JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Metabolic Profiling of a Fast Neutron Soybean Mutant Reveals an Increased Abundance of Isoflavones

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Cite This: J. Agric. Food Chem. 2023, 71, 9994−10003 Read Online

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ABSTRACT: A total of 718 metabolites were identified in leaves and seeds of the soybean (*Glycine max* (L.) Merr., Fabaceae) fast neutron (FN) mutant 2012CM7F040p05ar154bMN15, which was previously shown to have 21 genes deleted and higher protein content in seeds as compared to wild-type. Among the identified metabolites, 164 were found only in seeds, 89 only in leaves, and 465 in both leaves and seeds. Metabolites that exhibited higher abundance in the mutant leaf than in the wild type include the flavonoids afromosin, biochanin A, dihydrodaidzein, and apigenin. Mutant leaves also exhibited a higher accumulation of glycitein-glucoside, dihydrokaempferol, and pipecolate. The seed-only metabolites that were found in higher abundance in the mutant compared to the wild type included 3-hydroxybenzoate, 3-aminoisobutyrate, coenzyme A, *N*-acetyl-*β*-alanine, and 1-methylhistidine. Among several amino acids, the cysteine content increased in the mutant leaf and seed when compared to the wild type. We anticipate that the deletion of acetyl-CoA synthase created a negative feedback effect on carbon dynamics, resulting in increased amounts of cysteine and isoflavone-associated metabolites. Metabolic profiling provided new insight into the cascading effect of gene deletions that helps breeders to produce value-added nutritional seed traits.

KEYWORDS: *Glycine max (L.) Merr., nontargeted metabolomics, mass spectrometry, leaf, seed, metabolic signatures, isoflavones, cysteine*

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1. INTRODUCTION

Soybean (*Glycine max* [L.] Merr.) is considered a valuable source of raw materials in food processing[1] and pharmaceutical industries for bioactive phenolic compounds that can be beneficial for human health.[2,3] The bioactive properties of leaves and seeds depend on factors such as genomic composition, as well as the pathological and environmental conditions in which the plants are grown. In addition, isoflavones have many other useful roles. They are essential in nodule formation, affecting nitrogen fixation and protein production by attracting rhizobium bacteria and mycorrhizae. Isoflavones may also have phytoalexin activities and have been shown to enhance the resistance of legumes to pests and pathogens.[4]

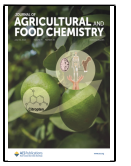
Alteration of the soybean genome has been achieved using several approaches, including transposon tagging,[5] chemical treatment,[6] gene editing,[7,8] and radiation mutagenesis.[9] Radiation mutagenesis, particularly fast neutron (FN) bombardment, tends to create large variations through deletions, duplications, and translocations, although numerous small indels have recently been shown to occur.[10] Taking advantage of this technique, Bolon et al.[9] created thousands of unique soybean FN mutants. Using comparative genomic hybridizations (CGHs) along with next-generation sequencing (NGS), they identified the major variations induced.[11]

Genomic, transcriptomic, and proteomic analyses have been applied to some of the FN soybean mutants to characterize their seed composition.[11−14] While some investigations were focused on root nodulation and other attributes of plant growth and response to the environment,[15,16] fewer studies have been performed on seed quality using metabolic profiling of soybean. Recently, an integrative transcriptomic and metabolomic approach has been directed toward improvement of seed quality in soybean.[17,18]

A global investigation of the changes in the metabolites of the FN mutants is paramount for seed improvement for the pharmaceutical industry, as well as for improvement of animal nutrition. Of particular interest for the pharmaceutical industry are metabolites such as phytoestrogens, which are known to have estrogenic effects and roles in preventing cancer, osteoporosis, and menopausal symptoms.[19] Among the phytoestrogens that are highly abundant in legume grains are lignans (secoisolariciresinol, matairesinol, pinoresinol, and lariciresinol), isoflavones (genistein, daidzein, glycitein, and formononetin), coumestans (coumestrol), and prenylflavonoids.[20,21] Among legumes, soybean seeds are particularly rich in isoflavonoid compounds,[21,22] where they also play a role in plant defense against invading biotic entities.[23,24] The genomic diversity associated with the FN mutants of soybean provides an opportunity to improve our understanding of the global network of metabolites in soybean, with the goal of improvement to the metabolic profiles found in seed and leaf.

In this study, we used a nontargeted metabolic approach to investigate the metabolic profile in leaves and seeds of the FN mutant, "M92-220" (2012CM7F040p05ar154bMN15), which

Received: March 16, 2023
Revised: May 22, 2023
Accepted: May 22, 2023
Published: June 21, 2023



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https://doi.org/10.1021/acs.jafc.3c01493
J. Agric. Food Chem. 2023, 71, 9994−10003

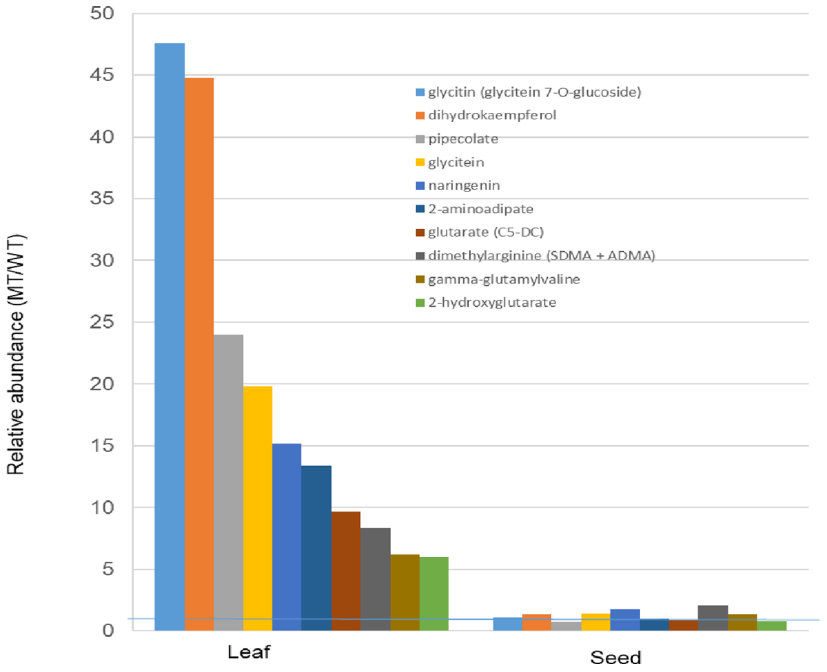

Figure 1. Ten metabolites with increased relative abundance in the mutant soybean leaf as compared to wild-type and the relative abundance of these same metabolites in the seed of the mutant as compared to wild-type. Four biological replicates were analyzed per sample. Student's *t*-test showed that the differences between mutant and wild-type in the leaf were significant. The *y* axis depicts the ratio of the abundance of mutant (MT) to that of wild-type (WT). The horizontal line above 0 indicates 1 on the *Y* axis.

was previously shown to have 21 genes deleted as compared to wild-type and accumulated 48% seed protein.[14] We found a higher abundance of several metabolites associated with human health and plant defense mechanisms in the mutant.

2. MATERIALS AND METHODS

2.1. Mutant Materials and Initial Seed Screening. The soybean line "M92-220" (2012CM7F040p05ar154bMN15), developed from a seed stock of the cultivar "MN1302"[25] by exposure of "M92-220" seeds to fast neutron radiation, was obtained from Robert M. Stupar, Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN, USA, and plants were grown in the field at the University of Missouri/USDA's Bradford Research Center (Columbia). The agronomic practices recommended for soybean cultivation in the midwest region of the United States were implemented for soybean production.[26] Four biological replicates of the leaf tissue and seed were harvested from wild-type and mutant plants. Samples consisting of fully expanded nonsenescent leaves and fully mature but green seeds were rapidly frozen in liquid nitrogen and freeze-dried prior to analysis.

2.2. Metabolic Profiling. Samples were prepared for metabolite extraction and analysis by Metabolon, Inc. (Morrisville, NC) as reported elsewhere[27,28] and at the Metabolon website (https://www.metabolon.com/). Briefly, a subsample of 20 mg of seed powder from each of the four biological replicates of wild and FN mutant was measured, and metabolites were extracted in methanol containing recovery standards using an automated MicroLab STAR system (Hamilton Company, Reno, NV). The supernatants were kept at −20 °C overnight to remove protein and other cell debris. After centrifugation at 10,000*g* for 10 min at 4 °C, the supernatants were collected and divided into five fractions.

All metabolite analyses were performed commercially by Metabolon, Inc. using a Thermo Scientific Q-Exactive high-resolution/accurate mass spectrometer (a hybrid quadrupole and Orbitrap mass analyzer) with heated electrospray ionization (HESI-II) sources (Thermo Fisher Scientific, Waltham, MA). The machine settings for each MS method were as reported elsewhere.[28,29] Instrument variability was corrected by the median relative standard deviation (RSD) for the standards, which were added to each sample prior to injection into the MS. The standard consisted of a cocktail of QC standards that were known to not interfere with any endogenous compound measurement. Additional quality control consisted of several technical replicates for each sample, a pooled matrix of samples, and buffer blank extraction.

Two fractions of each sample were analyzed by reverse-phase ultraperformance liquid chromatography−tandem mass spectrometry (RP/UPLC-MS/MS) with positive ion mode electrospray ionization (ESI). One fraction was analyzed by RP/UPLC-MS/MS with negative ion mode ESI. The fourth fraction was analyzed by hydrophilic interaction ultraperformance liquid chromatography−tandem mass spectrometry (HILIC/UPLC-MS/MS) with negative ion mode ESI. The fifth fraction was stored at −80 ° C for future analyses.

2.3. Data Extraction and Compound Identification. Quality control and informatics analyses were performed by Metabolon as described.[30] Metabolon's informatics system consists of four main components: the Laboratory Information Management System (LIMS), data extraction and peak identification software, data processing tools for QC and compound identification, and a collection of information interpretation and visualization tools.

The Metabolon system of compound identification is based on comparison between experimental data to their proprietary authentic standard library, an in-house generated database of more than 5400 biologically relevant small molecules. Putative identifications of almost all the known/named compounds detected are considered to meet the level 1 criteria for metabolite identification.[31,32]

Metabolon used three criteria for compound identification: retention index within a narrow RI window of the proposed identification, accurate mass match to their proprietary library ±10 ppm, and the MS/MS forward and reverse scores between the experimental data and authentic standards. The MS/MS scores and other associated information are based on a comparison of the ions present in the experimental spectrum to the ions present in the library spectrum.

2.4. Statistical Analyses and Data Visualization. Standard statistical analyses were performed in Array Studio (OmicSoft/Qiagen Bioinformatics) on log-transformed data. For those analyses not

https://doi.org/10.1021/acs.jafc.3c01493
J. Agric. Food Chem. 2023, 71, 9994−10003

standard in Array Studio, data were analyzed using R (http://cran.r-project.org/). Welch's two-sample *t*-test or Student's *t*-test was used to calculate *p*-values. Differences in abundance of metabolites were considered significant based on *p*-values and a ±20-fold change. R-studio was used for data visualization. The procedure used for metabolic mapping of omics data is described in refs 12, 33.

2.5. qRT-PCR Validation. RNA for probe-based quantitative reverse transcription PCR (qRT-PCR) was extracted as per the manufacturer's instruction (RNeasy Plant Mini Kit, cat. no./ID: 7490, Qiagen), and the concentration was measured using a Nanodrop spectrophotometer. A GoTaq 1-Step RT-qPCR system (Promega Corporation) was used for PCR reactions in a Stratagene MX3005p multiplex quantitative real-time PCR system (Agilent Technologies). The primers and probes were designed using Integrated DNA Technology (IDT) online tools (https://www.idtdna.com/pages/tools/primerquest). Two sets of primers and probes were used. The probes and the primers for Glyma.07G242200 were as follows: F-CTCCTCCTCCTTCTGTTCTTTC, P-TGGCATGTCCGAAA-GAGGATTTGGT, and R-AGACTTCTGAGAGGCCATAAAC and F-CACTAAAGCCAGGAGTGGATAAA, P-TGTCCTAAAGTT-CAGCAGGGCCAA, and R-TAGGTAAGGCTCCAAACACAAC. The specificity of the primers was confirmed using BLAST against *Glycine max* at Phytozome (https://phytozome-next.jgi.doe.gov/). The data were exported to Excel, and mRNA abundance was expressed using C_T values. Student's *t*-test ($p < 0.05$) ($Y = -3.7579X + 32.968$, $R^2 = 0.982$) was used to determine statistical significance of differences.

3. RESULTS

Eighteen of the deleted genes are found in one region of chromosome 07, and the remaining 3 genes are found in one region on chromosome 14. Information about these 21 genes is provided in Table 1S. The deleted genes encode enzymes related to energy metabolism (acyl-activating enzyme) and phosphorylation (phosphatidylinositol-4-phosphate 5-kinase family protein), as well as proteins involved in the biotic stress response (major latex protein) and signal transduction (SH3 domain of STAM 3). Genes encoding two transcription factors (myb-containing, B-type plant response regulator and GATA transcription factor 15-like) are also deleted.

A total of 718 metabolites were identified (Tables 2S−4S) in leaves and seeds of the soybean fast neutron mutant 2012CM7F040p05ar154bMN15, which was previously shown to have 21 genes deleted as compared to wild-type.[14] Of the 718 metabolites identified, 164 metabolites were found only in seed (Table 2S), 89 metabolites were found only in leaf (Table 3S), and 465 were found in both leaf and seed (Table 4S).

3.1. Identified Metabolites Found in Both Leaves and Seeds. Ten identified metabolites that exhibited relatively higher abundances in the mutant leaf but only moderately increased, or even decreased, in the seed are shown in Figure 1. These metabolites include flavonoids, fatty acids/lipids, and compounds associated with lysine metabolism. Two flavonoids, glycitin (glycitein 7-*O*-glucoside) and dihydrokaempferol, were found at 47.5-fold and 44.7-fold higher abundance in mutant leaves than in wild-type leaves. Two other metabolites derived from the flavonoid pathway, the aglycone of glycitin (glycitein) and naringenin, were also increased in the mutant leaf. In comparison, the abundance of these 10 metabolites changed very little in the mutant seed as compared to wild-type. Pipecolate, 2-amino adipate, and 2-hydroxyglutarate, metabolites associated with lysine metabolism, increased by greater than 5-fold in the leaves of the mutant; however, these metabolites were found at the same levels or decreased in the mutant seed as compared to wild-type. The dipeptide, γ-glutamyl valine, was also detected at a greater than 5-fold increase in the mutant leaves and increased as well in the seed but to less than 1.5-fold (Figure 1). The amino acid cysteine was detected at higher levels in both leaves (1.22×) and seeds (1.91×) of the mutant as compared to the wild type-soybean (Table 5S).

Ten identified metabolites that decreased in the mutant leaves as compared to wild-type are shown in Figure 2. Most of these metabolites also decreased in the seed, but to a lesser fold, except for 1-stearoyl-GPG (18:0), which was significantly higher in the mutant seeds when compared to the wild-type seed. Other identified metabolites that showed a relative increase in the mutant seed are listed in Table 6S.

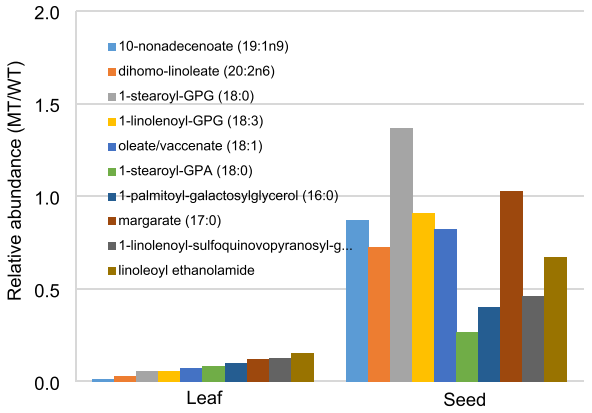

Figure 2. Ten metabolites that exhibited decreased relative abundance in the mutant soybean leaf as compared to wild-type. Most of these same metabolites also decreased in the mutant seed as compared to wild-type. Four biological replicates were analyzed per sample. Student's *t*-test showed that the differences between mutant and wild-type in the leaf and seed were significant. The *y* axis depicts the ratio of the abundance of mutant (MT) to that of wild-type (WT).

3.2. Identified Metabolites that Differed Only in the Leaf. The metabolites identified only in leaves that differed in abundance in the mutant as compared to the wild type are listed in Table 1. Of these, 19 metabolites were found that increased in the mutant leaf. Four of these 19 compounds are flavonoids. Afromosin was found in the mutant leaf at almost 60-fold the amount found in wild-type soybean, while biochanin A, dihydrodaidzein, and apigenin were about 30-, 9-, and 6-fold more abundant in the mutant than in wild-type, respectively. Other metabolites found at higher levels in the mutant leaf are primarily associated with carbohydrate and amino acid metabolism. Two dipeptides, leucylglutamine and alanylleucine, were also observed at higher levels in the mutant than wild-type soybean leaf. Metabolites associated with lipid metabolism were among those found at lower abundance in the mutant leaf. These include 1-dihomo-linoleoylglycerol (20:2), 3,4-dihydroxybutyrate, 1-linolenoyl-2-hexadecatrienoyl-galactosylglycerol, 1-palmitoyl-2-linolenoyl-galactosylglycerol, and dodecanedioate (C12-DC).

3.3. Identified Metabolites that Differed Only in the Seeds. The identified metabolites that were detected only in seed and exhibited a differential abundance between the mutant and wild-type are listed in Table 2. One of these metabolites, 3-hydroxybenzoate, increased almost 20-fold in the mutant seeds as compared to the wild type. Most of these

https://doi.org/10.1021/acs.jafc.3c01493
J. Agric. Food Chem. 2023, 71, 9994−10003

Table 1. Metabolites Identified Only in Leaves that Differed in Relative Abundance in the Mutant (MT) when Compared to the Wild Type (WT)

metabolites	ratio[a] (MT/WT)	*p*-value	metabolite class[b]	subclass or pathway[b]
Higher relative abundance				
afromosin	59.20	0.001	secondary	flavonoid
biochanin A	29.75	0.002	secondary	flavonoid
phosphoenolpyruvate (PEP)	8.79	0.023	carbohydrate	glycolysis
dihydrodaidzein	8.59	0.002	secondary	flavonoid
apigenin 7-*O*-(6-malonyl-*β*-D-glucoside)	5.50	0.013	secondary	flavonoid
*N*6,*N*6-dimethyllysine	3.87	0.004	amino acid	lysine metabolism
dihydroxyacetone phosphate (DHAP)	3.67	0.006	carbohydrate	Calvin cycle and PPP
phosphoethanolamine	3.58	0.014	lipid	phospholipid metabolism
UDP-glucose	3.02	0.008	carbohydrate	sugar and nucleotide
*N*1-methyladenosine	2.41	0.012	nucleotide	purine metabolism
quinolinate	2.38	0.028	pyridine	nicotinate and nicotinamide metabolism
glucuronate	2.35	0.042	carbohydrate	glycolysis
glycolate (hydroxyacetate)	2.26	0.014	carbohydrate	photorespiration
S-adenosylmethionine (SAM)	2.07	0.024	amino acid	aspartate family (OAA derived)
leucylglutamine	1.93	0.048	protein	dipeptide
acetyl-CoA	1.92	0.031	carbohydrate	glycolysis/lipid metabolism
alanylleucine	1.58	0.032	protein	dipeptide
caffeate	1.32	0.023	secondary	phenylpropanoid
glucoheptose	1.27	0.041	carbohydrate	sucrose, glucose, fructose metabolism
Lower relative abundance				
dodecanedioate (C12-DC)	0.78	0.032	lipid	fatty acid
1-palmitoyl-2-linolenoyl-galactosylglycerol	0.76	0.022	lipid	galactolipid
adenosine 2′-monophosphate (2′-AMP)	0.64	0.009	nucleotide	purine metabolism
1-linolenoyl-2-hexadecatrienoyl-galactosylglycerol	0.63	0.010	lipid	galactolipid
erythrulose	0.63	0.041	carbohydrate	sucrose, glucose, fructose metabolism
3,4-dihydroxybutyrate	0.55	0.002	lipid	fatty acid
anthranilate	0.51	0.006	amino acid	Shikimate
phenylglucopyranoside	0.48	0.015	carbohydrate	polysaccharide
glutamine conjugate	0.40	0.000	not available	not available
1-dihomo-linoleoylglycerol (20:2)	0.25	0.035	lipid	glycerolipid
2,4-di-*tert*-butylphenol	0.15	0.044	xenobiotic	alkyl-phenol

[a]Four biological replicates were analyzed, and Student's *t*-test was performed to determine significance of the difference between MT and WT. [b]The metabolite class and subclass or pathway are derived from pathway and subpathway designations provided by Metabolon to reflect plant metabolism.

seed metabolites that increased in the mutant are related to lipid metabolism (glycolipids, fatty acids, lyso-galactolipids, phospholipids, sulfolipids, and the phytosterol stigmastadienone). Other identified metabolites that occurred at relatively higher abundance in the mutant include 3-aminoisobutyrate, a metabolite of the pyrimidine thymine, galactinol, an oligosaccharide associated with plant stress responses, and the modified amino acids 1-methylhistidine and *N*-acetyl-*β*-alanine (Table 2).

Many of the identified metabolites that decreased in abundance in the mutant seed were also related to lipid metabolism. Deoxymugineic acid, which is a *γ*-amino acid derivative that acts as a phytosiderophore,[34] is decreased in the mutant seed, as are 3-hydroxyindolin-2-one and indolelactate, metabolites of chorismate in the shikimic acid pathway.

3.4. Pathway Analyses. Identified metabolites that exhibited significant differences in abundance between the mutant and wild-type leaves and seeds were divided into seven groups according to whether they increased or decreased in the mutant as compared to wild-type: (1) up in leaves, (2) down in leaves, (3) up in the seed, (4) down in the seed, (5) up in leaves and also up in the seed, (6) up in the leaf but down in the seed, and (7) down in leaves but up in seed. Using their KEGG accession numbers, these metabolites were mapped on the global metabolic pathway using color coding to indicate which group the metabolite belonged to (Figure 1S). Using their KEGG accession numbers, the seven deleted genes encoding enzymes were also located on the global pathway map. One enzyme (K01913, acetyl-CoA synthetase) was mapped on a glycolytic pathway where acetate is converted into acetyl-CoA by this enzyme (green line, Figure 1S). The enzyme was also located on another region of the glycolytic pathway where butanoic acid is converted to butanoyl-CoA. Figure 1S illustrates that most of the changes in metabolites were observed in the glycolytic, amino acid, and secondary metabolic pathways.

The amino acids and their derivatives that differed in abundance between mutant leaves and wild-type leaves and mutant seeds and wild-type seeds are shown in Figure 3 as they are derived from the primary carbon metabolism. In this scheme, fructose contributes carbon to the shikimic acid pathway leading to the aromatic amino acids tryptophan, tyrosine, and phenylalanine, which are present at increased levels in both the mutant seed and leaves. *α*-Ketoglutarate, which is elevated in the mutant, contributes carbon to metabolic precursors of arginine, and these are also elevated

https://doi.org/10.1021/acs.jafc.3c01493
J. Agric. Food Chem. 2023, 71, 9994−10003

Table 2. Metabolites Identified Only in Seed that Showed Higher or Lower Relative Abundance in the Mutant (MT) when Compared to the Wild Type (WT)

metabolites	ratio[a] (MT/WT)	*p*-value	metabolite class[b]	subclass or pathway[b]
Higher relative abundance				
3-hydroxybenzoate	18.55	1.45×10^{0}	secondary	phenylpropanoid
3-aminoisobutyrate	3.43	9.39×10^{-1}	nucleotide	pyrimidine metabolism
coenzyme A	3.30	1.21×10^{0}	carbohydrate	glycolysis/lipid metabolism
1-methylhistidine	2.25	1.12×10^{0}	amino acid	glutamate family (α-ketoglutarate)
N-acetyl-β-alanine	2.20	1.22×10^{0}	amino acid	aspartate family (OAA derived)
3′-dephosphocoenzyme A	2.05	1.34×10^{0}	carbohydrate	glycolysis/lipid metabolism
stigmastadienone	2.03	1.10×10^{0}	lipid	sterol
galactinol	1.98	1.03×10^{0}	carbohydrate	sucrose, glucose, fructose metabolism
2-aminoheptanoate	1.76	1.30×10^{0}	lipid	fatty acid
γ-glutamyl-α-lysine	1.75	1.47×10^{0}	amino acid	glutathione metabolism
2′-*O*-methyladenosine	1.70	1.31×10^{0}	nucleotide	purine metabolism
5-methylnorleucine	1.69	1.30×10^{0}	amino acid	branched chain amino acid (OAA derived)
1-stearoyl-2-oleoyl-GPI (18:0/18:1)	1.62	1.63×10^{0}	lipid	phospholipid
1-stearoyl-2-oleoyl-GPE (18:0/18:1)	1.61	1.38×10^{0}	lipid	phospholipid
γ-glutamylleucine	1.59	2.09×10^{0}	amino acid	glutathione metabolism
oleoyl-oleoyl-glycerol (18:1/18:1)	1.54	1.35×10^{0}	lipid	glycerolipid
1-palmitoyl-GPS (16:0)*	1.46	1.69×10^{0}	lipid	lyso-phospholipid
γ-glutamyltryptophan	1.44	1.43×10^{0}	amino acid	glutathione metabolism
1-palmitoyl-2-oleoyl-GPI (16:0/18:1)	1.43	9.10×10^{-1}	lipid	phospholipid
1,2-dioleoyl-GPC (18:1/18:1)	1.36	1.31×10^{0}	lipid	phospholipid
1-oleoyl-2-linolenoyl-digalactosylglycerol	1.33	1.41×10^{0}	lipid	galactolipid
NP-004685	1.32	1.16×10^{0}	xenobiotic	unknown
γ-glutamylphenylalanine	1.31	1.52×10^{0}	amino acid	glutathione metabolism
1-palmitoleoyl-2-linoleoyl-GPC (16:1/18:2)	1.29	1.34×10^{0}	lipid	phospholipid
1-palmitoyl-2-arachidonoyl-GPE (16:0/20:4)	1.29	1.22×10^{0}	lipid	phospholipid
hexadecanedioate (C16-DC)	1.28	1.49×10^{0}	lipid	fatty acid, dicarboxylate
1-oleoyl-GPI (18:1)	1.26	1.20×10^{1}	lipid	lyso-phospholipids
Lower relative abundance				
1-linoleoyl-sulfoquinovopyranosyl-glycerol	0.79	7.79×10^{-1}	lipid	sulfolipid
taurine	0.78	5.75×10^{-1}	amino acid	serine family (phosphoglycerate derived)
1-palmitoyl-2-oleoyl-GPA (16:0/18:1)	0.77	1.77×10^{0}	lipid	phospholipid
adipate (C6-DC)	0.76	3.56×10^{-1}	lipid	fatty acid
deoxymugineic acid	0.75	6.77×10^{-1}	secondary	siderophore
1-palmitoyl-2-linoleoyl-GPA (16:0/18:2)	0.74	4.95×10^{-1}	lipid	phospholipid
2′-deoxyuridine	0.74	9.31×10^{-1}	nucleotide	pyrimidine metabolism
acetylcholine	0.73	1.15×10^{0}	lipid	choline metabolism
3-methylglutarate/2-methylglutarate	0.73	7.01×10^{-1}	lipid	fatty acid
vanillate	0.71	1.15×10^{0}	secondary	phenylpropanoid
hypotaurine	0.70	9.79×10^{-1}	amino acid	serine family (phosphoglycerate derived)
phenyllactate (PLA)	0.69	8.97×10^{-1}	amino acid	aromatic amino acid metabolism (PEP derived)
azetidine-2-carboxylic acid	0.65	7.52×10^{-1}	amino acid	glutamate family (α-ketoglutarate derived)
stearoyl-linolenoyl-glycerol (18:0/18:3)	0.64	7.71×10^{-1}	lipid	glycerolipid
palmitoylcholine	0.64	8.85×10^{-1}	lipid	fatty acid ester
5-hydroxylysine	0.61	8.63×10^{-1}	amino acid	branched chain amino acid
oleoyl ethanolamide	0.59	8.01×10^{-1}	lipid	fatty acid amide
pterin	0.58	7.74×10^{-1}	cofactor	folate metabolism
isovalerate (i5:0)	0.55	6.41×10^{-1}	lipid	free fatty acid
3-hydroxyoctanoate	0.52	1.06×10^{0}	lipid	fatty acid, hydroxy
α-hydroxyisocaproate	0.52	8.46×10^{-1}	amino acid	branched chain amino acid
1-linoleoyl-digalactosylglycerol (18:2)	0.51	1.10×10^{0}	lipid	lyso-galactolipid
linolenoylcholine	0.48	7.63×10^{-1}	lipid	fatty acid ester
3-hydroxyindolin-2-one	0.47	7.21×10^{-1}	secondary	benzoxazinoid
palmitoyl ethanolamide	0.42	8.30×10^{-1}	lipids	fatty acid amide
N-acetyl-2-aminoadipate	0.37	8.16×10^{-1}	amino acid	aspartate family (OAA derived)
linolenoyl-linolenoyl-glycerol (18:3/18:3)	0.36	6.79×10^{-1}	lipids	glycerolipids - diacyl
2′-deoxycytidine	0.35	5.73×10^{-1}	nucleotide	pyrimidine metabolism
indolelactate	0.31	8.78×10^{-1}	amino acid	aromatic amino acid metabolism (PEP derived)
ergothioneine	0.23	8.89×10^{-1}	amino acid	glutamate family (α-ketoglutarate derived)
deoxycarnitine	0.21	9.91×10^{-1}	cofactor	carnitine metabolism

https://doi.org/10.1021/acs.jafc.3c01493
J. Agric. Food Chem. 2023, 71, 9994−10003

Table 2. continued

[a]Four biological replicates were analyzed, and Student's *t*-test was performed to determine significance of the difference between MT and WT. [b]The metabolite class and subclass or pathway are derived from pathway and subpathway designations provided by Metabolon to reflect plant metabolism.

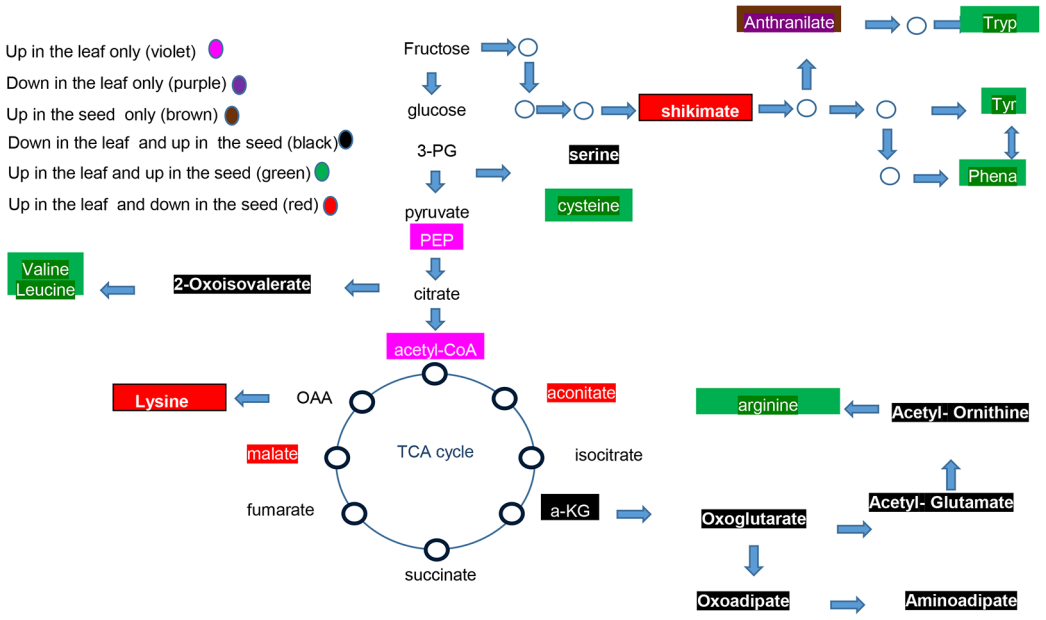

Figure 3. Broad map of the biosynthesis of identified amino acids and their derivatives from primary carbon metabolism, showing the differential abundance of each metabolite in leaves or seeds indicated by the color coding in the legend.

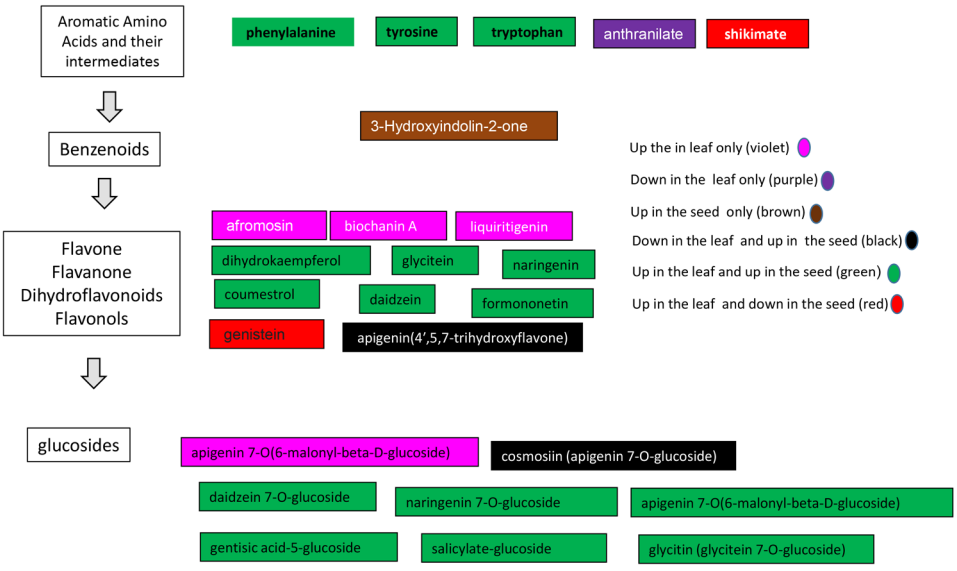

Figure 4. Enrichment of secondary metabolites and their derivatives. The differential abundances of the metabolites are shown by color coding in the legend.

in the mutant seed. Arginine is also elevated in the mutant leaves. Two other amino acids, valine and leucine, increased in abundance in the mutant leaves and seeds, whereas lysine was elevated in the leaves but was found at lower levels in the mutant seed than in wild-type.

The differentially abundant metabolites with KEGG accessions were separately mapped on the KEGG secondary metabolic pathways (Figure 2S). Of the 77 metabolites that were mapped on the global metabolic pathways (Figure 1S), 41 were mapped on the secondary biosynthetic metabolic pathways (Figure 2S). The majority of these metabolites were mapped on the biosynthetic pathways for the aromatic amino acids and to those producing secondary phytochemicals such as the flavanones and the flavonoids, as well as the monolignols that serve as the precursors for lignin.

The higher abundance in the mutant of several metabolites derived from the shikimate biosynthetic pathway led to the enrichment in the mutant of several compounds that are

https://doi.org/10.1021/acs.jafc.3c01493
J. Agric. Food Chem. 2023, 71, 9994−10003

nutraceuticals. Figure 4 classifies these compounds along the pathway and indicates whether the compounds were relatively increased or decreased in mutant versus wild-type. These compounds include afromosin, which increased nearly 60-fold, biochanin A (~30-fold), and liquiritigenin (~9-fold) only in the leaves. Among the compounds that appeared both in leaf and seeds are dihydrokaempferol, which increased nearly 45-fold, coumestrol (~27-fold), glycitein (~20-fold), naringenin (~15-fold), daidzein (~5-fold), formononetin (~4-fold), and genistein (~3-fold). Several glycosylated forms of these compounds were also relatively increased both in the mutant leaf and seed. This includes glycitin (glycitein 7-*O*-glucoside) (~48-fold), daidzin (daidzin 7-*O*-glucoside) (~26-fold), salicylate-glucoside (~6-fold), gentisic acid-5-glucoside (~1.6-fold), and naringenin 7-*O*-glucoside (~1.3-fold). Apigenin was relatively decreased in the mutant leaf, as was cosmosiin (apigenin 7-*O*-glucoside); however, another glycosylated form of apigenin, apigenin 7-*O*-(6-malonyl-*β*-D-glucoside), was relatively increased in the mutant leaves and seed.

Among the 21 deleted genes, two (Glyma.07G242100 and Glyma.07G242200) were annotated by Metabolon as acyl-activating enzyme 7. BLAST identified these genes in the NCBI database as peroxisomal acetate/butyrate-CoA ligase AAE7. Both of the proteins encoded by these genes have the type 1 plant peroxisomal targeting signal, [SA][RK][LMI],[35] at their C-termini. One of the two genes, Glyma.07G242200, corresponds to KEGG accession K01913 and was mapped on the KEGG global pathway map on the glycolytic pathway. A qRT-PCR analysis (Figure 5) of expression of this gene

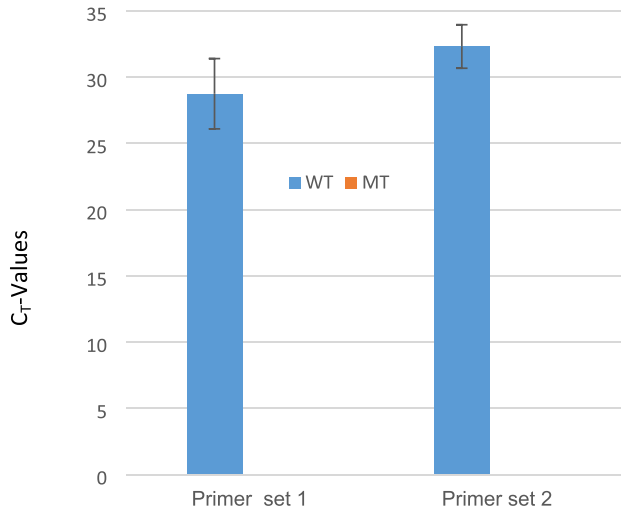

Figure 5. qRT-PCR analysis of expression of Glyma.07G242200 in leaves of the soybean FN mutant "M92-220" (2012CM7F040p05ar154bMN15). mRNA abundance is reflected by C_T values. As expected, mRNA from Glyma.07G242200 was not detected in the mutant. Data are the average ± SD, n = 3.

showed that it is expressed in the wild-type leaf, while no transcript was detected in the mutant, supporting CGHs' evidence that this gene is deleted in the mutant. Glyma.07G242100 was also annotated like Glyma.07G242200. No KEGG accession equivalent to Glyma.07G242100 was found. Alignment of the proteins encoded by these two genes shows that they arose by gene duplication with much of the sequence being conserved with the Arabidopsis ortholog (Figure 3S).

4. DISCUSSION

FN mutagenesis resulted in a widespread effect on the overall metabolome of the leaves and seeds of the mutant. The observed changes are most likely the result of two large deletions resulting in the loss of 21 annotated genes, although the extent of smaller indels in the mutant is not known at present. The deleted genes encode a wide variety of proteins, some functioning as metabolic enzymes but others, such as the transcription factors and signaling components, might be regulating expression of many other metabolic pathways. With our current knowledge, we have begun to analyze the resulting metabolome based on the relative abundances between the mutant and wild type of metabolically related compounds. Several of these compounds have been shown to have positive pharmaceutical effects or to act as plant protectants in response to biotic or abiotic stress.

Afromosin, a polyphenolic compound, is widely found in eukaryotes. It is a metabolite of phenylalanine, which is converted in the flavonoid pathway to the flavanones, liquiritigenin and naringenin. Flavanones are aromatic ketones that often occur in plants as glycosides. The flavanone is converted to the hydroxyisoflavanone and then dehydroxylated to the isoflavanone to eventually produce afromosin.[36]

Afromosin was first extracted from *Wisteria brachybotrys* (Leguminosae) and chemically characterized as 6,4′-dimethoxy-7-hydroxyisoflavone (Konoshima et al.[40]). An inhibitory effect of afromosin on the Epstein−Barr virus was reported.[37] Recently, afromosin was also isolated from *Dalbergia cultrata* (Leguminosae), and a strong antifungal activity was found when the compound was tested against *Pythium insidiosum* using a disc diffusion assay.[38] In addition to antiviral and antifungal activities, afromosin was shown to be effective against inflammation and have antitumor activities in a mammalian system.[39−41]

Biochanin A was identified only in the soybean leaf, and like afromosin, it was relatively much more abundant in the mutant than in wild-type soybean. Biochanin A is also derived from phenylalanine and is a precursor of afromosin.[36] While afromosin was reported to be effective against certain viruses and fungi, biochanin A was found to be effective against some bacteria, such as *Xanthomonas axonopodis*, which causes bacterial pustule disease in soybean.[42] Biochanin A has also been implicated in the antifertility syndrome discovered in Australian ewes in 1946. It can be metabolized into genistein in the ewes gut where it acts as an estrogenic endocrine disruptor.[43,44] A direct involvement of biochanin A against oxidative damage is reported elsewhere.[45]

The other two metabolites that showed a significant relative increase in abundance in the mutant seed as compared to wild-type are dihydrodaidzein and apigenin. Dihydrodaidzein is an intermediate product of the daidzein degradation pathway. Like most other isoflavones, daidzein is produced from the shikimate pathway and is primarily used by plants as signal carriers and for defense responses to pathogenic attacks.[46] A major portion of the isoflavones in soybeans consists of daidzein (37%), genistein (57%), and glycitein (6%). Soybean embryos contain more daidzein (48%) than other tissues.[47] Like daidzein, the flavonoid apigenin was shown to increase resistance to brown planthopper.[48]

Naringenin, glycitein, and glycitein 7-*O*-glucoside, which are also relatively more abundant in the mutant, are other isoflavones derived from phenylalanine metabolism.[49] An

https://doi.org/10.1021/acs.jafc.3c01493
J. Agric. Food Chem. 2023, 71, 9994−10003

intermediate product of phenylalanine metabolism is naringenin chalcone, which contributes to the synthesis of either naringenin, genistein, or isoliquiritigenin, which act as precursors for daidzein and glycitein.[49]

Similar to gentisic acid-5-glucoside, *N*-acetylhistidine and homocitrulline are also associated with health benefits in the mammalian systems. Among these metabolites, gentisic acid-5-glucoside belongs to the class of organic compounds known as hydroxybenzoic acid derivatives of gentisic acid. Gentisic acid and its derivatives are known to have a broad spectrum of biological activity, such as anti-inflammatory, antirheumatic, and antioxidant properties.[50]

Among the fatty acid-associated metabolites identified both in leaves and seeds, 1-stearoyl-GPG (18:0) was significantly higher in the mutant seeds when compared to wild-type. Recent studies have shown that this metabolite is effective against infection by the malaria parasite *Plasmodium falciparum*.[51,52]

Importantly, the level of cysteine, a sulfur-containing amino acid, was significantly increased in the mutant seed when compared to the wild type. Any increase in the amount of cysteine is considered noteworthy, as soybean is limited in sulfur-containing amino acids. An increase in sulfur-containing amino acids in soybean has been an area of interest for a while.[53−56] Several strategies involving biotechnology have been adopted to improve the sulfur content in soybean seeds. However, these strategies have encountered the challenges resulting from genome duplication in soybean. Attempts to increase sulfur-containing amino acids using genetics or biotechnology had limited success because of proteome rebalancing imparted by the genome duplication.[33,57,58] Despite the increased cysteine content, we did not detect significant changes in the methionine content in the mutant seeds. Although the sulfur in methionine is derived from cysteine, carbon in methionine is derived from the aspartate biosynthetic pathway, and metabolites in this pathway are not elevated in the mutant.

While analyzing the impact of all deleted genes on the metabolic profiling in the mutant is outside the scope of this investigation, we mapped the seven deleted genes that have corresponding KEGG accessions on the metabolic pathway to better understand the differential abundance of the metabolites between the wild type and the mutant due to gene deletion. Although two genes (Glyma.07G242100 and Glyma.07G242200) are annotated to encode acyl-activating enzymes, we could map only one of them, Glyma07G242200. The expression analysis of this gene supports the deletion of Glyma07G242200 in the mutant. It may be that the deletion of this gene affects carbon distribution from the TCA cycle to a wide range of secondary pathways leading to amino acids (Figure 4). The phenomenon of a feedback effect was reported after altering the genomes of other species.[59−61] While the genes encoding the acyl-activating enzyme are well-documented in diverting the carbon distribution to secondary metabolites, the cumulative effect of other deleted genes is likely to have contributed to the observed metabolite profile. Whatever the mechanism, it is apparent that there is a cascading effect of the deleted genes contributing to the increased abundance of metabolites associated not only with mammalian health and plant defense but also with some value-added nutrition traits in the seeds.

Metabolic profiling in leaves and seed in soybean is paramount to understanding the processes of their accumulation from leaves to the seed. Using an FN mutant, we identified a total of 527 metabolites in leaf and 595 metabolites in soybean seed. Metabolic mapping of the identified metabolites demonstrated an increased amount of metabolites associated with both beneficial or adverse effects on mammalian health and nutrition value of the soybean seeds. The results from our study will assist scientists and breeders in developing new value-added soybeans with improved protein quality traits.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.3c01493.

(Figure 1S) Metabolites that changed in abundance, mapped over global metabolic pathways from KEGG (https://www.kegg.jp/pathway/map); (Figure 2S) metabolites mapped over secondary metabolic pathways; (Table 1S) deleted genes, their annotation, some comments, and reported work; (Table 2S) total number of metabolites identified in seed only of wild type (WT) and mutant (MT); (Table 3S) total number of metabolites identified in leaf only of wild type (WT) and mutant (MT); (Table 4S) total number of metabolites identified in both leaf and seed of wild type (WT) and mutant (MT); (Table 5S) total number of metabolites that exhibited significant higher abundance in the leaf (L) and lower abundance in the mutant (MT) seeds (S) when compared to the wild type (WT); (Table 6S) total number of metabolites that exhibited significant lower abundance in the leaf (L) and higher abundance in the mutant (MT) seeds (S) when compared to the wild type (WT) (PDF)

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Funding

Funding for this research was provided by Agricultural Research Service, USDA.

Notes

The authors declare no competing financial interest.

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■ ACKNOWLEDGMENTS

The authors thank Dr. Robert Stupar at the University of Minnesota for providing FN soybean mutants. The authors also thank Metabolon, Inc. (Morrisville, NC) for their technical support to analyze the soybean metabolites.

■ ABBREVIATIONS

FN, fast neutron; MS, mass spectrometry; UPLC, ultra-performance liquid chromatography; qRT-PCR, quantitative real-time polymerase chain reaction; CGHs, comparative genomic hybridizations; NGS, next-generation sequencing; KEGG, Kyoto Encyclopedia of Genes and Genomes

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https://doi.org/10.1021/acs.jafc.3c01493
J. Agric. Food Chem. 2023, 71, 9994−10003