

RESEARCH PAPER



Compositional analysis of soybean event IND-ØØ41Ø-5

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ABSTRACT

Soybean (*Glycine max* L.) is the world's largest source of protein feed and the second largest source of vegetable oil. Water restriction is the main limiting factor to achieve maximum soybean yields. Therefore, development of varieties that maintain yield under environmental stresses is a major objective of soybean breeding programs. The *HaHB4* (*Helianthus annuus* homeobox 4) gene from sunflower encodes for a transcription factor involved in the plant's tolerance to environmental stress. The introduction of *HaHB4* in soybean led to the development of event IND-ØØ41Ø-5 (HB4[®] soybean), which displayed higher yield in environments having low productivity potential, compared with the parental control variety. Compositional analyses of soybean event IND-ØØ41Ø-5 were conducted both in Argentina and the United States. A total of 44 components were analyzed in grain and 9 components in forage. Based on the results of these studies it was concluded that soybean event IND-ØØ41Ø-5 was compositionally equivalent to its non-transgenic parental control.

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Introduction

Soybean is one of the most important crops worldwide. Processed soybeans are the world's largest source of protein feed and the second largest source of edible oil. The crop is grown on an estimated 6% of the world's arable land, and since the 1970s, the area allocated to soybean production has the highest percentage increase compared with any other major crop.¹ Combined, soybeans and their derivatives are the most traded agricultural commodities, accounting for over 10 percent of the total value of global agricultural trade.² At the current rates of soybean yield increase (1.3% per year), global production would be far below what is needed to meet projected demands in 2050.³ Furthermore, the increasing global population will need double the current food production by 2050 and, at the current rate it would achieve only ~55% of the estimated demand.³

Soybean yield is impacted by a variety of factors including regional genetic adaptability, diseases, insects, nutrient deficiencies, and others environmental stressors. Environmental stresses can be a major cause of yield reduction in soybean, affecting all production areas during a growing season.^{1,4–6}

Among the various environmental factors affecting the performance of the crop, water restriction is the major limitation leading to the failure to achieve maximum yields.^{7,8} In addition, it has been anticipated that water deficit, already a serious worldwide problem, is likely to increase as a consequence of climate change, further reducing arable land in rainfall-dependent regions.^{9–13} Against this background, the development of varieties that maintain yield under the broad array of environmental stresses, in particular under water restriction, is a major objective of soybean breeding programs.

To face environmental stress, plants have developed several response mechanisms including the activation of a variety of genes.¹⁴ The products of these genes are involved in self-protection to minimize the immediate consequences of unfavorable conditions as well as in adaptive response-signaling processes. Stress tolerant soybean varieties have been developed using conventional plant breeding methods. However, given the multigenic components involved in both yield and response to environmental stressors,^{15,16} efforts to develop stress tolerant genotypes by conventional breeding that can be

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applied across a variety of soybean production environments have met with limited success.¹⁷

Genetic engineering appears as an appropriate approach for the genetic improvement of soybean. Different single protein-based transgenesis approaches have been suggested over the last years. Among them, the expression of osmoprotectants, chaperones, transporters, membrane proteins and enzymes have been proposed¹⁸ but none of these products have reached the market yet. Considering that responses to environmental stress involve transcriptional regulation of several genes, transcription factors (TFs) appear as likely targets for engineering crops for stress tolerance.^{19,20} Following this line, some TF-based approaches have been published but they proved to have penalties under non-stressed conditions²¹ or have not reach the market yet.^{22,23}

The HD-Zip family of TFs, unique to the plant kingdom,^{24,25} display a set of distinctive physiological functions particularly related to developmental events in which abiotic factors generate stress. Specifically, the expression of genes of the HD-Zip subfamily I is regulated by external factors such as drought, extreme temperatures, osmotic stresses, and light conditions.^{24,25}

Expression of the *HaHB4* (*Helianthus annuus* homeobox 4), a member of the HD-Zip subfamily, provides increased tolerance to water deficit when expressed in *Arabidopsis*.²⁶ Consistent with this proof-of-concept, the introduction of the *HaHB4* gene led to expression of drought stress tolerance in wheat and soybean.^{27,28}

Similarly, *HaHB4*-expressing soybean also show a stress-tolerant phenotype. Field performance in different production environments allowed the selection of a soybean event (OECD Unique Identifier IND-ØØ41Ø-5) which increases yield under conditions of environmental stress with no penalty in high yield potential areas, suggesting a tight environment dependent HAHB4 regulation of the tolerance pathway.²⁷

From a food safety perspective, several relevant features result from the use of HAHB4 in soybean event IND-ØØ41Ø-5. First, the source of this protein (sunflower) has been in the food chain for a long time and therefore has a history of safe use. Second, HAHB4 acts as a transcriptional regulator of endogenous pathways, relaying on the natural physiology of the plant. Therefore, no

proteins or metabolites other than those of the natural plant are expressed in the transgenic event. Third, being a TF, HAHB4 is expressed at extremely low levels which makes its presence in foods a negligible safety risk. As expected from the low expression level of HAHB4 and from its mechanism of action,²⁹ the expression of this TF did not show significant grain and forage compositional changes when expressed in transgenic wheat.³⁰ The soybean event presented here also contains the *bar* gene from *Streptomyces hygroscopicus*, expressing the glufosinate-inactivating enzyme phosphinothricin N-acetyl transferase (PAT), which confers glufosinate herbicide tolerance. This protein has also a history of safe food use and shown not to modify the composition of the recipient plant.³¹

Assessment of the compositional equivalence between genetically engineered crops and their non-modified counterparts is mandatory for the food/feed safety evaluation in the regulatory approval process in many countries.^{32,33} Here, the results of the comparative compositional assessment of soybean event IND-ØØ41Ø-5 are presented.

Materials and Methods

Field Trials and Samples

Field trials were conducted in Argentina and the United States during the 2012 and 2013 growing seasons, at the major soybean production areas covering the diverse environmental conditions for the crop. Six locations in Argentina (in the Provinces of Buenos Aires, Córdoba, Entre Ríos, and Santa Fe) and five locations in the US (in the States of Illinois, Indiana, Iowa, Kansas, and Ohio) were chosen. A randomized complete block design with four replicate blocks was used in each trial. Entries were soybean event IND-ØØ41Ø-5, the near isogenic control variety Williams 82, and a set of commercial reference varieties already used by farmers and adapted for each site. These local varieties were used to estimate the natural compositional variability for the crop, giving the appropriate context for the interpretation of the experimental results in terms of their biological significance. They include: Biosoja 4.6 (Bioceres Semillas), DM 4670 and DM 4210 (Don Mario Semillas), SRM 3970 (Sursem), FN 3.85 (FN

Semillas), A 3731 RG and NS 4009 (Nidera Semillas), and SPS 3900 (Syngenta) for Argentine trials. Commercial reference varieties used in the United States were Dow 32R280 (Dow); Pioneer 93Y82, 93Y84, 93M94, and Dupont 93Y82 (DuPont); Asgrow (AG) 3832, Asgrow AG3931 (Monsanto); Stine 39LD02 (Stine Seeds); DynaGro 36RY38 (DynaGro); Hoffman H38-12CR2 (Hoffman Seed House); and NK S39-U2 (Syngenta Seeds). Forage and grain samples were taken at R3 (early pod development) and R8 stage (full maturity), respectively.

Statistical Analysis

The statistical analysis was conducted using SAS software (SAS Institute, Inc., Cary, NC) with the following model:

$$y_{ijk} = \mu + g_i + l_j + r_{k(j)} + (gl)_{ij} + e_{ijk}$$

$$l_j \sim iid N(0, \sigma^2 Location), r_{k(j)} \sim iid N(0, \sigma^2 Replicate),$$

$$(gl)_{ij} \sim iid N(0, \sigma^2 Location \sim Entry), \text{ and}$$

$$e_{ijk} \sim iid N(0, \sigma^2 Plot)$$

where y_{ijk} denotes the unique individual observation, μ denotes the overall mean, g_i denotes the mean of the i th entry, l_j denotes the effect of the j th location, $r_{k(j)}$ denotes the effect of the k th replicate within the j th location, $(gl)_{ij}$ denotes the interaction between the entries and locations, e_{ijk} denotes the effect of the plot assigned the i th entry in the k th replicate of the j th location (residual error), $\sim iid N(0, \sigma^2)$ indicates random variables that are identically and independently distributed (*iid*) as normal with zero mean and variance σ^2 .

Statistical significance for the genotype main effect was determined at an alpha level of 0.05 (5%) for each of the analytical measurements. Levels for each component in IND-ØØ41Ø-5 soybean were statistically compared to those measures in Williams 82. Mean values from the commercial varieties were calculated to establish the reference range of expected local variation. Additional reference ranges with data encompassing numerous variables (cultivar, country, environment) were taken from bibliography.^{34,35} Therefore, the biological relevance of the differences between IND-ØØ41Ø-5 and Williams 82 were analyzed considering the natural variation provided by both reference ranges.

Analytical Methods

Compositional analyses were conducted following the OECD guidelines.³⁵ Nutrients and micronutrients measured in grain (total 36 analytes) included proximates (moisture, protein, fat, ash, and carbohydrates), fiber (crude fiber, acid detergent fiber, ADF, and neutral detergent fiber, NDF), minerals (calcium and phosphorous), main fatty acids profile, vitamins (E and K1), and amino acid composition. Nutrients measured in forage (total 9 analytes) included proximates, fiber (ADF and NDF), and minerals (calcium and phosphorous). Anti-nutrients and other bioactive compounds measured in grain (8 in total) included isoflavones (daidzein, genistein, and glycitein), stachyose, raffinose, phytic acid, lectin, and trypsin inhibitors. All nutrients, anti-nutrients, and bioactive compounds for Argentina locations were measured at Melacrom Laboratories (Mercedes, Buenos Aires, Argentina) except lectin which was determined at Covance Laboratories (Madison, WI, United States). All nutrients, anti-nutrients, and bioactive compounds for locations in the US were measured at Covance Laboratories (Madison, WI, United States).

Proximate Analysis

Moisture was determined in grain and forage as the weight loss of samples heated for 72 hours at 130°C (AACC Method 44-15.02, Argentina sites) or dried in a vacuum oven at approximately 100°C (AOAC Methods 926.08 and 925.09, US sites). For ash content measurements, samples were incinerated in an oven at 585°C until constant weight (AOAC Method 923.03). Total protein nitrogen was determined through Kjeldahl analysis by digesting the sample in sulfuric acid-copper catalyst mixture (AACC Method 46-11A, Argentina sites) or with a similar process but using an instrument which automates the digestion, distillation, and titration processes (AOAC Method 979.09; AOCS Method Ac 4-91, US sites). The percent nitrogen was converted to equivalent protein using a factor of 6.25. Total fat in seeds was measured by Soxhlet extraction with diethyl ether (AACC Method 30-20.01, Argentina sites) or pentane (AOAC Methods 960.39 and 948.22, US sites). Total fat in forage was determined by first hydrolyzing the sample with hydrochloric acid, followed by extraction using ethyl ether and

hexane (AOAC Methods 922.06 and 954.02, US sites). Carbohydrates were calculated from the proximate analysis as the difference in percent weight using the following equation:

$$\begin{aligned} \% \text{ carbohydrates} &= 100\% \\ &- (\% \text{ protein} + \% \text{ fat} + \% \text{ moisture} + \% \text{ ash}) \end{aligned}$$

Acid Detergent Fiber (ADF)

The method used for samples from Argentina sites was AOAC Method 973.18. An acidified quaternary detergent solution was used to dissolve cell-solubles (hemicellulose and soluble minerals) leaving a residue of cellulose, lignin, damaged protein and a portion of cell wall protein, and minerals (ash). ADF was determined gravimetrically as the residue remaining after extraction with acetone (AOAC Method 973.18, Argentina sites). In the US, samples were placed in filter bags where fats and pigments are first extracted by acetone and then placed in an ANKOM Fiber Analyzer where the protein, carbohydrate, and ash contents were dissolved by boiling acidic detergent solution. Acid detergent fiber was determined gravimetrically after drying the residue.

Crude Fiber

Weighted samples were defatted by Soxhlet extraction and digested with sulfuric acid. The residue was then filtered, washed and digested with sodium hydroxide. The remaining residue, containing cellulose and lignin, was dried and weighed again. Crude fiber was calculated as the weight loss on ignition (AACC Method 32-10.01, Argentina sites). Crude fiber from US samples was quantified as the loss on ignition (2-3 hours at 600°C) of the dried residue remaining after digestion of the samples with 1.25% sulfuric acid and 1.25% sodium hydroxide solutions (AOAC Method 962.09).

Neutral Detergent Fiber

Samples were defatted by Soxhlet extraction, dried and washed with a neutral detergent solution. Extracted sample was rinsed with acetone to remove fat and pigments and the remaining residue was dried (AOAC Method 973.18, Argentina sites). An enzyme method was used for samples generated at

US sites. Fats and pigments were removed by an acetone wash from samples placed in filter bags. The filter bags were placed in an ANKOM Fiber Analyzer where the protein, carbohydrate, and ash content were dissolved in a boiling detergent solution at a neutral pH. Starch was removed via an alpha amylase treatment. Hemicellulose, cellulose, lignin, and insoluble protein fraction that were left in the filter bag were determined gravimetrically (AACC Method 32.20.01).

Minerals

Following conversion of the material into ash in a 500°C oven, residues were dissolved in nitric acid and analyzed by Inductively Coupled Plasma (ICP) emission spectroscopy. Minerals concentrations was determined by reading at 3179 Å and 2149 Å for calcium and phosphorus, respectively (AOAC Method 985.01, Argentina sites). AOAC Methods 984.27 and 985.01 were used for samples generated at US Sites. Samples were ashed overnight at 500°C, and then re-ashed with nitric acid, treated with hydrochloric acid, taken to dryness, and put into a hydrochloric acid solution. The amount of each element was determined by ICP emission spectrometry.

Vitamin E

Oil from soybean grains was extracted (Soxhlet) using hexane supplemented with butylated hydroxytoluene (BHT) to prevent oxidation. Alpha-tocopherol was quantified by HPLC and fluorescence detection (excitation 290 nm, emission 330 nm). For samples generated at US sites, samples were saponified to release vitamin E. The saponified mixtures were extracted with ethyl ether and tocopherols quantified by HPLC (AACC Method 86-06).

Vitamin K1

Samples were prepared by the addition of dimethyl sulfoxide and extraction with multiple portions of hexane. The combined hexane extracts were concentrated and reconstituted in dichloromethane and methanol. Analysis was done by reverse phase HPLC to separate the cis- and trans- vitamin K isomers. A post-column reduction assembly was used to produce a fluorescent derivative with detection at an

excitation wavelength of 243 nm and emission at 430 nm (Method USP 35-NF30 2012).

Fatty Acids Profile

Fat obtained from samples as described above was dissolved in hexane and saponified with potassium hydroxide in methanol. The fatty acid methyl esters (FAMES) were analyzed by gas chromatography (AOAC Method 996.06, Argentina Sites). For samples generated at US sites, lipids were extracted, saponified with sodium hydroxide and esterified with boron trifluoride in methanol. The resulting FAMES were extracted with heptane and analyzed by gas chromatography (AOCS Methods Ce 2-66 and Ce 1i-07).

Amino Acid Composition

After protein hydrolysis with 6 N hydrochloric acid, the amino acids were determined by derivatization with fluorenyl-methyl-oxycarbonyl chloride (FMOC-Cl) for proline and with orthophthalaldehyde/mercaptoethanol (OPA/ME) for the other amino acids. Amino acids were isolated and quantified using HPLC with a fluorescence detector and acetonitrile/water as organic and aqueous phase, respectively³⁶ (Argentina sites). Samples from US sites were hydrolyzed with 6 N hydrochloric acid with phenol added to prevent halogenation of tyrosine. Cystine and cysteine were converted to S-2-carboxyethylthiocysteine by the addition of dithiodipropionic acid. For the separated determination of tryptophan, proteins in the sample were hydrolyzed by heating in sodium hydroxide solution (AOAC Method 988.15). Samples were analyzed by HPLC after pre-injection derivatization with OPA and FMOC-Cl as indicated above.³⁶

Isoflavones Analysis

Grain samples were extracted with methanol/water (80:20, v/v) and heated at 65°C for 2 hr. The suspension was centrifuged and concentrated HCl was added to an aliquot of the supernatant. Aglycone content in the solution was analyzed by HPLC using an LC-ESI-MS/MS system with a tandem quadrupole detector (TQD). Targeted isoflavones

quantified were Daidzein, Glycitein and Genistein (AOAC Official Method 2001.10).

Lectin

The binding properties of soybean agglutinin (SBA, lectin) to N-acetylgalactosamine were utilized in an enzyme linked indirect sandwich assay.³⁷

Phytic Acid

Samples were extracted with trichloroacetic acid and centrifuged. A ferric chloride solution was added to an aliquot of the supernatant and boiled for phytate precipitation. Precipitate was dissolved in nitric acid, washed, diluted and potassium thiocyanate was added. The phytate ferric salt was measured colorimetrically at 480 nm. The phytate concentration was calculated using a 4:6 iron:phosphorus molar ratio³⁸ (Argentina sites). Samples generated at US sites were extracted using hydrochloric acid and sonication, purified using a silica-based anion exchange column, concentrated and injected onto a HPLC system with a refractive index detector.³⁹

Raffinose and Stachyose

Ground material was extracted with methanol, centrifuged, and the extracted sugars were collected. The procedure was repeated, and the supernatants combined and dried under vacuum. The product was dissolved in acetonitrile:water and analyzed by HPLC. The oligosaccharide content was measured with a refractive index detector⁴⁰ (Argentina Sites). For samples generated at US sites, sugars in the samples were extracted with a water:methanol solution. Aliquots were dried and reconstituted with a hydroxylamine hydrochloride solution in pyridine containing phenyl-β-D-glucopyranoside as the internal standard. The resulting oximes were converted to ester trimethylsilyl (TMS) derivatives, and then analyze by gas chromatography using a flame ionization detector.⁴⁰

Trypsin Inhibitors

Ground defatted (hexane extracted) samples were extracted with sodium hydroxide solution. Multiple dilutions and known amounts of trypsin and the substrate benzoyl-DL arginine-*p*-nitroanalide (BAPA)

hydrochloride were added, incubated, and quenched with acetic acid. The absorbance at 410 nm was measured in the filtered samples (AOCS Method Ba 12–75).

Results

Comparison of grain contents of proximates, ADF, NDF, crude fiber, minerals and vitamins has shown only one (Vitamin K1) statistically significant difference between the soybean event IND-ØØ41Ø-5 and the near-isogenic control variety Williams 82 (Table 1). However, the value for the event was within the range reported in the literature.^{34,35}

Significant differences were not found between the event IND-ØØ41Ø-5 and the control soybean for any of the six fatty acids measured (Table 2).

Analysis of the amino acids profile has shown only one (cysteine) statistically significant difference between the event IND-ØØ41Ø-5 and the non-transgenic control line Williams 82 (Table 3). However, the value for both genotypes fell within the range provided by both the reference varieties and the literature.^{34,35}

Data of the levels of anti-nutrients and other bioactive components showed five significant differences between soybean IND-ØØ41Ø-5 and Williams 82. These include phytic acid, stachyose, and the three isoflavones (Table 4). However, the values of all these analytes in IND-ØØ41Ø-5 soybean were within the range of the commercial reference varieties (Table 4).

No significant differences between IND-ØØ41Ø-5 and Williams 82 were found for the levels of any of the 9 analytes measured in forage (Table 5).

Table 1. Proximates, fiber, minerals and vitamins of soybean grain.

| Component ^a | IND-ØØ41Ø-5 Mean (SE) (Range) | Williams 82 Mean (SE) (Range) | Commercial Reference Range ^b | Literature Range ^c |
|-------------------------|----------------------------------|----------------------------------|---|-------------------------------|
| Ash | 5.69 (0.05) (5.20–6.36) | 5.68 (0.05) (5.03–6.42) | 4.83–6.35 | 3.9–7.0 |
| Carbohydrates | 35.84 (0.35) (32.27–41.52) | 35.19 (0.38) (32.16–40.97) | 31.46–38.11 | 29.6–50.2 |
| Moisture | 9.46 (0.18) (7.05–11.6) | 9.28 (0.18) (7.41–11.6) | 7.78–11.82 | 4.7–34.4 |
| Protein | 39.03 (0.30) (34.58–42.74) | 39.78 (0.24) (36.49–43.93) | 36.60–43.10 | 33.2–45.5 |
| Total Fat | 19.98 (0.19) (17.55–21.80) | 19.56 (0.28) (15.90–22.48) | 16.60–21.64 | 8.1–23.6 |
| Acid Detergent Fiber | 12.51 (0.40) (6.69–16.0) | 12.99 (0.34) (9.18–18.3) | 10.50–17.77 | 7.8–18.6 |
| Neutral Detergent Fiber | 16.88 (0.27) (14.30–21.23) | 16.83 (0.23) (13.80–21.16) | 14.10–18.07 | 8.5–21.3 |
| Crude Fiber | 7.35 (0.44) (3.21–12.50) | 7.74 (0.40) (4.66–13.20) | 4.61–13.60 | 4.12–18.5 ^d |
| Phosphorus | 0.56 (0.01) (0.35–0.69) | 0.57 (0.01) (0.38–0.68) | 0.36–0.61 | 0.50–0.94 |
| Calcium | 0.26 (0.01) (0.20–0.37) | 0.25 (0.01) (0.18–0.35) | 0.20–0.31 | 0.12–0.31 |
| Vitamin E | 1.87 (0.06) (0.11–2.78) | 1.81 (0.07) (0.95–2.93) | 1.37–3.13 | 0.19–6.17 |
| Vitamin K1 | 0.38 (0.02) * (0.31–0.91) | 0.43 (0.02) (0.31–0.61) | 0.44–0.85 | 0.06–1.76 ^d |

Numbers represent mean of 44 values measured in samples from field trials developed during 2012–2013 in 11 different locations, except for vitamin K1, which was only measured in the 20 samples from the 5 US trials.

^aResults are expressed as % dry weight, except for moisture (% fresh weight), vitamins E (mg/100 gr dwt) and K1 (mg/kg).

^bValues measured in commercial varieties grown in the same trials.

^cILSI values within OECD³⁵, unless otherwise indicated.

^dILSI Crop Composition database V7.0.³⁴

SE: standard error of the mean.

*Significant difference ($p < .05$).

Table 2. Fatty acid profile of soybean grain.

| Component ^a | IND-ØØ41Ø-5 Mean (SE) (Range) | Williams 82 Mean (SE) (Range) | Commercial Reference Range ^b | Literature Range ^c |
|------------------------|-------------------------------------|-------------------------------------|---|----------------------------------|
| Palmitic acid | 2.17 (0.03) (1.82–2.88) | 2.12 (0.03) (1.74–2.61) | 1.76–2.52 | 0.67–2.78 |
| Stearic acid | 0.85 (0.01) (0.68–1.02) | 0.84 (0.01) (0.62–1.06) | 0.61–1.15 | 0.28–1.13 |
| Oleic acid | 4.31 (0.07) (3.35–5.25) | 4.46 (0.10) (3.03–5.37) | 2.86–5.52 | 1.36–6.56 |
| Linoleic acid | 10.85 (0.10) (9.51–12.40) | 10.43 (0.14) (8.62–12.31) | 8.33–11.72 | 3.46–13.36 |
| Linolenic acid | 1.42 (0.02) (1.14–1.87) | 1.37 (0.02) (1.04–1.69) | 1.20–1.66 | 0.30–2.19 |
| Arachidic acid | 0.06 (0.00) (0.04–0.09) | 0.06 (0.00) (0.03–0.11) | 0.03–0.07 | 0.02–0.11 |

Numbers represent mean of 44 values measured in samples from field trials developed during 2012–2013 in 11 different locations.

^aResults are expressed as % of dry weight.

^bValues measured in commercial varieties grown in the same trials.

^cILSI values within OECD.³⁵

SE: standard error of the mean.

Discussion

Comparison of grain and forage composition between the transgenic event and the control demonstrated that the levels of most of the nutrients, micronutrients, anti-nutrients and other bioactive compounds were similar. In the few cases in which there were statistically significant differences between the event and the control, levels measured in IND-ØØ41Ø-5 soybean were either within the range of the reference varieties and/or the values reported in the literature, revealing that these differences were within the natural compositional variability of soybean.

Only few significant differences between IND-ØØ41Ø-5 soybean and its parental non-transgenic line Williams 82 were found. Particularly, some of the anti-nutrient (Table 4) showed an increase level in IND-ØØ41Ø-5 soybean when compared with the parental control Williams 82. These anti-nutrients, phytic acid, stachyose and the isoflavones, belong to 3 distinctive groups of compounds with quite different chemical structures, biosynthetic pathways, functions and modes of action. Therefore, it seems difficult to formulate a hypothesis implying an increase of all these anti-nutrients as a concerted, upregulated response associated to HAHB4 or PAT proteins expression. Moreover, considering that the levels of all

Table 3. Amino acid composition of soybean grain.

| Component ^a | IND-ØØ41Ø-5 Mean (SE) (Range) | Williams 82 Mean (SE) (Range) | Commercial Reference Range ^b | Literature Range ^c |
|------------------------|-------------------------------------|-------------------------------------|---|----------------------------------|
| Alanine | 1.85 (0.02) (1.57–2.14) | 1.86 (0.02) (1.65–2.17) | 1.63–2.10 | 1.51–2.10 |
| Arginine | 2.83 (0.04) (2.43–3.22) | 2.96 (0.02) (2.57–3.19) | 2.67–3.27 | 2.28–3.4 |
| Aspartic Acid | 4.51 (0.04) (4.04–5.07) | 4.56 (0.04) (4.04–5.08) | 4.17–4.91 | 3.81–5.12 |
| Cysteine | 0.55 (0.01) * (0.43–0.66) | 0.59 (0.01) (0.52–0.67) | 0.49–0.62 | 0.37–0.81 |
| Glycine | 1.73 (0.01) (1.59–2.00) | 1.69 (0.01) (1.53–1.83) | 6.28–7.41 | 1.46–1.99 |
| Glutamic Acid | 7.00 (0.05) (6.33–7.72) | 6.97 (0.05) (6.20–7.56) | 1.58–1.79 | 5.84–8.20 |
| Histidine | 1.00 (0.01) (0.85–1.19) | 1.01 (0.01) (0.84–1.13) | 0.90–1.10 | 0.87–1.17 |
| Isoleucine | 1.77 (0.02) (1.50–1.95) | 1.83 (0.01) (1.63–1.95) | 1.60–1.87 | 1.53–2.07 |
| Leucine | 3.02 (0.02) (2.62–3.30) | 3.02 (0.02) (2.70–3.26) | 2.80–3.15 | 2.59–3.62 |
| Lysine | 2.20 (0.05) (1.68–2.60) | 2.33 (0.02) (1.99–2.61) | 2.14–2.59 | 2.28–2.83 |
| Methionine | 0.52 (0.01) (0.44–0.60) | 0.52 (0) (0.44–0.57) | 0.45–0.55 | 0.43–0.68 |
| Phenylalanine | 1.96 (0.02) (1.56–2.18) | 1.99 (0.02) (1.67–2.19) | 1.77–2.20 | 1.63–2.34 |
| Proline | 2.00 (0.02) (1.69–2.37) | 2.01 (0.02) (1.70–2.30) | 1.85–2.29 | 1.68–2.28 |
| Serine | 1.94 (0.02) (1.64–2.33) | 2.03 (0.01) (1.78–2.24) | 1.80–2.19 | 1.10–2.48 |
| Threonine | 1.54 (0.02) (1.30–1.69) | 1.50 (0.02) (1.34–1.71) | 1.35–1.64 | 1.14–1.86 |
| Tryptophan | 0.50 (0.01) (0.34–0.61) | 0.52 (0.01) (0.40–0.62) | 0.41–0.60 | 0.36–0.50 |
| Tyrosine | 1.38 (0.03) (1.04–1.63) | 1.39 (0.03) (1.03–1.62) | 1.03–1.61 | 1.01–1.61 |
| Valine | 1.83 (0.02) (1.60–2.07) | 1.88 (0.01) (1.69–2.17) | 1.71–2.10 | 1.59–2.20 |

Numbers represent mean of 44 values measured in samples from field trials developed during 2012–2013 in 11 different locations.

^aResults are expressed as % of dry weight.

^bValues measured in commercial varieties grown in the same trials.

^cILSI values within OECD.³⁵

SE: standard error of the mean.

*Significant difference ($p < .05$).

these anti-nutrients in IND-ØØ41Ø-5 are within soybean natural variability, these differences would not have biological significance but still support the conclusion of the compositional equivalence of the transgenic event with conventional soybean.

When analyzed within the context of the natural variability provided by the commercial varieties cultivated along in the test sites and the range of values reported in the literature, it can be concluded that the transgenic event IND-ØØ41Ø-5 is compositionally equivalent to conventional soybean.

Table 4. Anti-nutrients and isoflavones composition of soybean grain.

| Component ^a | IND-ØØ41Ø-5 Mean (SE) (Range) | Williams 82 Mean (SE) (Range) | Commercial Reference Range ^b | Literature Range ^c |
|--------------------------------|-------------------------------|-------------------------------|---|-------------------------------|
| Phytic acid | 1.67 (0.10)* (0.62–3.09) | 1.35 (0.04) (0.68–1.88) | 0.54–1.69 | 0.63–1.96 |
| Lectins (mg/g) | 4.78 (0.13) (2.43–6.34) | 4.73 (0.15) (3.02–7.03) | 1.29–6.09 | 0.11–9.04 |
| Raffinose | 0.88 (0.03) (0.55–1.39) | 0.85 (0.02) (0.70–1.09) | 0.64–1.20 | 0.21–0.66 |
| Stachyose | 3.77 (0.07)* (2.50–4.85) | 3.39 (0.08) (2.27–4.32) | 2.56–4.76 | 1.21–3.50 |
| Trypsin Inhibitor ^d | 35.04 (1.78) (18.60–60.30) | 33.46 (1.60) (19.30–56.10) | 18.60–56.1 | 19.59–118.68 |
| Daidzein | 1240 (53)* (497–1870) | 1086 (48) (462–1700) | 533 – 2150 | 60.0–2453.5 |
| Genistein | 1402 (64)* (518–2130) | 1282 (61) (515–2060) | 671 – 2290 | 144.3–2837.2 |
| Glycitein | 276 (11)* (133–412) | 239 (8) (123–344) | 126 – 344 | 15.3–310.4 |

Numbers represent mean of 44 values measured in samples from field trials developed during 2012–2013 in 11 different locations.

^aResults are expressed as % of dry weight, except for lectins (mg/g), trypsin inhibitor units (TIU/mg dwt) and isoflavones (ppm dwt).

^bValues measured in commercial varieties grown in the same trials.

^cILSI values within OECD.³⁵

^dTIU = trypsin inhibitor units.

SE: standard error of the mean.

*Significant difference ($p < .05$)

Table 5. Proximates, fiber and minerals of soybean forage.

| Component ^a | IND-ØØ41Ø-5 Mean (SE) (Range) | Williams 82 Mean (SE) (Range) | Commercial Reference Range ^b | Literature Range ^c |
|-------------------------|-------------------------------|-------------------------------|---|-------------------------------|
| Ash | 9.12 (0.28) (6.43–15.90) | 9.04 (0.37) (6.50–20.50) | 6.96–19.10 | 6.71–10.78 |
| Carbohydrates | 49.76 (2.69) (30.43–75.40) | 50.54 (2.67) (30.72–77.30) | 32.40–73.90 | 27.8–80.6 |
| Moisture | 76.72 (0.72) (65.31–85.50) | 76.94 (0.67) (65.32–85.60) | 64.30–84.20 | 73.5–81.6 ^d |
| Protein | 20.85 (0.44) (14.80–26.90) | 20.68 (0.48) (13.70–29.20) | 15.60–24.70 | 14.37–24.71 |
| Total Fat | 2.46 (0.13) (1.15–4.70) | 2.47 (0.12) (1.32–4.33) | 1.38–3.48 | 1.30–5.13 |
| Acid Detergent Fiber | 33.08 (0.65) (24.50–42.50) | 33.17 (0.55) (27.30–41.20) | 20.30–36.78 | 12.85–64.10 ^d |
| Neutral Detergent Fiber | 41.64 (1.01) (29.50–52.40) | 41.87 (0.98) (26.30–53.70) | 25.60–52.30 | 19.26–82.00 ^d |
| Phosphorus | 0.25 (0.01) (0.20–0.35) | 0.26 (0.01) (0.18–0.35) | 0.21–0.37 | NA ^e |
| Calcium | 1.21 (0.02) (1.03–1.56) | 1.26 (0.03) (0.96–1.58) | 0.97–1.51 | NA |

Numbers represent mean of 44 values measured in samples from field trials developed during 2012–2013 in 11 different locations.

^aResults are expressed as % dry weight, except for moisture (% fresh weight).

^bValues measured in commercial varieties grown in the same trials.

^cILSI values within OECD³⁵, unless otherwise indicated.

^dILSI Crop Composition database V7.0.³⁴

^eNot available.

SE: standard error of the mean.

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Authors are or were all affiliated to INDEAR (a controlled subsidiary of Bioceres), which developed the soybean transgenic

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References

- Hartman GL, West ED, Herman TK. Crops that feed the World 2. Soybean—worldwide production, use, and constraints caused by pathogens and pests. *Food Secur.* 2011;3:5–17. doi:10.1007/s12571-010-0108-x.
- Lee T, Tran A, Hansen J, Ash M. Major factors affecting global soybean and products trade projections. Washington (USA): United States Department of Agriculture, Economic Research Service; 2016.
- Ray DK, Mueller ND, West PC, Foley JA. Yield trends are insufficient to double global crop production by 2050. *PLoS One.* 2013;8:e66428. doi:10.1371/journal.pone.0066428.
- Bray E, Bailey-Serres J, Weretilnyk E. Responses to abiotic stresses. In: Buchannan B, Gruissem W, Jones R, editors. *Biochemistry and molecular biology of plants*, first. Rockville (MD): American Society of Plant Physiologists; 2000. p. 1158–1203.
- Duque AS, de Almeida AM, Bernardes da Silva A. Abiotic stress responses in plants: unraveling the complexity of genes and networks to survive. In: Vahdati K, Leslie C, editors. *Abiotic stress - plant responses and applications in agriculture*. Rijeka (Croatia): InTech; 2013. p. 418.
- Ku Y-S, Au-Yeung W-K, Yung Y-L, Li M-W, Wen C-Q, Lam H-M. Drought stress and tolerance in soybean. In: Board JE, editor. *A comprehensive survey of international soybean research. Genetics, physiology, agronomy and nitrogen relationships*. 1st ed. London (UK): IntechOpen; 2013. p.209–237.
- Casagrande EC, Farias JRB, Neumaier N, OYA T, PEDROSO J, MARTINS PK, BRETON MC, NEPOMUCENO AL. Expressão gênica diferencial durante déficit hídrico em soja. *Rev Bras Fisiol Veg.* 2001;13:168–184. doi:10.1590/S0103-3131200100020006.
- Lesk C, Rowhani P, Ramankutty N. Influence of extreme weather disasters on global crop production. *Nature* [Internet] 2016 [Cited 2020 Feb 17]; 529:84–87.
- Ahuja I, de Vos RCH, Bones AM, Hall RD. Plant molecular stress responses face climate change. *Trends Plant Sci.* 2010;15:664–674. doi:10.1016/j.tplants.2010.08.002.
- Elliott J, Deryng D, Müller C, Frieler K, Konzmann M, Gerten D, Glotter M, Flörke M, Wada Y, Best N, et al. Constraints and potentials of future irrigation water availability on agricultural production under climate change. *Proc Natl Acad Sci U S A.* 2014;111:3239–3244. doi:10.1073/pnas.1222474110.
- Fernandes ECM, Soliman A, Confalonieri R, Donatelli M, Tubiello F. Climate change and agriculture in Latin America, 2020-2050: projected impacts and response to adaptation strategies. Washington: The World Bank; 2012.
- Kadam NN, Xiao G, Melgar RJ, Bahuguna RN, Quinones C, Tamilselvan A, Prasad PVV, Jagadish KSV. Agronomic and physiological responses to high temperature, drought, and elevated CO2 interactions in cereals. *Adv Agron.* 2014;127:111–256. doi:10.1016/B978-0-12-800131-8.00003-0.
- Vergara W, Rios AR, Trapido P, Malarín H. Agriculture and future climate in Latin America and the caribbean: systemic impacts and potential responses. Washington: Inter-American Development Bank; 2014.
- Zhao T, Aleem M, Sharmin R. Adaptation to water stress in soybean: morphology to genetics. [Internet]. In: Andjelkovic V, editor. *Plant, abiotic stress and responses to climate change*. London (UK): IntechOpen; 2018. p. 33–68. Available from: <https://www.intechopen.com/books/plant-abiotic-stress-and-responses-to-climate-change/adaptation-to-water-stress-in-soybean-morphology-to-genetics>. doi:10.5772/intechopen.72229
- Guimarães-Dias F, Neves-Borges AC, Viana AAB, Mesquita RO, Romano E, Grossi-de-Sá MDF, Nepomuceno AL, Loureiro ME, Alves-Ferreira M. Expression analysis in response to drought stress in soybean: shedding light on the regulation of metabolic pathway genes. *Genet Mol Biol.* 2012;35:222–232. doi:10.1590/S1415-47572012000200004.
- Hussain RM, Ali M, Feng X, Li X. The essence of NAC gene family to the cultivation of drought-resistant soybean (*Glycine max* L. Merr.) cultivars. *BMC Plant Biol.* 2017;17:55. doi:10.1186/s12870-017-1001-y.
- Sinclair TR. Challenges in breeding for yield increase for drought. *Trends Plant Sci.* 2011;16:289–293. doi:10.1016/j.tplants.2011.02.008.
- Valliyodan B, Nguyen HT. Understanding regulatory networks and engineering for enhanced drought tolerance in plants. *Curr Opin Plant Biol.* 2006;9:189–195. doi:10.1016/j.pbi.2006.01.019.
- Host R, David A, Ceasar SA, Ignacimuthu S. Genetic engineering of crop plants for drought tolerance: role

- of transcription factors. *South Indian J Biol Sci.* **2016**;2:272–286. doi:10.22205/sijbs/2016/v2/i2/100317.
20. Wang H, Wang H, Shao H, Tang X. Recent advances in utilizing transcription factors to improve plant abiotic stress tolerance by transgenic technology. *Front Plant Sci.* **2016**;7:67. doi:10.3389/fpls.2016.00067.
 21. Skirycz A, Vandenbroucke K, Clauw P, Maleux K, De Meyer B, Dhondt S, Pucci A, Gonzalez N, Hoerberichs F, Tognetti VB, et al. Survival and growth of Arabidopsis plants given limited water are not equal. *Nat Biotechnol.* **2011**;29(3):212–214. doi:10.1038/nbt.1800.
 22. Fuganti-Pagliarini R, Ferreira LC, Rodrigues FA, Molinari HBC, Marin SRR, Molinari MDC, Marcolino-Gomes J, Mertz-Henning LM, Farias JRB, de Oliveira MCN, et al. Characterization of soybean genetically modified for drought tolerance in field conditions. *Front Plant Sci.* **2017**;8:448. doi:10.3389/fpls.2017.00448.
 23. Shi W-Y, Du Y-T, Ma J, Min D-H, Jin L-G, Chen J, Chen M, Zhou Y-B, Ma Y-Z, Xu Z-S, et al. The WRKY transcription factor GmWRKY12 confers drought and salt tolerance in soybean. *Int J Mol Sci.* **2018**;19(12):4087. doi:10.3390/ijms19124087.
 24. Ariel FD, Manavella PA, Dezar CA, Chan RL. The true story of the HD-Zip family. *Trends Plant Sci.* **2007**;12:419–426. doi:10.1016/j.tplants.2007.08.003.
 25. Chan RL. The use of sunflower transcription factors as biotechnological tools to improve yield and stress tolerance in crops. *Phyt Int J Exp Bot.* **2009**;78:5–10.
 26. Dezar CA, Gago GM, Gonzalez DH, Chan RL. Hahb-4, a sunflower homeobox-leucine zipper gene, is a developmental regulator and confers drought tolerance to Arabidopsis thaliana plants. *Transgenic Res.* **2005**;14:429–440. doi:10.1007/s11248-005-5076-0.
 27. González FG, Capella M, Ribichich KF, Curín F, Giacomelli JJ, Ayala F, Watson G, Otegui ME, Chan RL. Field-grown transgenic wheat expressing the sunflower gene *HaHB4* significantly outyields the wild type. *J Exp Bot.* **2019**;70:1669–1681. doi:10.1093/jxb/erz037.
 28. Ribichich KF, Chiozza M, Ávalos-Britez S, Cabello JV, Arce AL, Watson G, Arias C, Portapila M, Trucco F, Otegui ME, et al. Successful field performance in dry-warm environments of soybean expressing the sunflower transcription factor HaHB4. *J Exp Bot.* **2020**. doi:10.1093/jxb/eraa064.
 29. Delaney B, Astwood JD, Cunny H, Conn RE, Herouet-Guicheney C, MacIntosh S, Meyer LS, Privalle L, Gao Y, Mattsson J, et al. Evaluation of protein safety in the context of agricultural biotechnology. *Food Chem Toxicol.* **2008**;46:S71–S97. doi:10.1016/j.fct.2008.01.045.
 30. Ayala F, Fedrigo GV, Burachik M, Miranda PV. Compositional equivalence of event IND-ØØ412-7 to non-transgenic wheat. *Transgenic Res.* **2019**;28:165–176. doi:10.1007/s11248-019-00111-y.
 31. Cromwell GL, Henry BJ, Scott AL, Gerngross MF, Dusek DL, Fletcher DW. Glufosinate herbicide-tolerant (LibertyLink) rice vs. conventional rice in diets for growing-finishing swine. *J Anim Sci.* **2005**;83:1068–1074. doi:10.2527/2005.8351068x.
 32. Kuiper HA, Kleter GA, Noteborn HP, Kok EJ. Assessment of the food safety issues related to genetically modified foods. *Plant J.* **2001**;27:503–528. doi:10.1046/j.1365-313X.2001.01119.x.
 33. Privalle LS, Gillikin N, Wandelt C. Bringing a transgenic crop to market: where compositional analysis fits. *J Agric Food Chem.* **2013**;61:8260–8266. doi:10.1021/jf400185q.
 34. ILSI. Crop composition database. **2019** [accessed May 8]. <https://www.cropcomposition.org/>
 35. OECD. Revised consensus document on compositional considerations for new varieties of soybean [Glycine Max (L.) Merr.]: key food and feed nutrients, antinutrients, toxicants and allergens. ENV/JM/MONO(2012)24. 48. Paris: Organization for Economic Co-operation and Development; **2012**.
 36. Zhou W, Zhanga X-Y, Duana G-L. Liquid-chromatography quantitative analysis of 20 amino acids after derivatization with FMOC-Cl and its application to different origin radix isatidis. *J Chinese Chem Soc.* **2011**;58:509–515. doi:10.1002/jccs.201190014.
 37. Wang T-H, Lee M-H, Su N-W. Screening of lectins by an enzyme-linked adsorbent assay. *Food Chem.* **2009**;113:1218–1225. doi:10.1016/j.foodchem.2008.08.024.
 38. Wheeler EL, Ferrel RE. A method for phytic acid determination in wheat and wheat fractions. *Cereal Chem.* **1971**;48:312–320.
 39. Lehrfeld J. HPLC separation and quantitation of phytic acid and some inositol phosphates in foods: problems and solutions. *J Agric Food Chem.* **1994**;42:2726–2731. doi:10.1021/jf00048a015.
 40. Muzquiz M, Hill GD, Cuadrado C, Pedrosa MM, Burbano C, editors. Recent advances of research in antinutritional factors in legume seeds and oilseeds. The Netherlands: Wageningen Academic Publishers; **2004**.
 41. Mason BS, Slover HT. Gas-chromatographic method for the determination of sugars in foods. *J Agric Food Chem.* **1971**;19:551–554. doi:10.1021/jf60175a006.