RESEARCH

Growing Location has a Pronounced Effect on the Accumulation of Cancer Chemopreventive Agent Bowman–Birk Inhibitor in Soybean Seeds

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ABSTRACT

Soybean [*Glycine max* (L.) Merr.] contains several health promoting compounds including phytosterols, isoflavones, phytic acid, and protease inhibitors. The two abundant protease inhibitors of soybean seeds are the Kunitz trypsin inhibitor and the Bowman-Birk inhibitor (BBI). Bowman-Birk inhibitor has been touted as a potential cancer chemopreventive agent for humans. Little information is available on the effect of growing location on the accumulation of this cancer chemopreventive agent. In this study we have examined the protein profile of eight soybean varieties that were grown in three Missouri locations in 2009 and 2010. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis demonstrated that soybean varieties that were grown in Grand Pass contained elevated levels of the β subunit of β-conglycinin and reduced amounts of BBI. This observation was further confirmed by Western blot analysis. This difference in the levels of BBI was also reflected in the chymotrypsin inhibitor activity. Growing location also influenced the overall S content of soybean seeds as evidenced by inductively coupled plasma mass spectrometry analysis. Seeds grown in Grand Pass had lower amounts of total S content in both 2009 and 2010. Our results demonstrate that growing location has a profound effect on the accumulation of BBI and it is possible to modulate the concentration of this cancer chemopreventive agent by simple changes in agronomic practices.

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Abbreviations: BBI, Bowman-Birk inhibitor; CTI, chymotrypsin inhibitor; ICP-MS, inductively coupled plasma mass spectrometry; mRNA, messenger RNA; OAS, *O*-acetyl-L-serine; SDS, sodium dodecyl sulfate; SDS-PAGE; sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

In the United States, soybean [*Glycine max* (L.) Merr.] is grown in 31 states with Iowa, Illinois, Minnesota, Indiana, and Ohio being the top soybean producing states, accounting for about 60% of the total U.S. soybean crop (American Soybean Association, 2012). In addition, soybean is also grown in the Mississippi Delta and Atlantic Coast regions. Consequently, soybean grown in these geographical regions is exposed to distinct environmental conditions, soil types, and cultural practices. Soybean seed composition is known to be influenced by several factors including genotype, environment, planting location, and planting date (Breene et al., 1988; Gibson and Mullen, 1996; Piper and Boote, 1999; Fehr et al., 2003; Thomas et al., 2003; Vollmann et al., 2000; Bennett and Krishnan, 2005; Jenkinson and Fehr, 2010). Numerous studies have examined the effect of environmental conditions on soybean seed composition and concluded that in general soybean grown in higher temperatures accumulates more protein while soybean grown in cooler conditions stores higher percentage of oil (Piper and Boote, 1999; Fehr et al., 2003; Wolf et al., 1982; Yaklich et al., 2002; Thomas et al., 2003).

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Soybean contains several health promoting compounds including phytosterols, isoflavones, phytic acid, and protease inhibitors (Messina and Barnes, 1991). Yamaya et al. (2007) examined the effect of genetic variability and planting locations on soybean phytosterol content. These authors determined the phytosterol content of 510 germplasm accessions of cultivated soybean by high performance liquid chromatography and concluded that even though genetic variability and planting location affected the phytosterol content it did not affect their composition (Yamaya et al., 2007). Effects of genotype, environment, and planting location on the accumulation of isoflavones have also been investigated (Eldridge and Kwolek, 1983; Wang and Murphy, 1994). Cool temperatures at seed fill have shown to increase the isoflavone content severalfold (Tsukamoto et al., 1995). Additionally, agronomic cultural practices such as planting date and irrigation has also been shown to have a profound effect on accumulation of isoflavones, daidzein, and genistein in soybean seeds (Bennett et al., 2004).

Soybean contains two types of protease inhibitors: the Kunitz trypsin inhibitor and the Bowman-Birk protease inhibitor (Birk, 1985; Nielsen, 1996). These protease inhibitors have been traditionally treated as antinutritional components because they interfere with the efficient feed usage (Clarke and Wiseman, 2000). Consequently, soybean is subjected to roasting to inactivate these antinutritional factors (Perez-Maldonado et al., 2003). The effect of genotype, environment, and fertilization on the trypsin inhibitor activity in soybean has been reported (Vollmann et al., 2003). A significant variation in trypsin inhibitor activity among different soybean genotypes was observed. Nitrogen application was shown to reduce the trypsin inhibitor activity by about 15% (Vollmann et al., 2003). However, no information on the Bowman-Birk protease inhibitor was reported in this study. Bowman-Birk protease inhibitors have received greater attention since it has been demonstrated that this bioactive peptide possess chemopreventive activity against different types of cancer (Yavelow et al., 1985; Billings and Habres, 1992; Kennedy, 1998; Zhang et al., 1999; Meyskens, 2001; Kennedy and Wan, 2002). Consequently, Bowman-Birk inhibitor (BBI) is intensively researched as a cancer chemopreventive agent. In spite of the important role of BBI in cancer prevention, very little is known on the effect of the environment on the accumulation of this bioactive peptide. In this study we report that growing location has profound effect on the accumulation of BBI. Furthermore, we demonstrate that geographical location also can significantly affect the nutritive quality of soybean seed.

MATERIALS AND METHODS

Plant Material

Soybean varieties used in this study were submitted by various companies to the University of Missouri's variety testing program in 2009 and 2010. They were grown at three locations in Missouri where significant number of acres of soybean are grown according to the Missouri Agricultural Statistics Service. These locations were Grand Pass, MO (Saline County), the Bradford Research and Extension Center, Columbia, MO (Boone County), and the Bob Burkemper farm, Annada, MO (Pike County). These locations will be referred to as Grand Pass, Columbia, and Annada, respectively. Eight soybean varieties were randomly chosen from those submitted to the University of Missouri's variety testing program for our study. Soybean varieties studied in 2009 include Asgrow AG3803, Crow's C3916R, Dyna-Gro 32X29, G2 Genetics 7341, Hubner H39-01R2, Kruger K2-3801, Lewis 380R2, and Midland 3738NRR. Soybean varieties investigated in 2010 include Kruger K2-4201, Asgrow AG3830, Power Plus 37T1, Midland 3861NR2, MPVC-450N, Morsoy RT 4457N, NK S35-T9, and Morsoy R2 4240N. Soybean cultural practices were very similar in all three locations and reflected those followed by farmers in these areas. Soybean varieties were planted using commercial equipment modified for small plot work. No fertilizer applications were made at these locations except in 2009 when the Columbia location received fertilizer applications of 10 kg N ha^{-1}, 46 kg P ha^{-1}, and 60 kg K ha^{-1}. A brief summary of soybean management is provided in Table 1.

Extraction of Proteins from Soybean Seeds

Seed samples from each of three replicates of all soybean varieties grown in three locations in 2009 and 2010 were ground to a fine powder using a mortar and pestle. Total seed proteins were isolated by extracting 10 mg of ground soybean powder in 1.0 mL of sodium dodecyl sulfate (SDS) buffer (125 mM Tris-HCL buffer pH 6.8, 4% SDS [w/v], 20% glycerol [v/v], 0.03 mM bromophenol blue, and 5% [v/v] 2-mercaptoethanol) followed by boiling for 5 min. After centrifugation, the supernatants were transferred to microfuge tubes and served as the source of total seed proteins. Isopropanol-soluble proteins from soybean seeds were extracted as previously described (Krishnan et al., 2005). Briefly, 100 mg of seed powder was extracted with 1 mL of 50% isopropanol in a 37°C shaker for 1 h. The slurry was clarified by centrifugation and isopropanol-soluble proteins recovered by acetone precipitation. Recovered proteins were solubilized directly in 200 μL of SDS buffer (Laemmli, 1970).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blot Analysis

Before electrophoresis protein samples were heated in a boiling water bath for 5 min and then cooled on ice. Aliquots of these samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Electrophoresis was performed on either a 12.5 or 15% resolving gel (w/v) at 20 mA for 1 h using the Hoefer SE 260 minigel apparatus (Amersham Biosciences). Protein bands were visualized with Coomassie Blue R-250.Western blot analysis was performed as described earlier (Krishnan et al., 2005). Polyclonal antibodies generated against a conserved region of BBI were used for detecting the relative concentration of BBI among soybean varieties grown at three locations. Proteins transferred to the nitrocellulose membranes were incubated overnight with the Bowman-Birk protease inhibitor antibody that was diluted

Table 1. Soybean management summary.

Dates					Herbicide†	
Year	Location	Planting	Harvest	Tillage	Preemergent	Postemergent
2009	Grand Pass	20 May	7 Oct.	No-till	Dual II Magnum, First Rate, Authority, Roundup, PowerMax	Roundup, PowerMax
2009	Columbia	19 May	29 Sept.	No-till	Dual II Magnum, First Rate	Roundup, PowerMax
2009	Annada	22 June	14 Nov.	Minimum	Dual II Magnum, First Rate, Authority, Roundup, PowerMax	Roundup, PowerMax
2010	Grand Pass	1 June	16 Oct.	No-till	Dual II Magnum, Sonic, Roundup, PowerMax	none
2010	Columbia	27 May	7 Oct.	No-till	Roundup, PowerMax	Shadow
2010	Annada	7 June	20 Oct.	Minimum	Dual II Magnum, Sonic, Roundup, PowerMax	none

†Dual II Magnum (Syngenta Crop Protection, Greensboro, NC); Fist Rate (Dow AgroSciences, Indianapolis, IN); Authority (FMC Corporation, Philadelphia, PA); Roundup (Monsanto, St. Louis, MO); PowerMax (Monsanto, St. Louis, MO); Sonic (Dow AgroSciences, Indianapolis, IN); Shadow (Arysta LifeScience Corporation, Tokyo, Japan).

1:5000 in Tris-buffered saline (TBS) (10 mM Tris-HCl, pH 7.5, and 500 mM NaCl) containing 5% (w/v) nonfat dried milk. Following several washes in TBST (TBS containing 0.3% Tween 20) the nitrocellulose membranes were incubated with goat anti-rabbit immunoglobulin G (IgG)–horseradish peroxidase conjugate for 1 h. Immunoreactive polypeptides were detected with an enhanced chemiluminescent substrate following the procedure provided by the manufacturer (Pierce Biotechnology).

Chymotrypsin Inhibitor Assay

To assay for the presence of chymotrypsin inhibitor (BBI), 50 mg of seed powder was extracted with 1 mL of 100 mM Tris-Cl, pH 8.0, followed by vortexing for 10 min. Extract was clarified at 16000 × *g* for 10 min and 400 μL of extract removed and diluted to 1 mL with extraction buffer. Assays were performed in 100 mM Tris-Cl, pH 8.0, with 25 mM calcium chloride dihydrate, using 0.55 mM N-benzoyl-L-tyrosine ethyl ester (Sigma-Aldrich Company) dissolved in 64% methanol and 36% water as substrate. An equal volume of extract was added to each assay after the addition of buffer and substrate. Assay volume was 1 mL and assay performed in a quartz cuvette and monitored at 256 nm. Assay solution was zeroed and then 0.1 U of α-chymotrypsin (Sigma-Aldrich Company) (dissolved in 1 mM HCl solution) was added to begin the reaction. The change in absorbance was monitored for at least 3 min. Chymotrypsin inhibitor units were calculated as the amount of inhibitor that reduced the absorbance per minute of the standard reaction by 0.01 (Stauffer, 1990) and was based on the amount of protein added. For accuracy the reaction was measured in the linear portion in the 40 to 60% inhibition range.

Inductively Coupled Plasma Mass Spectrometry Analysis

Single dry seeds were arrayed in 48-well plates and then loaded on a custom built weighing robot that deposited the weighed seeds into 16 by 100 mm Pyrex test tubes. Digestion was performed by adding 2.5 mL of concentrated HNO_3 (VWR AR Select American Chemical Society grade [VWR International]) containing Indium internal standard to the test tubes and incubating overnight at room temperature before heating the samples to 105°C over 2 h and then cooling to room temperature over 2 h. The digested samples were diluted in the test tubes to 10 mL by adding 18 MΩ water, and a second dilution was made in a second set of test tubes by taking 900 μL of the first dilutions to 5 mL with 18 MΩ water. Then 1.2 mL of the second dilutions was transferred to 96-well autosampler plates using an adjustable-width multichannel pipette. Sulfur analysis was performed using an Elan DRC - e inductively coupled plasma mass spectrometer (PerkinElmer Inc.) with an Apex Desolvation Nebulizer, FAST sampling valve, and SC4 DX autosampler (Elemental Scientific Inc.). 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 weights.

Gel Image and Statistical Analyses

Computer-assisted analysis was performed with the Advanced Analysis (version 4.01) module of Phoretix 1D gel analysis software (Nonlinear Dynamics Limited, 2009). Those settings recommended by Nonlinear USA were used for all band analysis and quantification. Automatic band acquisition, background subtraction, and Gaussian distribution of band intensity were used to generate a band and peak volume for each 1D gel band of interest. These numbers were applied to a linear regression of a 1D SDS-PAGE analysis of multiple lanes containing a known amount of bovine serum albumin (0–1000 ng) run previously and analyzed identically using Phoretix 1D. Protein band values generated in nanograms of protein for each sample of each cultivar at each location were then used for statistical analyses. Each year of data was analyzed separately, as different cultivars were used each year. The ANOVA procedure of SAS version 9.2 (SAS Institute, 2004) was used to conduct an analysis of variance, in which the model consisted of cultivar, locations, replications within location, and cultivar × location. The MEANS statement was used to obtain location means, and the Tukey's studentized range procedure was used to test for differences between locations.

RESULTS

Protein Composition of Soybean Seeds is Affected by Growing Location

To ascertain if growing environment influences the protein composition of soybean seeds we examined the total protein profile of soybean seeds that were grown at same three locations in the 2009 and 2010 growing seasons. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of soybean seed proteins revealed that the protein profile of the seeds were strikingly similar between all the soybean cultivars (Fig. 1). Soybean seeds accumulated several abundant

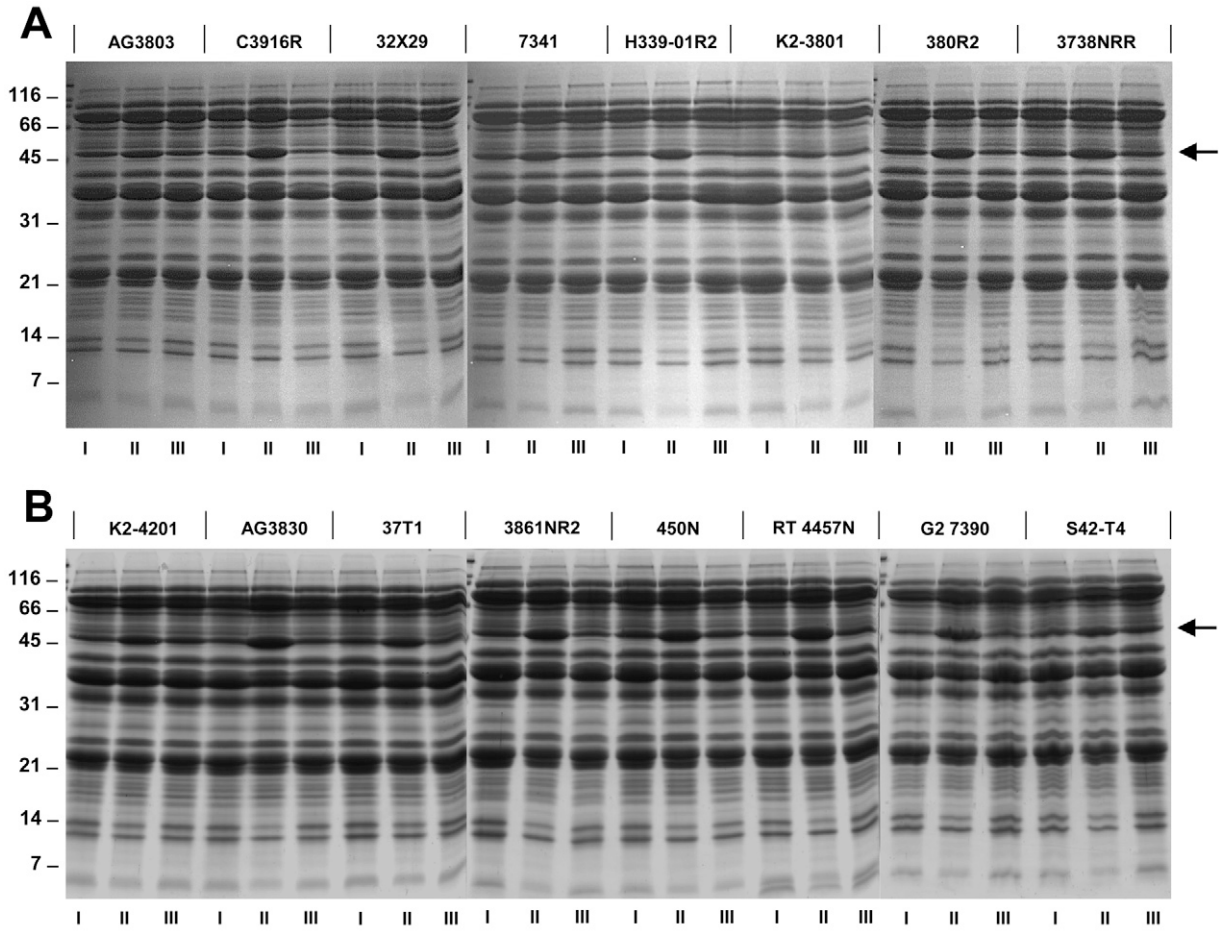

Figure 1. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoretic analysis of soybean seed proteins. Total proteins obtained on the basis of equal seed dry weight were separated on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Resolved proteins were stained with Coomassie Brilliant Blue. Names of soybean varieties are shown at the top of the figure and the numbers at the bottom of the gel refers to growing locations (I, Annada; II, Grand Pass; and III, Columbia). Sizes of protein standards are shown in kilodaltons. The arrow points to the β subunit of β-conglycinin. Panels A and B are protein profiles of soybean seeds grown in 2009 and 2010 growing season, respectively.

proteins with apparent molecular weights of 72, 70, 52, 40, and 20 kDa. The 72, 70, and 52 kDa proteins belong to the 7S globulins while the 40 and 20 kDa proteins represent the acidic and basic subunits of 11S globulins (Nielsen, 1996). Interestingly when the protein profile of soybean cultivars grown at three locations were compared the accumulation a 52 kDa polypeptide was significantly higher in seeds that were grown in Grand Pass (Fig. 1). Computer assisted analysis of the band intensity resulted in the quantification of the 52 kDa polypeptide. The mean value for the amount of this protein from all the cultivars grown at Annada, Grand Pass, and Columbia were 19.7, 27.2, and 20.5 ng per band, respectively. Tukey's studentized range procedure revealed that the concentration of the 52 kDa polypeptide at the Grand Pass location was significantly higher than the other two locations. All the cultivars grown in Grand Pass showed an increase, although at different levels, in the accumulation of this protein (Fig. 1). Our results demonstrate that irrespective of the cultivars used, the accumulation of the 52 kDa polypeptide is modulated by the growing environment.

To confirm the identity of the 52 kDa polypeptide we performed immunoblot analysis using antibodies raised against the purified β subunit of β-conglycinin (Krishnan et al., 2000). The antibodies specifically recognized the 52 kDa protein demonstrating that the 52 kDa polypeptide is the β subunit of β-conglycinin (data not shown).

Bowman-Birk Protease Inhibitor Accumulation is Influenced by Growing Location

We have previously shown that nonnodulating soybean accumulates low amounts of the β subunit of β-conglycinin and high amounts of Bowman-Birk protease inhibitor, indicating an inverse correlation (Krishnan et al., 2005). Since soybean cultivars grown in Grand Pass accumulate significantly higher amounts of β subunit of β-conglycinin, we wanted to examine if they accumulated less Bowman-Birk protease inhibitor in comparison to those grown in Columbia and Annada. Bowman-Birk protease inhibitor can be preferentially extracted with 50% isopropanol (Krishnan, 2004). Figure 2A shows

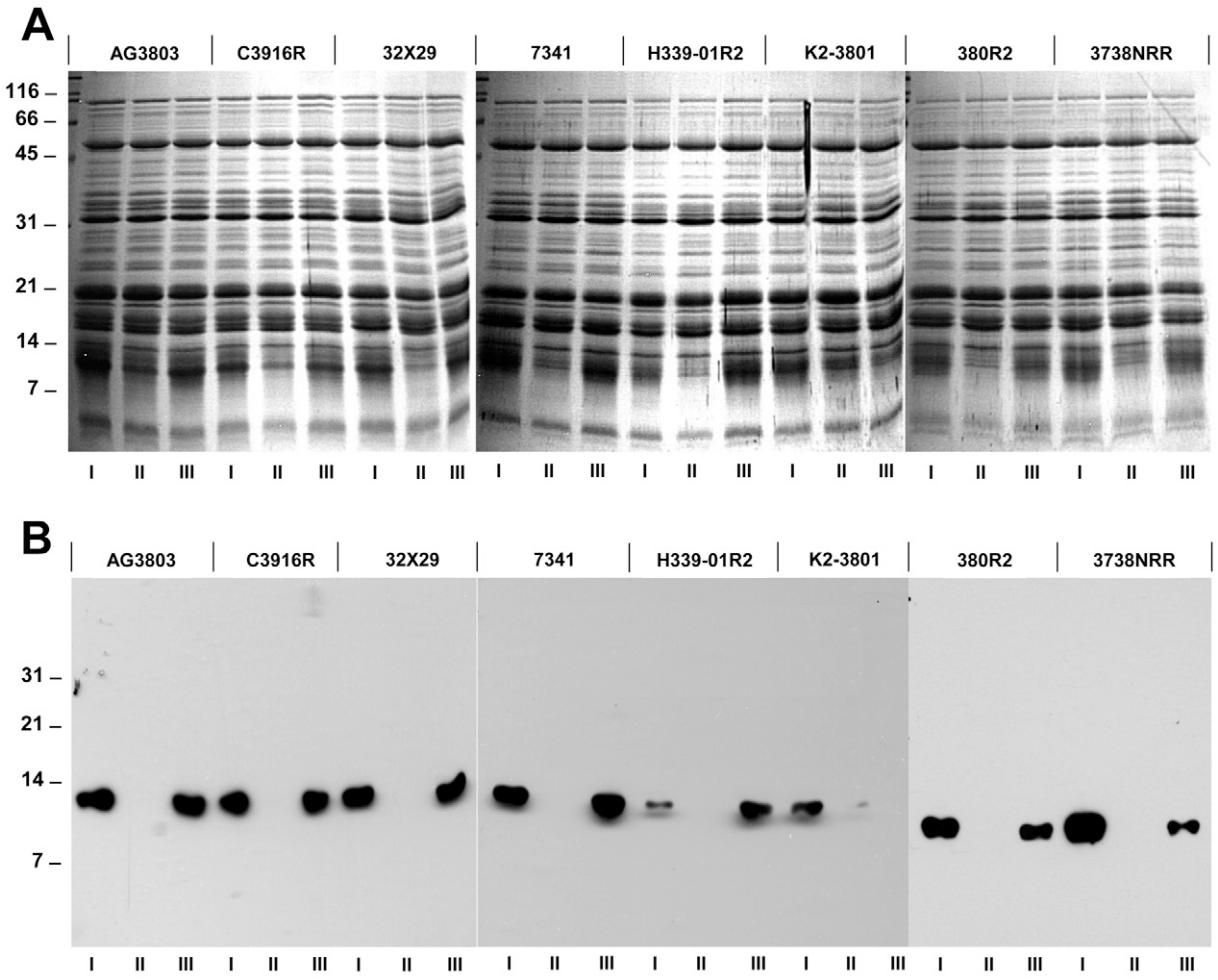

Figure 2. Panel A: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of 50% isopropanol-extracted proteins from soybean. Equal amounts of dry soybean seed powder was extracted with 50% isopropanol and the recovered proteins were resolved on 15% SDS-PAGE gels. Proteins were visualized by staining with Coomassie Brilliant Blue. Panel B: Immunoblot analysis of Bowman-Birk inhibitor (BBI) accumulation. Isopropanol-extracted proteins separated on a 15% SDS-PAGE were electrophoretically transferred to nitrocellulose membranes and probed with BBI-specific antibodies. Immunoreactive proteins were detected using anti rabbit immunoglobulin G (IgG)–horseradish peroxidase conjugate followed by chemiluminescent detection. Names of soybean varieties are shown at the top of the figure and the numbers at the bottom of the gel refers to growing locations (I, Annada; II, Grand Pass; and III, Columbia). Sizes of protein standards are shown in kilodaltons.

SDS-PAGE profile of 50% isopropanol soluble proteins from soybean seeds grown in three locations in the 2009 growing season. Several abundant proteins with molecular weights of 56, 34, 21, 18, 16, 14, and 10 kDa were present in all the soybean cultivars examined in this study. We have previously identified the 56 and 34 kDa proteins as β-amylase and dehydrin, respectively (Natarajan et al., 2009; Kim and Krishnan, 2010). Similarly, the 21 and 10 kDa protein has been identified as Kunitz trypsin inhibitor and Bowman-Birk protease inhibitor, respectively (Krishnan, 2004; Natarajan et al., 2009). A comparison of the 50% isopropanol-soluble protein profile of eight soybean varieties grown in three different locations showed low accumulation of low molecular weight proteins corresponding to 10 to 14 kDa in seeds grown in Grand Pass (Fig. 2A). Quantification of the band intensity by computer-assisted analysis indicated that the amount of the polypeptide corresponding to the BBI was much lower (19.7 ng per band) at the Grand Pass location when compared to Annada (47.0 ng per band) and Columbia (33.1 ng per band) locations. Tukey's studentized range procedure revealed that the BBI accumulation at all three locations were different with Grand Pass location being lowest.

To examine if the seeds grown in Grand Pass accumulated lower amounts of Bowman-Birk protease inhibitor than those grown in Annada and Columbia, we performed immunoblot analysis using peptide antibodies raised against conserved region of the Bowman-Birk protease inhibitor (Fig. 2B). Densitometer scan of the immunoblot analysis clearly showed that seeds grown in Grand Pass in 2009 accumulated low amounts of Bowman-Birk protease inhibitor (3881 arbitrary units) when compared to Annada (466127 arbitrary units) and Columbia (56334 arbitrary units) locations. Similar results were also obtained from all

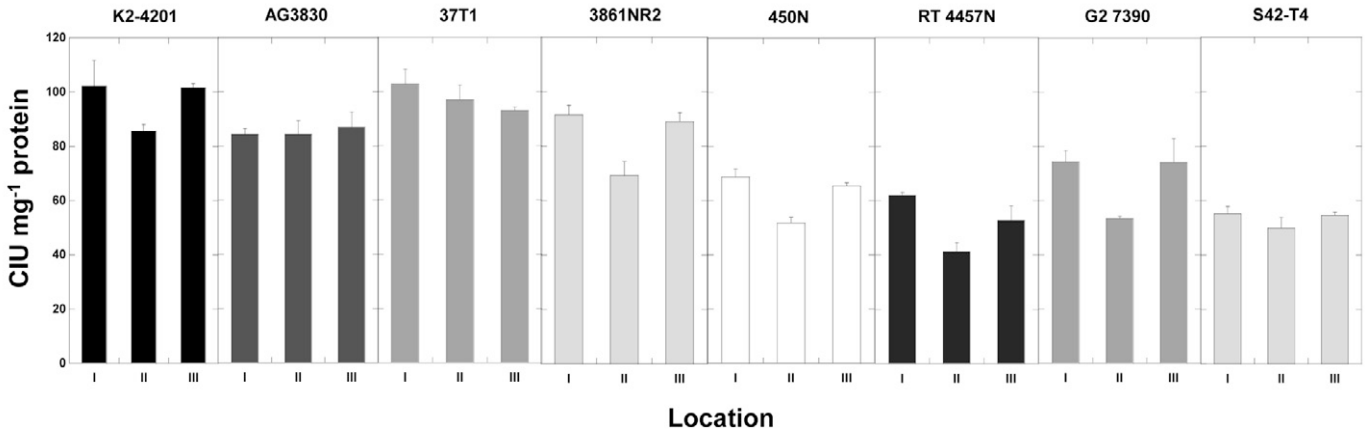

Figure 3. Chymotrypsin inhibitor activity of soybean seeds. Chymotrypsin inhibitor activity was measured under standard assay conditions as described under "Materials and Methods" using protein extracts from soybean seeds. The activity is expressed as chymotrypsin inhibitor units (CIU) and presented as the mean ± SE (n = 3). Names of soybean varieties are shown at the top of the figure and the numbers at the bottom of the figure refers to growing locations (I, Annada; II, Grand Pass; and III, Columbia).

the eight cultivars grown in the same locations in the 2010 growing season (data not shown).

Measurement of chymotrypsin activity is a method to identify Bowman-Birk protease inhibitor in soybean seeds. Since immunoblot analysis revealed that soybean cultivars grown in Grand Pass location accumulated significantly lower amounts of Bowman-Birk protease inhibitor we wanted to examine if there was also a noticeable decrease in the chymotrypsin inhibitor (CTI) activity. Consequently we measured the CTI activity of the different soybean varieties that were grown in three locations (Fig. 3). The CTI activity varied among different soybean varieties. The mean CTI activity of all the cultivars grown at Annada, Grand Pass, and Columbia locations were 80.1, 66.5, and 77.1, respectively. Tukey's studentized range procedure revealed that CTI activity in the Grand Pass location was significantly lower than the other two locations. While the reduction in CTI activity was quite evident in most of soybean varieties grown in Grand Pass, few varieties did not follow this trend (Fig. 3).

Sulfur Content is Significantly Lower in Soybean Seeds Grown at Grand Pass

Bowman-Birk protease inhibitor is a major contributor of the total S content in soybean seeds since this protein contains 14 cysteine residues (Birk 1985; Clarke and Wiseman, 2000). Since the immunoblot analysis clearly demonstrated that there are location-specific differences in the accumulation of Bowman-Birk protease inhibitor we also wanted to examine if there was also a similar change in total S content of these seeds. The total S content was measured with the aid of ICP-MS. The S content of soybean seeds grown in Annada and Columbia varied between 3000 and 3500 mg kg^{-1} while those grown in Grand Pass had less than 2500 mg kg^{-1} (Fig. 4). This trend was observed in both years, demonstrating that seeds grown in Grand Pass contain lower amounts of total S. Tukey's studentized range procedure confirmed that the S content of seeds harvested from Grand Pass was significantly lower than the other two locations.

DISCUSSION

In this study we have demonstrated that growing locations have a profound effect on protein composition of soybean seeds. Soybean seeds grown in Grand Pass accumulated significantly higher amounts of the β subunit of β-conglycinin. This particular protein is deficient in S-containing amino acids and its accumulation is elevated when the plants are grown under limited S supply (Holowach et al., 1984; Gayler and Sykes, 1985; Fujiwara et al., 1992). Additionally, the accumulation of this protein is also shown to be regulated by the N status of the plant (Sexton et al., 1998). When soybean plants are grown in presence of excess N the accumulation of the β subunit of β-conglycinin is preferentially enhanced (Paek et al., 1997). Using transgenic petunia (*Petunia* spp.) and *Arabidopsis thaliana* (L.) Heynh., it has been demonstrated that that the gene encoding the β subunit of β-conglycinin is upregulated during S deficiency (Fujiwara et al., 1992; Naito et al., 1994) and is coordinately regulated by both S and N nutrition (Kim et al., 1999). The precise reason why soybean grown at Grand Pass accumulates higher amounts of the β subunit of β-conglycinin remains yet to be answered. One possible factor could be that Grand Pass location contains higher N or lower S content, which will result in increased accumulation of the β subunit of β-conglycinin. However, analysis of the soil samples from these three locations in both 2009 and 2010 did not reveal any major differences in the levels of S and N in these locations (data not shown). Additionally, in 2009 the Columbia location received fertilizer applications of 10 kg N ha^{-1} and yet the accumulation of the β subunit of β-conglycinin was lower than the Grand Pass location. This observation suggests that the enhanced accumulation of the β subunit of β-conglycinin in seeds grown

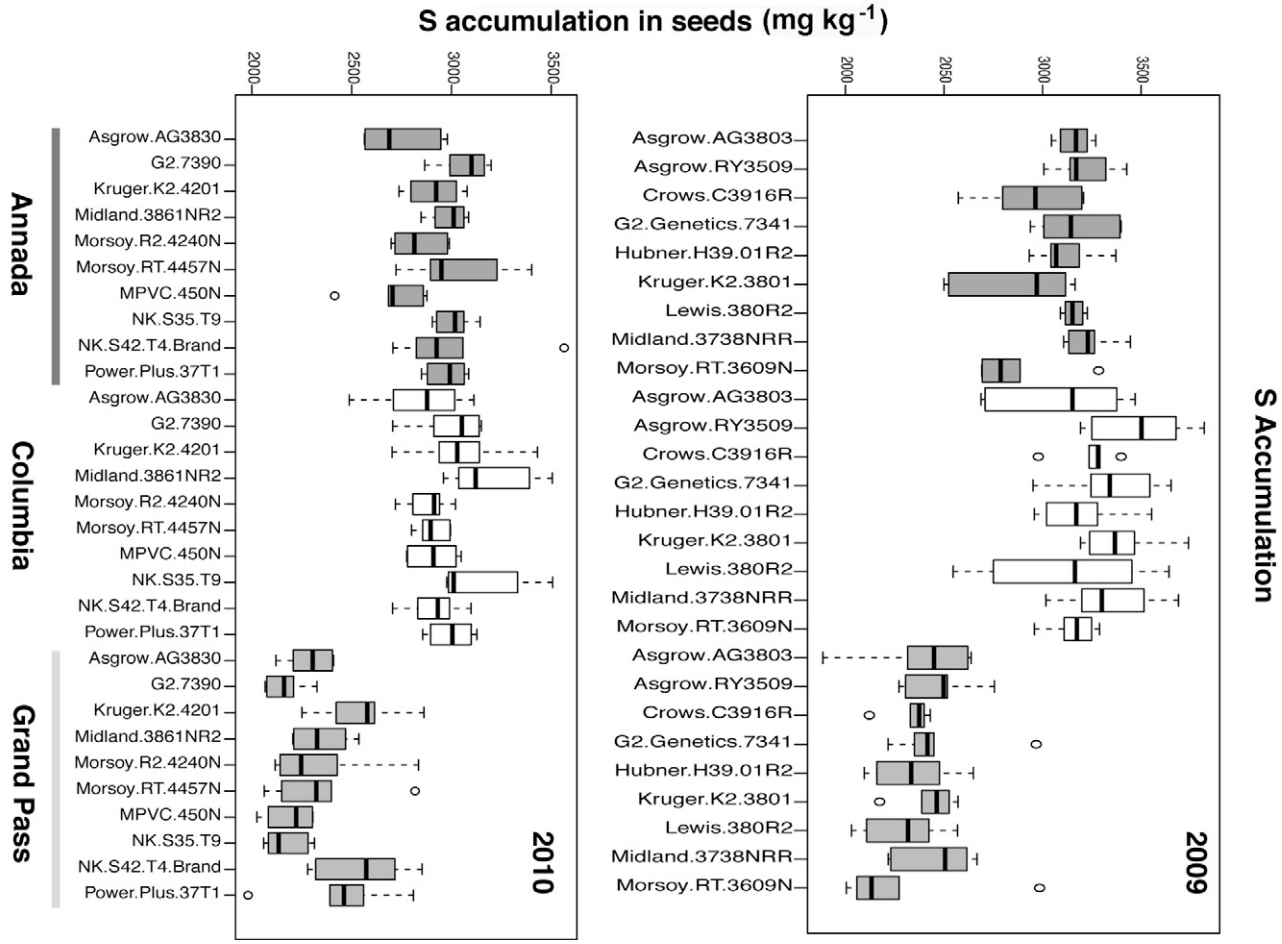

Figure 4. Sulfur content of soybean seeds. Elemental S content of soybean seeds grown in three different locations was determined by inductively coupled plasma-mass spectroscopy. Note soybean grown in Grand Pass in both 2009 and 2010 contain significantly lower concentration of S. Data is shown as a five number summary (the minimum, first quartile, median, third quartile, and maximum) for each line with outliers indicated by small circles and is summarized from an average of seeds for each line at each location.

in Grand Pass may be regulated by other yet unidentified factors. One potential candidate is *O*-acetyl-L-serine (OAS), a key metabolite involved in cysteine synthesis (Kim et al., 1999). A previous study has demonstrated that OAS is a key mediator of S- and N-nutrition-regulated expression of soybean seed storage-protein genes (Kim et al., 1999). In this context it will be interesting to examine if there are differences in the concentration of OAS in soybean seeds grown in Grand Pass in comparison to other two locations.

Our study also confirms that there is an inverse relationship between the accumulation between the β subunit of β-conglycinin and Bowman-Birk protease inhibitor. All the soybean varieties that were grown in Grand Pass accumulated elevated amounts of β subunit of β-conglycinin but low levels of Bowman-Birk protease inhibitor. Our results are consistent with an earlier study that demonstrated that nonnodulating soybean, which accumulate very low levels of β subunit of β-conglycinin, contained elevated amounts of Bowman-Birk protease inhibitor (Krishnan et al., 2000). Previous studies have also shown a reduction in messenger RNA (mRNA) encoding Bowman-Birk protease inhibitor when soybean seeds were grown with excess N nutrition (Krishnan et al., 2005). In contrast Bowman-Birk protease inhibitor mRNA levels were increased in soybean cotyledons grown on methionine-supplemented medium (Holowach et al., 1984). Supplementation of sulfate to greenhouse-grown soybean also increased the amount and activity of protease inhibitor (Holowach et al., 1984). Our immunoblot analysis clearly demonstrates that there is a drastic reduction in the accumulation of Bowman-Birk protease inhibitor in seeds grown in Grand Pass but this reduction is not precisely correlated with the CTI activity. This result, however, is not surprising given the fact that Bowman-Birk protease inhibitor is encoded by a multigene family (Deshimaru et al., 2004). At least seven BBI isoinhibitors, A, B, C-I/II, D-I/II, and E-I, have been identified and purified from soybean seeds (Deshimaru et al., 2004). Therefore, it is possible that other BBI isoinhibitors may contribute to CTI activity reported in our study.

Soybean varieties with higher amounts of β subunit of β-conglycinin are also desirable since this protein provides health benefits. Bioactive peptides are released when β-conglycinin is subjected to either enzymatic hydrolysis or fermentation. These bioactive peptides have antimicrobial

properties (Yang et al., 2008), promote the growth of Bifidobacteria (Zuo et al., 2005), inhibit L1210 leukemia cell growth (Wang et al., 2008), and possess anxiolytic activity (Ohinata et al., 2007). Soybean with lower concentrations of BBI can have a positive effect on the animal feed industry. Traditionally, protease inhibitors are considered anti-nutritional components of soybean meal (Clarke and Wiseman, 2000; Perez-Maldonado et al., 2003). Bowman-Birk inhibitor in soybean meal is known to inhibit the activity of intestinal enzymes resulting in reduced nutrition absorption, which translates into poor animal performance. In this regard soybean varieties that accumulate low levels of BBI will be desirable in animal feed. In our study we have shown that growing locations have profound effect on the accumulation of the BBI. Therefore, soybean seeds that were grown in Grand Pass will be a better nutritive source for animal feed. The fact that one can manipulate the levels of BBI in soybean seeds by regulating the availability of N bodes well for lowering this anti-nutritional component of soybean seeds. In the last two decades BBI has received substantial attention as a potential anticancer compound. The effectiveness of BBI in suppressing different forms of cancer has been demonstrated in both in vitro and in vivo animal model systems (Messina and Barnes, 1991). Currently, BBI is being tested in large-scale human trials as an anticarcinogenic agent (Armstrong et al., 2000, 2003; Meyskens, 2001). Therefore, soybean containing a higher amount of BBI will be useful with respect to human health. We suggest that one can modulate the concentration of BBI in soybean seeds by simple changes in the agronomic practices. For example, for obtaining soybean with low concentration of BBI the farmers can fertilize their fields with N during the reproductive phase. Alternatively, nonnodulating soybean plants, which are unable to carry out symbiotic N-fixation, can be exploited to obtain seed with higher content of BBI.

Acknowledgments

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