

Comparison of the seed nutritional composition between conventional varieties and transgenic soybean overexpressing *Physaria FAD3-1*

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Abstract

BACKGROUND: *PffAD3* transgenic soybean expressing omega-3 fatty acid desaturase 3 of *Physaria* produces increased level of α -linolenic acid in seed. Composition data of non-transgenic conventional varieties is important in the safety assessment of the genetically-modified (GM) crops in the context of the natural variation.

RESULTS: The natural variation was characterized in seed composition of 13 Korean soybean varieties grown in three locations in South Korea for 2 years. Univariate analysis of combined data showed significant differences by variety and cultivation environment for proximates, minerals, anti-nutrients, and fatty acids. Percent variability analysis demonstrated that genotype, environment and the interaction of environment with genotype contributed to soybean seed compositions. Principal component analysis and orthogonal projections to latent structure discriminant analysis indicated that significant variance in compositions was attributable to location and cultivation year. The composition of three *PffAD3* soybean lines for proximates, minerals, anti-nutrients, and fatty acids was compared to a non-transgenic commercial comparator (Kwangankong, KA), and three non-transgenic commercial varieties grown at two sites in South Korea. Only linoleic and linolenic acids significantly differed in *PffAD3-1* lines compared to KA, which were expected changes by the introduction of the *PffAD3-1* trait in KA.

CONCLUSION: Genotype, environment, and the interaction of environment with genotype contributed to compositional variability in soybean. *PffAD3-1* soybean is equivalent to the conventional varieties with respect to these components.

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Supporting information may be found in the online version of this article.

Keywords: natural variation; omega-3; nutrition; soybean; environment; genotype; GMO

INTRODUCTION

Soybean (*Glycine max* (L.) Merr.) is an economically important crop worldwide as a source of vegetable oil and protein for human and animal consumption. Soybean is the world's largest genetically-modified (GM) crop due to agronomic, nutritional, and industrial interest and its amenability to genetic transformation, comprising 50% of global biotech crop production.^{1,2} GM soybeans with superior nutritional traits, including modifications in protein quality and quantity, essential amino acids, oils and fatty acids, functional secondary metabolites, and mineral content, have been meticulously developed with great effort.³

Molecular breeding has been utilized to produce soybean seeds containing high levels of oleic acid and low levels of linoleic acid to increase oxidative stability to address the needs of both food companies and consumers.^{4,5} GM soybeans have also been

developed to improve their functional lipid content, such as omega-3 very long chain polyunsaturated fatty acids (stearidonic acid and eicosapentaenoic acid) and omega-6 fatty acids

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(γ -linolenic acid), which are essential for human health.^{6–9} Currently, 12 transgenic soybeans with modified oils and fatty acids are listed in the GM Approval Database (isaaa.org/gmapprovaldatabase/default.asp). However, only two events, DP305423 and MON87705 developed by Dupont (Pioneer Hi-Bred International Inc., Johnston, IA, USA) and Monsanto company, respectively are available. α -Linolenic acid (ALA, omega-3) is an essential fatty acid and is converted to omega-3 eicosapentaenoic acid and omega-3 docosahexaenoic acid in the human body.^{10–12} Soybean seeds contain relatively low levels of ALA, ~8% of total fatty acids. It has been reported that three omega-3 fatty-acid desaturase 3 (*FAD3*) genes are expressed in soybean seeds to control ALA content.^{13,14} Recently, transgenic soybean lines overexpressing the *PffAD3-1* gene from *Physaria fendleri* using the β -conglycinin promoter were successfully developed.¹⁵ The content of ALA was increased seven-fold in the T1 seeds of *PffAD3-1* soybeans compared to that of the parental plant, resulting in a 52.6% increase in total fatty acids.

For commercialization, compositional studies are required for the food safety assessment of genetically modified crops. Comparative compositional analyses based on substantial equivalence have been conducted by comparing the analyte levels of GM crops and their non-GM conventional counterparts to detect any differences caused by intended and unintended effects.¹⁶ Reference data of non-transgenic conventional commercial varieties (reference varieties) are important to evaluate whether any observed differences between the GM crops and their direct comparators are within the natural range of variation. It is recommended to include reference varieties in the same field trials of GM crops in order to obtain reliable reference values.^{17,18} Reference data are also available from the consensus documents by the Organization for Economic Co-operation and Development (OECD) for the assessment of new crops,¹⁹ Agriculture and Food Systems Institute Crop Composition Database (www.cropcomposition.org), and peer-reviewed scientific literature. Recently, the National Institute of Agricultural Sciences (South Korea) has developed a crop composition database that provides analytical data on conventional commercialized crops such as rice, soybean, and red pepper.²⁰ The latter database can be used to obtain reference data for comparative compositional assessment of GM crops.

In this study, we aimed to conduct a comparative compositional analysis of *PffAD3-1* transgenic soybean lines with their conventional counterpart and three non-transgenic commercial soybean reference varieties. In addition, natural variations in compositional components in 13 commercial soybean varieties that are widely used for food in South Korea were studied based on genotype, cultivation location, cultivation year, and the interactions between genotype and environment. The compositional data generated from 13 soybean varieties were used as references for the biological relevance evaluation of *PffAD3-1* soybeans. Compositional analyses included measurements of eight proximates, nine minerals, four anti-nutrients, and thirteen fatty acids. In this article, we first present natural variations in the components of the 13 reference varieties and then present the comparative compositional analysis of the *PffAD3-1* transgenic soybeans.

MATERIALS AND METHODS

Soybean materials and growing conditions

Thirteen non-transgenic commercial varieties were grown in Suwon (37°27'50.02" N, 126°98'49.59" E), Iksan (35°94'40.02" N,

126°99'36.60" E), and Dalseong (35°90'66.92" N, 128°44'76.59" E) of South Korea during the 2017 and 2018 growing seasons. Two field experiments were conducted in 2019 for the compositional analyses of *PffAD3-1* soybeans (*PffAD3-1*, Accession No. Mf611845). The field sites were in Jeonju (35°83'08.57" N, 127°06'62.29" E) and Gunwi (36°11'24.08" N, 128°64'16.66" E), located in the western and eastern regions of South Korea, respectively. Three soybean *PffAD3-1* transgenic lines (T10-1, T11-8, and T12-1; *Glycine max* L. 'Kwangankong'; T₂ seeds), the conventional comparator Kwangankong (parental line, KA), and three non-transgenic commercial varieties (DP-2, PSN, and PW) were cultivated simultaneously in the same field. Plots at each site were arranged in a balanced strip design. Each plot consisted of two 10 m-long rows with 20-cm seed spacing. Rows were approximately 0.6 m apart and plots were separated by at least 0.8 m. Seeds were collected from individual plants during the R8 (full maturity) growth stage, and then pooled and stored at room temperature. Weather conditions, including rainfall, at the cultivation sites are presented in Tables S1–S3 of the Supporting Information. Information of these commercial varieties is shown in Table S4.

Compositional analyses

Proximates

All components of proximates were analyzed according to standard methods of the Korea Ministry of Food and Drug Safety (MFDS). Moisture content was measured by gravimetric analysis using a hot-air oven at 105 °C.²¹ Crude fat was analyzed using the Soxhlet extraction method,²² while crude protein content was calculated from total nitrogen using the Kjeldahl method.²³ Ash content was determined by incinerating the sample in a furnace at 600 °C for 22 h to constant weight.²⁴ Carbohydrate content was calculated as 100% – (% protein + % lipid + % ash + % moisture). Crude fiber content was determined according to the Association of Official Analytical Chemists method 962.09.²⁵ Acid detergent fiber (ADF) and neutral detergent fiber (NDF) contents were determined by enzymatic-gravimetric methods using amylase, protease, and amyloglucosidase according to the MFDS food code.²⁶

Minerals

Calcium, magnesium, phosphorus, potassium, sulfate, copper, iron, manganese, sodium, and zinc were determined using inductively coupled plasma optical emission spectrometry (Inegra XL; GBC Co., Melbourne, Australia) according to the MFDS food code.²⁷

Anti-nutrients

Phytic acid content was determined based on the method of Park *et al.*²⁸ using pre-filled Poly-Prep® chromatographic columns (Bio-Rad Laboratories, Richmond, CA, USA) containing AG-1-X8 anion exchange resin (100–200 mesh chloride form, 0.8 cm × 4 cm) to allow the isolation of phytate from the soybean seed extracts. Trypsin inhibitor activity (TIU) in the soybean seeds was determined using American Oil Chemists' Society (AOCS) official method Ba 12-75.²⁹ Each grain powder (0.5 g) was suspended in dilute sodium hydroxide and subjected to a series of dilutions. The final diluted suspension was incubated at 37 °C with trypsin and the synthetic substrate, benzoyl-DL-arginine-*p*-nitroanilide (BAPNA). The action of trypsin was stopped by the addition of acetic acid after 10 min. The mixture was filtered through a syringe membrane filter (0.22 μ m), and the absorbance of the filtrate was measured at 410 nm. One unit of trypsin inhibitor was defined as the amount of inhibitor that reduced the optical

density reading of trypsin-digested BAPNA by 0.01. The raffinose and stachyose assays were based on two methods.^{30,31} The samples were extracted in 50% ethanol with shaking at 200 rpm for 15 min using a Thermomixer composit (Eppendorf AG, Hamburg, Germany), and then sonicated at 80 °C in a water bath for 25 min. The extracts were centrifuged at 10 000 × *g* for 10 min and the

supernatants were filtered through syringe membrane filters (0.22 μm). The samples were injected into an Agilent-1100 high-performance liquid chromatography instrument (Agilent Technologies, Santa Clara, CA, USA) equipped with a refractive index detector and Shodex SUGAR SC1011 column (8.0 mm × 300 mm, inner diameter 6 μm).

Table 1. Proximate composition of grain from 13 soybean varieties across three locations for 2 years by variety and across 13 varieties by location and year

| Component (%) | Moisture | Ash | Protein | Fat | Carbohydrate | Fiber | Neutral detergent fiber | Acid detergent fiber |
|------------------|-----------------------------------|------------------------------------|--------------------------------------|---------------------------------------|--|-------------------------------------|-------------------------------------|------------------------------------|
| <i>Varieties</i> | | | | | | | | |
| CHO | 8.66 ^a (5.89–13.6) | 6.06 ^{abc} (5.51–7.28) | 39.93 ^{ab} (37.76–42.14) | 14.67 ^{ab} (11.39–19.14) | 30.67 ^{cd} (23.75–35.53) | 8.00 ^{ab} (5.50–11.08) | 16.22 ^a (7.61–24.53) | 10.16 ^a (4.64–14.2) |
| CJ-3 | 8.70 ^a (5.78–13.38) | 5.96 ^{abc} (5.49–6.64) | 38.28 ^{bc} (36.60–40.79) | 13.64 ^c (10.03–19.16) | 33.42 ^{abc} (24.0–37.81) | 9.26 ^{ab} (5.37–12.35) | 14.97 ^a (7.45–22.67) | 8.96 ^a (4.16–13.15) |
| DC | 8.45 ^a (5.56–13.64) | 5.81 ^{bc} (5.24–6.28) | 35.57 ^{de} (32.69–38.92) | 16.50 ^{ab} (12.49–22.05) | 33.67 ^{abc} (36.52–46.91) | 10.09 ^{ab} (5.01–16.09) | 15.81 ^a (8.10–22.18) | 10.07 ^a (4.78–13.90) |
| DP-2 | 8.21 ^a (5.61–13.14) | 5.61 ^c (4.93–6.18) | 37.09 ^{cd} (34.69–39.55) | 16.9 ^a (10.75–26.1) | 32.19 ^{bcd} (26.4–35.7) | 10.13 ^{ab} (5.01–15.43) | 16.65 ^a (7.89–26.27) | 10.32 ^a (4.88–13.57) |
| DW | 8.31 ^a (5.69–12.73) | 5.74 ^{bc} (5.06–6.79) | 36.07 ^{cd} (33.92–38.75) | 16.91 ^a (11.74–22.61) | 33.0 ^{abcd} (24.08–37.21) | 7.80 ^{ab} (5.26–12.8) | 14.38 ^a (8.30–22.62) | 8.70 ^a (4.54–12.96) |
| MS | 8.49 ^a (5.67–12.54) | 5.88 ^{bc} (5.35–7.11) | 38.16 ^{bc} (36.15–39.97) | 13.75 ^{abc} (9.62–18.93) | 33.73 ^{abc} (25.66–38.83) | 9.71 ^{ab} (5.89–12.81) | 16.66 ^a (8.14–21.75) | 10.03 ^a (5.27–13.17) |
| PSN | 8.54 ^a (5.29–15.01) | 5.79 ^{bc} (4.77–6.72) | 37.12 ^{cd} (33.56–38.84) | 14.43 ^{abc} (10.57–17.65) | 34.13 ^{ab} (24.81–41.15) | 10.94 ^a (6.72–16.0) | 17.44 ^a (10.47–28.34) | 10.77 ^a (5.95–17.07) |
| PW | 8.07 ^a (5.68–13.16) | 5.87 ^{bc} (5.33–7.59) | 37.27 ^{cd} (34.4–39.16) | 15.49 ^{ab} (11.8–21.75) | 33.31 ^{abcd} (22.93–38.33) | 10.11 ^{ab} (5.42–15.71) | 13.78 ^a (8.57–20.27) | 8.67 ^a (5.24–14.34) |
| SCJ | 8.87 ^a (5.96–14.84) | 6.43 ^a (5.67–7.01) | 38.53 ^{bc} (36.6–41.05) | 11.41 ^c (6.68–15.41) | 34.76 ^a (24.73–41.59) | 10.94 ^a (7.40–15.64) | 16.94 ^a (12.56–22.36) | 10.77 ^a (6.73–14.73) |
| SO | 8.12 ^a (4.88–14.84) | 6.19 ^{ab} (5.64–6.80) | 40.94 ^a (37.89–43.79) | 14.20 ^{abc} (10.95–22.57) | 30.54 ^d (21.71–35.69) | 7.47 ^b (5.69–9.96) | 13.36 ^a (8.51–16.80) | 7.65 ^a (5.69–9.96) |
| SP | 8.17 ^a (5.16–13.27) | 5.63 ^c (4.89–6.74) | 36.16 ^{de} (34.42–37.88) | 14.75 ^{ab} (12.35–20.49) | 35.29 ^{ab} (25.089–39.43) | 9.03 ^{ab} (5.55–12.41) | 13.54 ^a (7.82–17.34) | 8.04 ^a (4.78–9.85) |
| TG | 8.21 ^a (6.14–13.51) | 5.69 ^c (5.16–6.67) | 35.01 ^e (33.22–36.60) | 16.33 ^{ab} (11.78–19.64) | 34.77 ^{ab} (27.52–41.43) | 9.33 ^{ab} (6.52–12.78) | 16.60 ^a (9.98–32.71) | 10.15 ^a (6.65–18.74) |
| WR | 8.21 ^a (5.71–13.61) | 5.92 ^{bc} (5.45–6.51) | 35.56 ^{de} (32.55–39.29) | 15.92 ^{ab} (9.28–22.70) | 34.39 ^{ab} (24.83–42.9) | 8.51 ^{ab} (4.13–12.0) | 14.89 ^a (7.23–24.4) | 9.25 ^a (4.37–15.02) |
| <i>P-Value</i> | NS | *** | *** | *** | *** | *** | - | * |
| <i>Location</i> | | | | | | | | |
| Suwon | 6.75 (5.29–8.42) | 6.12 (5.33–7.29) | 36.82 (32.55–43.12) | 15.72 (6.68–26.1) | 34.59 (25.7–41.59) | 16.11 (9.96–26.27) | 9.83 (5.89–14.73) | 9.95 (5.26–16.09) |
| Iksan | 11.72 (7.91–15.01) | 5.81 (4.98–6.64) | 36.49 (33.08–40.29) | 15.84 (10.75–22.61) | 30.14 (21.71–38.33) | 7.46 (4.13–15.71) | 12.4 (7.23–24.53) | 7.52 (4.16–14.11) |
| Dalseong | 6.68 (4.88–8.02) | 5.74 (4.77–7.59) | 38.78 (33.56–43.79) | 13.42 (9.28–19.37) | 35.38 (28.75–42.90) | 10.59 (5.42–16.0) | 17.93 (10.62–32.71) | 11.16 (6.39–18.74) |
| <i>P-Value</i> | *** | * | NS | *** | *** | *** | *** | NS |
| <i>Year</i> | | | | | | | | |
| 2017 | 9.45 (6.19–15.01) | 5.81 (5.06–6.77) | 37.19 (32.55–43.79) | 15.86 (9.40–26.10) | 31.68 (21.71–41.43) | 9.39 (4.13–15.12) | 13.61 (7.23–23.06) | 8.67 (4.16–15.03) |
| 2018 | 7.32 (4.88–11.85) | 5.97 (4.77–7.59) | 37.53 (33.08–43.12) | 14.12 (6.68–19.64) | 35.06 (29.57–42.90) | 9.28 (5.26–16.09) | 17.34 (9.98–32.71) | 10.34 (6.39–18.74) |
| <i>P-Value</i> | *** | *** | *** | *** | NS | *** | *** | *** |

Data are converted from fresh weight to dry weight basis using given moisture level. Data are the mean and range (parenthesis), expressed as percent dry weight except moisture. NS not significant; **P* < 0.05; ***P* < 0.01; ****P* < 0.001. The means in the same column followed by the same letter(s) are not significantly different at *P* < 0.05 by least significant difference.

Fatty acids

Fatty acids were extracted from 50 mg of grain powder with a chloroform–methanol (v/v 2:1) solution containing an internal standard (pentadecanoic acid solution) and then saponified with toluene, 5 N sodium hydroxide, and methanol. The saponification mixture was methylated with 14% boron trifluoride. The resulting methyl esters were resolved in hexane and analyzed using a

7890B gas chromatograph (Agilent Technologies) coupled to a flame ionization detector and a 100 m × 0.25 mm (inner diameter) HP-FFAP column (Agilent Technologies).³²

Statistical analyses

Statistical treatment of the data was carried out with SAS Enterprise Guide 7.0. One-way analysis of variance (ANOVA) was

Table 2. Minerals composition of grain from 13 soybean varieties across three locations for 2 years by variety and across 13 varieties by location and year

| Component | Calcium | Copper | Iron | Magnesium | Manganese | Phosphorus | Potassium | Sodium | Zinc |
|------------------|------------------------------------|--------------------------------------|------------------------------------|-------------------------------------|---------------------------------------|--------------------------------------|-------------------------------------|------------------------------------|---------------------------------------|
| <i>Varieties</i> | | | | | | | | | |
| CHO | 1.41 ^g (1.11–1.90) | 10.87 ^{abc} (4.91–15.64) | 114.2 ^a (723–174.9) | 2.32 ^{ef} (1.83–2.92) | 20.72 ^{bc} (16.95–25.36) | 7.27 ^{ab} (5.90–8.43) | 17.79 ^a (13.83–23.91) | 74.81 ^a (0–264.8) | 38.22 ^{ab} (27.62–47.98) |
| CJ-3 | 2.45 ^{ab} (2.09–2.88) | 11.33 ^{abc} (7.34–15.86) | 122.7 ^a (76.4–183.8) | 3.03 ^a (2.78–3.39) | 27.06 ^a (20.38–34.67) | 7.00 ^{abcde} (6.55–7.56) | 16.63 ^a (13.0–20.93) | 63.62 ^a (0–148.6) | 37.0 ^{abc} (27.95–48.33) |
| DC | 2.24 ^{bc} (1.96–2.63) | 6.32 ^{abc} (5.63–6.72) | 119.5 ^a (78.6–175.2) | 2.63 ^{bcd} (2.41–3.08) | 20.75 ^{bc} (13.79–28.08) | 6.63 ^{bcd} (5.42–7.77) | 16.72 ^a (12.87–21.70) | 64.40 ^a (0–170.3) | 32.66 ^{bcd} (19.52–48.57) |
| DP-2 | 1.79 ^{def} (1.34–2.87) | 11.54 ^{abc} (7.05–16.97) | 116.0 ^a (70.4–171.4) | 2.28 ^f (2.05–2.67) | 20.0 ^{bc} (14.69–26.27) | 6.35 ^{cd} (5.23–7.03) | 16.05 ^a (12.33–19.55) | 100.9 ^a (0–521.0) | 30.01 ^{cd} (22.39–40.74) |
| DW | 2.20 ^{bc} (1.70–2.51) | 10.51 ^{abc} (1.69–17.98) | 108.0 ^a (71.9–151.4) | 2.56 ^{bcd} (2.10–2.80) | 21.08 ^{bc} (14.29–30.71) | 6.83 ^{abcde} (4.95–8.03) | 17.32 ^a (11.95–22.42) | 67.51 ^a (0–162.3) | 31.42 ^{bcd} (22.2–45.68) |
| MS | 1.45 ^{fg} (1.20–1.91) | 10.81 ^{abc} (6.06–15.12) | 106.0 ^a (62.6–157.4) | 2.36 ^{ef} (1.98–2.86) | 19.69 ^{bc} (16.39–23.37) | 7.12 ^{abc} (5.58–8.61) | 17.33 ^a (12.89–24.93) | 67.26 ^a (0.31–266.0) | 33.22 ^{bcd} (25.87–39.75) |
| PSN | 2.0 ^{cde} (1.87–2.17) | 8.62 ^c (1.0–12.68) | 119.2 ^a (62.0–336.5) | 2.41 ^{cdef} (2.20–2.63) | 18.08 ^c (13.95–26.64) | 7.22 ^{ab} (6.32–8.08) | 16.63 ^a (13.19–22.13) | 68.26 ^a (0–180.7) | 28.39 ^d (23.50–37.59) |
| PW | 2.61 ^a (1.99–3.69) | 11.38 ^{abc} (2.95–16.18) | 107.4 ^a (74.0–135.8) | 2.65 ^{bc} (2.26–3.03) | 23.58 ^{ab} (18.11–31.74) | 6.52 ^{bcd} (5.68–7.43) | 1636 ^a (14.07–21.54) | 57.68 ^a (0–170.9) | 31.81 ^{bcd} (20.48–42.07) |
| SCJ | 1.90 ^{cde} (1.61–2.16) | 14.33 ^{ab} (10.91–21.27) | 130.0 ^a (84.7–177.6) | 2.73 ^b (2.54–2.94) | 21.75 ^{bc} (15.18–27.24) | 7.49 ^a (6.52–8.64) | 18.12 ^a (14.60–22.0) | 75.32 ^a (0–187.8) | 36.35 ^{abc} (28.3–56.68) |
| SO | 1.70 ^{efg} (1.23–2.94) | 14.66 ^a (1.09–19.02) | 128.0 ^a (77.7–186.0) | 2.38 ^{def} (2.10–3.03) | 21.18 ^{bc} (14.80–30.66) | 7.47 ^a (6.46–8.37) | 18.04 ^a (15.65–24.16) | 66.36 ^a (0–197.9) | 43.18 ^a (32.89–54.82) |
| SP | 2.11 ^{bcd} (1.72–2.62) | 9.05 ^c (0.66–14.9) | 121.1 ^a (64.1–215.9) | 2.44 ^{cdef} (1.94–2.74) | 22.03 ^{abc} (14.97–28.17) | 6.26 ^e (4.74–7.43) | 17.13 ^a (11.92–23.19) | 79.92 ^a (0–256.3) | 30.52 ^{cd} (24.29–38.57) |
| TG | 2.24 ^{bc} (1.68–2.67) | 10.90 ^{abc} (3.17–16.95) | 109.4 ^a (72.3–153.1) | 2.71 ^b (2.13–3.17) | 22.21 ^{abc} (15.74–31.75) | 7.07 ^{abcd} (5.48–8.36) | 16.61 ^a (12.47–21.09) | 67.47 ^a (0–183.8) | 34.53 ^{bcd} (21.92–48.56) |
| WR | 1.94 ^{cde} (1.51–2.35) | 10.19 ^{abc} (2.74–15.47) | 107.4 ^a (64.6–149.0) | 2.56 ^{bcd} (2.33–2.84) | 24.45 ^{ab} (17.81–38.54) | 6.30 ^{de} (5.66–6.87) | 17.89 ^a (13.51–22.08) | 68.75 ^a (0–131.7) | 30.47 ^{cd} (22.62–42.99) |
| <i>P-Value</i> | *** | *** | NS | *** | *** | *** | NS | NS | *** |
| <i>Location</i> | | | | | | | | | |
| Suwon | 2.21 (1.21–3.69) | 11.21 (0.66–21.27) | 124.2 (64.1–215.9) | 2.66 (2.19–3.17) | 26.21 (16.63–38.54) | 7.30 (6.0–8.61) | 19.33 (14.70–24.92) | 86.82 (8.90–521.0) | 37.28 (20.48–56.68) |
| Iksan | 1.92 (1.21–2.88) | 11.25 (1.69–17.63) | 111.7 (79.0–158.0) | 2.55 (1.83–3.39) | 19.52 (14.69–34.67) | 6.82 (4.74–8.64) | 16.94 (11.92–21.63) | 75.91 (0–266.0) | 34.33 (22.39–51.05) |
| Dalseong | 1.88 (1.11–3.31) | 10.72 (7.23–19.02) | 112.3 (62.0–336.5) | 2.42 (2.0–3.14) | 19.48 (13.79–28.79) | 6.55 (4.95–8.37) | 15.09 (11.95–20.21) | 50.08 (0–183.8) | 29.41 (19.52–54.82) |
| <i>P-Value</i> | *** | NS | *** | *** | NS | *** | *** | *** | *** |
| <i>Year</i> | | | | | | | | | |
| 2017 | 1.90 (1.11–2.87) | 11.53 (7.23–21.27) | 147.6 (110.7–336.5) | 2.43 (1.83–2.98) | 21.55 (14.29–38.54) | 6.52 (4.74–8.61) | 15.44 (11.92–20.21) | 111.4 (46.4–266.0) | 30.18 (19.52–41.73) |
| 2018 | 2.11 (1.20–3.69) | 10.59 (0.66–19.02) | 8.45 (62.0–153.8) | 2.66 (2.05–3.38) | 21.92 (13.79–34.67) | 7.26 (6.24–8.64) | 18.80 (13.57–24.92) | 30.42 (0–521.0) | 37.17 (23.50–56.68) |
| <i>P-Value</i> | *** | NS | NS | *** | *** | *** | *** | * | *** |

Data are converted from fresh weight to dry weight basis using given moisture level. Data are the mean and range (parenthesis). Calcium, magnesium, phosphorus, and potassium were expressed as gram per kilogram dry weight basis. Others were expressed as milligram per kilogram dry weight basis. NS not significant; **P* < 0.05; ***P* < 0.01; ****P* < 0.001. The means in the same column followed by the same letter(s) are not significantly different at *P* < 0.05 by least significant difference.

conducted to identify differences by soybean varieties, locations, and cultivation years. Mean discriminations were performed by using Bonferroni-corrected *t*-tests and statistically significant differences were determined at the probability level of $P < 0.05$. In order to determine the difference between each *PfFAD3-1* transgenic line and KA, a mixed model ANOVA was employed across test sites.^{31,33,34} Entry was considered a fixed effect; location and the location-by-entry interaction were considered random effects. A range of observed values from the reference varieties was determined for each analytical component. Statistically significant differences between *PfFAD3-1* lines and KA were declared at $P < 0.05$. A range of observed values from the non-transgenic commercial varieties was also determined for each analytical component. The random effects of varieties, location, year, and the interaction of these factors on the nutritional variation were evaluated using a linear mixed model in R statistics. The quantification data for proximates, minerals, anti-nutrients, and fatty acids were subjected to principal component analysis (PCA) and orthogonal projections to latent structure discriminant analysis (OPLS-DA) using SIMCA version 13 (Umetrics, Umeå, Sweden) to evaluate the differences among groups of multivariate data. The PCA and OPLS-DA output consisted of score plots to visualize the contrast between different samples and loading plots to explain the cluster separation.

RESULTS AND DISCUSSION

Variance analysis of compositions in 13 commercial soybean seeds

Soybean seed composition is known to be influenced by genotype and environmental conditions such as cultivation location and cultivation years, as well as management strategy.^{35–39} Recently, it was demonstrated that genetics, management strategies, and environmental factors could influence seed protein and oil composition in US soybean seeds.⁴⁰ Soybean seed proteins, oils, fatty acids, sugars, and minerals were also shown to be altered by agricultural practices in the Mid-South region of the United States.³⁹ In the present study, the contents of eight proximates, nine minerals, four anti-nutrients, and 13 fatty acids were measured and/or calculated for the 13 varieties grown in Suwon, Iksan, and Dalseong during the 2017 and 2018 growing seasons. The combined data for each analyte from the 13 varieties across three locations and 2 years are presented by variety, location, and cultivation year in Tables 1–4. All data for each variety in different year and location are presented in Tables S5–S10.

Proximates

The levels of proximates are presented in Table 1. Carbohydrate (~42.7%) and crude protein (~37.4%) were the major proximate components, followed by NDF (~15.5%) and crude fat (~15%). ADF and total fiber were present at similar levels (~10%). Moisture and ash were present at ~8.4% and ~5.9%, respectively. Statistical significance ($P < 0.05$) was observed for ash, crude protein, crude fat, carbohydrate, total fiber, and ADF among the 13 varieties across locations and cultivation years, indicating a genetic contribution to the variation in these compounds (Table 1). The location effect across the 13 soybean varieties and cultivation years was statistically significant for all proximate components, except for protein and ADF (Table 1). All proximates across the 13 soybean varieties and locations were significantly influenced by cultivation year, except for

Table 3. Anti-nutrients composition of grain from 13 soybean varieties across three locations for 2 years by variety and across 13 varieties by location and year

| Component | Trypsin | | | |
|------------------|--|---------------------------------------|---------------------------------------|------------------------------------|
| | Phytic acid | inhibitor | Stachyose | Raffinose |
| <i>Varieties</i> | | | | |
| CHO | 21.35 ^a (15.98–27.18) | 42.13 ^{abc} (29.0–51.74) | 33.75 ^{cde} (27.93–38.88) | 6.94 ^{ab} (5.47–10.22) |
| CJ-3 | 19.06 ^{abcd} (15.75–23.64) | 33.56 ^{cd} (19.78–45.42) | 32.73 ^{cde} (27.80–36.08) | 7.27 ^a (5.82–8.50) |
| DC | 17.44 ^{bcd} (10.98–21.52) | 47.64 ^a (31.41–63.44) | 30.04 ^{de} (25.48–34.53) | 6.22 ^{abc} (5.03–7.80) |
| DP-2 | 17.19 ^{cd} (13.50–21.78) | 42.23 ^{abc} (27.27–52.55) | 40.26 ^a (35.27–45.14) | 4.54 ^d (3.75–5.73) |
| DW | 18.49 ^{abcd} (13.25–25.35) | 42.73 ^{abc} (29.41–56.99) | 40.54 ^a (33.09–44.67) | 5.50 ^{bcd} (4.63–8.42) |
| MS | 19.98 ^{abc} (15.49–25.53) | 38.06 ^{bcd} (23.0–51.88) | 34.54 ^{bcd} (31.28–37.50) | 6.66 ^{ab} (4.84–8.16) |
| PSN | 19.35 ^{abcd} (15.88–23.56) | 42.63 ^{abc} (30.21–54.57) | 35.02 ^{bc} (24.09–45.41) | 6.46 ^{abc} (4.30–8.80) |
| PW | 17.89 ^{bcd} (14.3–20.7) | 41.83 ^{abc} (26.47–53.36) | 38.65 ^{ab} (35.82–43.95) | 6.45 ^{abc} (4.54–8.13) |
| SCJ | 19.57 ^{abcd} (16.28–23.42) | 30.16 ^d (17.38–38.71) | 29.81 ^e (21.47–34.03) | 6.87 ^{ab} (4.97–8.64) |
| SO | 20.43 ^{ab} (16.27–25.7) | 37.93 ^{bcd} (22.32–46.10) | 32.79 ^{cde} (28.06–37.29) | 7.53 ^a (5.26–11.43) |
| SP | 16.61 ^d (13.57–22.04) | 43.78 ^{abc} (30.48–57.39) | 31.84 ^{cde} (22.86–38.36) | 7.04 ^a (4.93–10.06) |
| TG | 18.94 ^{abcd} (14.06–23.91) | 45.87 ^{abc} (32.62–57.52) | 32.08 ^{cde} (24.76–37.94) | 6.38 ^{abc} (4.69–9.34) |
| WR | 16.87 ^{cd} (13.52–20.95) | 42.47 ^{abc} (33.69–55.91) | 36.33 ^{abc} (32.56–38.40) | 5.10 ^{cd} (4.39–6.44) |
| <i>P-Value</i> | *** | *** | *** | *** |
| <i>Location</i> | | | | |
| Suwon | 21.0 (16.28–27.18) | 35.87 (17.38–52.55) | 36.14 (28.41–45.41) | 6.04 (4.30–8.63) |
| Iksan | 19.04 (14.67–23.56) | 45.22 (29.94–63.44) | 31.80 (21.47–42.13) | 5.95 (3.89–8.13) |
| Dalseong | 16.07 (10.98–21.84) | 41.45 (28.21–54.97) | 35.54 (28.06–44.93) | 7.15 (3.75–11.43) |
| <i>P-Value</i> | * | *** | *** | NS |
| <i>Year</i> | | | | |
| 2017 | 19.13 (10.98–27.18) | 44.95 (27.01–63.44) | 33.33 (24.09–43.95) | 6.41 (3.75–11.43) |
| 2018 | 18.28 (14.11–23.42) | 36.74 (17.38–52.27) | 35.66 (21.47–45.41) | 6.36 (3.89–10.06) |
| <i>P-Value</i> | *** | *** | *** | NS |

Data are converted from fresh weight to dry weight basis using given moisture level. Data are the mean and range (parenthesis), expressed as g per kg dry weight basis except trypsin inhibitor (TIU/mg). NS not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. The means in the same column followed by the same letter(s) are not significantly different at $P < 0.05$ by least significant difference.

carbohydrate (Table 1). The high moisture levels in the soybean samples from Iksan across the 2 years compared to those from Suwon and Dalseong are believed to be caused by the crop

Table 4. Fatty acid composition of grain from 13 soybean varieties across three locations for 2 years by variety and across 13 varieties by location and year

| Varieties | Component (%) | | | | | | | | | | | | | |
|-----------|-----------------------------------|------------------------------------|-----------------------------------|----------------------------------|-------------------------------------|------------------------------------|---|--|-----------------------------------|--------------------------------------|------------------------------------|---------------------------------|------------------------------------|------------------------------------|
| | C14:1 | C16:0 | C16:1 | C17:0 | C17:1 | C18:0 | C18:1 | C18:2 | C18:3 | C20:0 | C20:1 | C20:4 | C20:5 | C22:0 |
| CHO | 0.07 ^{ef} (0.07-0.08) | 11.09 ^{cd} (10.8-11.3) | 0.11 ^b (0.09-0.12) | 0.04 ^c (0-0.10) | 0.05 ^{cd} (0-0.08) | 3.17 ^{ef} (2.93-3.40) | 20.4 ^{bcdde} (16.8-22.7) | 55.1 ^{ab} (53.4-57.1) | 8.83 ^{cd} (8.04-10.4) | 0.28 ^e (0.23-0.31) | 0.16 ^{bcd} (0.14-0.16) | 0.05 ^{ab} (0-0.06) | 0.17 ^b (0.14-0.21) | 0.40 ^c (0.31-0.47) |
| CJ-3 | 0.08 ^{de} (0.07-0.09) | 10.2 ^{ef} (9.74-10.83) | 0.09 ^{cd} (0.08-0.11) | 0.07 ^{bc} (0-0.13) | 0.05 ^{cd} (0-0.73) | 3.71 ^{bc} (3.43-4.24) | 20.6 ^{bcdde} (17.1-23.9) | 54.5 ^{bc} (51.5-57.4) | 9.46 ^{bc} (8.12-10.7) | 0.31 ^{bcdde} (0.27-0.38) | 0.17 ^{bc} (0.15-0.18) | 0.05 ^a (0-0.06) | 0.19 ^a (0.16-0.22) | 0.45 ^{abc} (0.38-0.54) |
| DC | 0.07 ^g (0.06-0.07) | 10.4 ^e (10.2-10.7) | 0.08 ^{de} (0.07-0.10) | 0.11 ^a (0.10-0.13) | 0.07 ^{abcd} (0.06-0.09) | 3.33 ^{def} (3.08-3.61) | 18.1 ^{def} (14.9-20.9) | 55.1 ^{ab} (52.9-58.1) | 11.6 ^a (10.4-12.9) | 0.28 ^{de} (0.25-0.32) | 0.15 ^{cde} (0.14-0.17) | 0.04 ^{abc} (0-0.05) | 0.15 ^{bcd} (0.14-0.17) | 0.41 ^{bc} (0.37-0.45) |
| DP-2 | 0.08 ^{bc} (0.07-0.09) | 11.0 ^{cd} (10.6-11.3) | 0.09 ^{de} (0.07-0.10) | 0.11 ^a (0.10-0.14) | 0.07 ^{abcd} (0.05-0.08) | 3.70 ^{bcd} (3.35-3.99) | 17.5 ^{ef} (15.4-20.1) | 57.5 ^a (55.4-60.0) | 9.09 ^c (8.29-10.3) | 0.27 ^e (0.24-0.29) | 0.15 ^{cde} (0.14-0.19) | 0.02 ^{bc} (0-0.05) | 0.13 ^d (0.12-0.18) | 0.33 ^d (0.3-0.37) |
| DW | 0.08 ^{de} (0.07-0.09) | 10.4 ^e (9.91-10.63) | 0.08 ^e (0.06-0.10) | 0.12 ^a (0.10-0.14) | 0.07 ^{abcd} (0-0.09) | 3.70 ^{bcd} (3.26-4.39) | 20.1 ^{b^{ce}} (16.6-24.3) | 55.2 ^{ab} (51.6-58.1) | 9.32 ^{bc} (8.28-10.6) | 9.04 ^{abcd} (7.83-10.6) | 0.17 ^b (0.15-0.19) | 0.03 ^{abc} (0-0.05) | 0.16 ^b (0.15-0.18) | 0.45 ^{abc} (0.39-0.56) |
| MS | 0.09 ^a (0.08-0.10) | 12.4 ^a (11.8-12.7) | 0.10 ^{bc} (0.09-0.11) | 0.12 ^a (0.10-0.13) | 0.07 ^{abc} (0.06-0.09) | 4.20 ^a (3.62-5.14) | 20.9 ^{bcd} (15.5-26.1) | 49.70 ^{def} (45.6-53.2) | 11.4 ^a (9.63-13.8) | 0.34 ^{ab} (0.29-0.43) | 0.14 ^e (0.13-0.16) | 0.01 ^{abc} (0-0.06) | 0.15 ^{bcd} (0.13-0.18) | 0.41 ^{bc} (0.37-0.51) |
| PSN | 0.07 ^g (0.06-0.07) | 11.1 ^{cd} (10.5-11.4) | 0.11 ^b (0.09-0.13) | 0.12 ^a (0.10-0.13) | 0.08 ^a (0.07-0.10) | 3.70 ^{bcd} (3.41-4.33) | 23.5 ^b (19.1-28.9) | 51.4 ^{d^e} (47.0-55.0) | 8.78 ^{cd} (7.56-10.4) | 0.33 ^{abc} (0.28-0.40) | 0.15 ^{cde} (0.14-0.17) | 0.03 ^{abc} (0-0.05) | 0.17 ^b (0.15-0.20) | 0.40 ^{bc} (0.34-0.51) |
| PW | 0.08 ^{cd} (0.07-0.10) | 11.0 ^{cd} (10.5-11.6) | 0.09 ^{de} (0.08-0.10) | 0.09 ^{ab} (0-0.12) | 0.06 ^{bcd} (0-0.08) | 3.44 ^{cde} (2.6-3.95) | 19.9 ^{cdef} (15.8-23.9) | 54.5 ^{bc} (51.7-58.1) | 9.73 ^{bc} (8.44-11.4) | 0.32 ^{abcd} (0.19-0.38) | 0.16 ^{bc} (0.13-0.18) | 0.04 ^{ab} (0-0.05) | 0.14 ^{cd} (0-0.18) | 0.46 ^{abc} (0.24-0.54) |
| SCJ | 0.09 ^a (0.08-0.10) | 11.3 ^c (10.8-11.6) | 0.09 ^{de} (0.07-0.11) | 0.11 ^a (0-0.15) | 0.05 ^d (0-0.07) | 3.98 ^{ab} (3.84-4.28) | 19.3 ^{cdef} (15.7-25.6) | 54.6 ^{bc} (50.2-56.5) | 9.33 ^c (7.42-11.1) | 0.36 ^a (0.32-0.40) | 0.16 ^{bcd} (0.14-0.17) | 0.04 ^{abc} (0-0.06) | 0.17 ^b (0.14-0.20) | 0.43 ^{abc} (0.37-0.51) |
| SO | 0.09 ^{ab} (0.08-0.09) | 11.7 ^b (11.3-12.0) | 0.13 ^a (0.12-0.14) | 0.04 ^c (0-0.11) | 0.08 ^{ab} (0-0.1) | 3.06 ^f (2.59-3.38) | 27.4 ^a (46.4-51.0) | 48.5 ^f (46.4-51.0) | 7.88 ^b (7.07-9.69) | 0.31 ^{cde} (0.26-0.35) | 0.19 ^a (0.17-0.21) | 0.05 ^a (0-0.06) | 0.16 ^{bc} (0.15-0.18) | 0.46 ^{ab} (0.41-0.51) |
| SP | 0.08 ^{cd} (0.08-0.08) | 12.3 ^a (11.4-12.9) | 0.11 ^b (0.09-0.12) | 0.10 ^{ab} (0-0.13) | 0.07 ^{abcd} (0-0.09) | 3.03 ^f (2.87-3.25) | 16.9 ^f (13.2-18.8) | 55.8 ^{ab} (54.3-60.6) | 10.6 ^{ab} (9.55-11.7) | 0.29 ^{cde} (0.22-0.33) | 0.15 ^{de} (0.12-0.16) | 0.03 ^{abc} (0-0.05) | 0.14 ^{cd} (0.10-0.16) | 0.42 ^{bc} (0.30-0.49) |
| TG | 0.07 ^g (0.06-0.08) | 9.92 ^f (9.56-10.5) | 0.09 ^{de} (0.07-0.10) | 0.06 ^{bc} (0-0.11) | 0.08 ^{ab} (0.06-0.09) | 4.04 ^{ab} (3.32-4.79) | 27.6 ^a (20.3-32.9) | 49.0 ^{ef} (44.5-54.8) | 7.88 ^d (6.89-9.30) | 0.34 ^{ab} (0.28-0.41) | 0.20 ^a (0.18-0.22) | 0.04 ^{abc} (0-0.52) | 0.15 ^{bcd} (0.13-0.18) | 0.49 ^a (0.42-0.58) |
| WR | 0.08 ^{cd} (0.07-0.09) | 10.9 ^d (10.4-11.3) | 0.09 ^{cd} (0.08-0.11) | 0.09 ^{ab} (0-0.12) | 0.06 ^{abcd} (0-0.08) | 3.99 ^{ab} (3.51-4.94) | 21.8 ^{bc} (17.4-26.3) | 52.2 ^{cd} (48.5-55.7) | 9.66 ^{bc} (7.80-11.5) | 0.32 ^{abcd} (0.28-0.41) | 0.16 ^{bc} (0.15-0.19) | 0.02 ^c (0-0.05) | 0.15 ^{bcd} (0.13-0.19) | 0.42 ^{bc} (0.36-0.51) |
| P-Value | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** |
| Location | | | | | | | | | | | | | | |
| Suwon | 0.08 (0.06-0.10) | 11.1 (9.58-12.6) | 0.09 (0.07-0.14) | 0.09 (0-0.15) | 0.07 (0-0.1) | 3.54 (2.59-4.72) | 20.5 (13.2-31.5) | 54.1 (45.7-60.6) | 9.32 (6.89-13.8) | 0.31 (0.19-0.41) | 0.16 (0.1-0.21) | 0.04 (0-0.06) | 0.15 (0-0.20) | 0.42 (0.24-0.58) |
| Iksan | 0.08 (0.06-0.10) | 11.2 (9.56-12.9) | 0.09 (0.06-0.14) | 0.10 (0-0.13) | 0.06 (0-0.08) | 3.55 (2.87-4.26) | 20.1 (15.3-32.9) | 53.8 (44.5-58.6) | 9.88 (7.23-13.1) | 0.31 (0.25-0.36) | 0.16 (0.13-0.22) | 0.04 (0-0.06) | 0.16 (0.12-0.22) | 0.42 (0.31-0.52) |
| Dalseong | 0.08 (0.06-0.09) | 10.8 (9.62-12.5) | 0.10 (0.07-0.13) | 0.08 (0-0.14) | 0.07 (0.06-0.10) | 3.76 (2.90-5.14) | 22.7 (17.3-29.8) | 52.0 (45.6-56.6) | 9.24 (7.09-12.5) | 0.32 (0.24-0.43) | 0.16 (0.13-0.20) | 0.04 (0-0.06) | 0.16 (0-0.21) | 0.44 (0.32-0.56) |
| P-Value | * | ** | - | *** | *** | ** | *** | *** | ** | * | - | - | ** | * |
| Year | | | | | | | | | | | | | | |
| 2017 | 0.08 (0.06-0.10) | 11.0 (9.56-12.7) | 0.10 (0.07-0.14) | 0.10 (0-0.15) | 0.07 (0-0.10) | 3.83 (2.87-5.14) | 23.1 (15.7-32.9) | 51.9 (44.5-60.0) | 8.67 (6.89-11.1) | 0.34 (0.27-0.43) | 0.17 (0.15-0.22) | 0.05 (0-0.06) | 0.16 (0-0.22) | 0.46 (0.32-0.58) |
| 2018 | 0.08 (0.06-0.09) | 11.1 (9.56-12.7) | 0.09 (0.07-0.14) | 0.07 (0-0.15) | 0.06 (0-0.10) | 3.4 (2.87-5.14) | 19.0 (15.7-32.9) | 54.8 (44.5-60.0) | 10.3 (6.89-11.1) | 0.28 (0.27-0.43) | 0.15 (0.15-0.22) | 0.03 (0-0.06) | 0.15 (0-0.22) | 0.69 (0.32-0.58) |

Table 4. Continued

| Component (%) | | | | | | | | | | | | | |
|---------------|-------------|-------------|----------|----------|-------------|-------------|-------------|-------------|-------------|-------------|----------|----------|-------------|
| C14:1 | C16:0 | C16:1 | C17:0 | C17:1 | C18:0 | C18:1 | C18:2 | C18:3 | C20:0 | C20:1 | C20:4 | C20:5 | C22:0 |
| (0.06–0.10) | (9.74–12.9) | (0.06–0.14) | (0–0.14) | (0–0.09) | (2.59–4.07) | (13.2–29.8) | (46.4–60.6) | (7.33–13.8) | (0.19–0.34) | (0.12–0.21) | (0–0.06) | (0–0.19) | (0.24–0.46) |
| — | — | *** | *** | ** | *** | *** | *** | *** | *** | *** | *** | *** | *** |
| P-Value | — | *** | *** | ** | *** | *** | *** | *** | *** | *** | *** | *** | *** |

Data are the mean and range (parenthesis), expressed as percent to total lipid. NS not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. The means in the same column followed by the same letter(s) are not significantly different at $P < 0.05$ by least significant difference. C14:1, myristoleic; C16:0, palmitic; C16:1, palmitoleic; C17:0, heptadecanoic; C17:1, heptadecanoic; C18:1, oleic; C18:2, linoleic; C18:3, linolenic; C20:0, arachidic; C20:1, eicosenoic; C20:4, eicosatetraenoic; C20:5, eicosapentaenoic; C22:0, behenic.

history at the cultivation site. The Iksan site was used for the cultivation of paddy rice for many years; thus, it contained more water content than the Suwon and Dalseong sites, which have been used consistently for field crops.

Minerals

The contents of minerals are provided in Table 2. Potassium was the most abundant mineral, followed by phosphorus, magnesium, and calcium. Calcium, copper, magnesium, manganese, phosphorus, and zinc showed significant differences among the 13 varieties across the three locations and two cultivation years, while iron, potassium, and sodium did not vary. The levels of minerals varied significantly by location and cultivation year, except for copper and manganese for location and copper and iron for year (Table 2). The mineral levels were found to be the highest in soybeans from Suwon (Table 2).

Anti-nutrients

Mature soybean seeds contain a number of anti-nutritional components with various levels of biological activity. The levels of phytic acid, trypsin inhibitor activity, raffinose, and stachyose were measured in soybean seeds. Phytic acid forms phytic acid–metal complexes with nutritionally important metals, especially zinc, calcium, and magnesium, resulting in poor absorption in the intestine.⁴¹ Through non-specific binding to proteins, phytate has been shown to inhibit the action of a number of enzymes important in digestion.⁴² The phytic acid levels in soybeans were shown to be dependent on genotypic variability, growing location, and application of phosphate fertilizer.^{38,43} Trypsin inhibitor activity is a measure of the degree of protease inhibition present in a soybean sample. Vollman *et al.*⁴⁴ presented evidence of a significant effect of environment, fertilizer treatment, and genotypes on trypsin inhibitor activity. In our study, phytic acid levels and trypsin inhibitor activity varied in the soybean varieties across the three locations and two cultivation years (Table 3). The levels of phytic acid were highest at Suwon across the varieties and cultivation years (Table 3). The levels of trypsin inhibitor activity were highest at Iksan across the varieties and cultivation years. Phytic acid levels and trypsin inhibitor activities were both higher in year 2017 than in year 2018 across the varieties and locations (Table 3).

Raffinose and stachyose are not digested in the human gastrointestinal tract due to the absence of appropriate digestion enzymes, causing abdominal discomfort and diarrhea.⁴⁵ Therefore, soybean genotypes with reduced levels of these anti-nutrients are desired to enhance the utilization of soybeans in food as well as in animal feed.^{46,47} The contents of stachyose and raffinose were significantly influenced by soybean variety, location, and year.^{44,46} In accordance, our results showed that the levels of raffinose and stachyose significantly differed in varieties across the locations and cultivation years (Table 3). Varied levels of stachyose, but not raffinose, were observed depending on the location and cultivation year (Table 3).

Fatty acid profiles

The fatty acid contents of 13 soybean varieties across three locations for 2 years are presented in Table 4. Linoleic acid was the most abundant fatty acid, followed by oleic acid, palmitic acid, and linolenic acid. All fatty acids showed significant differences among the 13 soybean varieties across the three locations and two cultivation years (Table 4). The widest range was found in

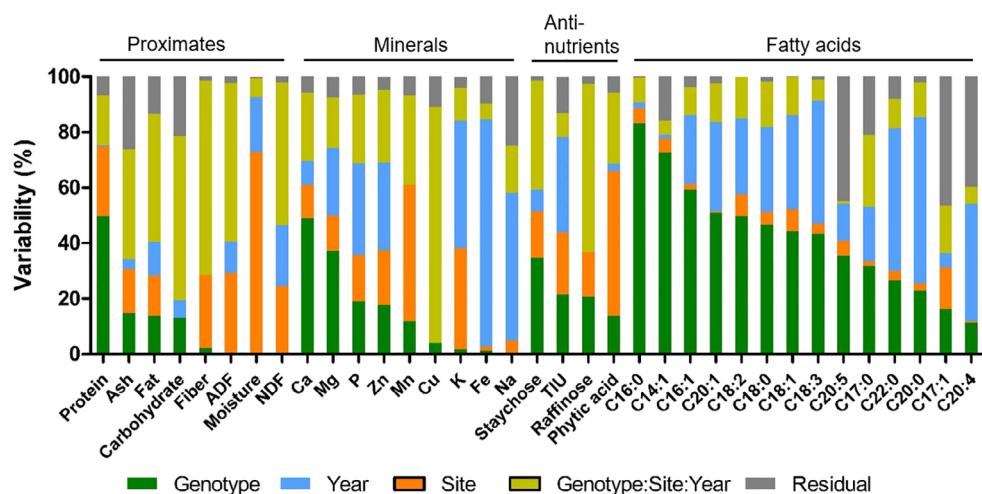


Figure 1. Variability proportion (percent variability) affecting natural variation in analytes. The random effects of varieties, location, year, and the interaction of these factors on the nutritional variation were evaluated using a linear mixed model in R statistics. C14:1, myristoleic; C16:0, palmitic; C16:1, palmitoleic; C17:0, heptadecanoic; C17:1, heptadecenoic; C18:0, stearic; C18:1, oleic; C18:2, linoleic; C18:3, linolenic; C20:0, arachidic; C20:1, eicosenoic; C20:4, eicosatetraenoic; C20:5, eicosapentaenoic; C22:0, behenic.

oleic acid (16.9–27.4%), followed by linoleic acid (48.5–57.5%) and linolenic acid (7.88–11.6%). In addition, the levels of fatty acids varied significantly by location and cultivation year, except for palmitoleic, heptadecenoic, eicosenoic, and eicosatetraenoic acid for location, and myristoleic acid and palmitic acid for cultivation year (Table 4).

Percent variability analysis in soybean seed composition

The proportion of random effects of individual variety (V), site (S), and cultivation year (Y), as well as their interaction ($G \times S \times Y$) contributing to the total variance was estimated using variance component analysis in R statistics software. This model was previously demonstrated suitable for describing the impact of random effects on the nutritional variation in field-grown crops.^{48–50} The variance components of the nutritional constituents were apportioned among the effects of variety, site, cultivation year, $G \times S \times Y$, and that which could not be explained by these factors (termed the residual).

The results of percentage variability are presented in Fig. 1. Among the proximate components, protein was highly influenced by genotype, accounting for 49.7% to the total variance. Ash, fat, carbohydrate, fiber, ADF, and NDF contents were highly influenced by the $G \times S \times Y$ effect, and accounted for 39.4%, 46.1%, 59.2%, 70.1%, 57.2% and 51.6% of the total variance, respectively. The cultivation site highly contributed to the total variance in moisture content (72.9%). Calcium and magnesium were mainly affected by genotype, accounting for 49% and 37.4%, respectively. Potassium, iron, and sodium were mainly affected by the cultivation year, accounting for 46.8%, 81.7%, and 53.3%, respectively. The $G \times S \times Y$ effect and the cultivation site highly contributed to the total variance in copper (71.8%) and manganese (49.1%), respectively.

With respect to the percentage variability in anti-nutrients, genotype (33.6%) and the $G \times S \times Y$ effect (29.8%) contributed to the variation in staychlose in similar proportions. Raffinose (60.7%) was mainly affected by the $G \times S \times Y$ effect, while phytic acid (52.4%) was influenced by the cultivation site. Trypsin inhibitor was highly determined by the cultivation year (34.3%), followed by cultivation site (22.3%) and genotype (21.6%). Overall, genotype and cultivation year mainly contributed to the total variance for fatty acids. Genotype notably contributed to palmitic (83.2%), myristoleic (72.8%), and palmitoleic (59.2%). Eicosenoic, linoleic, steric, oleic, and linolenic were

highly determined by genotype and the cultivation year. The cultivation year highly contributed to the total variance in behenic (51.4%), arachidic (60%) and eicosatetraenoic contents.

PCA and OPLS-DA of soybean seed nutrient composition

It was shown previously that chemometric methods such as PCA and OPLS-DA are useful for classifying compositional data sets from different environments or genotypes.^{36,38,49} We used PCA and OPLS-DA to investigate the degree to which compositions are separated by the factors of cultivars, locations, and cultivation years (Fig. 2). In addition, factor-loading scores of PCA and OPLS-DA were employed to identify nutritional components responsible for data variance (Supporting Information Fig. S1). The PCA results for proximates, minerals, anti-nutrients, and fatty acids by varieties showed no apparent separation among the 13 soybean varieties (Fig. 2(a)). These results demonstrated that these components were not significantly differentiated by genotype.

OPLS-DA has better separation than PCA and, thus, was used for location and cultivation year. The OPLS-DA showed good separation of proximates and minerals by location and by cultivation year (Fig. 2(b, c)). We performed the analysis with two predictive and two orthogonal components for location. The values R^2 and Q^2 indicate explained variance of the total variance and the prediction goodness parameter, respectively. The OPLS1 separated proximates of the soybeans grown in Iksan and Dalseong ($R^2 = 0.622$, $Q^2 = 0.571$) (Fig. 2(b)). The variation in OPLS1 of the proximates was mainly attributable to moisture (Fig. S1(b)), which is consistent with the level of moisture being higher in soybeans grown in Iksan than in those grown in Dalseong and Suwon (Table 1). OPLS1 also separated minerals of the soybeans grown in Suwon and Dalseong ($R^2 = 0.721$, $Q^2 = 0.365$) (Fig. 2(b)). The model showed one orthogonal component which was mainly attributable to potassium and manganese (Fig. S1(b)). Consistently, it was observed that the contents of potassium and manganese were higher in Suwon than in Dalseong (1.28-fold for potassium; 2.44-fold for manganese). We performed the analysis with one predictive and one orthogonal components for location. OPLS1 separated the proximates ($R^2 = 0.375$, $Q^2 = 0.267$) and minerals ($R^2 = 0.871$ and $Q^2 = 0.853$) of the soybeans grown in 2017 and 2018 (Fig. 2(c)). The variation in OPLS1 of the proximates was mainly attributable to moisture (Fig. S1(b)). Accordingly, the

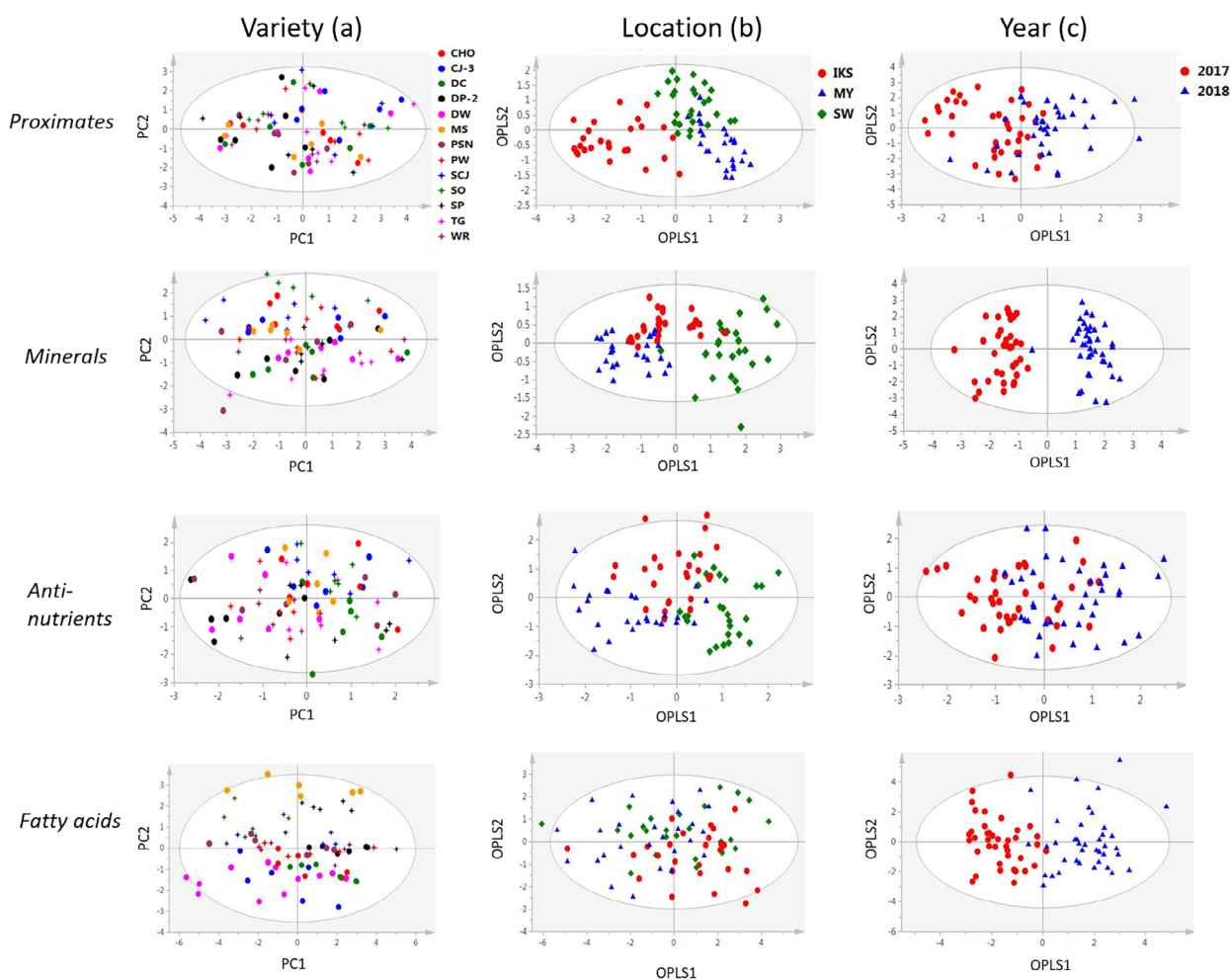


Figure 2. Principal component analysis (PCA) and orthogonal projection to latent structure discrimination analysis (OPLS-DA) score plots of compositional data on seeds from 13 soybean varieties. Proximates, minerals, anti-nutrients, and fatty acids data were subjected to PCA by variety (a), and to OPLS-DA by location (b) and by cultivation year (c). PCA model was obtained with five principal components. OPLS-DA model was obtained with one predictive and two orthogonal components for location, and one predictive and one orthogonal component for year.

level of moisture was lower in soybeans grown in 2017 than in soybeans grown in 2018. Iron and sodium contributed to OPLS1 variation in minerals between the two cultivation years. Both of these minerals were higher in 2017 than in 2018 (Table 1).

The OPLS-DA showed separation of anti-nutrients in soybeans produced from Dalseong and Suwon by OPLS1, and the variation in OPLS1 of anti-nutrients was attributable to phytic acid (Fig. S1 (b)). The anti-nutrients in soybeans grown in 2017 and 2018 formed small but some extent of separation (Fig. 2(c)). The model showed one orthogonal component, with $R^2 = 0.313$ and $Q^2 = 0.252$ in anti-nutrients by cultivation year. The variation in OPLS1 of anti-nutrients by cultivation year was attributable to TIU (Fig. S1(c)). With respect to fatty acids, the OPLS-DA showed no apparent separation among three locations (Fig. 2(b)), but it showed separation of fatty acids by cultivation year by OPLS1 (Fig. 2(c)). The model showed one orthogonal component, with $R^2 = 0.746$ and $Q^2 = 0.68$ in fatty acids by cultivation year. The variations in OPLS1 of fatty acids by cultivation year were attributable to linoleic and linolenic acid (Fig. S1(c)). These results indicate that the variations in proximates, minerals and anti-nutrients are greatly influenced by location and cultivation year.

Comparative compositional analysis of *PffAD3-1* soybeans

In the present study, we compared the levels of six proximates, nine minerals, four anti-nutrients, and 13 fatty acid profiles between three different *PffAD3-1* soybean lines and their non-transgenic conventional counterpart, KA, at two field sites in South Korea (Jeonju and Gunwi) in 2019. In addition to *PffAD3-1* soybeans and KA, three non-transgenic commercial soybean varieties (hereafter described as reference 1) were planted simultaneously to address the range of natural variation. We also included the range of values obtained from 13 commercial soybean varieties reported earlier in this manuscript as reference data (hereafter described as reference 2) to enhance the reference ranges. All the original data are for three *PffAD3-1* soybean lines, KA and commercial varieties in Jeonju and Gunwi are presented in Tables S11 and S12.

No significant differences ($P < 0.05$) were observed for proximate components between the *PffAD3-1* lines and KA using ANOVA (Table 5). A significant difference in mean mineral values was observed only for calcium between the *PffAD3-1* line (T10-1) and KA (Table 6). However, the calcium value for the *PffAD3-1* (T10-1) was within both the reference 1 and reference

Table 5. Proximate composition of *PffAD3-1* soybean seeds across two locations

| Component | T10-1 mean (min–max) ^a | T11-8 mean (min–max) ^a | T12-1 mean (min–max) ^a | Kwangankong mean (min–max) ^a | Reference 1 (min–max) ^b | Reference 2 (min–max) ^c |
|--------------|--------------------------------------|--------------------------------------|--------------------------------------|--|---------------------------------------|---------------------------------------|
| Moisture | 7.75 (6.25–9.37) | 6.90 (5.35–8.58) | 6.80 (5.70–7.89) | 6.86 (6.72–7.00) | 5.56–7.69 | 4.88–14.84 |
| Ash | 5.94 (5.80–6.04) | 5.76 (5.66–5.85) | 5.83 (5.67–5.97) | 5.74 (5.53–5.94) | 5.24–5.98 | 4.77–7.59 |
| Carbohydrate | 27.39 (26.39–28.46) | 28.65 (27.34–29.94) | 27.08 (26.16–27.54) | 28.74 (28.01–29.81) | 24.52–29.89 | 21.71–42.90 |
| Protein | 45.74 (44.00–47.65) | 45.51 (43.98–47.21) | 46.56 (44.66–48.63) | 46.31 (44.68–48.17) | 41.22–44.36 | 32.55–43.79 |
| Fat | 13.19 (12.06–14.10) | 13.18 (11.96–14.44) | 13.73 (13.07–14.17) | 12.35 (10.45–14.24) | 14.42–21.35 | 6.68–22.70 |
| Fiber | 7.43 (7.25–7.69) | 7.67 (6.96–8.44) | 7.34 (6.91–7.63) | 8.62 (7.74–9.12) | 5.17–8.74 | 4.13–16.09 |

^a Data are the mean and range (parenthesis) values across two sites (Jeonju, Gunwi) with three replicates at each site. Data are converted from fresh weight to dry weight basis using given moisture level. Data are the mean and range (parenthesis), expressed as percent dry weight except moisture.

^b Minimum (min) and maximum (max) values observed from three reference varieties (termed as Reference 1) planted in the same site with test and comparator.

^c Min and max values obtained from 13 commercial varieties (termed as Reference 2) in this study.

2 values (Table 6). No significant differences were found for phytic acid, trypsin inhibitor, stachyose, and raffinose between each of the three *PffAD3-1* lines and KA (Table 7).

PffAD3-1 transgenic lines has an intentional change to the fatty acid profile compared to KA, producing a lower content of oleic

and linoleic acids, and a higher content of linolenic acid, as a consequence of the overexpression of *PffAD3-1*.¹⁵ In the current study, the levels of linoleic and linolenic were significantly changed between KA and three *PffAD3-1* transgenic lines, indicating that the introduced *PffAD3-1* gene has been successfully

Table 6. Mineral composition of *PffAD3-1* soybean seeds across two locations

| Component | T10-1 mean (min–max) ^a | T11-8 mean (min–max) ^a | T12-1 mean (min–max) ^a | Kwangankong mean (min–max) ^a | Reference 1 (min–max) ^b | Reference 2 (min–max) ^c |
|------------|--|--------------------------------------|--------------------------------------|--|---------------------------------------|---------------------------------------|
| Calcium | 3.15^d (3.04–3.33) | 2.73 (2.58–2.88) | 3.04 (2.99–3.12) | 2.90 (2.82–2.94) | 1.98–3.68 | 1.11–3.69 |
| Copper | 19.66 (16.63–23.26) | 16.38 (15.99–16.87) | 17.75 (16.63–18.93) | 15.69 (14.14–17.21) | 13.18–16.78 | 0.66–21.27 |
| Iron | 107.1 (96.86–118.0) | 99.25 (99.49–105.2) | 105.1 (98.26–111.3) | 100.3 (98.3–105.2) | 78.24–180.2 | 62.0–336.5 |
| Magnesium | 2.64 (2.63–2.65) | 2.63 (2.56–2.71) | 2.69 (2.64–2.72) | 2.61 (2.46–2.72) | 2.19–2.84 | 1.83–3.38 |
| Manganese | 37.3 (35.58–39.26) | 44.55 (43.7–46.28) | 40.96 (38.87–43.86) | 45.5 (38.65–52.85) | 25.86–47.44 | 13.79–38.54 |
| Phosphorus | 11.30 (10.52–12.14) | 10.19 (10.08–10.29) | 10.56 (10.06–11.00) | 10.73 (10.22–11.14) | 8.81–10.53 | 4.74–8.64 |
| Potassium | 17.87 (17.36–18.47) | 17.53 (17.29–17.58) | 17.58 (18.04–18.49) | 17.82 (16.89–18.60) | 17.88–19.22 | 11.92–24.93 |
| Sodium | 251.9 (129.1–419.6) | 272.1 (206.5–318.4) | 268.1 (161.8–374.6) | 269.7 (199.4–421.1) | 116.1–322.3 | 0.0–521.0 |
| Zinc | 59.83 (56.61–63.65) | 51.69 (48.21–54.04) | 57.12 (55.20–58.63) | 55.84 (48.98–62.27) | 42.37–60.90 | 19.52–56.68 |

^a Data are the mean and range (parenthesis) values across two sites (Jeonju, Gunwi) with three replicates at each site. Data are converted from fresh weight to dry weight basis using given moisture level. Data are the mean and range (parenthesis). Calcium, magnesium, phosphorus, and potassium were expressed as gram per kilogram dry weight basis. Others were expressed as milligrams per kilogram dry weight basis.

^b Minimum (min) and maximum (max) values observed from three reference varieties (termed as Reference 1) planted in the same site with test and comparator.

^c Min and max values obtained from 13 commercial varieties (termed as Reference 2) in this study.

^d Statistically significant difference with Kwangankong ($P < 0.05$).

Table 7. Anti-nutrient composition of *PffAD3-1* soybean seeds across two locations

| Component | T10-1 mean (min–max) ^a | T11-8 mean (min–max) ^a | T12-1 mean (min–max) ^a | Kwangankong mean (min–max) ^a | Reference 1 (min–max) ^b | Reference 2 (min–max) ^c |
|-------------------|--------------------------------------|--------------------------------------|--------------------------------------|--|---------------------------------------|---------------------------------------|
| Phytic acid | 23.98 (21.28–25.53) | 22.97 (21.29–23.98) | 23.68 (21.63–25.89) | 22.55 (20.75–23.81) | 18.66–23.55 | 10.98–27.18 |
| Trypsin inhibitor | 42.0 (37.6–44.3) | 47.9 (42.8–53.8) | 46.6 (41.3–52.4) | 44.5 (36.6–51.3) | 36.6–51.3 | 17.38–63.44 |
| Stachyose | 42.17 (40.74–44.41) | 41.50 (40.19–43.15) | 40.83 (40.37–41.35) | 43.54 (42.24–46.16) | 25.03–45.48 | 21.47–45.41 |
| Raffinose | 4.10 (3.56–4.71) | 5.23 (4.87–5.71) | 4.39 (3.16–5.54) | 4.68 (4.26–5.18) | 2.74–7.64 | 3.89–11.43 |

^a Data are the mean and range (parenthesis) values across two sites (Jeonju, Gunwi) with three replicates at each site. Data are converted from fresh weight to dry weight basis using given moisture level. Data are the mean and range (parenthesis), expressed as gram per kilogram dry weight basis except trypsin inhibitor (TIU/mg).
^b Minimum (min) and maximum (max) values observed from the three reference varieties (termed as Reference 1) planted in the same site with test and comparator.
^c Min and max values obtained from the 13 commercial varieties (termed as Reference 2) in this study.

expressed (Table 8). The linoleic acid contents were decreased about 76.1%, 72%, and 72.3% in T10-1, T11-8, and T12-1 lines, respectively compared with that in KA. The linolenic acid contents

were significantly increased about 336%, 327%, and 327% in T10-1, T11-8, and T12-1 lines, respectively compared with that in KA. In contrast to previous studies,¹⁵ the oleic acid contents were

Table 8. Fatty acid composition of *PffAD3-1* soybean seeds across two locations

| Component | T10-1 mean (min–max) ^a | T11-8 mean (min–max) ^a | T12-1 mean (min–max) ^a | Kwangankong mean (min–max) ^a | Reference 1 (min–max) ^b | Reference 2 (min–max) ^c |
|--------------------------|---|---|---|--|---------------------------------------|------------------------------------|
| C14:1 (myristoleic) | 0.08 (0.08–0.09) | 0.08 (0.08–0.09) | 0.08 (0.08–0.09) | 0.08 (0.08–0.09) | 0.07–0.08 | 0.06–0.10 |
| C16:0 (palmitic) | 10.2 (10.0–10.4) | 10.4 (10.4–10.5) | 10.4 (10.2–10.6) | 10.9 (10.8–11.1) | 10.5–11.3 | 9.56–12.9 |
| C16:1 (palmitoleic) | 0.09 (0.09–0.11) | 0.09 (0.09–0.10) | 0.09 (0.09–0.10) | 0.09 (0.09–0.09) | 0.09–0.10 | 0.06–0.14 |
| C17:1 (heptadecenoic) | 0.07 (0.0–0.11) | 0.09 (0.08–0.10) | 0.09 (0.08–0.10) | 0.08 (0.07–0.09) | 0.07–0.08 | 0.0–0.10 |
| C18:0 (stearic) | 3.0 (2.66–3.20) | 3.13 (2.87–3.38) | 3.04 (2.86–3.20) | 3.28 (3.02–3.33) | 2.91–3.63 | 2.59–5.14 |
| C18:1 (oleic) | 48.4 (46.8–49.5) | 47.4 (44.7–50.1) | 47.6 (45.1–49.0) | 45.8 (44.2–47.1) | 23.0–33.1 | 13.2–32.9 |
| C18:2 (linoleic) | ^d 7.48 (7.17–7.80) | ^d 8.75 (7.83–9.63) | ^d 8.66 (7.8–9.52) | 31.3 (29.8–33.0) | 44.6–54.5 | 44.5–60.6 |
| C18:3 (linolenic) | ^d 29.1 (27.9–30.8) | ^d 28.5 (27.1–30.1) | ^d 28.5 (27.7–29.9) | 6.67 (6.41–6.74) | 7.16–7.45 | 6.89–13.8 |
| C20:0 (arachidic) | 0.43 (0.34–0.46) | 0.44 (0.42–0.47) | 0.44 (0.41–0.46) | 0.45 (0.44–0.47) | 0.27–0.34 | 0.19–0.43 |
| C20:1 (eicosenoic) | 0.05 (0.0–0.27) | 0.0 (0.0–0.0) | 0.0 (0.0–0.0) | 0.30 (0.30–0.31) | 0.18–0.24 | 0.12–0.22 |
| C20:4 (eicosatetraenoic) | 0.048 (0.0–0.073) | 0.066 (0.065–0.068) | 0.072 (0.071–0.073) | 0.067 (0.064–0.071) | 0.027–0.058 | 0.0–0.06 |
| C20:5 (eicosapentaenoic) | 0.06 (0.0–0.07) | 0.06 (0.06–0.07) | 0.07 (0.06–0.07) | 0.07 (0.06–0.07) | 0.03–0.06 | 0.0–0.22 |
| C22:0 (behenic) | 0.23 (0.13–0.26) | 0.22 (0.21–0.23) | 0.23 (0.22–0.24) | 0.25 (0.24–0.28) | 0.14–0.21 | 0.24–0.58 |

^a Data are the mean and range (parenthesis) values across two sites (Jeonju, Gunwi) with three replicates at each site, and expressed as percent to total fatty acids.
^b Minimum (min) and maximum (max) values observed from the three reference varieties (termed as Reference 1) planted in the same site with test and comparator.
^c Min and max values obtained from the 13 commercial varieties (termed as Reference 2) in this study.
^d Statistically significant difference with Kwangankong ($P < 0.05$).

not altered in *PffAD3-1* lines. Linoleic and linolenic acids for *PffAD3-1* lines were not within the ranges of the reference varieties. Notably, KA exhibited higher linoleic and linolenic acids compared to the commercial varieties while it had higher oleic, arachidic, and eicosenoic acids compared to the commercial varieties. Additionally, the levels of linoleic and linolenic acids were lower in KA than those of the commercial varieties. None of the fatty acids with the exception of linoleic and linolenic acids differed significantly between KA and three *PffAD3-1* transgenic lines.

Taken together, when compared to the corresponding KA values, only three analytes (calcium, linolenic and linoleic acids) out of 31 components differed significantly in the *PffAD3-1*. However, the calcium value in the *PffAD3-1* was within the range of both reference 1 and reference 2, indicating that it was within the range of natural variation. Statistically significant differences for linoleic and linolenic acids in the three *PffAD3-1* soybean lines compared to KA are attributed to the introduction of the *PffAD3-1* trait in KA (intended differences). Our data exhibited that the insertion site or insertion number of introduced gene did not make unintended differences for the measured components.

CONCLUSION

Thirteen Korean soybean varieties grown in three locations in South Korea during 2017 and 2018 were characterized for their natural variations in the seed levels of proximates, minerals, anti-nutrients, and fatty acids. Statistical analysis of combined data showed significant differences by variety and environment (location and cultivation year) for the measured components. Percent variability analysis demonstrated that genotype, environment and the interaction of environment with genotype contributed to the nutritional contents. PCA and OPLS-DA indicated that the significant variance in these compounds was attributable to cultivation site and cultivation year. Taken together, genotype, growth environment, and their interaction all exerted influences on these components. The results of these 13 soybean varieties could be used to expand conventional compositional data sets for future composition studies of GM soybean crops. Comparative compositional analysis of three *PffAD3-1* soybean lines was conducted with KA and three reference varieties cultivated at Jeonju and Gunwi during the 2019 growing season in South Korea. The values obtained from the 13 commercial soybean varieties reported earlier in the study were also used as reference data (reference 2). The results showed that the composition of proximates, minerals, anti-nutrients, and fatty acids in *PffAD3-1* soybean seeds is equivalent to that in conventional soybean varieties, exception with intended differences in linoleic and linolenic acids. Nevertheless, further nutrient composition studies of *PffAD3-1* in multiple growing regions and cultivation years is warranted in order to fully address its equivalence to the conventional soybean.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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