B), indicating that this FAD3C<sub>P266S</sub> polymorphism is likely to be the cause of the low linolenic acid phenotype.

To examine the degree of additivity between FAD3C and FAD3A mutations, and to determine to what extent linolenic acid levels could be reduced by this combination of mutations, we crossed the FAD3C<sub>P266S</sub> mutant to three different FAD3A mutants (Thapa et al., 2018). The F<sub>2</sub> individuals for FAD3C<sub>P266S</sub> × FAD3A<sub>W81STOP</sub> were planted in the field in 2016. Each individual was genotyped, and seeds were analyzed for fatty acid content (therefore genotype corresponds to F<sub>2</sub> individuals and fatty acid composition was determined from five-seed bulk samples from each individual). We observed that double mutants occurred in the expected frequency and ranged from 2.1 to 2.5% linolenic acid (Fig. 3A). FAD3C<sub>P266S</sub> × FAD3A<sub>W81STOP</sub> and FAD3A<sub>227RD</sub> × FAD3C<sub>P266S</sub> F<sub>1</sub> plants were grown in the greenhouse and F<sub>2</sub> seeds were chipped for GC analysis and DNA extraction (therefore fatty acid composition phenotype data and genotype are from one individual seed). Figure 3B and C show that the combination of FAD3C<sub>P266S</sub> with FAD3A<sub>W81STOP</sub> or FAD3A<sub>227RD</sub> resulted in double mutants with 2.2 to 3.5% linolenic acid. The subtle differences in linolenic acid levels between double mutants including the FAD3A<sub>W81STOP</sub> allele and the FAD3A missense alleles may be a result of different growing environments.

Fig. 2. Multiple sequence alignment for soybean and Arabidopsis thaliana FAD genes. Sequences retrieved from Phytozome 12. AT3G11170 is Arabidopsis FAD3, AtFAD2 (At3g12120), GmFAD3A-1 (Glyma.10g278000), GmFAD3A-2 (Glyma.20g111000), GmFAD3A-3 (Glyma.14g193400), and GmFAD3B (Glyma.02g027200). *Indicates proline (P) 266, which is mutated to serine (S) in the FAD3C<sub>P266S</sub> mutant. Shading indicates sequence identity >70%.

Fig. 3. Additive interaction of fad3c and fad3a mutations in low linolenic acid double mutants. (A) Composition in 73 F<sub>2</sub> individuals from the FAD3C<sub>P266S</sub> × FAD3A<sub>W81STOP</sub> cross. (B) Composition in 44 F<sub>2</sub> individuals from the FAD3C<sub>P266S</sub> × FAD3A<sub>227RD</sub> cross. (C) Composition in 48 F<sub>2</sub> individuals from the FAD3A<sub>W81STOP</sub> × FAD3C<sub>P266S</sub> cross.
In this study, we have described the isolation of a novel mutant allele of the \textit{FAD3C} gene. The \textit{FAD3C} \textit{p2362} substitution affects a highly conserved proline residue. This amino acid is located in the paralogous position affected in mutant alleles from two other soybean fatty acid desaturases identified previously: \textit{FAD2-1A} \textit{p2044} (Thapa et al., 2016a) and \textit{FAD2-1B} \textit{p2362} (Sweeney et al., 2017). This interesting coincidence underscores the importance of this region of the desaturase molecule for proper enzyme conformation and function on multiple substrates. The 3-D crystal structure indicates that this amino acid is within the region of the protein containing multiple α helices between the second and third histidine boxes, and in the vicinity of the active site of the enzyme (Zhu et al., 2015). Two of the missense mutations that were previously identified in the \textit{FAD3A} gene are also in this region (Thapa et al., 2018).

Low linolenic soybean oil is defined as containing no more than 3% linolenic acid, and ultralow linolenic soybean is defined as containing <2% linolenic acid in the total seed oil fraction (Warner and Fehr, 2008). In isolation, the content of 2% linolenic acid in the vicinity of the active site of the enzyme (Zhu et al., 2015). Of this, the ratio of linolenic acid to oleic acid in the region containing the FAD3 gene is important when measuring in bulk seed) can be misleading.

In our observations, \textit{fad3A} and \textit{fad3C} mutations are not completely recessive, and when combining two mutant alleles seed phenotype alone (particularly when measured in bulk seed) can be misleading for the selection of double homozygous mutants; therefore, accurate genotyping assays will continue to be important for breeding efforts in this area. In conclusion, our results suggest that the \textit{FAD3C} \textit{p2362} allele, facilitated by the codominant PCR based genotyping marker described, can be a useful tool for breeders to use in combination with mutant alleles of \textit{FAD3} or \textit{FAD2} genes to develop soybean plants with improved oil functionality.

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