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Identification of a New Soybean Kunitz Trypsin Inhibitor Mutation and Its Effect on Bowman−Birk Protease Inhibitor Content in Soybean Seed

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Supporting Information

ABSTRACT: Soybean seed contains antinutritional compounds that inactivate digestive proteases, principally corresponding to two families: Kunitz trypsin inhibitors (KTi) and Bowman−Birk inhibitors (BBI). High levels of raw soybean/soybean meal in feed mixtures can cause poor weight gain and pancreatic abnormalities via inactivation of trypsin/chymotrypsin enzymes. Soybean protein meal is routinely heat-treated to inactivate inhibitors, a practice that is energy-intensive and costly and can degrade certain essential amino acids. In this work, we screened seed from 520 soybean accessions, using a combination of sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and immunoblots with anti-Kunitz trypsin inhibitor antibodies. A soybean germplasm accession was identified with a mutation affecting an isoform annotated as nonfunctional (*KTi1*), which was determined to be synergistic with a previously identified mutation (*KTi3*−). We observed significant proteome rebalancing in all KTi mutant lines, resulting in dramatically increased BBI protein levels.

KEYWORDS: *soybean, Kunitz trypsin inhibitor, Bowman−Birk inhibitor, seed composition improvement*

INTRODUCTION

Although soybean (*Glycine max*. L. Merr.) is classified as an oilseed, the major economic value of the seed is due to the protein component, which comprises ∼40% of dry seed weight. Except for soybeans intended for export or the small amount used for human food (∼1%), soybeans in the United States are processed to extract seed oil, and the resulting protein meal undergoes complex processing to yield defatted soybean meal. Soybean meal is a predominant source of protein meal (∼67%) utilized worldwide, and the major use is in animal feed mixtures for poultry (50%) and swine (26%) (http://soystats.com).

Soybean is a remarkably productive plant, and the seed meal is used as a complete source of essential amino acids.[1] Soybean seed, however, also contain a number of antinutritional factors that have been demonstrated to reduce animal weight gain.[2] The inclusion of large amounts of raw soybeans, or partially defatted raw soybean meal, in animal diets can also result in hypoglycemia, pancreatic hypertrophy, liver damage, and other complications.[3] Although other proteins/compounds that interfere with proper digestion have been identified (e.g., lectins, phytate/phytic acid), two major protein inhibitor molecules have been identified that interact with and inactivate the digestive enzymes trypsin and/or chymotrypsin: the 20 kDa Kunitz trypsin inhibitor protein family (KTi)[4] and the 6−21 kDa Bowman−Birk inhibitor protein family (BBI).[5,6] KTi proteins are thought to be largely specific for trypsin inhibition,[7] whereas the major isoform of BBI contains domains that interact with and inhibit both trypsin and chymotrypsin.[5] In addition, isoinhibitors of BBI that can inhibit both trypsin and porcine elastase have also been described.[8−10] A recent study has demonstrated that BBI and KTi content of soybean seed can be reduced by germination and hydrolysis.[11] Sensitivities to trypsin/chymotrypsin inhibition vary from species to species. An examination of 10 different vertebrate species revealed at least a 10-fold variation in sensitivity; salmonoids (fish) were shown to be the most sensitive, poultry and swine were intermediate, and humans were the least sensitive.[12]

Both KTi and BBI proteins can be irreversibly inactivated by heat, although BBI proteins are more resistant to heat inactivation as compared to KTi proteins.[13] As a result, soybean seed meal is routinely heat-processed. This practice has the potential to reduce protein quality and digestibility due to the Maillard reaction,[14] and heat processing is energy-intensive and costly. One enduring goal of soybean breeding efforts has been the development of high-yielding lines that would require reduced heat processing.

Germplasm screens have been carried out to identify accessions with reduced or absent BBI or KTi proteins. A *Glycine microphylla* accession has been identified that lacks detectable BBI proteins in mature seed; this effect is due to a single loss-of-function frameshift mutation affecting one BBI gene.[15] Unfortunately, development of a *G. max* line with reduced BBI content from this *G. microphylla* genetic source has not been reported, and substantial hybridization barriers exist between *G. microphylla* and *G. max*. Substantial variation in BBI content has been found in a study of *G. max* and perennial species;[16] however, no *G. max* germplasm has been identified that has complete or consistent reduction in BBI

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protein content. This is most likely due to presence of multiple, seed-expressed BBI genes[17,18] and a pronounced environmental effect on BBI protein accumulation.[19] An alternate, successful approach to reduce BBI protein levels was the overexpression of a mutated, inactive BBI transgene, which was able to outcompete endogenous BBI transcripts and significantly reduced BBI protein levels.[20]

By far the largest success in reducing trypsin inhibitor activity in soybean is due to the identification of a soybean accession (PI 157740) with dramatically reduced (~40%) trypsin inhibitor activity.[21] The molecular genetic cause is a frameshift mutation (termed *KTi3*−) that affects one of three seed-expressed *KTi* genes.[22] This accession has been used in extensive backcrossing and feeding trials,[3,23−25] and raw extruded protein meal lacking KTi3 protein is superior for animal weight gain as compared to raw soybeans.[3,23,24] However, weight gain for young animals fed non-heat-treated soybean materials (including *KTi3*−) is inferior to those fed conventional heat-treated soybeans, although soybean seed lacking KTi3 protein has been shown to require less heat processing in comparison to conventional soybeans.[3,23,24]

In this study, we report identification of a *G. max* line with reduced Kunitz trypsin inhibitor protein and decreased trypsin inhibition, which is not due to mutations affecting the previously identified *KTi3* gene. The objectives of this work were (1) to identify the specific genetic cause for this reduction in trypsin inhibition, (2) to evaluate if this reduction was synergistic with the previously identified *KTi3*− mutation, and (3) to ascertain what effect these mutations had on seed proteome and BBI protein accumulation in mature seed.

MATERIALS AND METHODS

Plant Materials. Seed for soybean lines examined was obtained from the USDA-ARS GRIN collection. Out of 520 lines examined, two were Kunitz trypsin inhibitor (KTi) mutants (PI 68679, *KTi1*−, and PI 542044, Kunitz, *KTi3*−). A wild-type line, Williams 82 (PI 518671, *KTi1*, *KTi3*)[26] was used as a control line. The Kunitz trypsin inhibitor 3 (*KTi3*−) mutation in this line is derived from a landrace identified by Theodore Hymowitz (PI 157740) backcrossed to Williams 82 for five generations.[21]

Population Development. PI 68679 (*KTi1*−) and PI 542044 (*KTi3*−) were crossed in the summer of 2012 at the Southfarm experimental research station, and F_1 seed was advanced in greenhouses to produce F_2 seed. Seed chips from individual F_2 seed were examined via SDS−PAGE analysis and two lines were selected for absence of both KTi1 and KTi3 proteins.

***KTi3*− SimpleProbe Assay.** A SimpleProbe assay was developed to track the *KTi3*− allele,[22] designed by use of Lightcycler probe design software, version 1 (Roche Applied Sciences, Indianapolis, IN). Genotyping PCR reactions were performed with a 5:1 asymmetric mix of primers (5′-TTGCAGTTATAATGCTGTGTGTTG-3′, 5′-AA-TACTAATCCCAATATCCCCACA-3′) in the presence of a non-extensible fluorescently labeled SimpleProbe (5′-fluorescein-SPC-TGAATTCATCATCAGAAACTCTCTCAAGTC-phosphate-3′) using Titanium Taq polymerase (Clonetech, Mountain View, CA) and 5−50 ng of genomic DNA in a Lightcycler 480 II instrument (Roche) under the following conditions: 95 °C for 5 min, followed by 45 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Following amplification (and a 1 min denaturation step at 95 °C and 2 min at 55 °C), melting curve analysis of 20 reads/deg from 50 to 70 °C was performed. For *KTi3*− homozygote samples, a single melting peak was observed at ~56−57 °C, whereas wild-type lines show a single peak at ~64 °C and heterozygotes showed both peaks (Figure S1, Supporting Information).

SDS−PAGE and Immunoblot Analysis of Extracted Soybean Seed Proteins. Protein extraction and SDS−PAGE analysis was performed as previously described.[19] Briefly, mature soybean seed samples were ground with a mortar and pestle, and proteins were extracted from a 10 mg portion (for analysis of F_2 lines, a 10 mg chip from each seed was used instead) in SDS buffer [125 mM Tris-HCl, pH 6.8, 20% glycerol (v/v), 5% (v/v) 2-mercaptoethanol, 4% SDS (w/v), and 0.03 mM bromophenol blue], boiled for 5 min, and centrifuged to remove insoluble material. Protein extract supernatant was separated by SDS−PAGE with 15% (w/v) total acrylamide monomer in the resolving gel for 1 h at 20 mA on a Hoefer SE 260 minigel apparatus. Protein bands were visualized with Coomassie blue R-250. Immunoblots were performed as previously described with a 1:5000 dilution of an anti-BBI antibody[19] or 1:10 000 anti-KTi (non-isoform-specific) antibody[27] in Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.5, and 150 mM NaCl). After being washed with TBST buffer (Tris-buffered saline with 0.3% Tween-20), membranes were incubated with goat anti-rabbit IgG−horseradish peroxidase conjugate for 1 h and detected with enhanced chemiluminescent substrate according to manufacturer's recommendations (Pierce, Rockford, IL).

Two-Dimensional Protein Electrophoresis. Two-dimensional electrophoresis was carried out essentially as previously described.[28] Protein samples (300 μg) were initially separated by use of isoelectric focusing IPG strips (pH 3−10, 13 cm in length) in an IPGPhor system (GE Healthcare, Little Chalfont, U.K.). Following electrophoresis, the protein in the IPG strips was resolved in the second dimension with a Hoefer SE 600 Ruby electrophoresis unit according to the manufacturer's specifications (GE Healthcare). After completion of electrophoresis, the gels were removed from the cassette and stained with Coomassie blue R-250. Coomassie-stained gels were scanned on a UMAX PowerLook 2100XL scanner running Adobe Photoshop version 7.0 (Adobe Systems, San Jose, CA), and image files were analyzed via Phoretix 2-D Advanced software, version 6.01 (Nonliner Dynamics Ltd., Newcastle-upon-Tyne, U.K.).

DEAE Anion-Exchange Chromatographic Separation of Extracted Soybean Seed Proteins. Mature soybean seed was ground into a fine powder with a mortar and pestle. To 20 g of seed powder was added 200 mL of 20 mM Tris-HCl/0.1 mM EDTA buffer, pH 6.8, and the mixture was placed in an orbital shaker (150 rpm, 30 °C) for 15 min. The slurry was clarified by centrifugation at 13320*g* for 20 min. To the clear supernatant was added 100 mM $CaCl_2$ stock solution, to a final concentration of 10 mM of calcium chloride. After incubation at room temperature for 10 min, the solution was clarified by centrifugation as stated above. Protein from the supernatant was precipitated by addition of solid $(NH_4)_2SO_4$ to 70%. The resulting protein was recovered by centrifugation, resuspended in 20 mL of 10 mM sodium phosphate/0.1 mM EDTA buffer, pH 6.5, and dialyzed overnight against the same buffer. The concentrated protein solution was loaded onto a column of DEAE-cellulose (2.5 × 20 cm) previously equilibrated with the dialysis buffer. The column was washed with 2 volumes of buffer and then subjected to a step gradient of 50, 100, 200, 300, 400, and 500 mM NaCl, prepared in dialysis buffer. Fractions of 6 mL were collected, and alternate fractions were examined by SDS−PAGE.

Trypsin and Chymotrypsin Inhibitor Assays. Trypsin and chymotrypsin inhibitor assays were done in duplicate as previously described.[29] Briefly, 10−50 μg of seed protein were used in inhibitor assays, using *N*-benzoyl-L-arginine ethyl ester (BAEE) and *N*-benzoyl-L-tyrosine ethyl ester (BTEE; obtained from Sigma) as trypsin and chymotrypsin reaction substrates, respectively. Absorbance at 256 nm was monitored for ~3 min, and trypsin and chymotrypsin inhibitor activities were calculated as the difference between absorbance change in control and samples containing seed protein.

RNA Extraction and qRT-PCR Analysis. F_3 lines were also grown in a growth chamber in a random complete block design with four biological replicates for each genotype. Immature seed was collected and separated into size categories based on fresh weight. Two seed stages representing midmaturation (200−300 mg/seed fresh weight) and late maturation (400−500 mg/seed fresh weight) were selected for mRNA extraction and qRT-PCR analysis.

Each genotype was evaluated with four biological replicates consisting of three pooled immature seeds. RNA isolation, cDNA

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production and qRT-PCR were carried out as previously described[28] with 1 μg of DNase-treated total seed RNA. A subsample of $^1/_{20}$ of the total RT reaction was used for qRT-PCR reaction. Samples were compared by the ΔΔ*CT* method,[26] with the *cons6* gene as a reference gene.[31]

Statistical Analyses. JMP version 9 software (SAS Institute Inc., Cary, NC) was used to perform one-way analysis of variance (ANOVA) tests to compare the effect of genotype on gene expression or trypsin/chymotrypsin inhibition activity of seed extracts. For ANOVA tests that displayed significant differences at the $p < 0.05$ level, Tukey's honest significant differences (HSD) ad hoc test was performed ($\alpha = 0.05$ significance level cutoff).

RESULTS AND DISCUSSION

Identification of a Germplasm Line with Reduced Kunitz Trypsin Inhibitor Content. To identify soybean lines with reduced trypsin and chymotrypsin inhibitor content, we screened a total of 520 soybean accessions, obtained from the USDA Soybean Germplasm Collection, Urbana, IL, by SDS–PAGE and Western blot analysis with antibodies raised against purified soybean KTi protein.[27] Among these soybean accessions, one germplasm line, PI 68679, was identified that had reduced KTi protein levels (Figure 1).

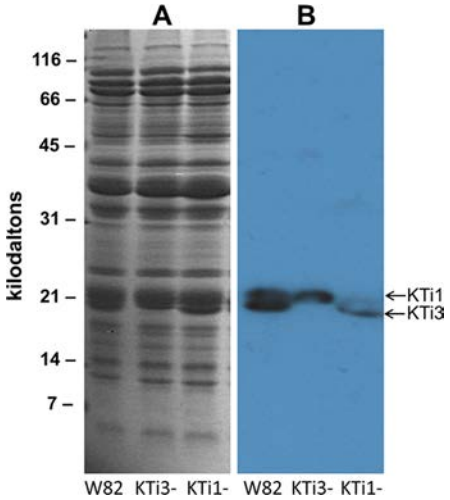

Figure 1. SDS–PAGE protein fractionation of mature soybean seed. Equivalent amounts of protein (50 μg) of clarified seed protein were separated on polyacrylamide gels. (A) Coomassie brilliant blue-stained polyacrylamide gel; (B) Western blot probed with anti-Kunitz trypsin inhibitor antibody (non-isoform-specific). Genotypes are indicated below chart: W82 = Williams 82 (PI 518671, wild type); KTi3– = Kunitz (PI 542044, *KTi3*– mutant); KTi1– = PI 68679 (*KTi1*– mutant). The bands corresponding to KTi1 and KTi3 proteins are indicated by arrows.

Determination of Molecular Genetic Basis for Reduced KTi Levels in PI 68679. In soybean, three *KTi* isoforms (*KTi1*, *KTi2*, and *KTi3*) have been reported to be expressed at the mRNA level in developing seed.[22,32] Cloning of the *KTi3* gene from PI 68679 revealed no mutations that could affect the coding region (Figure 2A). *KTi1/2* were annotated as tightly linked and highly similar (>90%), and were suggested to lack trypsin inhibitor activity based on sequence analysis alone,[32] but this assertion was not experimentally validated. The Williams 82 reference sequence (http://www.phytozome.net/search.php?method=Org_Gmax) does not contain a second locus linked to *KTi1*, which displays high (>90%) similarity reported for *KTi2* in the initial characterization.[22,32] BLAST searches with the *KTi1/KTi2* gene fragment (NCBI accession no. S45035.1), revealed only a single gene (Glyma01g10900/Glyma01G095000) on chromosome 1. Sanger sequencing with cloned *KTi1* PCR products revealed a single-base deletion (GGG356 → 358GG, relative to start codon, NCBI accession no. KC460320), which introduces a frameshift mutation at position 119 (Figure 2B). In concordance with the previously named *KTi3*– allele (Figure 2A), we have termed this new mutant allele *KTi1*–.

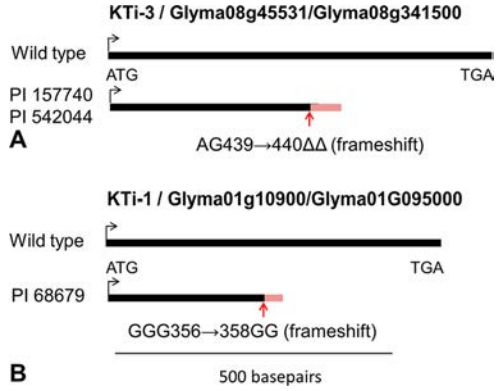

Figure 2. Cartoon depiction of Kunitz trypsin inhibitor genes and the impact of loss-of-function mutations. (A) *KTi3*; (B) *KTi1*.

Development of Lines Containing Two Genetically Distinct Mutations Affecting KTi Encoding Genes. Using traditional hybridization methods, we crossed the line bearing a *KTi1*– mutation (PI 68679) to a line bearing the previously identified *KTi3*– mutation (PI 542044). A SimpleProbe-based genotyping assay for the *KTi3*– mutation was developed (Figure S1, Supporting Information) and this assay was completely predictive for absence/accumulation of KTi3 protein (data not shown). However, attempts to develop a routine molecular marker assay for the novel *KTi1*– mutation were not successful. The mutation is a deletion of one guanine residue within a string of three guanines (GGG > GG), which is extremely difficult to detect by hybridization-based genotyping. We explored other genotyping methods that rely on size polymorphism (e.g., CAPS, dCAPS[33,34]) but no suitable restriction enzymes were identified.

A single F_1 line was advanced and F_2 seed was examined for accumulation of KTi (Figure 3), via SDS–PAGE with seed produced in a greenhouse. Homozygosity for both *KTi1*– and *KTi3*– mutations was completely correlated with absence of both KTi protein bands (Figure 3, lane 8). PCR and sequencing verified that all lines with absent KTi1 and KTi3 protein were homozygous for both *KTi*– mutations and this trait was consistent for at least two generations.

Proteomic Investigations of Mutant Lines Bearing *KTi*– Mutations. Seed of the line homozygous for both the *KTi1*– and *KTi3*– mutations (E16) lacked any KTi protein, as detectable by anti-KTi antibodies. (Figure 4A,B). These results were also concordant with results for F_2 seed chips (Figure 3). However, BBI proteins accumulate to a much greater degree (≥10-fold higher) in mature seed bearing either or both *KTi* mutations, relative to Williams 82 (Figure 4C). Similar seed proteome rebalancing has been documented in previous studies. For example, the silencing of β-conglycinin, 7S seed storage protein, resulted in higher accumulation of the 11S glycinin.[35] Similarly, a soybean β-conglycinin mutant also

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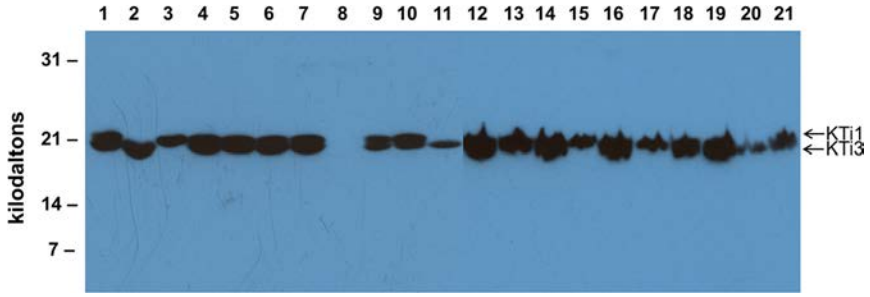

Figure 3. Immunoblot analysis of clarified protein from F_2 seed chips, produced from a single F_1 plant heterozygous for *KTi1*− and *KTi3*−. Immunoblots were probed with a non-isoform-specific anti-Kunitz trypsin inhibitor antibody. Numbers above lanes indicate individual F_2 seed from which seed chips were taken. The bands corresponding to KTi1 and KTi3 proteins are indicated by arrows.

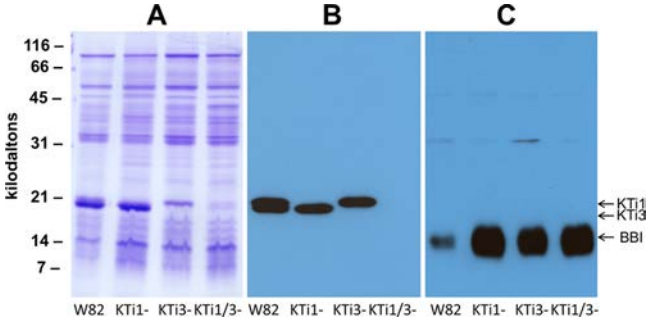

Figure 4. Western blot analysis of Kunitz and Bowman−Birk trypsin inhibitors in soybean seed of selected genotypes. (A) Coomassie brilliant blue R-250-stained PAGE gel. Seed proteins remaining in the supernatant after fractionation with 10 mM calcium were resolved by SDS−15% PAGE and stained with Coomassie brilliant blue. Protein for this analysis was obtained from equal amounts (20 mg) of dry seed powder. (B) Western blot probed with anti-Kunitz trypsin inhibitor antibodies (non-isoform-specific). (C) Western blot probed with anti-Bowman−Birk inhibitor antibody (non-isoform-specific). Bands corresponding to KTi1, KTi3, and BBI proteins are indicated by arrows. Genotypes are indicated below images: W82 = Williams 82 (PI 518671, wild type); KTi3− = Kunitz (PI 542044, *KTi3*− mutant); KTi1− = PI 68679 (*KTi1*− mutant); and KTi1/3− = E16 F_3 progeny of line homozygous for *KTi1*−, *KTi3*− mutant alleles.

exhibited proteome rebalancing by accumulating higher amounts of glycinin.[36]

Proteins extracted from mature seed of Williams 82 or *KTi* mutant lines were separated by DEAE-cellulose chromatography. The elution profile of soybean KTi protein is shown in Figure 5. When DEAE-cellulose-eluted fractions from Williams 82 and *KTi1*− were resolved by SDS−PAGE, an abundant 21 kDa protein was observed in fractions eluted with approximately 250 mM NaCl (Figure 5). Western blot analysis with KTi antibodies reacted strongly with the 21 kDa protein(s), confirming it as KTi (Figure S2, Supporting Information). This abundant protein was drastically reduced in the protein extracts from *KTi3*− and *KTi1/3*− mutant lines (Figure 5). We also fractionated total seed proteins from Williams 82 and *KTi* mutant lines by two-dimensional PAGE (Figure 6). We have previously identified soybean seed proteins resolved by 2D gels by MALDI-TOF-MS.[37] These analyses have demonstrated that the KTi proteins in Williams 82 seed resolves into at least three spots, KTi1/KTi2 and KTi3 (Figure 6, arrows and box indicate KTi protein spots). The protein spot corresponding to KTi3 is much greater in abundance as compared with the KTi1 spots. The *KTi1*− mutant accumulates abundant KTi3 but lacks the KTi1 spot, while the *KTi3*− mutant lacks the abundant KTi3 spot (Figure 6). The homozygote double-mutant line E16 has no detectable KTi protein in mature seed (Figure 6). Although there are at least four *KTi* genes in the soybean genome, the combination of two point mutations (*KTi1*− and *KTi3*−) resulted in complete absence of KTi protein (at a minimum below the threshold level of detection by immunoblot analysis).

Trypsin and Chymotrypsin Activities in Seed of Mutant Lines. The canonical KTi3 protein has been conclusively demonstrated to have trypsin inhibitor activity, and the *KTi3*− nonsense mutation reduces trypsin inhibitory activity by ∼40%.[32] In contrast, the relative contribution of the *KTi1* gene to overall trypsin inhibition activity was unclear.[32] We performed trypsin and chymotrypsin inhibitor assays, and significant genotypic differences were noted by one-way ANOVA (F = 16.8616 and p = 0.0098 for trypsin; F = 7.2156 and p = 0.0432 for chymotrypsin). Seed of all *KTi*− genotypes showed significantly (α = 0.05) decreased trypsin inhibitor activity relative to wild-type seed, and the combination of both mutations in line E16 had the lowest trypsin inhibitor activity (Figure 7A), although the mean trypsin inhibition among *KTi*− genotypes was not significantly different. Soybean genotype E16 revealed only about 50% trypsin inhibitor activity relative to the wild-type Williams 82 (Figure 7A). This observation is consistent with our proteomics data, which show no detectable KTi proteins in E16 (Figure 6). In spite of the complete absence of KTi in E16 genotype, the overall decrease in trypsin inhibitor activity is not significantly different from that of KTi3− mutant. This may be due to E16 seeds compensating for the mutation by overproducing BBI protein (Figures 4C and 8). Mean chymotrypsin activity was lower in all lines bearing *KTi1*− or *KTi3*− mutations, but these differences were significant (α = 0.05) only for the E16 genotype (Figure 7B) as compared to wild-type seed. A previous study has shown that a soybean line lacking KTi3 was nutritionally superior to unheated raw soybean but was inferior to processed soybean meal.[38]Heat treatment reduced trypsin and chymotrypsin inhibitor activities of the KTi3-free soybeans by approximately half and three-fourths in comparison with commercial untoasted soy flour.[38] It will be interesting to compare the effect of heat treatment on trypsin and chymotrypsin inhibitor activities of the E16 genotype created in the current study with those reported for the KTi3− mutant.

qRT-PCR Analysis of Developing Seed of *KTi* Mutant Lines. We examined gene expression in immature seed for all four lines (*KTi1*−, *KTi3*−, *KTi1/3*−, and Williams 82) by qRT-PCR with mRNA isolated from growth-chamber-produced seed at two seed development stages: (1) 200−300 mg/seed fresh weight and (2) 400−500 mg/seed fresh weight. Four of the

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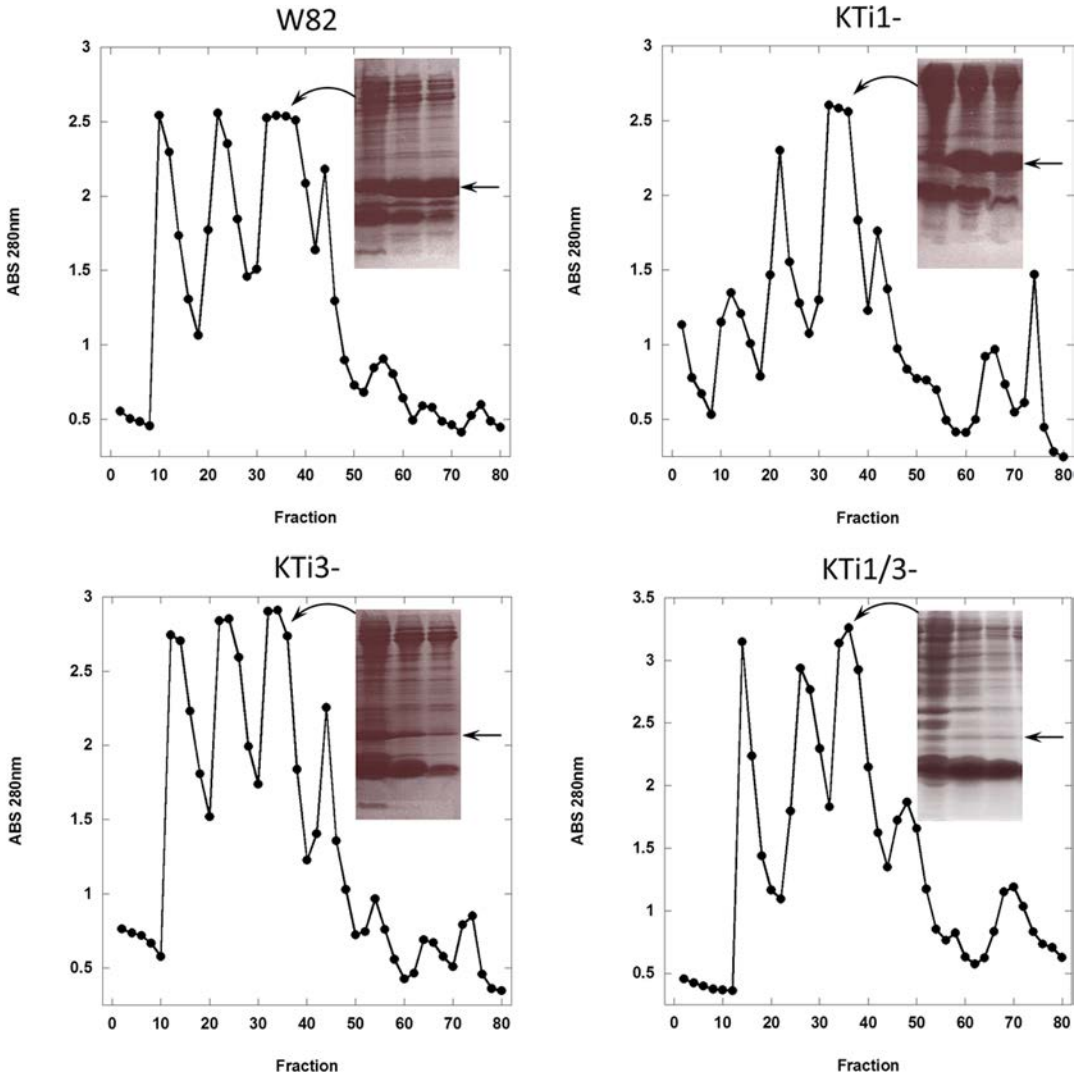

Figure 5. Elution profile of KTi fractionated by DEAE-cellulose chromatography. Soybean KTi was eluted from the column by use of a NaCl step gradient of 50 mM (fractions 1−10), 100 mM (fractions 11−21), 200 mM (fractions 22−31), 250 mM (fractions 32−41), 300 mM (fractions 42−51), 400 mM (fractions 52−61), and 500 mM (fractions 62−82). (Insets) SDS−PAGE images of fractions enriched in KTi. Arrows point to the abundant 21 kDa protein. Genotypes are indicated above each panel as described in the caption for Figure 4.

major seed storage protein-encoding genes present in soybean seed were selected for qRT-PCR analysis: glycinin 4 (*Gy4*), *KTi1*, *KTi3*, and a non-gene-specific primer pair that amplified several BBI genes (full details in Materials and Methods). One-way ANOVA was performed to compare the effect of genotype on each gene's expression level.

We observed no significant differences in *Gy4* mRNA levels between any genotypes at either seed developmental stage (Figure 8A, $F = 0.4091$ and $p = 0.7496$; Figure 8B, $F = 1.0407$ and $p = 0.4127$). No significant differences were noted for *KTi1* at the early developmental stage for any of the genotypes (Figure 8A, $F = 1.5404$ and $p = 0.2591$), and differences at the late developmental stage were not significant (Figure 8B, $F = 2.7633$ and $p = 0.0922$). Significant differences were noted for *KTi3* gene expression at both early and late developmental stages (Figure 8A, $F = 234.9889$ and $p < 0.001$; Figure 8B, $F = 56.8767$ and $p < 0.0001$). We observed significant differences in accumulation of BBI gene transcripts at the early seed developmental stage (Figure 8A, $F = 5.994$ and $p = 0.0113$).

Post hoc analyses were done with the Tukey HSD test ($\alpha = 0.05$) to compare genotype means (Figure 8) for *KTi3* and *BBI*. We noted significant near-absence of *KTi3* gene transcripts in lines bearing the *KTi3*− mutation at both early (Figure 8A) and late (Figure 8B) seed development stages, which is consistent with previous reports.[22] *KTi3* gene expression was similar between Williams 82 and *KTi1*− genotypes and between *KTi3*− and *KTi1/3*− genotypes. BBI gene expression was higher for both the *KTi1*− mutant (~2.75-fold higher) and the *KTi1/3*− mutant (~3.75-fold higher) as compared to Williams 82 at the early seed developmental stage, although only the *KTi1/3*− mutant was significantly different from Williams 82. *BBI* gene expression for Williams 82 and *KTi3*− genotypes were insignificantly different (Figure 8), and no significant differences were found at the later developmental stage for *BBI* gene expression (Figure 8B).

At least 10 BBI isoforms exist in soybean seed, which have been shown to have different specific inhibitory activities on trypsin, chymotrypsin, and/or elastase.[39,40] Some BBI isoforms inhibit only trypsin, or inhibit elastase and trypsin but not chymotrypsin. The specific proportion of these isoforms has been shown to vary from cultivar to cultivar.[41] Enhancement of BBI isoforms that inhibit only trypsin or inhibit elastase and

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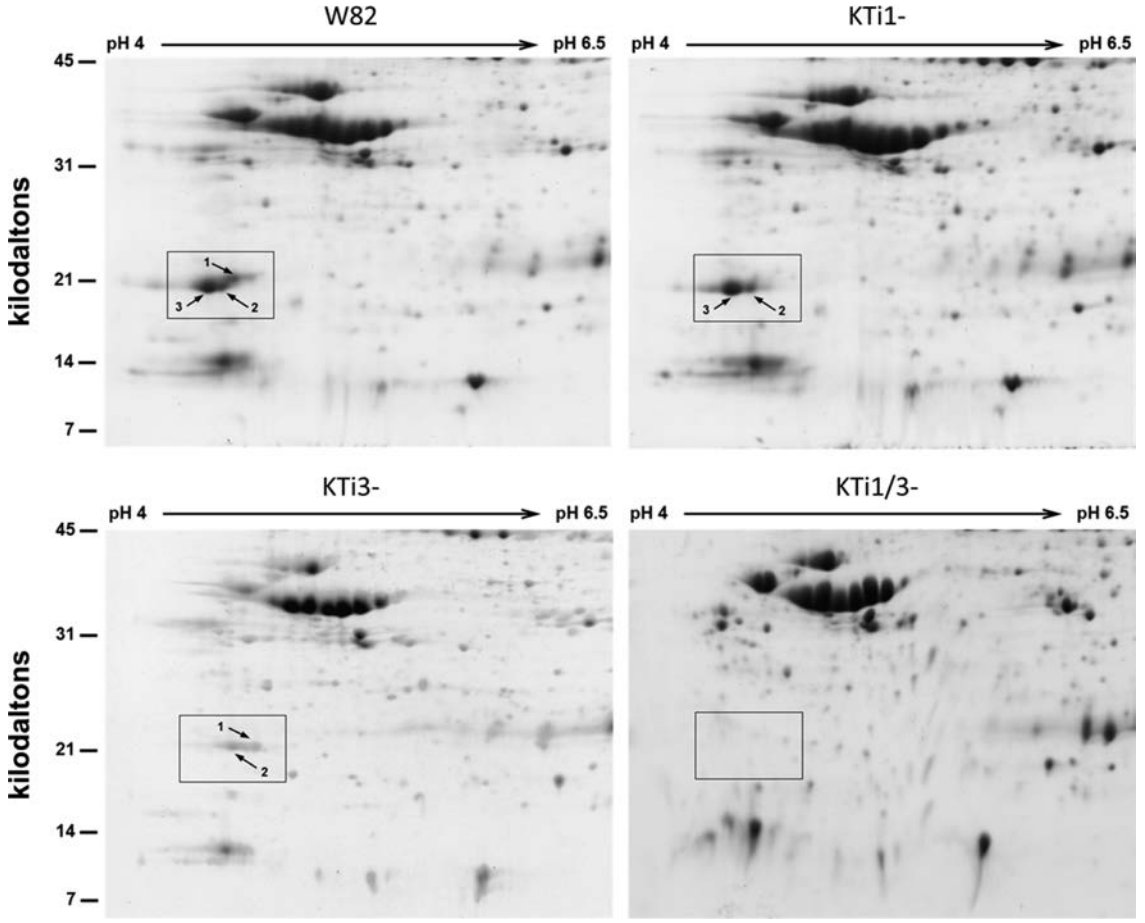

Figure 6. Two-dimensional gel electrophoresis of soybean total seed protein stained with Coomassie brilliant blue. Equivalent amounts of protein (300 μg) were separated on the basis of isoelectric point (p*I*) and then by SDS−PAGE. Genotype is indicated above each image, as described in the caption for Figure 4. The position of KTi proteins is indicated by numbered arrows and box.

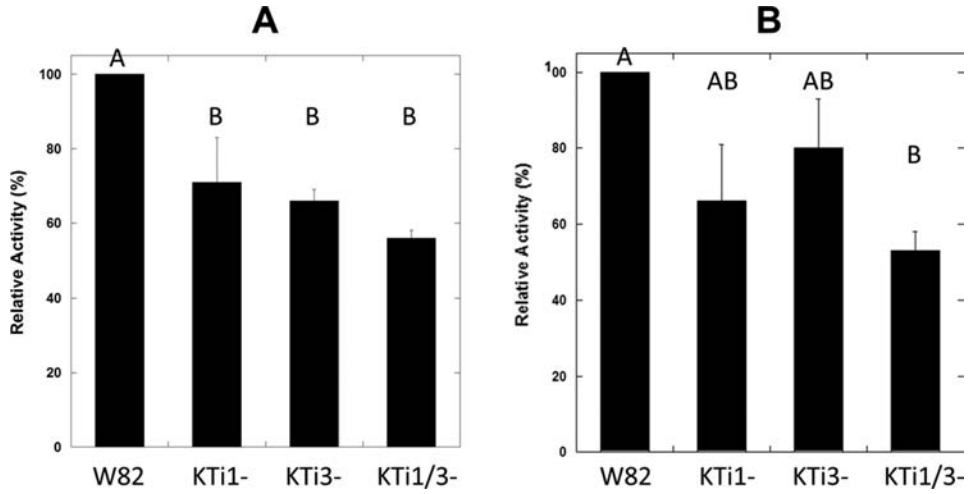

Figure 7. (A) Trypsin and (B) chymotrypsin inhibitor activity levels present in seed of selected genotypes. Letters above columns indicate result of one-way ANOVA ($p < 0.05$) and Tukey's HSD test ($\alpha = 0.05$); common letters indicate insignificant differences between means. Trypsin and chymotrypsin inhibitor activity were measured under standard conditions containing 10−50 μg of soybean seed protein with *N*-benzoyl-L-arginine ethyl ester and *N*-benzoyl-L-tyrosine ester as substrates, respectively. Genotypes are indicated below bars, as described in the caption for Figure 4.

chymotrypsin would compensate for the loss of trypsin inhibitor activity due to the KTi mutations without affecting chymotrypsin inhibitor activity. We observed no change in mRNA abundance of total BBI transcripts in seed of the *KTi3*− mutant (Figure 8A), which suggests that increased BBI protein is not likely due to increased transcription and/or enhanced mRNA stability in *KTi3*− lines. In contrast, we observed significantly higher levels of BBI transcripts in *KTi1*− lines. These results suggest that developing seed responds to *KTi*− lesions to maintain protein homeostasis through two different

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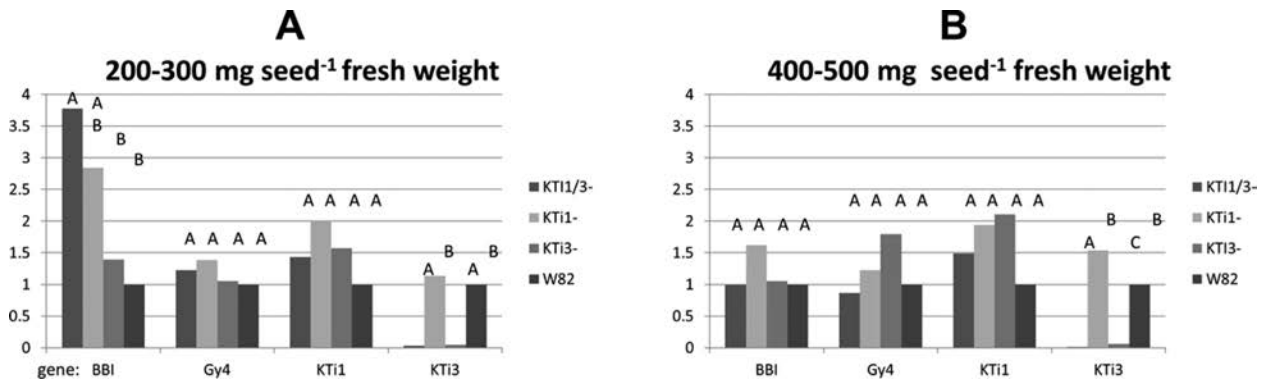

Figure 8. qRT-PCR analysis of mRNA from immature seed of two developmental stages examined with seed storage protein-specific primers: (A) early stage, isolated from immature seed between 200 and 300 mg/seed, and (B) late stage, isolated from immature seed between 400 and 500 mg/seed. Genes examined are *BBI* (non-isoform-specific), *KTi1*, *KTi3*, and glycinin subunit 4 (*Gy4*), and results were normalized to Williams 82 gene expression. Letters above columns indicates result of one-way ANOVA and Tukey's HSD test (α = 0.05); common letters indicate insignificant differences between means.

methods: elevated BBI mRNA levels (*KTi1*−) or proteome rebalancing (*KTi3*−), though the exact mechanisms are still incompletely understood. Enhanced accumulation of BBI proteins, however, does not translate to increased chymotrypsin inhibitor activity, which suggests that only a subset of BBI proteins are upregulated, in contrast to the major BBI isoform, which has functional chymotrypsin inhibition domains.[5] Similar results were obtained by transgenic overexpression of a mutated, nonfunctional BBI transgene, where the nonfunctional version effectively outcompetes translation of an endogenous, chymotrypsin-inhibitory BBI protein.[2016] In this study we have observed a reduction in chymotrypsin inhibitor activity in the KTi mutants (Figure 7B). It has been reported that soybean KTi can inhibit bovine chymotrypsin.[39] The loss of both KTi1 and KTi3 in the E16 genotype could result in greater inhibition of chymotrypsin inhibitor activity.

If substantial reduction in trypsin/chymotrypsin inhibitors can be achieved, beyond that currently possible with the *KTi1*− and/or *KTi3*− mutations, it may be possible to utilize raw soybean derivatives without the pancreatic complications or impaired weight gain issues of conventional soybean seed. This aim is particularly relevant for certain fish species (e.g., salmonoids), which are extremely sensitive to trypsin proteinase inhibitors, as compared to poultry and swine.[12] Even conventional heat-treated soybean meal bears residual trypsin/chymotrypsin inhibitor activity, which has limited inclusion of soybean meal in salmonoid diet mixtures.[42] As the current use of fishmeal is unsustainable in the long term,[43] reducing antinutritional trypsin inhibitory content of soybean seed may facilitate a more sustainable source of protein meal for fish production.

■ ASSOCIATED CONTENT

Supporting Information

Two figures, showing typical genotyping SimpleProbe assay for the *KTi3*− allele and immunoblot confirmation of KTi fractionated by DEAE-cellulose chromatography, and two tables, listing primers used in this work and expression of BBI genes in developing seed, as determined by RNA sequencing analysis. Data are taken from ref 44. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

BBI, Bowman−Birk proteinase inhibitor; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; *KTi1*, Kunitz trypsin inhibitor isoform 1 gene; *KTi2*, Kunitz trypsin inhibitor isoform 2 gene; *KTi3*, Kunitz trypsin inhibitor isoform 3 gene; PAGE, polyacrylamide gel electrophoresis; qRT-PCR, quantitative real-time polymerase chain reaction; SDS, sodium dodecyl sulfate

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