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Development of soybean experimental lines with enhanced protein and sulfur amino acid content

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ABSTRACT

Soybean is the preferred protein source for both poultry and swine feed. However, this preferred status is being challenged due to competition from alternative feed ingredients. To overcome this, it becomes necessary for breeders to develop soybean cultivars that contain higher protein and better nutritional composition. In this study, we have developed experimental soybean lines that not only contain significantly higher amounts of protein but also improved sulfur amino acid content. This objective was achieved by crossing a O-acetylserine sulfhydrylase (OASS) overexpressing transgenic soybean line with elevated levels of sulfur amino acid content (CS) with a high protein Korean soybean cultivar (Lee 5). Introgression of high protein and overexpression of OASS was monitored in the experimental lines at each successive generation (F2-F6) by measuring protein content and OASS activity. The average protein content of transgenic CS and Lee 5 seeds were 34.8 % and 44.7 %, while in the experimental soybean lines the protein content ranged from 41.3 %–47.7 %, respectively. HPLC and inductively coupled plasma-mass spectrometry analyses revealed that all the experimental lines developed in this study contained significantly higher amounts of sulfur containing amino acids and elemental sulfur in the seeds. The sulfur amino acid (cysteine + methionine) content of the experimental lines ranged from 1.1 % to 1.26 % while the parents Lee 5 and CS had 0.79 % and 1.1 %, respectively. SDS-PAGE and western blot analysis demonstrated that the accumulation of Bowman-Birk protease inhibitor and lunasin, two sulfur amino acid rich peptides, were elevated in experimental soybean lines. High-resolution 2D-gel electrophoresis and Delta2D gel analysis validated that an overall increase in the different subunits of 7S β-conglycinin and 11S glycinin were mainly responsible for the observed increase in the total amount of protein in experimental lines.

1. Introduction

Soybean is an excellent source of protein for both humans and livestock. In a commercial cultivar, soybean seed contains about 36–40 % crude protein and 18–21 % oil. A vast majority of soybean protein is processed into soybean meal and used for livestock feed, especially for poultry and swine [1]. Despite the large protein content, the nutritional quality of soybean protein remains suboptimal due to lower concentrations of sulfur containing amino acids cysteine and methionine [2–4]. Sulfur containing amino acids are critical in the human diet [5]. Methionine is an essential amino acid and cysteine is a semi-essential amino acid that can be converted from methionine. Neither amino acid can be synthesized in mammals' bodies, thus mammals require a diet rich in both cysteine and methionine to grow and develop properly. A deficiency of cysteine and methionine in feeding meal causes a lack of physical growth and susceptibility to some diseases [5]. Soybean meal is a good choice for livestock and poultry feeding because of its relatively high protein content. However, a proper animal diet must include all nutrition that is required for growth and development. The minimum requirement of these two amino acids for monogastric animals is 3.5 g per 100 g of protein [6]. Therefore, a mixture of corn with synthetic methionine is added to soybean to maintain the optimal growth and

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development of poultry and livestock.

Due to the importance of soybean protein in animal feed, increasing sulfur containing amino acid content in soybean has been a critical goal for breeders to achieve [7]. Several QTLs have been used to improve the efficiency of selecting soybean cultivars with improved methionine and cysteine content [3,8–12]. Even though several QTL and candidate alleles associated with amino acid content have been reported, they have not resulted in the commercial development of soybean cultivars with improved sulfur amino acid content.

Several attempts to increase sulfur containing amino acids in soybean were applied by expressing heterologous methionine-rich proteins such as 2S albumin from Brazil nut and δ and γ-zein protein from maize in soybean [2,4]. However, the overall improvement in methionine content for these transgenic soybeans has been modest because of the insufficient accumulation of heterologous protein in soybean seed [4]. This accumulation represents about 1 % of the total seed weight in the transgenic soybean [13–15]. Moreover, in many cases, the accumulation of the heterologous protein is not significant when compared to the endogenous soybean seed storage proteins. Therefore, improving the sulfur containing amino acid profile using this transgenic approach requires improving the accumulation of heterologous sulfur-rich protein in soybean seed [4].

Another approach to improve soybean nutritional value is to manipulate some of the key enzymes involved in the sulfur assimilatory pathway to increase cysteine and methionine contents of soybean seed. Manipulation of key enzymes in sulfur assimilatory and methionine biosynthesis pathway has resulted in the improvement of overall sulfur amino acid content in soybean and other plants [4]. Transgenic soybean plants that overexpress a cytosolic isoform of O-acetylserine sulfhydrylase (OASS) exhibited a four to tenfold increase in OASS, resulting in a significant increase in the cysteine level: about 58–74 % in protein-bound cysteine [16]. Furthermore, these transgenic soybeans overexpressing OASS showed a noticeable increase in the accumulation of Bowman–Birk protease inhibitor (BBI), a cysteine-rich protein. The overall increase in soybean total cysteine content satisfies the recommended levels required for the optimal growth of monogastric animals [16].

In addition to protein quality the recent trend of lower levels of protein concentration among soybean cultivars possess an additional challenge. In the past, soybean breeders have mainly focused on increasing the yield. This has resulted in a gradual decrease in the overall protein content due to the existence of a negative correlation between yield and seed protein concentration [12]. Hence, there is a critical need to develop soybean cultivars with relatively higher levels of protein with improved nutritional quality. One of the major concerns for soybean quality is the sulfur containing amino acid deficiency in the soybean seed. In this study, we report the development of soybean experimental lines with elevated protein content along with improved cysteine and methionine profiles.

2. Materials and methods

2.1. Plant material and population development

The experimental lines were developed by crossing 'CS' and 'Lee 5'. The transgenic line CS [16], which overexpresses a sulfur assimilatory enzyme O-acetylserine sulfhydrylase (OASS), has a 'Maverick' cultivar background. This line was used as a high cysteine content parent and Lee 5, the high protein Korean soybean line, was the second parent. The parents were crossed in summer 2014 at Bradford Research Center at the University of Missouri, Columbia. A total of 19 F_1 seeds were produced. The $F_{1\text{-}2}$ generation were grown at Bradford Research Center in spring 2015 for seed increase. The F_2 population confirmed that each F_1 plant was the product of cross-pollination with segregation for flower color and an increase in the OASS activity as expected. In summer 2016, 10 $F_{2\text{-}3}$ progenies were grown in Bradford Research Center, and five individual plants were randomly tested for OASS activity in each progeny row during the summer. In fall 2016, F_3 seeds were harvested and threshed individually for further selection based on their protein content and OASS activities. In summer 2017, 27 F_3 progeny rows were grown in a completely randomized design at Bradford Research Center, and 16 individuals were selected based on their protein content and OASS activities. In summer 2018, the selected 16 individuals of F_4 were grown in single-row plots in randomized complete block design with three replicates. Samples were harvested and threshed individually for further analysis.

A back cross was made in summer 2015 while the plants were growing at Bradford Research Center with 9 F_1 plants from the single cross between CS and Lee 5. These seeds were grown in fall 2015 at Sears Greenhouse and tested for OASS activities for selection. Five individual seeds were carrying the transgene and were selected to advance. BC_1F_2 plants were grown in summer 2016 at Bradford Research Center and harvested individually in fall 2017. In summer 2017, BC_1F_3 seeds were grown at Bradford Research Center and summer 2018, BC_1F_4 seeds were grown at Bradford Research Center and harvested individually in Fall 2018 for further analysis.

2.2. Protein and oil analyses

Protein analysis of early generations (F_2- F_4) of soybean was accomplished using a LECO truSpec model FP-428 nitrogen analyzer (St. Joseph, MI, USA). Two hundred milligrams of soybean seed powder from each sample were combusted at 900 °C in a chamber in the presence of oxygen, which resulted in the release of carbon dioxide, water, and nitrogen from the sample. The gases were then passed over a potassium hydroxide aqueous solution to eliminate carbon dioxide and water. The sample was then subjected to a thermal conductivity detector in a column to separate the nitrogen from any carbon dioxide and water residual, and the remaining nitrogen content was measured. The instrument was calibrated using ultrapure EDTA of known nitrogen content. The protein content of the seeds was inferred from its nitrogen content using a protein correction factor of 6.25. Three separate biological replicates were evaluated per line, and the results were compared using standard deviation from the mean [17].

Oil analysis of $F_{2\text{-}4}$ seeds was accomplished using an Oxford Instruments America MQC Oilseeds Analyzer with a 26 mm probe (Concord, MA, USA). A 15-point calibration curve was built using data obtained from both pure soybean oil and soybean seeds of known oil content ($11 - 21$ %) as determined by the University of Missouri Experiment Station Chemical Laboratories (AOCS Official Method Ca 5b-71). Three separate biological replicates were evaluated per line, and the results were compared using standard deviation from the mean [17].

Protein and oil analysis for F_5 were determined by scanning the samples on a NIRS monochromator model FOSS 6500 (FOSS North America, Eden Prairie, MN) using a transport quarter cup (dimension 97 mm × 55 mm). The reflectance (R) spectra were collected at 2 nm intervals in the NIRS region of 400–2500 nm at room temperature. Calibrations previously developed by FOSS were used to estimate moisture content.

2.3. Seed protein extraction

The soybean protein was extracted from soybean seed powder using three different methods: total seed protein was extracted using SDS-sample buffer, lunasin was extracted with 30 % ethanol [18], and Bowman-Birk protease inhibitor (BBI) was extracted with 50 % isopropanol [19]. For seed total protein extraction, 10 mg of seed powder was placed in a 2-mL Eppendorf tube and extracted with 1 mL of sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-HCl, 2 % SDS, 10 % glycerol, 30 nM Bromophenol Blue, pH 6.8, 2 % (v/v) β-mercaptoethanol). First, the solution was placed in a vortex at room temperature for 10 min. Then, it was subjected to centrifugation at 15,800xg for 10 min.

Finally, the supernatant was collected.

Lunasin enriched fraction was obtained by extracting the protein from 50 mg of seed powder with 1 mL of ethanol and placing the tube in the shaker for 30 min at room temperature. The slurry was centrifuged at 15,800xg for 10 min. An aliquot of 300 μL of the clear supernatant was transferred to a new tube, and 20 μL of 100 mM calcium chloride was added. This solution was thoroughly mixed and left on the bench top for 5 min then centrifuged at 15,800xg for 10 min. The resulting pellet was air-dried and resolved in 300 μL of 1X SDS-sample buffer. BBI was obtained by extracting 50 mg of seed powder with 1 mL of 50 % isopropanol and placing the tube in a shaker for 30 min at room temperature. The slurry was centrifuged at 15,800xg for 10 min. A 500 μL of the clear supernatant after centrifugation was placed in a 2-mL and 1.5 mL acetone was added and placed at −20 °C for 15 h. The solution was then centrifuged at 15,800xg for 10 min. The resulting pellet was air-dried and resolved in 200 μL of 1X SDS-sample buffer. After all protein extractions were complete, all samples were heated in a boiling water bath for 5 min before further analysis.

2.4. One-dimensional gel electrophoresis

For 1-D gel electrophoresis separation, samples were loaded onto 10 % or 15 % resolving gels using the Mini250 apparatus (GE Healthcare, Piscataway, NJ, U.S.A.). Separation was achieved with a constant 20 mA per gel and a typical run time of 1.2 h. Gels were removed from the cassette and placed immediately in 0.1 % Coomassie Blue R-250 staining solution. Images of Coomassie stained gels were obtained with an Epson V700 scanner, and images were scanned at resolution of 600 dpi. Gel Analyzer software was used to analyze the images and quantify bands.

2.5. Two-dimensional gel electrophoresis

Isolation of soybean proteins and their fractionations on 2-D gels were performed as previously described [20]. For isoelectric focusing, 300 μg of extracted protein based on equal dry bases of seed powder from the samples were loaded per strip using overnight in-gel rehydration.

2.6. Immunoblotting analysis

Soybean protein samples were first resolved on 10 % and 15 % SDS-PAGE gels as described in the one-dimensional gel electrophoresis process. The resolved proteins were electrophoretically transferred to nitrocellulose membranes for an hour. Following this, the nitrocellulose membrane was incubated with 3 % dry milk powder dissolved in Tris-buffered saline (TBS; pH 7.5) for 1 h at 25 °C with gentle shaking. Antibodies against soybean OASS, BBI, the β-subunit of β-conglycinin, and lunasin have been previously reported [18,21,22]. The nitrocellulose membranes were incubated with β-conglycinin (1:40,000 dilution), BBI (1:10,000 dilution), lunasin (1:10,000 dilution), and OAAS (1:10,000 dilution) antibodies in TBS containing 3 % dry milk powder for 15 h. Nonspecific binding was eliminated by washing the membrane four times (10 min each wash) with TBS containing 0.05 % Tween-20 (TBST). The membrane-bound antibodies were detected by incubating the nitrocellulose membrane with 1:20,000 of goat anti-rabbit IgG–horseradish peroxidase conjugate antibody (Bio-Rad) for 1 h. Then, the membrane was washed three times in the same TBST noted above. Immunoreactive polypeptides were visualized by incubation of the membrane with an enhanced chemiluminescent substrate (Super Signal West Pico Kit; Pierce Biotechnology, Rockford, IL).

2.7. O-acetylserine sulfhydrylase assay

Leaf samples collected from young seedlings (6 weeks after germination) were used to measure the OASS activity according to the ninhydrin method [23]. Protein extracts were obtained by grinding 50 mg of leaf samples in a chilled mortar and pestle with 1 mL of ice-cold extraction buffer [100 mM Tris–HCl pH 8.0, 100 mM KCl, 20 mM $MgCl_2$, 0.5 % Tween 80 and 10 mM dithiothreitol (DTT)]. The samples were transferred to microcentrifuge tubes and centrifuged at 4 °C for 10 min at 12,000 *g*. The clear supernatant was saved and used immediately to measure the OASS activity. The protein concentration from plant extracts was determined spectrophotometrically using a Coomassie Plus Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). Each assay was repeated three times.

2.8. Mineral analysis

Mineral analysis was performed at Donald Danforth Plant Science Center. Inductively coupled plasma-mass spectrometry was performed as previously described [24]. Individually weighed dry seeds were digested overnight in 2.5 mL of concentrated HNO_3 at room temperature before the samples were heated for 2 h to 105 °C and then cooled to room temperature over 2 h. Following this step, the samples were diluted with ultrapure water and transferred to 96-well autosampler plates. A PerkinElmer Elan DRC-e inductively coupled plasma-mass spectrometer (Waltham, MA, USA) with an Apex Desolvation Nebulizer, a FAST sampling valve, and an Elemental Scientific SC4 DX autosampler (Omaha, NE, USA) was used to analyze the sulfur content. A liquid reference material composed of pooled samples of soybean digests was run every ninth sample to correct for ICP-MS run-to-run variation and within-run drift. All samples were normalized to the recorded dry weights.

2.9. Amino acid analysis

Amino acid analysis was performed at the University of Missouri Agriculture Experiment Station Chemical Laboratories, University of Missouri, Columbia, MO. Briefly, two technical replications of soybean F_5 dry seed lines, which were grown in Summer 2018 at Bradford Research Center, were ground to a fine powder. Two hundred milligrams of the seed powder were subjected to hydrolysis for 16 h at 155 °C in 6.0 N HCl. Amino acids were separated on a Beckman 6300 Amino Acid Analyzer (Beckman Instruments, Fullerton, CA, USA) equipped with a high-performance, cation exchange resin column.

2.10. Statistical analyses

Comparisons were made using a model that accounted for genotype and plot replicate using a least square mean analysis in JMP 14 (SAS Institute Inc., Cary, NC, USA). If ANOVA results indicated significant differences, means were compared using the student's *t*-test.

3. Results

3.1. Protein and oil content of soybean hybrids derived from crosses between a high protein and high sulfur amino acid containing line

In our previous work we have generated a transgenic soybean line (CS) that overexpress OASS, a key sulfur assimilatory enzyme. This transgenic soybean plant contains significantly higher amount of sulfur containing amino acids than the conventional soybean cultivars [16]. We utilized this line to cross it with a high protein Korean soybean line (Lee 5) with a goal to develop soybean experimental lines with high protein and high sulfur amino acid content. The protein and oil content of the hybrid seeds were monitored during each generation utilizing analytical tools. The average protein and oil contents of transgenic CS seeds were 34.8 % ± 0.9 (Fig. 1A) and 20.3 % ± 0.7 (Fig. 1B), respectively. The protein content of Lee 5 seeds was 44.7 % ± 2.6 (Fig. 1A) and the oil content was 13.3 % ± 1.7(Fig. 1B). The hybrid seeds showed a significant increase in protein content, while the oil content was significantly lower when compared to the CS parent ($p < 0.05$). The

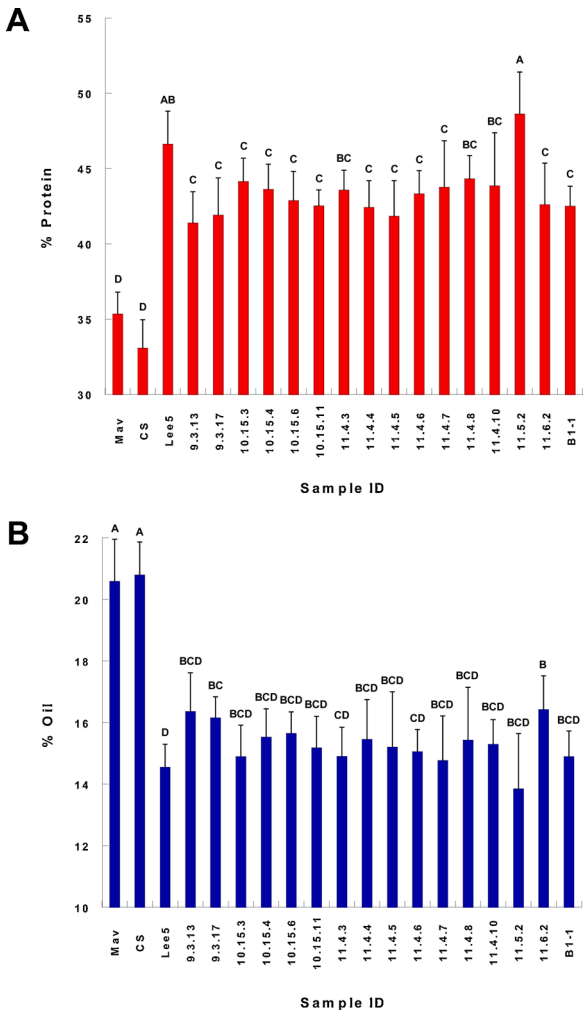

Fig. 1. Protein and oil content of soybean seeds. The experimental line population was developed by crossing 'CS' and 'Lee5'. Progenies from16 individual crosses (9.3.13 to B1-1) were selected and analyzed for their protein and oil content. Mean protein (Panel **A**) and oil (Panel **B**) concentration of $F_{4:5}$ experimental line seeds from individual plants measured by NIRS monochromator. The protein and oil concentration of Maverick (Mav), the soybean cultivar used to generate 'CS', is also shown. The error bars indicate the standard error of the mean (n = 3). Values followed by the same letter are not significantly different.

increased protein content was observed at each successive generation (F2-F6) indicating that the experimental lines have acquired the high protein trait. Least square mean analysis showed that there were no significant differences among plots for $F_{5:6}$ seeds (Supplemental Fig. 1). However, there was a significant difference between the crosses and CS in terms of protein and oil content as well as a significant difference among them ($P < 0.05$).

Also, the seed weight of the parent and the experimental lines were measured, and a significant difference was detected between CS and Lee 5 with an average of 111.5 ± 4.7 mg/seed and 190.3 ± 9 mg/seed respectively. The experimental lines were significantly greater than CS with a minimum 129.0 ± 2.9 mg/seed of and a maximum of 183.8 ± 3.8 mg/seed. Interestingly, there was a positive correlation between protein content and seed weight r = 0.57 ($p < .0001$). A picture of the seed parental lines and select experimental lines developed in this study is shown in Supplemental Fig. 2.

3.2. O-acetylserine sulfhydrylase (OASS) activity and its accumulation is elevated in high protein experimental soybean lines

One of the parents, CS, employed in generating the experimental lines overexpresses a key sulfur assimilatory enzyme OASS. To verify this trait is incorporated in the experimental lines as well we first measured the OASS activity in leaves. OASS activity in parents Lee 5 and CS was 63 and 793 mmol min^{-1} mg^{-1}, respectively, while in the experimental lines it ranged from 383 to 1274 mmol min^{-1} mg^{-1} (Table 1). OASS activities in these crosses showed a significant increase ($P < 0.05$) when compared to Lee 5 and Maverick. This represents a 6 to 20-fold increase in the OASS activity in these experimental lines when compared to Lee 5. To examine if the increase in the OASS activity is also reflected in its accumulation we performed western blot analysis. For this purpose, we utilized seeds from 6 representative individuals from each independent cross along with the parents. Total seed protein fractionated by 10 % SDS-PAGE (Fig. 2A) were subjected to immunoblot analysis using antibodies raised against soybean OASS [21] (Fig. 2B). Soybean OASS antibody strongly reacted against a 34 kDa protein from the total protein of soybean dried seed extracts in all selected experimental lines and CS. The 34 kDa protein corresponds to the molecular weight of OASS [21]. The non-transgenic parent, Lee 5, and Maverick, the wild type of CS, showed no reaction when compared with the transgenic lines. Thus, our results confirm the successful integration of the target traits (high protein and high OASS expression) in the experimental lines.

3.3. Sulfur containing amino acids and elemental sulfur are significantly higher in the seeds of experimental lines

Previously, we have shown that overexpression of OASS results in a significant increase in the sulfur amino acid content (cysteine and methionine) of soybean seeds [16]. To verify if the experimental soybean lines developed in this study also contain high sulfur amino acid content, we first determined the amino acid content of seeds by HPLC (Supplemental Table 1). The result showed that protein-bound cysteine was significantly increased in the crosses when compared to Lee 5 and Maverick. The cysteine content of the parent CS and Lee 5 were 1.08 % and 0.795 % respectively, while the minimum and the maximum cysteine content of the crosses were 1.1 % and 1.25 % respectively

Table 1
O-acetylserine sulfhydrylase (OASS) activity in leaf tissue of soybean parental lines and their crosses. Protein extracted from leaf tissue from soybean lines (parents and experimental lines) were assayed for OASS activity. Results are the mean of 6 samples (n = 6) reported with the standard deviation (SD) of the mean. Letters indicate the result of Tukey-Kramer comparison for a significant ANOVA result at ($p = 0.05$).

Sample ID	OASS activity (nmol/min/mg protein) ± SD	Tukey-Kramer HSD
Mav	56.69 ± 12.03	F
CS	792.80 ± 232.69	BCD
Lee5	62.91 ± 29.10	F
9.3.13	683.01 ± 435.11	BCD
9.3.17	925.79 ± 429.19	ABC
10.15.3	871.69 ± 341.10	BC
10.15.4	764.22 ± 203.62	BCD
10.15.6	740.71 ± 355.29	BCD
10.15.11	909.57 ± 172.60	B
11.4.3	463.81 ± 70.49	DE
11.4.4	712.77 ± 206.72	BCD
11.4.5	658.59 ± 174.82	BCDE
11.4.6	383.60 ± 271.92	E
11.4.7	872.20 ± 254.82	BC
11.4.8	543.38 ± 108.51	BCDE
11.4.10	572.79 ± 207.23	CDE
11.5.2	880.18 ± 267.81	BC
11.6.2	1,274.30 ± 209.30	A
B1−1	701.20 ± 463.59	BCD

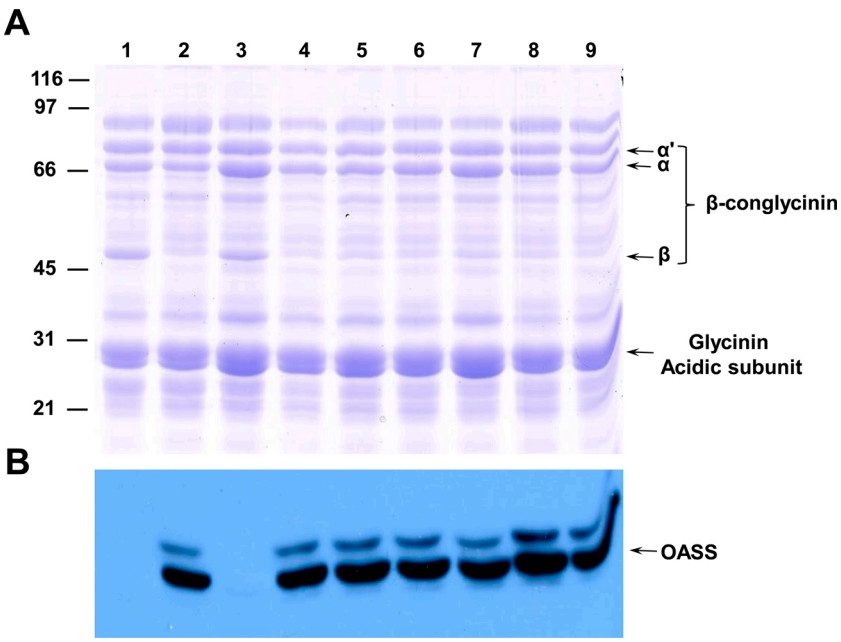

Fig. 2. Western blot detection of O-acetylserine sulfhydrylase in soybean seeds. SDS-PAGE/Immunoblot analysis of soybean seed protein of selected experimental lines and the parents. **A.** Total protein seed protein from Maverick (lane 1), CS (lane 2), Lee 5 (lane 3), experimental line 9.3.17 (lane 4), 10.15.6 (lane 5), 11.4.7 (lane 6), 11.5.2 (lane 7), 11.6.2 (lane 8), and B1-1 (lane 9) were resolved by SDS-PAGE on a 10 % gel and stained with Coomassie Blue. The molecular weight markers whose sizes in kilodaltons are displayed on the left side of the figure. **B.** Immunological detection of proteins showed in panel A that were transferred to a nitrocellulose membrane and probed with an antibody specific for soybean OASS. The arrow points to the 34 kDa protein OASS (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Table 2
Sulfur containing amino acids cysteine and methionine content (w/w %) in soybean seeds of the parental lines and their crosses. Dry seeds powder from duplicate samples from soybean lines (parents and experimental lines) were used to measure sulfur containing amino acids cysteine and methionine content by HPLC. Results are the average of two samples reported with the standard deviation (SD) of the mean.

Sample ID	Cysteine ± SD	Methionine ± SD
Mav	0.660 ± 0.014	0.535 ± 0.007
CS	1.080 ± 0.014	0.640 ± 0.000
Lee5	0.795 ± 0.007	0.630 ± 0.014
9.3.13	1.160 ± 0.014	0.740 ± 0.000
9.3.17	1.160 ± 0.014	0.770 ± 0.014
10.15.3	1.250 ± 0.028	0.770 ± 0.014
10.15.4	1.110 ± 0.000	0.730 ± 0.014
10.15.6	1.175 ± 0.007	0.745 ± 0.007
10.15.11	1.260 ± 0.000	0.770 ± 0.007
11.4.3	1.100 ± 0.014	0.725 ± 0.007
11.4.4	1.165 ± 0.007	0.750 ± 0.014
11.4.5	1.110 ± 0.000	0.730 ± 0.014
11.4.6	1.230 ± 0.014	0.760 ± 0.014
11.4.7	1.220 ± 0.000	0.750 ± 0.014
11.4.8	1.130 ± 0.014	0.770 ± 0.014
11.4.10	1.215 ± 0.007	0.760 ± 0.014
11.5.2	1.065 ± 0.007	0.750 ± 0.014
11.6.2	1.260 ± 0.014	0.765 ± 0.007
B1-1	1.140 ± 0.014	0.710 ± 0.014

Table 3
Elemental sulfur content in soybean seeds of the parental lines and their crosses. Dry seed powder from soybean lines (parents and experimental lines) in 2017 and 2018 was analyzed by inductively coupled plasma-mass spectrometry to measure sulfur content. Results are the mean of 6 samples (n = 6) reported with the standard deviation (SD) of the mean. Letters indicate the result of Tukey-Kramer comparison for a significant ANOVA result at ($p =$ 0.05).

Sample ID	Elemental sulfur content (ppm) ± SD	Tukey-Kramer HSD
Mav	2,621.18 ± 34.20	H
CS	4,368.56 ± 157.40	F
Lee5	3,150.55 ± 160.20	G
9.3.13	4,717.05 ± 138.70	DE
9.3.17	5,077.41 ± 143.00	ABC
10.15.3	4,850.57 ± 162.70	CDE
10.15.4	4,626.71 ± 234.20	EF
10.15.6	4,867.72 ± 220.30	BCDE
10.15.11	4,982.99 ± 62.80	ABCD
11.4.3	4,724.14 ± 54.50	DE
11.4.4	4,965.90 ± 201.40	ABCD
11.4.5	4,942.37 ± 171.10	BCDE
11.4.6	5,028.57 ± 194.90	ABCD
11.4.7	5,036.38 ± 76.40	ABCD
11.4.8	5,078.69 ± 85.10	ABC
11.4.10	5,186.20 ± 45.10	AB
11.5.2	4,902.10 ± 184.40	BCDE
11.6.2	5,272.75 ± 59.70	A
B1-1	4,752.74 ± 100.30	CDE

(Table 2). Furthermore, methionine content for the crosses showed a range between 0.71 to 0.77 % and was significantly higher than both parents CS (0.64 %) and Lee 5 (0.63 %). Interestingly, there was a strong positive correlation between cysteine and methionine $r = 0.907$ ($p < .0001$).

In addition to sulfur containing amino acids, we also measured the elemental sulfur content of soybean seed seeds that were grown in 2017 and 2018 by inductively coupled plasma-mass spectrometry (Table 3). CS, the transgenic parent, accumulated significantly higher elemental sulfur (average 4,368 ppm) than Maverick, its wildtype, which had the lowest amount of sulfur (average 3,150 ppm). Interestingly, Lee 5 parent had greater accumulation of sulfur than Maverick, but it was significantly lower than CS. Elemental sulfur in the crosses was significantly higher than all parents in both years ranging from 4,626 to 5,272 ppm. Our observation demonstrates that the experimental soybean lines developed in this study not only contains high protein but also has enhanced sulfur containing amino acids and elemental sulfur.

3.4. Accumulation of the β-subunit of β-conglycinin, a protein devoid of sulfur amino acids, is lower in experimental soybean lines

Earlier we have demonstrated that overexpression of sulfur assimilatory enzymes results in lower accumulation the β-subunit of β-conglycinin [25]. To verify if a similar trend is also found in our newly developed experimental high-protein lines, we compared the total seed protein profiles of the parents along with the experimental lines. For this comparison, we isolated total seed protein from the parent and six selected crosses and separated them by SDS-PAGE. This protein extraction was based on equal dry seed weight. The result showed that Lee 5 and the experimental lines accumulated more protein when

compared to Maverick and CS (Fig. 3A). Unlike Lee 5, all experimental lines showed a significant decrease ($p < 0.05$) in the accumulation of the β sub-unit of β-conglycinin (54 kDa). This unique characteristic was observed in CS as well and was further confirmed by western blot analysis (Fig. 3B). Antibodies specific to the β-subunit of β-conglycinin recognized a 52 kDa polypeptide from Maverick and Lee 5, while the CS and the selected experimental lines showed a faint reaction against the β-subunit (Fig. 3B). This observation confirms that the β-subunit of β-conglycinin accumulates less in CS and all the selected experimental lines which was further verified by quantification of the immunoreactive bands using Gel Analyzer software.

3.5. The accumulation of Bowman-Birk protease inhibitor and lunasin, two sulfur amino acid rich peptides, are elevated in experimental soybean lines

In addition to major seed storage proteins (7S β-conglycinin and 11S glycinin) soybean also accumulate few sulfur-rich proteins, albeit at lower levels. Prominent among them are Bowman-Birk protease inhibitor (BBI) and lunasin. Bowman-Birk protease inhibitor is a cysteine rich peptide and a major contributor to the overall sulfur amino acid content of the seed [26,27]. Lunasin, a 2S albumin, is synthesized as a precursor protein and contains a signal peptide, a methionine-rich larger subunit, an aspartic acid-rich small subunit corresponding to lunasin, and a linker peptide [28]. We wanted to examine if the accumulation of these two sulfur-rich bioactive peptides was altered in these experimental soybean lines. This possibility was verified by western blot analyses using antibodies raised against soybean BBI and lunasin. SDS-PAGE analysis of 50 % isopropanol extracted proteins, which preferentially extracts BBI, revealed a remarkable increase in the accumulation of a 14 kDa protein in the experimental lines when compared to Lee 5, the non-transgenic parent (Fig. 4A). Immunoblotting analysis with an antibody generated against Bowman-Birk peptide showed a reaction with this specific protein at 14 kDa (Fig. 4B). Though slight differences in the relative abundance of BBI in the experimental lines was evident, still it was several-fold greater when compared to Lee 5. An examination of the stained gel revels that some lanes (example lane 6) contained less protein than others. This may account for the relatively lower accumulation of BBI in select experimental lines. Gel Analyzer software was used to determine the differences in band intensity of Bowman-Birk Protease Inhibitor in parents and the six selected experimental lines. A significant increase in band intensity was detected in the crosses compared to Lee 5 (data not shown).

We also investigated the accumulation of lunasin in the selected experimental lines from each cross and the parents. A lunasin-enriched protein fraction obtained by extracting seed powder with 30 % ethanol and fractionated on SDS-PAGE (15 % gel) showed a 5 kDa protein that accumulated at higher amounts in CS and the crosses when compared to Maverick and Lee 5 (Fig. 4C). Western blot analysis utilizing lunasin specific antibodies specifically reacted with the 5 kDa protein (Fig. 4D). The lunasin peptide was detected in all the crosses and the parent; however, the reaction intensity was noticeably greater in CS and the crosses when compared to Maverick and Lee 5. To determine the differences in the accumulation of lunasin, the band intensity of this 5 kDa peptide was analyzed using Gel Analyzer software. A significant increase in band intensity ($p < 0.05$) (n=3) was detected in the crosses and CS when compared to Lee 5 (data not shown).

3.6. Identification of seed proteins that contribute to the higher protein content of experimental lines

Immunoblot analyses have clearly shown that the soybean experimental lines with high protein and high sulfur amino acid accumulate significantly higher amounts of BBI and lunasin. In order to further characterize the changes in the protein composition and to investigate the role of individual proteins to the overall increase in the protein content we performed high-resolution 2-D gel analysis of seed proteins. For this purpose, we isolated seed proteins from CS, Lee 5 and a single experimental line 11.5.2 and resolved them by 2-D gel electrophoresis (Fig. 5). The differences in the intensity of protein spots resolved by 2-D gels were analyzed by overlaying two separate two-dimensional gels using Delta2D software (Supplementary Fig. 3). This analysis confirmed that the seed storage globulins were mainly responsible for the increase in the total amount of protein in experimental line 11.5.2, which is a trait inherited from the Lee 5 parent. For this analysis, an equal volume of protein from experimental line 11.5.2 was separated and compared with an equal volume of protein from Lee 5. Similarly, comparison was made between CS and Lee 5. The result from the fusion of Lee 5 gel image overlaid with that of CS makes it clear that many protein spots are significantly higher in Lee 5 when compared to CS. Protein spots in this

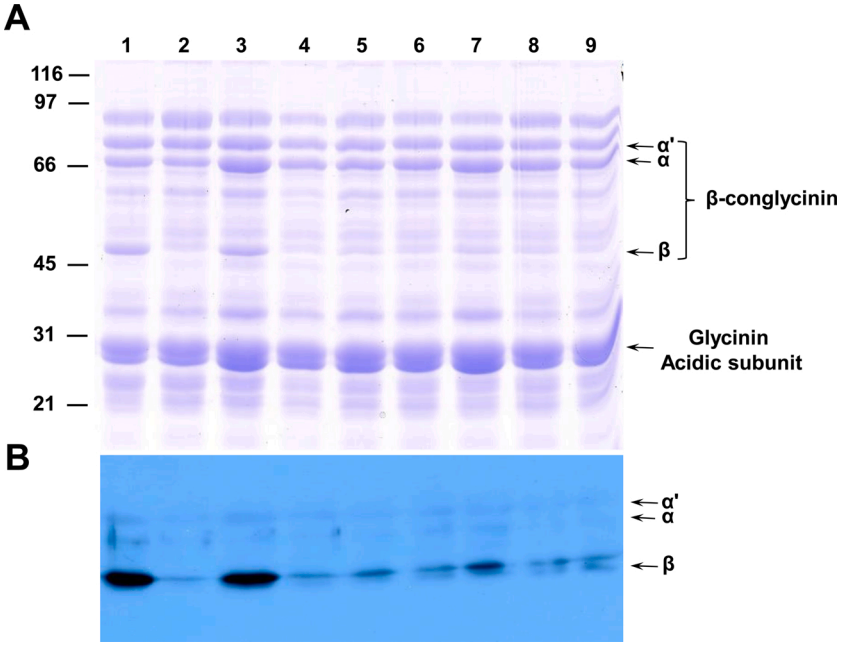

Fig. 3. Western blot analysis of β-conglycinin accumulation in selected experimental lines and the parents. **A.** Total seed proteins from Maverick (lane 1), CS (lane 2), Lee 5 (lane 3), experimental line 9.3.17 (lane 4), 10.15.6 (lane 5), 11.4.7 (lane 6), 11.5.2 (lane 7), 11.6.2 (lane 8), and B1-1 (lane 9) were resolved on a 10 % SDS-PAGE and stained with Coomassie Blue The molecular weight markers whose sizes in kilodaltons are displayed on the left side of the figure. **B.** Immunological detection of proteins showed in panel A that were transferred to a nitrocellulose membrane and probed with an antibody specific for β-conglycinin proteins (Panel B). The arrows point to the α, α' and β-subunits of β-conglycinin proteins (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

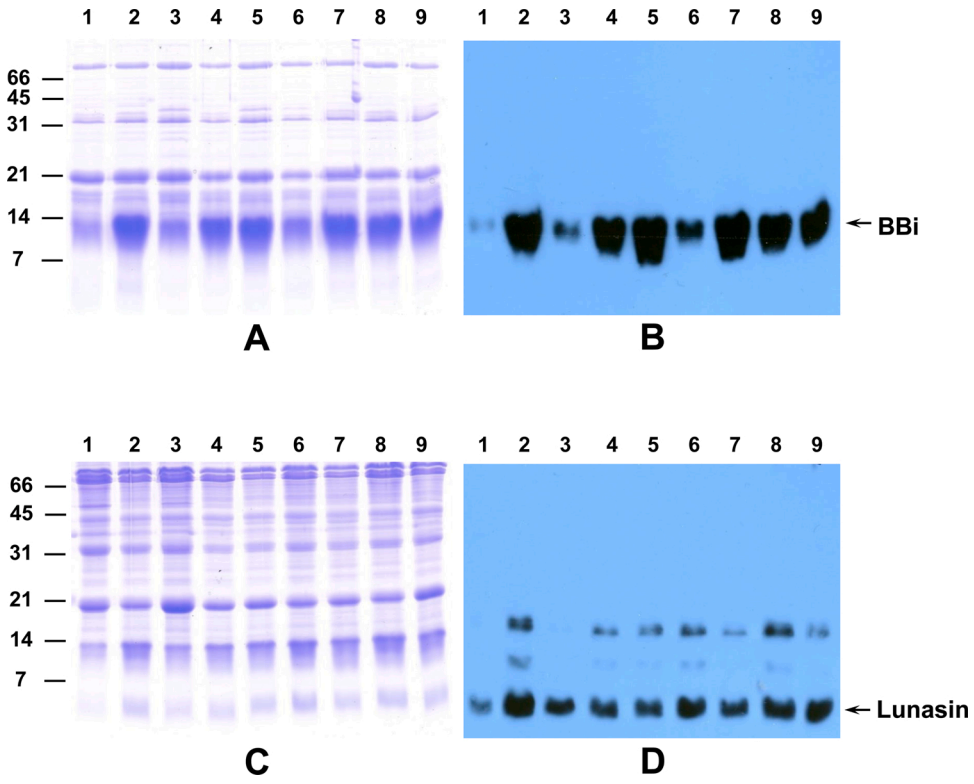

Fig. 4. Western blot analysis of Bowman-Birk protease inhibitor and lunasin accumulation in selected experimental lines and the parents. 50 % isopropanol (**A**) or 30 % ethanol (**C**) soluble seed proteins from Maverick (lane 1), CS (lane 2), Lee 5 (lane 3), experimental line 9.3.17 (lane 4), 10.15.6 (lane 5), 11.4.7 (lane 6), 11.5.2 (lane 7), 11.6.2 (lane 8), and B1-1 (lane 9) were resolved on a 15 % SDS-PAGE and stained with Coomassie Blue. The molecular weight markers whose sizes in kilodaltons are displayed on the left side of the figure. **B** and **D.** Immunological detection of proteins showed in panel A and C that were transferred to a nitrocellulose membrane and probed with an antibody specific for BBI (**B**) and lunasin (**D**) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

experiment were compared to soybean seed protein spots from previous work in our lab [35]. Forty-two protein spots known to include the many subunits of seed storage globulins as well as other abundant proteins were selected for this analysis. Supplemental Table 2 shows the percent spot volume of those 42 spots generated from the percent spot volume data collected using Delta2D image analysis software. Protein spot numbers shown in supplemental Fig. 3 also correspond to those presented in Supplemental Table 2, where the identity of each protein is listed. Clearly, the data indicate that protein number 1 (β-conglycinin α-subunit) is relatively higher in Lee 5 when compared to CS and 11.5.2, as is protein numbers 10 and 11 (glycinin β-subunit), 38 (glycinin), 40, 41 (glycinin A2B1a), 42 (glycinin A1aBx precursor), 43, (proglycinin (A1ab1b), and especially 79 (Bowman-Birk proteinase inhibitor) are substantially higher in percent spot volume in CS and 11.5.2 when compared to those of Lee 5.

4. Discussion

It has long been documented that among legumes, soybean seeds contain a high percentage of protein, i.e., 35–40% depending on the cultivar and growing conditions [3,12]. Protein meal is one of the major by-products of soybean processing, and high seed protein levels allow processors to derive meal with high nutritional value. Most of the soybean value is derived from the protein component of the seed [29]. As a result, generating high-protein soybean cultivars is an important goal for the breeders. However, a major impediment to the development of high protein cultivars is the negative relationship between seed yield and seed protein. Nevertheless, soybean breeders have made significant progresses in overcoming this negative correlation by employing advanced breeding techniques resulting in the development of agronomically viable high-protein cultivars [12].

Several quantitative trait loci (QTLs) that influence seed protein have been identified and mapped to different chromosomes [3,12]. Some of these QTLs have only small effect, while others like those located on chromosomes 15 and 20 have large effect resulting in about 2 % increase in protein. For example, researchers have successfully transferred a high protein allele from PI407788A at the chr 15 locus into two elite soybean backgrounds. They reported that these experimental lines showed increased seed protein and had only a nonsignificant effect on yield [30]. Similarly, researchers at Georgia have introgressed a high-protein allele from 'Danbaekkong' located on chr 20 into an elite soybean cultivar [31]. These newly developed near-isogenic lines (NILs) showed a significant increase in protein without any significant reduction in yield. In our study, we have developed experimental soybean lines with significantly high protein content utilizing a high-protein Korean line. Currently, we do not know if the high protein trait of the Korean line is associated with QTLs located at chr 15 or chr 20 or on elsewhere. Additional studies will enable us to identify the genomic location of the high protein QTL and utilize marker-assisted approaches to introgress this valuable trait into high yielding elite soybean cultivars.

Glycinin and β-conglycinin account 70–80 % of the seed storage protein and together they determine the quantity and quality of the seed protein because of their combined abundance and differences in amino acid content [32,33]. Relatively, the 11S glycinin accumulates greater amount of cysteine and methionine than β-conglycinin which exhibit a paucity of methionine and cysteine [32,33]. A recent study utilized tandem mass tag-based proteomic profiling of a high protein fast neutron mutant line and reported an increase in the abundance of 206 proteins and a decrease in 214 proteins [34]. The high protein fast neutron mutant line showed an increase in seed storage proteins especially the basic 7S globulin which increased fourfold, followed by vacuolar-sorting receptor and protein transporters [34]. Additionally, this study also identified 29 ribosomal proteins, 16 endoplasmic reticular proteins, and several proteins in export metabolic pathways that were elevated in the mutant line. Earlier, we investigated the relative contribution of various seed proteins from nine soybean accessions whose seed protein content was greater than 45 % of the seed dry weight [35]. High-resolution two-dimensional gel electrophoretic analysis in combination with mass spectrophotometry indicated that a significant increase in the seed 11S and 7S globulins in Lee 5 was responsible for the high protein trait. Our study reveals that the experimental soybean lines accumulate some subunits of β-conglycinins and glycinins at higher

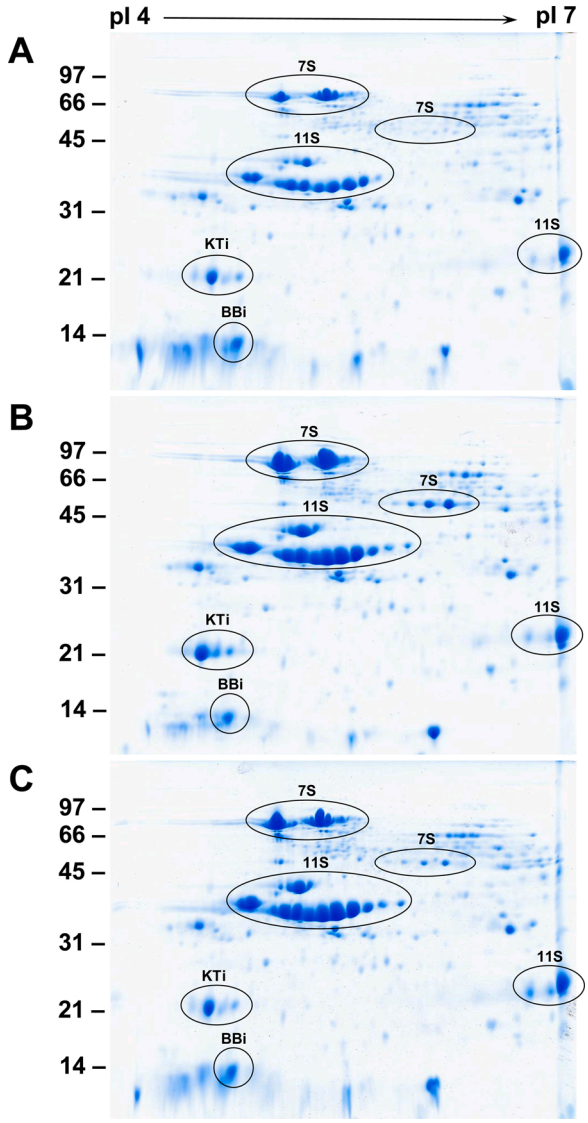

Fig. 5. Two-dimensional gel electrophoresis separation of seed protein extracted from the parents (CS and Lee 5), and experimental line 11.5.2. Seed protein from each line were separated using 2-DE and compared with each other. The first dimension was run using a pH gradient from 4.0 to 7.0. The second dimension was a 13 % SDS-PAGE. Gels were stained with colloidal Coomassie blue stain G-250. Panel **A** shows seed protein extracted from CS, panel **B** shows seed protein extracted from Lee 5, Panel **C** shows seed protein extracted from experimental line 11.5.2. Prominent seed proteins (7S and 11S globulins, KTi and BBi) are enclosed in circles. The molecular weight markers whose sizes in kilodaltons are displayed on the left side of the figure (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

levels, while other subunits at lower levels when compared to the parental lines. The accumulation of β-subunit of β-conglycinin in these experimental lines showed significant reduction when compared to the high protein line Lee 5. This reduction in the accumulation of the β-subunit of β-conglycinin is probably due to the high levels of methionine and cysteine present in these experimental lines. Previous studies have shown that the accumulation of the β-subunit of β-conglycinin can be negatively impacted by sulfur amino acids, sulfur and nitrogen nutrition [36,37].

The experimental lines developed by this study contain higher amounts of Bowman-Birk protease inhibitor when compared with that of the high protein soybean parent Lee 5. The protease inhibitor family contains 14 cysteine residues and contributes significantly to the overall sulfur content of soybean seeds [26,27]. The increased level of cysteine in the experimental lines is inherited from the transgenic soybean CS that overexpress OASS, which promotes the synthesis of Bowman-Birk protease inhibitor in these lines [16]. Interestingly, this increased cysteine availability positively affects the accumulation of other sulfur-rich proteins, such as lunasin in these experimental lines.

Both protein and oil are commercially important components of soybean seed. It is well established that protein and oil content are inversely correlated and an increase in either lowers the concentration of the other. Not surprisingly the high protein experimental lines developed in this study revealed lower oil content. Earlier studies have established that competition for carbon skeletons between storage protein synthesis and oil synthesis may account for this negative correlation [38]. Metabolite studies of soybean NIL lines with contrasting seed oil content have shown that the abundance of 28 metabolites, especially free Asn and free 3-cyano-Ala, was positively correlated with protein and negatively correlated with oil content [38]. It was speculated that the high levels of free Asn, Asp, and Glu found in high-protein lines may promote storage-protein synthesis, thereby depleting carbon skeletons required for oil synthesis, leading to lower seed oil content. This possibility was augmented by a recent study which employed fast neutron (FN) mutagenized soybean populations with deletions in central carbon metabolic genes [39]. It was demonstrated that a delayed switch in carbon allocation towards raffinose family oligosaccharides biosynthesis resulted in extended lipid accumulation [39]. These studies emphasize the need for ample supply of carbon skeleton especially at the later stages of seed development to meet the demand for both protein and oil biosynthesis.

Increasing the overall seed protein concentration alone does not address the quality of soybean protein. For optimal animal growth the balance and composition of the amino acids that makes the protein is most important than the overall protein content. An important role of soybean protein in animal feed is to provide adequate amounts of required amino acids [40]. Our laboratory has targeted manipulation of key enzymes involved in sulfur assimilatory pathway as a potential avenue to improve the overall sulfur amino acid content of soybean seeds [41–43]. ATP sulfurylase (ATPS) and O-acetylserine sulfhydrylase (OASS) are two critical enzymes in the sulfur assimilation pathway. We were successful in individually overexpressing these two enzymes in transgenic soybeans that resulted in a significant increase in the overall sulfur amino acid content of soybean seeds [16,25]. Interestingly, these transgenic soybeans and the experimental lines with high protein and high sulfur amino acid developed in this study, also contain high amount of elemental sulfur. Our observation suggests a positive correlation between the amount of elemental sulfur and total sulfur amino acid content. Based on this assumption, one should be able perform rapid high throughput ionomic phenotyping of soybean lines from germplasm repositories to identify lines with high sulfur content. Our earlier work has shown that the elemental content of soybean seed is a determined by both genetic and environmental factors [44]. Analyses of the elemental profile of seeds from 1653 lines in the USDA Soybean Germplasm Collection led to the identification of several soybean lines that showed the highest and lowest sulfur content [44]. However, it needs to be established if the soybean lines with the high sulfur content also accumulates high amounts of cysteine and methionine.

In this study, we have utilized OASS overexpressing soybean line and crossed it with a Korean high-protein line. Our approach has resulted in the development of experimental soybean lines that not only have elevated protein content but also enhanced levels of sulfur containing amino acid cysteine and methionine. The development of such high protein soybean lines with enhanced sulfur containing amino acids represents a significant achievement that bodes well for retaining the prominence of soybean as the eminent protein source in animal feed. These newly developed lines can be exploited to develop elite's soybean lines with desirable agronomic characteristics and marketable components in the future.

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Author contributions

HK conceived and designed the experiments. AA, BS, NO, WW, TM and HK performed the experiments. AA and HK analyzed the results and wrote the paper. All authors read and approved the manuscript.

Declaration of Competing Interest

The authors declare that there are no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.plantsci.2021.110912.

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