As the building blocks of the majority of plant lipids, fatty acids are needed for membrane functions and plant growth and development. In plants, fatty acids are synthesized in plastids prior to their transportation into the endoplasmic reticulum (ER), where they are assembled into various lipids (Li et al., 2016). Although the seeds of oil seed crops also contain cellular membrane-type phospholipids, the majority of seed lipids are produced and stored in the form of high-energy density molecules, triacylglycerides (TAGs).
The first committed step in fatty acid biosynthesis is catalyzed by the enzyme acyl-CoA carboxylase (EC6.4.1.2). Fatty acids are produced in plastid stroma by the fluxing of carbon, with the most important regulator of carbon flux being the ACCase enzyme (Nikolau et al., 2003). An extra-plastid isozyme contributes to the malonyl-CoA for fatty acid elongation, which happens in a four-reaction cycle localized in acyl carrier protein (acyl-ACP), with the initial cycle yielding a four-carbon acyl chain and each subsequent cycle adding two carbons to the acyl chain. After a seven-cycle acyl elongation by acyl-ACP, the product is in the form of saturated 16-carbon acyl-ACP. The termination of fatty acid synthesis can then occur by elongation to 18:0-ACP through β-ketoacyl-ACP synthase II (KASII) and then desaturation to 18:1-ACP and hydrolysis via FATA thioesterase. Fatty acid synthesis termination can also occur without further elongation via fatty acid thioesterase to form the 16:0 fatty acid (Li-Beisson et al., 2013).

Furthermore, in the plastid stroma, Type II fatty acids with multiprotein complexes are associated with different intermediate reactions. As shown in Fig. 1, in the plastid inner envelope, acyl-ACP is in charge of free fatty acid (FFA) biosynthesis, and the fatty acid exporter 1 (FAX1) is associated with the transport of the FFA towards the plastid outer envelope. Through a yet unknown system (shown as “?” in Fig. 1), FFAs are then sent to the plastid membrane where, with the help of long-chain acyl-CoA synthetase 9 (LACS9), they enter the cytosol for acyl-CoA biosynthesis. Another transporter, ATP-binding cassette A9 (ABCA9), is associated with the transport of acyl-CoA through the ER membrane and inside of the ER, where diacylglycerol (DAG) biosynthesis takes place (Fig. 1).

During the final step of the Kennedy pathway (Kenny, 1961), the membrane-bound enzyme diacylglycerol acyltransferase1 (DGAT1) catalyzes the esterification of fatty acids to TAGs by transferring the acyl group from acyl-ACP to DAG via the Kennedy pathway (Kennedy’s cycle). From the Kennedy pathway, the fatty acids are then condensed to form fatty acyl-CoA, which is then converted to diacylglycerol (DAG) by acyl-CoA:Diacylglycerol acyltransferase (DGAT1). DAG is then converted to TAG by acyl-CoA thioesterase (FATA).

Transcription factors have played an important role in the developmental regulation of seed fatty acid synthesis (Baud and Lepiniec 2010). One of the most significant transcription factors in the regulation of fatty acid biosynthesis is WRI1, which is related to the ethylene-responsive element-binding family. Focks and Benning (1998) characterized the mutant line of WRI1 with a phenotype of “wrinkled surface” with 80% fewer TAGs than the wild-type. Several scientists (Ruuksa et al., 2002; Baud et al., 2007, 2009; Maeo et al., 2009) reported the control of fatty acid biosynthesis–related genes by overexpressing this transcription factor. Additionally, the overexpression of AtWRI1 or its orthologs resulted in an increase of seed oil accumulation in transgenic Arabidopsis, maize (Zea mays L.), and rapeseed by 10 to 30% (Cernac and Benning 2004; Liu et al., 2010; Shen et al., 2010; Wu et al., 2014). Shen et al. (2010) suggested that the gene stacking of the WRI1 and Dgat1 could be a breakthrough for the future of increased oil production in transgenic crops.

After being synthesized, TAGs are stored while they await degradation mostly into fatty acids by lipases. According to Barros et al. (2010), when TAGs are not degraded, they are usually packaged in the form of “oil bodies” or “lipid droplets” (Fig. 1). Oleosin (16–24 kDa) molecules are known to naturally develop a single-layer membrane around each of the oil bodies in pollen grains, seeds, and tapetum cells. Each oleosin molecule has a hairpin–like hydrophobic portion that is inserted inside the TAG molecules, and another hydrophilic part that is left outside of the oil bodies. Together, these structures enable oleosins to stabilize the oil bodies from degradation (Hsieh and Huang, 2005). Siloto et al. (2006) reported that, with an inverse correlation between oleosin expression levels and oil body size, oleosins are also determinants of the size of Arabidopsis seed oil bodies. Xu and Shanklin (2016) reported that, in Arabidopsis seeds, oleosin controls the stabilization and regulation of the size of smaller oil droplets by not allowing them to become larger and less stable.

The objective of the present investigation was to study the outcome of the overexpression of a combination of AtDgat1, AtWRI1, and AtOleosin genes, with the hypotheses that: (i) doing so will result in an increase in the maize seed total oil and TAG contents, and (ii) the fatty acid profile will be shifted according to acyl chain length and/or the degree of saturation.

In this study, we selected to increase the DGAT1 because this enzyme is the last and key enzyme (e.g., as compared with phospholipid diacylglycerol acyltransferase 1 [PDAT1]; Zhang et al., 2009) in the conversion of DAG to TAG. We also selected the WRII1 transcription factor because it has been the most studied among other fatty acids and lipid transcription factors, and because its overexpression does not interfere with starch biosynthesis (Shen et al., 2010). We also selected oleosin because this protein has not only been proven block the TAG degradation, but it has also been shown to have a role in stabilization of smaller oil bodies (Xu and Shanklin, 2016).
Constitutive promoters were specifically chosen in all three transgene constructs to test whether it is possible to produce seed-like TAGs in the vegetative tissues (stover) of maize plants (data to be published elsewhere).

**MATERIALS AND METHODS**

**Constructs**

Three different constructs were used in this research (Fig. 2). The first construct is the pHA-AtDGAT1 containing two cassettes. This construct was synthesized by exchanging the bacterial mannitol-1-phosphate dehydrogenase (mutD) coding sequences of the first cassette of the Pj101 plasmid (Nguyen et al., 2013) with the AtDGAT1 (GenBank NM_127503.2) to have the AtDGAT1 regulated by rice (*Oryza sativa* L.) actin promoter (*Act1*) and potato (*Solanum tuberosum* L.) protease inhibitor II (*ppiII*) terminator. The second cassette of the Pj101 plasmid contains the bar herbicide resistance selectable marker gene (Nguyen et al., 2013) sequences regulated by the cauliflower mosaic virus (35S) promoter and the bacterial Nos terminator. The second construct, pAtWR1 (Ma et al., 2013), also contains two cassettes. The first cassette of the second construct contains the *A. thaliana* Wri1 (AtWR1) coding sequences regulated by the cauliflower mosaic virus (CaMV 35S) promoter and the Nos terminator, and its second cassette contains the bar herbicide resistance selectable marker coding sequences regulated by the CaMV 35S promoter and Nos
terminator. The third construct (Fan et al., 2013), pAtOle, only contains one cassette with the AtOle gene regulated by the CaMV 35S promoter and the Nos terminator.

Maize Metabolic Engineering

Hi-II (hybrid) maize seeds were produced by cross breeding of the maize T940A with maize T940B parental lines. Immature embryos were collected 10 to 13 d after pollination and surface sterilized in 70% ethanol for 1 min, followed by 25 min of 50% commercial bleach (5.25% hypochlorite). The surface-sterilized immature embryos were rinsed three times with cold autoclaved water and cultured on medium containing 4 g L\(^{-1}\) N6 salts, 1 mL L\(^{-1}\) (1000×) N6 vitamin (Bio-World), 2 mg L\(^{-1}\) 2,4-D, 100 mg L\(^{-1}\) myo-inositol, 2.76 g L\(^{-1}\) proline, 30 g L\(^{-1}\) sucrose, 100 mg L\(^{-1}\) casein hydrolysate, 2.5 g L\(^{-1}\) gelrite, pH 5.8, and 25 μM silver nitrate. Cultures were then incubated in the dark under room temperature (22°C) for 2 to 3 d for proliferation.

Well-proliferating immature-embryo-derived somatic embryogenic calli were co-bombarded with a 1:1:1 combination ratio of the pHAtDGAT1, pWRII, and pOle using the BioRad PSD-1000/He Particle Delivery device. The bombarded calli were transferred onto Petri dishes containing the Murashige and Skoog (Murashige and Skoog, 1962) medium, supplemented with 2 mg L\(^{-1}\) 2,4-dichlorophenoxyacetic acid (2,4-D) growth regulator and 2 mg L\(^{-1}\) glufosinate ammonium. Cultures were then maintained under dark conditions for another 6 to 8 wk. The well-proliferating, herbicide-resistant calli pieces were transferred into fresh in vitro culture media, where they regenerated into plantlets following our previous work (Biswas et al., 2006). The herbicide-resistant plantlets were acclimated in a growth chamber under plastic covers to eliminate dehydration and then transferred to a greenhouse with 23°C and 16/8-h light/dark period, where they grew to maturity. The leaf samples from each bioengineered versus a wild-type plant leaf were then painted with 0.1% phosphonothricin (PPT) (i.e., the active ingredient of glufosinate ammonium [Liberty]) herbicide to assure the presence of the AtDGAT1 and/or AtWRI1-containing plasmids, as these two plasmids also contained the bar selectable marker cassette. Nonbombarded calli were included in all tissue culture steps without selection to produce the wild-type negative control plants for comparison with metabolically engineered plants.

Transgene Integration, Transcription Analyses, and Breeding of Bioengineered Plants

PCR

Total DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (Xin and Chen, 2012) from both herbicide-resistant plantlets and Hi-II nontransgenic plantlets. The polymerase chain reaction (PCR) was conducted using forward and reverse primers specific to DGAT1 (5′-GCTCCTACAAATGCCATCA-3′) coding sequence and ppiII (5′-CCACATTATAGTGATTAGCATGTC-3′), which were used to detect the pAtDGAT1-js101 construct integration, amplifying ~750 bp, as well as 35S promoter forward primer 5′-GCTCCTACAAATGCCATCA-3′ and oleosin reverse 5′-AACGATTAGCGGCATCATAC-3′, amplifying ~650 bp. However, 35S forward promoter and Wrinkle reverse 5′-GCAGCTTCCTCCTCTCGATATT-3′ were used to amplify ~700 bp.

qPCR

An RNA isolation kit system (EZNA plant RNA Kit, Omega Bio-tec) was used to extract total RNAs from the leaves of three individual randomly selected plants of each transgenic line, as well as from three randomly selected plants of Hi-II nontransgenic tissue culture regenerated plants. Then, a complementary
DNA strand was synthesized using GoScript Reverse Transcrip-
tase (Promega cat no. A5003), and the SYBR Green quantitative
PCR (qPCRs) were conducted to test the expressions of the
PCR positive plants that contained all three transgenes (DGAT1,
Oleosin, and WR11) to determine their transcript levels

The total qPCR reaction solution (20 μl) included 10 ng
complementary DNA, 0.3 μM of each primer, and 1× Fast-
SYBR Green Master Mix (Applied Biosystems). All reactions
were done in triplicates of the metabolically engineered plant
lines, as well as for the control plants, using an ABI 7900 HT
real-time PCR system (Applied Biosystems) under the condi-
tions of 94°C for 30 s, 40 cycles of 95°C for 15 s, 60°C for 60
s, and 72°C for 20 s to calculate the cycle threshold (Ct) values.
Ubiquitin–conjugating enzyme E2 gene was used as a house-
keeping gene to standardize the gene expression. The primers
were specifically designed to detect each Arabidopsis–based
integrated transgene in maize genome (Table 1). The Relative
Quantification of Gene Expression method (ΔΔCt) was
used to calculate the results using 2−ΔΔCt formula (Livak and
Schmittgen, 2001).

Maize Breeding and Seed Collection
In cases where less than two transgenes proved integrated in
the maize genome, plants were crossbred to combine all three
transgenes. The first and second generation of transgenic (T0
and T1) and tissue-culture–grown wild-type control plants
were grown under the same greenhouse conditions. Fer-
tile plants expressing all three transgenes were self-pollinated
towards homozygosity, and seeds of each ear were separately
harvested after the plant had reached maturity (35–45 d after
pollination) and dried overnight in a 50°C oven.

Oil Extraction
Mature dried seed samples of three different ears of metabolically
eengineered line 91-8 versus those of three ears of control
wild-type plants were premilled prior to extrusion using a
2-mm pore size Foss mill system. Milled samples were incu-
bated overnight in an oven at 105°C to obtain 6.8 ± 0.73%
motion before oil extraction.

The maize total seed oil was extracted using hexane in
an accelerated solvent extraction (ASE) system ( Dionex ASE
200), following a modified procedure developed by Dionex.
The detailed procedure involved placing ~1.3 g of fine corn
seed milled powder in a cellulose fiber filled in a high-pres-
sure stainless steel extraction cell. Conditions set during each
extraction cycle for the ASE system included: the oven temper-
ure stainless steel extraction cell. Conditions set during each
seed milled powder in a cellulose fiber filled in a high-pres-
sure stainless steel extraction cell. Conditions set during each

Table 1. List of quantitative polymerase chain reaction primers.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>UBO forward</td>
<td>5<code>-ACACCTTGTACGCCAAGCCTACT-3</code></td>
</tr>
<tr>
<td>UBO reverse</td>
<td>5<code>-ACCGCTAAGGCTGCTCCTT-3</code></td>
</tr>
<tr>
<td>DGAT1_Q_F</td>
<td>5<code>-GGTTCAATAAGGTAGTGTTCACATAT-3</code></td>
</tr>
<tr>
<td>DGAT1_Q_R</td>
<td>5<code>-GATAATGGCGAGTGTCTTTGGTATC-3</code></td>
</tr>
<tr>
<td>Oleosin_Q_F</td>
<td>5<code>-AACCGTTATAGCGGCAATGC-3</code></td>
</tr>
<tr>
<td>Oleosin_Q_R</td>
<td>5<code>-GCAGCTTCTCCTCGGTGATATT-3</code></td>
</tr>
</tbody>
</table>

After the extraction, hexane was recovered using a rotary
evaporator (Rotavapor R, Buchi) and reused. The extracted oil
was air dried overnight to remove residual hexane and to allow
the calculation of dry mass and oil percentage. The moisture
content in the biomass was measured by the infrared moisture
analyzer (Denver Instrument, IR-30). Dry oil from each sample
was collected by dissolution in 1 mL of hexane. The extracted
samples were then securely capped and kept in −20°C condi-
tions until further analysis.

Seed Oil Sample Preparation
The maize seed oil samples were methyl esterified after evapora-
tion of the hexane in vacuo. One milliliter of methanol:H2SO4
(5:1 by volume) was added to each solid oil sample, which was
then agitated for a few minutes and stored at room temperature
for 14 h to allow the reaction to proceed. For the separation
of the esterified fatty acids from other components, 1 mL of
chloroform and 5 mL of deionized water were added to each
sample. The organic phase containing fatty acid methyl esters
(FAMEs) was collected. Fatty acids were separated from other
components through preparation methods and kept as FAMEs
for analysis. A 37-component FAME standard mixture was
used as an external standard source; this mixture was used to
generate a six-point standard curve by serial dilution for FAME
quantification. The standard curves generated from each com-
ponent in the FAME mixture ranged from about 6000 ng μL−1
down into about 0.1 ng μL−1, with the exact range being species
specific.

FAME Analysis via Gas Chromatography–
Mass Spectrometry
Fatty acid methyl esters were quantified using gas chromatog-
raphy coupled with mass spectrometry (GC–MS) on a system
composed of a Thermo Trace GC Ultra coupled to a Thermo
DSQ II mass spectrometer. The GC was equipped with a
Thermo Scientific Triplus AS autosampler. For the GC separa-
tions, a DB-23 column featuring a stationary phase composed
of 50% cyanopropyl groups and 50% dimethylpolysiloxane
groups was chosen (Agilent J&W P/N 112-2332; 30 m × 0.25
mm i.d. × 0.25 μm film thickness). Parameters of the GC
method included syringe washes with ethyl acetate and hexane,
a 100:1 split injection volume of 1.0 μL, an inlet temperature
of 250°C, a 24.67-min method run time, a transfer line tem-
perature of 250°C, and a helium flow rate of 1.3 mL min−1.
The oven ramp was as follows: start at 40°C (hold 1 min), then
40°C min−1 ramp to 160°C (no hold), then 3°C min−1 ramp to
210°C, then 40°C min−1 to 250°C (hold for 3 min). The mass
spectrometer used in the system featured an electron ionization
source operated at 70 eV and a single quadrupole mass analyzer.
Operating parameters for the mass spectrometer included:
a solvent delay of 3 min, an ion source temperature of 250°C, and
scanning from 210 to 400 m/z on a scan rate of 1.3 mL min−1.
The GC–MS system was controlled using Thermo Xcibi-
lar version 3.0. The retention times of the known standards in
the 37-component FAME mix were identified by automated
library matching against the NIST 2011 Mass Spectral Database
in combination with spectral interpretation and comparisons
with published elution orders for the standard mixture of the

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same type of column (Frank et al., 2005). Information on the analytes quantified, their retention times, and the m/z from which the extracted ion chromatograms were constructed are provided in Supplemental Table S1.

The samples were analyzed in a manner that allowed minimization and assessment of the risk of carryover or systematic bias. That is, the analysis order was as follows: solvent blank, IS (internal standard as C19 methyl ester) blank, calibration curve (from lowest to highest concentration), IS blank, biological samples (in randomized order), and IS blank.

**GC–MS Data Processing**

The acquired data was converted from the Thermo.raw file format into the universal.cdf file format using the File Converter program featured in Xcalibur. From there, the .cdf files were imported into the Waters MassLynx 4.1 program suite using the DataBridge program by Waters. Automated quantitative analyses were performed on extracted ion chromatograms (EICs) for each analyte using the Waters QuanLynx program for peak smoothing, for peak integration, to create calibration curves (linear curve fitting with 1/x weighting) and to calculate IS-normalized concentration measurements for each of the targeted analytes.

**Starch Content Determination**

The total starch contents were measured based on the Association of Official Analytical Chemists (AOAC) Official Method 996.11, using YSI glucose analyzer following Latimer (2012).

**Confocal Laser Microscopy**

Nile Red dye solution was used according to Gao et al. (2014) to distinguish the cellular lipids. Three randomly selected dry seeds of T1 plants that had shown the transcripts of all three transgenes and three randomly selected dry seeds of a wild-type control plant were horizontally sliced in the middle to observe their embryos. The sliced sections were then incubated in 100 μg mL⁻¹ of Nile Red solution for 30 min, embryos were gently removed from the sliced seeds using forceps, and the samples were observed under a Zeiss LSM5 Pascal confocal laser scanning microscope (Carl Zeiss) at 10 mM using an argon ion laser (λex = 488 nm).

**RESULTS**

A total of 98 first-generation (T0) transgenic plants survived the herbicide selection, and the herbicide-resistant plants were tested via PCR (results not shown) to determine the integration of each transgene construct. A total of 40 plants were selected based on the presence of all transgenes. Then, the T1 generation of these lines were developed and retested via PCR for the stability of transgenes presence. Two lines (62-4 and 91-8) among T1 plants that showed the integration of all three transgenes were further examined for their levels of transcripts using the real-time (q)PCR technology (Fig. 3), and the qPCR analysis of both lines confirmed the transcriptions of all three *Arabidopsis* transgenes (Dgat1, Wri1, and Oleosin) in maize genome. The relative increases in the expression of Dgat1 transcript for lines 62-4 and 91-8 were 7.4 and 5.5, for the Wri1 transcript were 21.8 and 44.3, and for Oleosin were 5.0 and 2.4, respectively. Because line 91-8 showed higher transgenes transcripts, three randomly selected replicates of the same line were used for seed oil extraction and analysis (Fig. 4A), exhibiting significant (α = 0.05) differences as compared with those of the three wild-type control total seed oil. Figure 4A shows that the total oil in seeds of metabolically engineered plants increased from 3.6 to 4.5% (i.e., 25%) as compared with their respective wild-type control plant seeds.

The GC–MS method of separating the oil volatile compounds into their fatty acids components was used to determine the fatty acid profiles of the T1 versus the wild-type control plants seed oils. A total of 23 fatty acids were in the T1 seed oil extract, representing ~99.9% of the total fatty acids. In this experiment, the percentage of the total saturated and monounsaturated fatty acids increased, whereas the percentage of polyunsaturated fatty acids decreased (Table 2). As the GC–MS data show, there were 13 saturated, 4 monounsaturated, and 6 polyunsaturated fatty acids present in corn seeds (Fig. 5). The main fatty acids included: saturated (palmitic [C16:0] and stearic acids [C18:0]), and unsaturated (oleic [C18:1] and linoleic acid [C18:2], as shown in Tables 3 and 4.

This study reveals that the percentage of oleic acid (C18:1) increased by 73.3% and linoleic acid (C18:2) decreased by 51.1% in transgenic seeds. The oleic acid concentration was negatively correlated with linoleic acid. Figure 5 shows that the maize seed oil oleic acid and stearic acid increased, the linoleic acid decreased, and the level of linolenic acid was not significantly changed in metabolically engineered maize seeds. These results indicate that the production of polyunsaturated fatty acids from their precursors or other categories of fatty acids, such as monounsaturated and saturated fatty acids, has been decreased.

According to GC–MS analysis, the TAG fatty acid components of the T1 seeds increased from 10.9 mg g⁻¹
Fig. 4. (A) Percentage total oil in T1 seed dry matter compared with the total oil of the wild-type (WT) control maize seed of the same age. According to student’s t test ($P < 0.05$), letters above the bars show significant differences between the total oil contents of the T1 vs. the WT control maize seeds. (B) T1 vs. the WT control maize seed total fatty acid (TAGs) contents. The “a” and “b” letters above the bars represent statistically significant differences based on student’s t test ($P < 0.05$). (C) T1 vs. the WT control maize seeds starch content. Note: The same letters above the two bars indicate statistically insignificant differences based on student’s t test ($P < 0.05$). All errors bars are based on standard error of means.

Table 2. Percentage of seed fatty acids categories in T1 (91-8 line) vs. the wild-type control.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>SFA†</th>
<th>MUFA</th>
<th>PUFA</th>
<th>PUFA:SFA</th>
<th>ω6:ω3‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>29.70b§</td>
<td>15.40b</td>
<td>55.40a</td>
<td>1.86</td>
<td>8.84</td>
</tr>
<tr>
<td>91-8</td>
<td>31.86a</td>
<td>26.59a</td>
<td>41.80b</td>
<td>1.31</td>
<td>6.31</td>
</tr>
</tbody>
</table>

† SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
‡ ω6:ω3, ratio of Omega6 to Omega3.
§ Letters following means in each class indicate that their differences are statistically significant at $P < 0.01$.

Fig. 5. The percentage of different fatty acids in triplicates of T1 vs. the wild-type (WT) control seeds. The * and ** above the bars represent statistically significant differences based on student’s t test at $P < 0.05$ and 0.01, respectively. There is no statistical significance between other components.

Table 3. Composition and percentages of saturated fatty acids in maize seeds.

<table>
<thead>
<tr>
<th>Saturated fatty acid†</th>
<th>Genotype</th>
<th>C6:0</th>
<th>C8:0</th>
<th>C10:0</th>
<th>C12:0</th>
<th>C14:0</th>
<th>C16:0</th>
<th>C17:0</th>
<th>C18:0</th>
<th>C20:0</th>
<th>C22:0</th>
<th>C23:0</th>
<th>C24:0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.05</td>
<td>0.21</td>
<td>0.04</td>
<td>0.04</td>
<td>0.12</td>
<td>0.04</td>
<td>23.14</td>
<td>0.12</td>
<td>3.98</td>
<td>0.60</td>
<td>0.05</td>
<td>0.11</td>
<td>0.14</td>
</tr>
<tr>
<td>91-8</td>
<td>0.11</td>
<td>0.33</td>
<td>0.13</td>
<td>0.05</td>
<td>0.11</td>
<td>0.04</td>
<td>23.43</td>
<td>0.19</td>
<td>5.78</td>
<td>0.57</td>
<td>0.03</td>
<td>0.13</td>
<td>0.10</td>
</tr>
</tbody>
</table>

† C6, caproic acid; C8:0, caprylic acid; C10:0, capric acid; C11:0, undecylic acid; C12:0, lauric acid; C14:0, myristic acid; C15:0, pentadecylic acid; C16:0, palmitic acid; C17:0, margaric acid; C18:0, stearic acid; C20:0, arachidic acid; C21:0, heneicosylic acid; C22:0, behenic acid; C23:0, tricosylic acid; C24:0, lignoceric acid. There is significant difference only for mean values of C:18 at $P < 0.05$.

Table 4. Composition and percentages of unsaturated fatty acids in maize seeds.

<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.11</td>
<td>15.12</td>
<td>0.11</td>
<td>40.12</td>
<td>4.53</td>
<td>0.03</td>
<td>0.03</td>
<td>8.53</td>
<td>2.18</td>
<td></td>
</tr>
<tr>
<td>91-8</td>
<td>0.11</td>
<td>26.21</td>
<td>0.23</td>
<td>26.53</td>
<td>4.20</td>
<td>0.01</td>
<td>0.04</td>
<td>9.29</td>
<td>1.73</td>
<td></td>
</tr>
</tbody>
</table>

† C16:1, palmitoleic acid; C18:1, oleic acid; C18:2, linoleic acid; C18:3 ω3, α-linolenic acid; C20:3, dihomo-γ-linolenic acid; C20:5, eicosapentaenoic acid; C22:6, docosahexaenoic acid. Differences between mean values of C18:1 and C18:2 were significant at $P < 0.01$, and differences of C20:1 was significant at $P < 0.05$. Differences between other classes were not significant.
dry weight in wild-type lines to 23.7 mg g$^{-1}$ dry weight in line 91-8 (~117%), as shown in Fig. 4B, and the total seed oil content increased from 3.6 ± 0.08 in wild-type control seed fatty acids to 4.5 ± 0.21% (~25%) in the those of the bioengineered line 91-8 (Fig. 4A). This finding was supported by images produced by confocal laser microscopy using the Nile Red-stained dry seed embryos of T1 versus the wild-type control maize plants, showing the apparent increased levels of oil bodies in the metabolically engineered embryo cells (Fig. 6). Furthermore, Fig. 4C shows that the starch contents of milled seeds of the T1 versus the wild-type control plants were not significantly reduced (from 63.2% in wild-type control to 62.3% in T1 seeds) due to maize oil metabolic engineering.

**DISCUSSION**

Although the words lipids, fatty acids, oils, and TAGs are often used interchangeably in the literature, lipids include a class of nutrients that contain a combination of TAGs (fats and oils), phospholipids, and sterols. However, fatty acids are long chains of lipid-carboxylic acids that are present in fats, oils, and cell membranes as a component of phospholipids and glycolipids. Also, despite the fact that the plant seed fatty acid profiles represent a combination of TAG and phospholipids, we anticipate that the increases in the fatty acid in maize seeds in our study are largely due to increases in TAG content.

The study presented here demonstrated an increase in maize seeds storage fatty acids (TAGs) by ~117% and an increase in seed total oil by ~25% (Fig. 2). Such increases can add an additional 90 kg of maize seed oil, worth US$72 ha$^{-1}$, given the current yield of 10 t ha$^{-1}$ and an oil price of $0.80 kg$^{-1}$.

It is important to note that the approach described here resulted in increasing the oil content of the Hi-II maize (i.e., a low oil-producing genotype, ~1.1% oil per dry weight). The same technology can be used to double the TAG content of presently available high oil-producing maize genotypes. United States corn farmers plant ~35 million ha of land with high-oil maize. When the metabolic engineering described here is applied to these crops, it could result in ~3 million t of additional oil production.

Taylor et al. (2009) field tested bioengineered double-haploid canola plants that were overexpressing the At$Dgat1$ and Bn$Dat1$ genes regulated by the $B. napus$ seed-specific napin promoter, reporting a 2.5 to 7% increase in seed oil content per dry weight as compared with the seeds of nontransgenic plants. Comparing the research reported by Taylor et al. (2009) with our research, they probably had the benefits of regulating the $Dgat$ gene under a seed-specific promoter. Therefore, in the research presented here, we would expect higher levels of total oil and TAG contents had we chosen a maize seed-specific promoter, such as that of the zein gene. Additionally, compared with the work presented on canola and Arabidopsis seeds (Taylor et al., 2009), the research presented here may support the argument that the overexpression of oleosin in the maize genome has helped to enhance TAG levels by blocking the newly synthesized oil bodies from degradation.

The increase in seed TAGs via metabolic engineering can result in carbon flux in the form of lower seed starch content, which can affect the seed germination rate. Previously, Shen et al. (2010) overexpressed the maize leafy cotyledon 2 (ZmLEC2) transcription factor in the maize genome, reporting 48% increase in maize seed total oil content. However, such increase in seed total oil resulted in reduction of starch content in embryos by 60% and reduced seed germination and leaf growth due to the LEC2 pleiotropic nature. However, when they overexpressed the Zm$Wri1$ transcription factor in maize, seed

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**Fig. 6.** Confocal laser microscopy of an embryo of a half-sliced seed of second generation of metabolically engineered maize (left) versus an embryo of a half-sliced seed of a wild-type control maize (right) showing an apparent increase in oil bodies (white arrows). Note that the embryos of sliced seeds were first stained with 100 μg mL$^{-1}$ of oil-specific dye (i.e., Nile Red) before microscopy. Therefore, the more oil bodies in a cell, the bulkier and the brighter the color of red are under the microscope.
oil increased at the same percentage without affecting the starch level, seed germination, seedling growth, or grain yield all the way to the field level. Comparing the value of overexpressing the ZmLEC2 versus ZMWri1, we decided to use the Wri1 in our study, which resulted in no significant negative effects on starch biosynthesis (Fig. 4C) and seed germination, and no apparent negative effects on plant growth and development.

In the research presented here, the polyunsaturated fatty acids such as linoleic acid (18:2) and α-linolenic acid (18:3) decreased. The data presented here also show an increase in the monounsaturated fatty acid oleic acid (18:1) in bioengineered maize seeds. The polyunsaturated fatty acids naturally produced in developing seeds by the desaturation of oleic acid (18:1) has been reported (Lu et al., 2009) to be unhealthy cis fats. However, as per Aranceta and Pérez-Rodrigo (2012) and Dyson et al. (2011), foods rich in monounsaturated fatty acids is considered important to improve glucose-insulin profiles and reduce the effects of Type 2 diabetes. Also, partial hydrogenation of polyunsaturated oils is used to produce high-oleic acid corn oil, a technology with potential of capturing >40% of the domestic consumption of vegetable oil in the United States by 2020 due to its health benefits (Wilson 2012).

In the data presented here (Table 4), the ratio of linoleic acid (omega 6) to α-linolenic acid (omega 3) has been reduced from 10:1 in the seeds of control plants to 6:1 in the seeds of the bioengineered line. Simopoulos (2008) reported that the lower ratio of omega 6 to omega 3 correlates with the suppression of many diseases, especially cardiovascular. According to Simopoulos (2008), the 4:1 ratio of omegas 6:3 resulted with a 70% decrease in mortality, and the ratio of 2.5:1.0 lowered the rectal cell proliferation in colorectal cancer patients. Therefore, the dramatic decrease in the ratio of omega 6 to omega 3 observed in the study described herein suggests a route to the production of maize culinary oil with improved health benefits. Furthermore, in the research presented here, the ratio of polyunsaturated fatty acids to saturated fats (polyunsaturated:saturated) decreased from 1.86 to 1.31 (Table 2). It has been reported (Kang et al., 2005) that the ratio of polyunsaturated:saturated is also an important index used to evaluate oils for human health, and an oil index of polyunsaturated:saturated ratio between 1 and 1.5 is a favorable range for the human diet. In summary, the increase in maize seed TAGs and changes in their fatty acid profiles presented here can increase the maize oil caloric content and enhanced health benefits.

We selected the line 91-8 for fatty acids and lipids analysis due to its higher level of transcripts as compared with the other metabolically engineered lines. The primers designed for such real-time PCRs were to detect the Arabidopsis-driven transcripts in maize while avoiding any conserved regions of the native maize genes. However, we did not compare sequences with the same amplification efficiency. Therefore, more research might be needed to compare those sequences between the Arabidopsis-derived transcripts in maize and those of the wild-type control maize plants with the same amplification frequency. Furthermore, it is assumed that the different levels of transcripts and the higher TAG contents in line 91-8, as compared with other metabolically engineered lines might be related to the insertion sites of each of the three transgenes in the maize genome and might be due to somaclonal variations during plants in vitro cultures, and/or due to other unintentional consequence of the transformation processes, especially during transgenes bombardments.

The new concept is to increase the crop seed TAGs via overexpressing of certain subcellular membrane transporters in plant genome. For example, the overexpression of FAX1 transporter (Fig. 1) increased TAGs via mediating the transport of fatty acids from the plastid (Li et al., 2015a, 2015b). Also, the overexpressing of the ABCA9, a transporter that supplies fatty acids to ER for lipid biosynthesis, increased TAGs in Arabidopsis seed (Kim et al., 2013). It might even be possible to further increase the maize seed TAGs to match the level of TAGs produced in soybean [Glycine max (L.) Merr.] seed, which is ~20% of total dry weight. This can potentially be achieved via: (i) increasing the supply of fatty acids biosynthesis, (ii) overexpressing other genes associated with TAG biosynthesis and accumulation, (iii) improving the expression of transporters of fatty acids and lipids out of plastid and towards the ER, for TAG biosynthesis, and (iv) further blocking the degradation of TAGs using other technologies.

In addition to the research conducted thus far, more studies are needed in lipidomics and in the interactions between metabolic engineering and synthetic biology to lead scientists to discover and understand the fundamentals of other genes associated with TAG biosynthesis and accumulation. Additionally, most of metabolic engineering research so far performed is on the model plant Arabidopsis. It is important that future funds are allocated among scientists to apply the concepts of fundamental research to real crops, especially cereals, which vary tremendously from model plants such as Arabidopsis. The research presented here is on the Hi-II maize. More studies are also needed on metabolic engineering of other maize genotypes to determine whether important farmers’ maize can produce higher total oils, higher TAGs, and healthier fatty acids via metabolic engineering.

**Supplemental Material Available**

Supplemental material for this article is available online.
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References


