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Oil, Fatty Acid, Flavonoid, and Resveratrol Content Variability and *FAD2A* Functional SNP Genotypes in the U.S. Peanut Mini-Core Collection

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Supporting Information

ABSTRACT: Peanut seeds contain high amounts of oil and protein as well as some useful bioactive phytochemicals which can contribute to human health. The U.S. peanut mini-core collection is an important genetic resource for improving seed quality and developing new cultivars. Variability of seed chemical composition within the mini-core was evaluated from freshly harvested seeds for two years. Oil, fatty acid composition, and flavonoid/resveratrol content were quantified by NMR, GC, and HPLC, respectively. Significant variability was detected in seed chemical composition among accessions and botanical varieties. Accessions were further genotyped with a functional SNP marker from the *FAD2A* gene using real-time PCR and classified into three genotypes with significantly different O/L ratios: wild type (G/G with a low O/L ratio <1.7), heterozygote (G/A with O/L ratio >1.4 but <1.7), and mutant (A/A with a high O/L ratio >1.7). The results from real-time PCR genotyping and GC fatty acid analysis were consistent. Accessions with high amounts of oil, quercetin, high seed weight, and O/L ratio were identified. The results from this study may be useful not only for peanut breeders, food processors, and product consumers to select suitable accessions or cultivars but also for curators to potentially expand the mini-core collection.

KEYWORDS: *peanut (Arachis hypogaea L.), seed chemical composition, variability, nuclear magnetic resonance (NMR), gas chromatography (GC), high performance liquid chromatography (HPLC), gene for fatty acid desaturase (FAD2), real-time polymerase chain reaction (PCR), single nucleotide polymorphism (SNP), oleic/linoleic acid (O/L) ratio*

INTRODUCTION

Peanut (*Arachis hypogaea* L.) is an important oilseed crop worldwide. Peanut seeds contain about 50% oil, 25% protein, and some useful phytochemicals such as folic acids, tocopherols, flavonoids, and resveratrol.[1−6] Peanut oil is mainly composed of eight major fatty acids: palmitic (C16:0), stearic (C18:0), oleic (18:1), linoleic (C18:2), arachidic (C20:0), eicosenoic (C20:1), behenic (22:0), and lignoceric (24:0) acids. However, oleic acid (monounsaturated fatty acid, MUFA) and linoleic acid (polyunsaturated fatty acid, PUFA) comprise over 80% of the oil. Consuming oil with high oleic acid and low linoleic acid can reduce blood pressure and risk of cardiovascular diseases.[7] Therefore, the ratio of oleic acid and linoleic acid (O/L) is an important parameter for determining peanut oil and seed quality. To enhance the oil quality, high oleate peanut varieties have been developed and cultivated for peanut production. For example, a high oleate cultivar, SunOleic 95R, contains 49% oil composed of 80.6% oleic acid and 2.8% linoleic acid.[8]

Flavonoids are a class of secondary plant phenolics. To date, over 4000 flavonoids have been identified. As natural compounds, flavonoids can be divided into six major classes: flavones, flavonols, flavanones, catechins, anthocyanins, and isoflavones based on variation in the C-ring.[9] Flavonoids have multiple hydroxyl groups which are responsible for antioxidant, chelating, and pro-oxidant activities. Flavonoids can alter seed quality by reducing digestibility but also have some positive effects on human health. A wide range of beneficial effects (through antioxidant, antiestrogenic, and antiproliferative activities) to human health have been reviewed,[9−12] including reducing the risk of certain forms of cancer, heart disease, and old age-linked diseases. Thorough research on flavonoids has been conducted in soybean, but relevant information on flavonoids in peanuts is very limited.[13] Some flavonoids have been quantified in peanut testa, meal, and seeds from a limited number of accessions.[4,14,15] In general, soybean seeds contain higher amounts of daidzein and genistein, while peanut seeds contain higher amounts of quercetin.[4]

Resveratrol (3,5,4′-trihydroxystilbene) is a polyphenolic compound that was first isolated from white hellebore.[16] However, it was initially classified as a phytoalexin.[17] Recently,

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resveratrol was found as a natural product which can contribute to human health by potentially preventing cardiovascular heart diseases, diabetes, neurodegenerative diseases, and certain types of cancer.[18] There are two conformational forms of resveratrol: *trans*-resveratrol and *cis*-resveratrol. Under bright light, *trans*-resveratrol can easily convert to *cis*-resveratrol;[19] however, *trans*-resveratrol is thought to be the biologically active conformational form. *trans*-Resveratrol has shown antioxidant, antifungal, anti-inflammatory, and anticancer activities, as well as chemopreventive, cardioprotective, and estrogenic effects.[18] Resveratrol has been found in over 70 plant species (including grapes and mulberries), but the natural level of resveratrol present in some species, e.g., peanut, is low. There are two possible ways to enhance the level of resveratrol in peanut: bioproduction (use of biotechnology through recombinant microorganisms or large-scale culture of plant cells[20]) and traditional breeding.

The peanut mini-core consisting of 112 accessions and representing ~1% of the U.S. peanut germplasm collection is an important genetic resource for breeders to use in improving seed quality and other traits for developing new cultivars.[21] This mini-core has limited information for seed biochemical composition. Associating seed composition information with DNA markers will help breeders choose suitable accessions to use in their breeding programs and increase the mini-core use efficiency. The genetic diversity of this mini-core was first evaluated with 31 SSR markers along with DNA sequencing.[22] The frequency of the *FAD2A* mutant allele in the mini-core was also estimated using a cleaved amplified polymorphic sequence (CAPS) marker.[23] Some seed nutrients of this mini-core were also analyzed from a single year of harvested seeds,[6] but information is lacking for multiple years' harvests (oil content and fatty acid composition) as well as new information on flavonoids and resveratrol. The evaluation of these multiple seed quality traits will be useful for breeders and consumers. Recently, the population structure and marker-trait association (especially for chemical composition traits) were conducted using 94 peanut accessions from the mini-core collection,[24] and four subpopulations were revealed that corresponded with botanical varieties. The objectives of this study were to (i) determine the variability in seed chemical composition traits among accessions within the mini-core, (ii) determine the variability in seed chemical composition traits among botanical varieties and *FAD2A* functional SNP mutant genotypes, (iii) detect correlations among seed chemical composition traits, and (iv) identify accessions with optimum seed chemical composition traits for germplasm enhancement and product consumption.

MATERIALS AND METHODS

Collection of Accession Samples. Seeds from 102 available accessions within the U.S. peanut mini-core collection were requested from the USDA-ARS, Plant Genetic Resources Conservation Unit (PGRCU) in Griffin, GA. Twenty seeds from each accession were planted in two-row 10-feet long plots in Dawson, GA during 2008 and 2009. Botanical variety classification for each accession was confirmed in the field at 10 and 14 wks after planting following the peanut descriptor's handbook.[25] The plant introduction (PI) number, botanical variety type, and country of origin or collection site for these accessions are listed in Table S1 in the Supporting Information. Seeds were harvested at physiological maturity with the aid of a small peanut harvester. After drying, pods were shelled, and seeds were cleaned by removing broken or split seeds. Chemical analysis was conducted on seeds from each accession in duplicate for statistical data analysis.

Oil Content by NMR Analysis. A Maran pulse nuclear magnetic resonance (NMR, Resonance Instruments, Whitney Oxfordshire, UK) was used to determine oil percentage following the published method.[26] Oil percentage was determined from physiologically mature seeds (~10 g) on a 0% H_2O basis by using the formula (100(% oil))/(100 − % H_2O). An average of the two subsamples was used for the reported value of oil content in this study.

Fatty Acid Composition by GC Analysis. The fatty acid composition analysis was adopted and modified from a previously published method.[27] Three to five peanut seeds were ground to a fine powder in a coffee bean mill. Oil from a small amount (~50−75 mg) of meal was extracted in 5.0 mL of heptane (Fisher Scientific) and converted to FAMEs with 500 μL of 0.5 N sodium methoxide ($NaOCH_3$) in methanol. Water was then added to separate the organic layer containing fatty acids from the peanut meal, and a portion of this layer was transferred to a vial for injection. Fatty acid composition was determined on an Agilent 7890A gas chromatograph (GC) with a flame ionization detector (FID). Peak separation was performed on a DB-225 capillary column (15 m × 0.25 mm i.d. with a 0.25 μm film) from Agilent Technologies. The inlet and detector temperatures were set to 280 and 300 °C, respectively. The carrier gas was helium set to a flow rate of 1.0 mL/min. One microliter of sample was injected at a 60:1 split ratio onto the column with the following thermal gradient: 195 °C for 3 min, 195 to 200 °C at 2.5 °C/min, 200 to 230 °C at 5 °C/min, and 230 to 235 °C at 1.5 °C/min for a total run time of 14 min. A fatty acid methyl ester (FAME) standard mix RM-3 plus four additional FAMES (all from Sigma) were mixed and used to establish peak retention times. Fatty acid composition was determined by identifying and calculating relative peak areas. The O/L ratio was determined by % oleic acid/% linoleic acid.

Iso/Flavonoid Content by HPLC Analysis. Peanut samples were prepared following the published method.[4] About 30 seeds (with uniform size) from each accession were ground into a fine powder using a coffee blender. Ground seed tissue (0.5 g) was transferred into 5 mL of polypropylene tubes and mixed with 2.5 mL of 80% methanol containing 1.2 M hydrochloric acid for hydrolysis. The mixture was vortexed briefly and then incubated at 80 °C for 2 h with tube inversion to mix the seed powder with the extract solution at 15-min intervals. After incubation, the samples were centrifuged at 14,000 rpm for 3 min, and the supernatant was transferred into 2 mL Eppendorf tubes. The supernatant containing flavonoids was filtered through a syringe with a 0.2 μm filter prior to injection into high performance liquid chromatography (HPLC) system. Separations were performed by reverse phase (RP)-HPLC using a Zorbax Eclipse, 4.6 × 150 mm, 5 μm, C18 column (Agilent Technologies) at 40 °C on an Agilent 1100 with a binary pump and autosampler. The mobile phase consisted of HPLC-grade acetonitrile (B) and 0.1% formic acid in filtered, sterile water (A). The flow rate was 2.0 mL/min at the following gradient: 15% B at time zero to 35% B at 20 min. The column was washed with 95% B for 5 min and equilibrated at 15% B for 7 min between injections. Sample injection volume was 10 μL, and analytes were monitored with a diode-array detector (DAD) at 260 nm (isoflavones) and 370 nm (flavonols). Flavonoid standards (Sigma, St. Louis, MO) including myricetin, quercetin, kaempferol, genistein, and daidzein were injected to generate external standard curves for peak identification and quantification.

Resveratrol Content by HPLC Analysis. The sample preparation was modified following a previously published method.[2] Approximately 8 g of air-dried seeds were ground to a fine powder in a coffee blender. Ground seed tissue (3 g) was transferred into 15 mL Falcon tubes and homogenized with 9 mL of 80% ethanol using a Power Gen 125 homogenizer (Fisher Scientific, Loughborough, UK). The homogenized samples were centrifuged (Eppendorf, 5415D, Hamburg, Germany) at 12,000*g* for 3 min. The supernatant (2 mL) was cleaned by solid-phase extraction using a Poly-Prep chromatography column (0.8 cm × 4 cm, Bio-Rad, Hercules, CA) packed with an ~1 mL mixture (1:1 w/w) of Al_2O_3 (EM Industries Inc., Gibbstown, NJ) and silica gel 60 RP-18 (EMD Chemicals Inc., Gibbstown, NJ). The

packed column was conditioned with 80% ethanol. The supernatant was applied to the equilibrated column, and the effluent was collected into a 4 mL vial. The column was washed with an additional 2 mL of 80% ethanol, and the effluent was collected into the same vial. The collected solvent was evaporated at 50 °C until dry with a nitrogen gas stream. The extracted compounds were dissolved in 1 mL of 20% acetonitrile and filtered (using a 0.45 μm filter) prior to injection for HPLC analysis. Separation of metabolites was performed on the RP-HPLC system (Agilent 1100 series) using a C_{18} column (4.6 mm × 150 mm, 5 μm, Agilent Technologies) at 40 °C with a binary pump and autosampler. The mobile phase consisted of A, filtered sterile water containing 0.1% formic acid at pH 2.5, and B, HPLC-grade acetonitrile. The flow rate was 1.5 mL/min with the following gradient: 10% B for 2 min, 10−30% B for 8 min, 30% B for 1 min, followed by column wash at 95% B for 6 min and 10% B for 9 min prior to the next injection. The volume for sample injection was 30 μL, and the analytes were monitored with a diode-array detector (DAD) at 310 nm absorbance. *trans*-Resveratrol in the extract of each accession was quantified at 310 nm by reference to the peak area of an external authentic standard of resveratrol (Sigma). The amount of resveratrol was only quantified from the first-year harvested seeds.

Seed Weight and Seed-Coat Color. Two samples of 100 seeds each were weighed. The average value for each accession was expressed in g per 100 seeds. Seed coat colors were noted and simply classified into one of six categories: 1 (red), 2 (purple), 3 (pink/tan), 4 (red/white mottled), 5 (red/tan mottled), and 6 (purple/tan mottled).

Genotyping *FAD2A* Functional SNPs by Real-time PCR. A real-time PCR assay for genotyping the *FAD2A* functional mutation on the A genome followed a previously published method.[28] For DNA extraction, peanut seed slices (75−150 mg) were transferred into a 2 mL microcentrifuge tube along with two 3 mm tungsten carbide beads (Qiagen, Valencia, CA) and 600 μL of P1 buffer from the Omega-BioTek kit (Doraville, GA). The tissue was then pulverized using a Retsch Mixer Mill 301 (Leeds, UK) at 30 Hz for 3 min. Extracts were quantified on a DyNA Quant 200 fluorometer from Hoefer Pharmacia Biotech (San Francisco, CA). In addition, all samples were loaded on a 1% agarose gel along with a Low DNA Mass Ladder from Invitrogen (Carlsbad, CA) to evaluate quantity and quality of each extraction. Samples were subsequently diluted to 10 ng/μL for real-time PCR. Genome specific SNPs identified from publically available sequence data derived from the wild progenitors of cultivated peanut for *FAD2* were incorporated into the probe/primer design to select the A genome rather than the B genome in this assay. Primers for this assay were synthesized by MWG Operon (Huntsville, AL). The sequence of the forward and reverse primers were 5′ GCC GCC ACC ACT CCA ACA C 3′ and 5′ GTT ATA CCA TGA TAC CTT TGA TTT TGG TTT TG 3′, respectively. Two TaqMan probes with 5′ reporter fluorophores, 3′ minor groove binders (MGB) and 3′ nonfluorescent quenchers (NFQ) were synthesized by Applied Biosystems (Foster City, CA). The sequences of the probes to discriminate the G448A mutation were 5′ 6FAM CCT CGA CCG CAA CG MGBNFQ 3′ and 5′ VIC CCT CGA CCG CGA CG MGBNFQ 3′, respectively. The VIC probe targeted the wild-type allele (Ol_1) and the 6FAM probe targeted the mutant allele (ol_1). The total product size was 83 base pairs long. Optimum probe and primer concentrations for this assay were determined by testing a matrix of various primer/probe concentrations and choosing the condition which maximized the normalized reporter fluorescence (ΔR_N) and minimized the cycle threshold (C_T). The total volume of the PCR reaction was 25 μL and consisted of 1× TaqMan Genotyping Master Mix (Applied Biosystems), 0.16 μM forward primer, 0.16 μM reverse primer, 0.4 μM VIC probe, 0.3 μM 6FAM probe, and 0.4 ng/μL of DNA. The TaqMan Genotyping Master Mix includes AmpliTaq Gold polymerase and ROX, a passive internal reference to correct for signal variation between wells. All PCR reactions were performed on an ABI StepOne real-time PCR machine (Applied Biosystems). The cycling conditions consisted of 1 cycle of 60 °C for 30 s, 1 cycle of 95 °C for 10 min, 50 cycles of 95 °C for 15 s and 62 °C for 1 min, and a final cycle of 60 °C for 30 s. Each PCR run included nontemplate controls to ensure that reagents were free of contaminants. In addition, several positive controls were included in each run (such as F435) to represent the homozygous recessive mutant genotype, normal oleate lines to represent the wild type, and heterozygotes containing both the mutant and wild-type alleles. StepOne version 2.0 (Applied Biosystems) was utilized to analyze and score genotypes using the default parameters. A similar real-time PCR method[29] was used for detection and classification of the functional mutant allele on the B genome.

Statistical Analysis. A Pearson's coefficient analysis was performed to determine significant correlations among different seed chemical composition traits. An analysis of variance was performed on the data, and means were separated using Tukey's multiple comparison procedure (SAS, 2008, Online Doc 9.2. Cary, NC: SAS Institute Inc.).

RESULTS AND DISCUSSION

Variability in Investigated Traits. Significant variability was identified for the seed chemical composition traits among accessions within the U.S. peanut mini-core collection (Table 1). There was significant variation in seed weight (ranging from 31.17 to 88.52 g/100 seeds with an average of 51 g). Variation in seed oil content ranged from 43.69 to 54.22% with an average of 49.17%. This means that there is potential in breeding programs to develop new peanut cultivars with small or large seeds as well as low or high oil content using accessions within the mini-core collection. Variation in oleic and linoleic acid content ranged from 38.97 to 62.04% and from 19.03 to 37.97% with an average of 47.26% and 31.12%, respectively. Significant variability was also detected in the amounts of quercetin (1.32−129.29 μg/g), kaempferol (0−6.07 μg/g), and genistein (0−1.37 μg/g). For example, PI 259836 contained eight times more quercetin than PI 290536 (59.2 μg/g vs 6.5 μg/g). Peanut seeds contained a relatively high amount of quercetin (mean = 15.85 μg/g) and low amounts of kaempferol (mean = 1.47 μg/g) and genistein (mean = 0.29 μg/g). These results were consistent with previously published results[4] in

Table 1. Variability of Seed Weight, Oil Content, Fatty Acid Composition, Flavonoid, and Resveratrol within the U.S. Peanut Mini-Core Collection[a]

variable	mean	SD	minimum	maximum	MSD
oil (%)	49.17	2.376	43.69	54.22	2.654
C16:0 (%)	10.65	1.252	7.98	13.34	0.141
C18:0 (%)	3.69	1.058	1.69	7.50	0.054
C18:1 (%)	47.26	6.562	38.97	62.04	0.352
C18:2 (%)	31.12	5.161	19.03	37.97	0.239
C20:0 (%)	1.63	0.279	1.04	2.48	0.022
C20:1 (%)	1.03	0.227	0.70	1.82	0.016
C22:0 (%)	3.13	0.498	2.33	6.34	0.043
C24:0 (%)	1.50	0.226	1.14	2.14	0.029
O/L ratio	1.61	0.552	1.03	3.28	0.027
quercetin (μg/g)	15.85	21.050	1.32	129.29	4.668
kaempferol (μg/g)	1.47	0.888	0	6.07	0.728
genistein (μg/g)	0.29	0.509	0	1.37	0.847
resveratrol (μg/g)[b]	0.10	0.043	0.03	0.26	0.019
seed weight (g/100 seeds)	51.03	12.067	31.17	88.52	6.681

[a]Trait value is averaged from the data from two years. Fatty acid composition is expressed as % (ME) = % of total methyl esters found in oil samples. SD, standard deviation. MSD, minimum significant difference among accessions. [b]The resveratrol value is from the data from one year.

which a higher amount of quercetin and a lower amount of kaempferol were detected in peanut seeds. Peanut seed contained a low amount of resveratrol (0.1 μg/g), but significant variation was detected with a 9-fold difference (0.03−0.26 μg/g) among accessions. Therefore, based on the variability there is potential to enhance flavonoid and resveratrol content in peanut seeds via breeding programs.

The variation of all 14 investigated traits (except for resveratrol) demonstrated significant year effect and year × genotype interaction (Table S2 in Supporting Information). When comparing the results from two years (Table 2), seed weight, oil content, oleic acid, and O/L ratio were significantly higher in seeds from year 1 (53.8 g/100 seeds, 50.19%, 48.1%, and 1.68, respectively) than seeds from year 2 (48.14 g/100 seeds, 48.18%, 46.4%, and 1.53, respectively). Quercetin, kaempferol, and genistein content were also significantly higher in seeds from year 1 (17.0, 1.56, and 0.46 μg/g, respectively) than in seeds from year 2 (14.72, 1.38, and 0.11 μg/g, respectively). However, the contribution to variation (Type I SS) from the year × genotype interaction was greater than the year effect for all investigated traits except oil content. These differences may be explained if oil content can be significantly affected by environmental conditions (i.e., years), or the adaptability of plant accession to the environment (genotype × year) can significantly affect the trait value. Overall, genotype was the major factor for determining the trait value. The values for all investigated traits of each accession are listed in Table S3 in the Supporting Information and can be compared among accessions using minimum significant differences (MSD) presented in Table 1.

Table 2. Comparison of Seed Weight and Chemical Composition Value between Year 1 and Year 2[a]

trait	year 1	year 2	MSD
oil	50.19 a	48.18 b	0.1662
C16:0	10.49 b	10.81 a	0.0089
C18:0	3.52 b	3.86 a	0.0034
C18:1	48.11 a	46.4 b	0.0221
C18:2	30.48 b	31.77 a	0.015
C20:0	1.61 b	1.66 a	0.0014
C20:1	1.08 a	0.97 b	0.001
C22:0	3.20 a	3.05 b	0.0027
C24:0	1.50 a	1.49 b	0.0018
O/L ratio	1.68 a	1.53 b	0.0017
quercetin	17.00 a	14.72 b	0.2935
kaempferol	1.56 a	1.38 b	0.0457
genistein	0.46 a	0.11 b	0.0533
seed weight	53.8 a	48.14 b	0.4185

[a]Means with different letters within the same row are significantly different. MSD stands for minimum significant difference.

Variability in Investigated Traits among Botanical Varieties. There are six botanical varieties (var.) in cultivated peanut, but only four of these, *fatigiata* var. fastigiata (ff), *fastigiata* var. peruviana (fp), *fatigiata* var. vulgaris (fv), and *hypogaea* var. hypogaea (hh), are represented in the mini-core collection.[24] Significant variability in all investigated traits except kaempferol and resveratrol content was detected among the botanical varieties, and the comparison results are listed in Table S4 in the Supporting Information. There was significant variability in seed weight and oil content (Figure 1). The 100-seed weights of var. hypogaea and var. peruviana were 55.08 and 54.51 g, respectively, which were much higher than those reported for var. fastigiata and var. vulgaris (47.17 and 45.28 g, respectively). The oil content of var. hypogaea and var. vulgaris was 49.57% and 49.84%, respectively, which were significantly higher than those reported for var. fastigiata and var. peruviana (48.52% and 47.35%, respectively). When considering seed weight and oil content together, the botanical variety of hypogaea may be more economically useful than the three other botanical varieties. Furthermore, the botanical variety hypogaea had a higher O/L ratio with a significantly higher amount of oleic acid (C18:1, 49.78%) and lower amounts of linoleic acid (C18:2, 29.19%) and palmitic acid (C16:0, 10.25%) than the other botanical varieties. However, the botanical variety hypogaea had a significantly lower amount of kaempferol (1.13 μg/g, similar to var. vulgaris 1.25 μ g/g) than var. fastigiata (1.89 μg/g) and var. peruviana (3.56 μg/g) (Figure 1 and Table S4, Supporting Information). This botanical variety may not be a good source to use for increasing kaempferol content. There was no significant variability in quercetin and resveratrol content among the four botanical varieties.

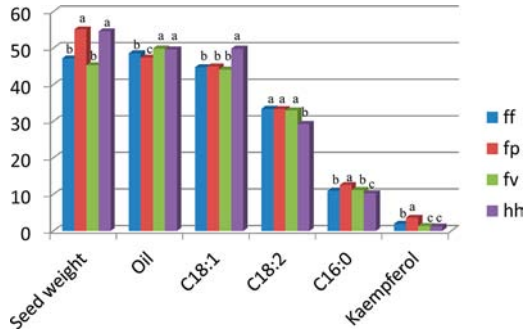

Figure 1. Variability in seed weight, major fatty acids, oil, and kaempferol content among different botanical varieties. On the *y*-axis, seed weight is in g/100 seeds; oil content, C18:1, C18:2, and C16:0 are in percentage; kaempferol is in μg/g. The abbreviations "ff", "fp", "fv", and "hh" represent for "*fatigiata* var. fastigiata", "*fastigiata* var. peruviana", "*fatigiata* var. vulgaris", and "*hypogaea* var. hypogaea", respectively.

Variability in Investigated Traits among Different *FAD2A* Functional Mutation Genotypes. Functional mutations of the *FAD2* genes (G448A *FAD2A* and 442insA *FAD2B*) were assayed by real-time PCR. No functional mutation was detected on the B genome, and thus, all 102 accessions belonged to the wild type. Three genotypes (wild type, heterozygous, and homozygous mutant) were identified with the *FAD2A* functional mutant SNP marker and designated as G/G, G/A, and A/A, respectively. Among 102 accessions, 58, 2, and 42 were identified as wild type, heterozygous, and mutant, respectively. Since peanut is mainly a self-pollinated species, heterozygous accessions should in theory not exist in the mini-core collection because these accessions had gone through several generations of field observation and purification during the mini-core establishment. There are two possible explanations for the heterozygosity. One is that these two accessions were heterogeneous when they were selected for the mini-core establishment; and another is that these two accessions received pollen from neighboring accessions in the field during seed increase. The latter is the more likely explanation because cross-pollination occurs when bees visit peanut flowers.[30,31]

The frequency of the *FAD2A* mutant allele (i.e., G→A) among different botanical varieties within the U.S. mini-core collection is significantly different. They were 0%, 1.96%, 6.37%, and 33.82% for vars. peruviana, vulgaris, fastigiata, and hypogaea, respectively. The difference in the frequency of the mutant allele for each botanical variety may be real or due to the uneven representation of each botanical variety within the mini-core collection. The percent representation within the mini-core collection for vars. peruviana, vulgaris, fastigiata, and hypogaea is 2.94% (3/102), 10.78% (11/102), 34.31% (35/102), and 51.96% (53/102), respectively. Because only three accessions are var. peruviana types, the low frequency of representation may explain why no mutant allele was identified in this botanical variety purely based on chance. More accessions from var. peruviana may need to be tested for determining whether this mutant allele occurs in this botanical variety. Over half the accessions (51.96%) are from var. hypogaea. This may explain why the mutant allele frequency from the var. hypogaea is much higher (33.82%) than those from the other botanical varieties.

Although there was no insertion mutation identified on the B genome, significant variability was identified in the amount of oleic acid and linoleic acid, and the O/L ratio among the three genotypes (Figure 2 and Table S4, Supporting Information).

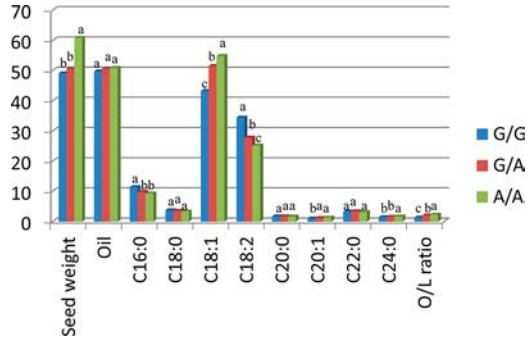

Figure 2. Variability in seed weight, oil, and fatty acid composition among different *FAD2A* functional SNP mutation genotypes. On the *y*-axis, seed weight is in g/100 seeds; oil content, C16:0, C18:0, C18:1, C18:2, C20:0, C20:1, C22:0, and C24:0, is in percentage. The base symbols "G" and "A" for nucleotides are "guanine" and "adenine".

Accessions with the homozygous mutant genotype (A/A) had the highest level of oleic acid (54.81%), lowest level of linoleic acid (34.41%), and highest O/L ratio (2.26). Accessions with the heterozygous genotype (G/A) had a higher level of oleic acid (51.50%), lower level of linoleic acid (27.76%), and higher O/L ratio (1.97) than the wild type (G/G, 43.20%, 34.41%, and 1.26). The ranges of the O/L ratio for the three genotypes were wild type (G/G with a low O/L ratio <1.7), heterozygote (G/A with an O/L ratio >1.4 but <1.7), and mutant homozygote (A/A with a high O/L ratio >1.7). These results were expected because fatty acid desaturase (*FAD2A*) is responsible for the conversion of oleic acid (C18:1) to linoleic acid (C18:2). The functional mutation (G→A) of the *FAD2A* gene reduces the fatty acid desaturase activity and leads to a higher level of oleic acid, lower level of linoleic acid, and higher O/L ratio. Because of the environmental effect, there was some overlap for the O/L ratios between wild type and heterozygote, but most accessions from the wild genotype had an O/L ratio <1.4. This mutant allele did not affect the percentage of stearic acid (C18:0), arachidic acid (C20:0), and behenic acid (C22:0), but it did have some effect on reducing the level of palmitc acid (C16:0) and increasing the levels of eicosenoic acid (C20:1) and lignoceric acid (C24:0) (Figure 2). There was no significant variability in oil content among the homozygous or heterozygous genotypes, but accessions with the homozygous mutant genotype (A/A) had significantly higher seed weight per 100 seeds (60.77 g) than the heterozygote (G/A, 50.63 g) and wild type (G/G, 49.04 g). This means that the *FAD2A* functional mutation can not only lead to a relatively high level of oleic acid in seeds but may also play a role in increasing seed weight (r = 0.40 at p < 0.0001 level). This correlation is desirable for peanut breeding programs. Since the correlation value was not very high, it needs to be further confirmed by testing more germplasm accessions. There was no significant variability in the amounts of quercetin and kaempferol, but there is significant variability in genistein among the three genotypes. Wild-type accessions (G/G) had a higher amount of genistein (0.65 μg/g) than heterozygotes and mutant homozygotes (0.13 and 0.22 μg/g, respectively). Accessions with the homozygous genotype (A/A) also had a significantly higher amount of resveratrol (0.12 μg/g) than the wild type and heterozygote (0.09 and 0.09 μg/g, respectively).

Correlations among Investigated Traits. Correlation coefficients were calculated from 15 investigated traits (Table 3). Seed oil content and seed weight are two important factors affecting peanut oil yield (gallon/acre). In this study, they were not highly significantly correlated. They were also not highly significantly correlated with other investigated traits except that both had a moderate positive correlation with oleic acid content (r = 0.40 for oil % and r = 0.42 for seed weight) and a moderate negative correlation with linoleic acid content (r = −0.40 and r = −0.40; p < 0.0001). Seed coat color was not correlated with many traits, but it did have a moderate negative correlation with quercetin and genistein content (r = −0.39 and r = −0.39) and a moderate positive correlation with resveratrol content (r = 0.45; p < 0.0001). In soybean, pigmented seed coats result from the accumulation of proanthocyanidins.[32] In peanut, the relationship between seed coat color and the amounts of flavonoids and resveratrol is not clear. Further study on this issue is needed. Overall, the relative levels of fatty acids were not highly significantly correlated with the levels of flavonoids and resveratrol. The amount of resveratrol was negatively correlated with the amounts of quercetin and genistein (r = −0.38 and r = −0.31; p < 0.0001), but the correlation values were low.

Significant correlations were identified among eight major fatty acids. Palmitic acid (C16:0) was highly significantly correlated with linoleic acid (r = 0.83; p < 0.0001) and negatively correlated with oleic acid and eicosenoic acid (r = −0.85 and r = −0.48; p < 0.0001). Stearic acid (C18:0) was highly significantly correlated with arachidic acid (r = 0.92) and negatively with eicosenoic acid (r = −0.74; p < 0.0001). Oleic acid (C18:1) was correlated with eicosenoic acid (r = 0.52) and, as expected, highly negatively correlated with linoleic acid (r = −0.98; p < 0.0001). Linoleic acid (C18:2) was negatively correlated with eicosenoic acid (r = −0.44; p < 0.0001). Arachidic acid (C20:0) was significantly correlated with behenic acid (r = 0.51) and negatively correlated with eicosenoic acid (r = −0.61) at the p < 0.0001 level. Lignoceric acid (C24:0) was significantly correlated with eicosenoic acid and behenic acid (r = 0.76 and r = 0.41, respectively; p < 0.0001). Similar correlations among fatty acids were also observed in our previous study of different peanut lines.[33] Seed chemical

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Table 3. Pearson Correlation Coefficients and Probability Values for Seed Weight, Fatty Acid Composition, and Oil, Flavonoid, and Resveratrol Content among Accessions within the U.S. Mini-Core Collection[a]

	SdWt	C16:0	C18:0	C18:1	C18:2	C20:0	C20:1	C22:0	C24:0	querc	kaemp	genist	resv	SdCol
oil	0.17	−0.30	−0.11	0.40	−0.40	−0.07	0.16	−0.14	−0.10	−0.17	−0.35	−0.04	0.18	0.04
	<0.0006	<0.0001	0.0332	<0.0001	<0.0001	0.1778	0.0010	0.0062	0.0395	0.0008	<0.0001	0.3743	0.0121	0.5554
SdWt		−0.30	−0.19	0.42	−0.40	−0.13	0.18	−0.18	0.06	−0.03	−0.22	−0.16	0.07	0.04
		<0.0001	0.0001	<0.0001	<0.0001	0.0119	0.0002	0.0003	0.2192	0.4957	<0.0001	0.0017	0.3257	0.6193
C16:0			0.10	−0.85	0.83	−0.00	−0.48	0.16	0.31	−0.08	0.18	0.07	−0.30	0.04
			0.0381	<0.0001	<0.0001	0.9753	<0.0001	0.0017	<0.0001	0.0949	0.0002	0.1490	0.0076	0.5307
C18:0				−0.35	0.18	0.92	−0.74	0.30	−0.39	0.18	−0.18	0.06	−0.19	−0.09
				<0.0001	0.0002	<0.0001	<0.0001	<0.0001	<0.0001	0.0003	0.0003	0.2532	0.0054	0.2034
C18:1					−0.98	−0.28	0.52	−0.36	0.16	−0.09	−0.20	−0.23	0.31	0.13
					<0.0001	<0.0001	<0.0001	<0.0001	0.0015	0.0558	<0.0001	<0.0001	<0.0001	0.0741
C18:2						0.10	−0.44	0.22	−0.15	0.08	0.25	0.25	−0.33	−0.18
						0.0531	<0.0001	<0.0001	0.0024	0.1365	<0.0001	<0.0001	<0.0001	0.0097
C20:0							−0.61	0.51	0.19	0.22	−0.18	0.09	−0.15	−0.05
							<0.0001	<0.0001	<0.0001	<0.0001	0.0004	0.0858	0.0311	0.4489
C20:1								0.02	0.76	−0.04	0.07	−0.09	0.28	0.15
								0.6443	0.0001	0.4539	0.1332	0.0570	<0.0001	0.0283
C22:0									0.41	0.18	0.02	0.10	−0.01	0.15
									<0.0001	0.0002	0.6832	0.0561	0.8720	0.0297
C24:0										0.06	0.04	−0.06	0.19	0.17
										0.2693	0.4029	0.2133	0.0059	0.0140
querc											0.13	0.20	−0.38	−0.39
											0.0086	<0.0001	<0.0001	<0.0001
kaemp												0.32	−0.08	0.08
												<0.0001	0.3063	0.2600
genist													−0.31	−0.39
													<0.0001	<0.0001
resv														0.45
														<0.0001

[a]SdWt, seed weight (g/100seeds); querc, quercetin; kaemp, kaempferol; genist, genistein; resv, resveratrol.

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composition trait correlations are important for breeders in choosing parents for making crosses and selecting progenies to develop new peanut cultivars.

Evaluation of the U.S. Mini-Core Collection. This U.S. peanut mini-core collection has been useful for breeders, geneticists, and other users; but it has some limitations and needs to be expanded. There are six botanical varieties in cultivated peanut, but this mini-core includes only four of these varieties. For maximum coverage of genetic diversity, the two other botanical varieties, aequatoriana and hirsuta, should be represented in the mini-core. The results for all investigated traits from 102 accessions are listed in Table S3 (Supporting Information) and sorted from high to low oil content. Among the 102 accessions, PI 497318 and PI 370331 (collected from Bolivia and Israel, respectively) belonging to var. hypogaea and containing oil above 54% were significantly higher than the remaining 91 accessions. Accession PI 370331 had the highest amount of oleic acid (62.04%), lowest amount of linoleic acid (19.03%), highest O/L ratio (3.28), highest seed weight (88.52 g/100 seeds), and a relatively high amount of quercetin (42.35 μg/g). Although there were no double mutants identified (i.e., functional mutations on both *FAD2A* and *FAD2B*), we recommend these two accessions as parents to make crosses with high oleate lines (i.e., double mutant for *FAD2* genes) for developing high oil content, high oleate peanut cultivars in breeding programs. Furthermore, PI 370331 was previously observed to be tolerant to TSWV, leaf hoppers, and spidermites (http://www.ars-grin.gov/cgi-bin/npgs/acc/obs.pl?1277355). Accession PI 292950 (collected from South Africa) had the highest amount of quercetin (129.29 μg/g, 10 times the average) and contained the homozygous mutant genotype (A/A) with a high O/L ratio (2.29). This accession may be good breeding material to use for increasing quercetin content in peanut seeds. Raw peanut seeds contain a low level of resveratrol, and enhancing its level through traditional breeding programs may be limited. To enhance the level of resveratrol, other alternative approaches, such as development of transgenic peanut or food processing, may need to be considered and exploited.[34]

■ ASSOCIATED CONTENT

Supporting Information

Information on 102 accessions of the U.S. peanut mini-core; variation sources and significance from statistical analysis; and average value for seed weight, seed-coat color, and chemical composition from two year data for each accession with the genotype from functional SNP of *FAD2A*. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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