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RAPID COMMUNICATION

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A four-nucleotide base-pair deletion in the coding region of the Bowman–Birk protease inhibitor gene prevents its accumulation in the seeds of *Glycine microphylla* PI440956

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Abstract The Bowman–Birk protease inhibitor (BBI), an abundant soybean [*Glycine max* (L.) Merr.] seed protein, is a major antinutritional factor. Nulls for the major soybean BBI have been reported in several of the wild perennial *Glycine* species including *G. microphylla* (Benth.) Tind PI440956. This perennial *Glycine* species does not accumulate the major BBI and the molecular basis for the absence of the major BBI in this plant introduction (PI) line is not known. We have cloned the BBI gene from *G. microphylla* PI440956, *G. microphylla* PI505188, and *G. max* cv. Jefferson and determined its nucleotide sequences. Analysis of the *G. microphyllla* PI505188 and *G. max* cv. Jefferson nucleotide sequences revealed a complete open-reading frame encoding the BBI. In contrast, the BBI coding region of *G. microphylla* PI440956 contained a frameshift mutation that resulted in the introduction of a stop codon at the amino terminal region of the protein. Reverse transcription–polymerase chain reaction analysis revealed that the BBI gene was expressed in developing seeds of *G. microphylla* PI505188 and *G. max* cv. Jefferson, but not in developing seeds of *G. microphylla* PI440956. In contrast, a BBI-related isoinhibitor gene was expressed at similar levels in all three *Glycine* species. Our results suggest that the frameshift mutation in the BBI coding region is responsible for the absence of BBI in the seeds of *G. microphylla* PI440956.

Keywords Anti-nutritional proteins · *Glycine* · Mutation · Trypsin inhibitor

Abbreviations BBI: Bowman–Birk protease inhibitor · KTi: Kunitz trypsin inhibitor · PI: plant introduction

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Introduction

The United States is the leading producer of soybeans (*Glycine max* [L.] Merr.). The majority of soybeans produced in the United States are crushed for oil and meal. Most of the soybean meal is used as animal feed, especially in poultry, swine, and cattle diets. In oriental countries, soybeans are regularly used in human foods and serve as an important protein source. Soybeans contain approximately 40% protein and 20% oil (Krober and Cartter 1962; Nielsen 1996; Krishnan 2000). In spite of the relatively high concentration, the quality of soybean protein is not optimal for human and animal nutrition. Soybean proteins are deficient in sulfur-containing amino acids (cysteine and methionine) and their nutritional value is lower than meat proteins. In addition, soybeans contain significant amounts of anti-nutritional factors such as protease inhibitors, lectins, tannins, alkaloids, and phytic acid (Friedman and Brandon 2001).

Soybeans contain two types of protease inhibitor, Kunitz trypsin inhibitor (KTi) and Bowman–Birk inhibitor (BBI) of chymotrypsin and trypsin (Kunitz 1946; Koide and Ikenaka 1973; Birk 1985). Soybean seeds also accumulate a BBI-related family of isoinhibitors (Hwang et al. 1977; Odani and Ikenaka 1977; Tan-Wilson et al. 1987). These protease inhibitors account for approximately 6% of the total soybean seed protein (Rackis et al. 1986). Numerous studies have demonstrated that diets containing soybean protein have beneficial effects on human nutrition (Messina et al. 1994). As a consequence the U.S. Food and Drug Administration has approved claims that consumption of soybean protein is efficacious in reducing coronary heart disease (FDA 1999). Recent studies have also established that BBI exhibits anticarcinogenic properties (Yavelow et al. 1985; Kennedy 1998). In several different animal systems it has been shown that BBI can prevent carcinogenesis with no adverse side effects (Kennedy et al. 1996; Kennedy 1998).

In the United States, soybean meal is primarily used as an adjuvant for animal feed, especially in the formulation of diets for non-ruminants, such as poultry and swine (Baldwin and Fulmer 1985). Feeding studies have demonstrated that protease inhibitors can cause pancreatic hypertrophy in rats (Booth et al. 1960). Similar effects on the pancreas have also been reported in chickens and growing guinea pigs (Friedman and Brandon 2001). The protease inhibitors are generally inactivated during commercial processing by heat treatment of soybean meal. However, protease inhibitors are fairly heat-stable and commercially heated soybean meals may retain significant amounts of protease inhibitor activity. Heat inactivation of protease inhibitor may also destroy some essential amino acids such as cysteine, methionine, and lysine (Friedman and Brandon 2001). Identification of soybean cultivars that are deficient in protease inhibitors should alleviate these problems. Orf and Hymowitz (1979) identified a KTi null and utilized this plant introduction (PI) line in a breeding program resulting in the release of soybean cultivars that had reduced amounts of trypsin inhibitor activity (Bernars et al. 1991). Even though the KTi nulls lacked the major isoform of the KTi, they retained a significant amount of protease inhibitor activity. This could be attributed to the presence of other isoforms of KTi and BBI. Therefore, it will be desirable to identify soybean cultivars and plant PI lines that lack both the KTi and BBI.

Hymowitz and his colleagues conducted an extensive screening of the genus *Glycine* for lines that naturally lack BBI (Domagalski et al. 1992). They screened 11,692 soybean and 678 wild soybean accessions by enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody against the major soybean BBI. All tested soybean cultivars and accessions contained significant amounts of BBI, even though they were able to detect considerable variation in the BBI content among different cultivars. In contrast, they reported BBI nulls in several of the wild perennial *Glycine* species. Immunoblot analysis revealed that *G. microphylla* PI440956 did not contain any immunologically reactive polypeptides, thereby confirming PI440956 as a BBI null (Domagalski et al. 1992). The reason why *G. microphylla* PI440956 fails to accumulate the major BBI was not known. The objective of this study is to provide a molecular basis for the apparent lack of BBI in this PI line.

Materials and methods

Plant material

Seeds of *Glycine max* [L.] Merr. cv. Jefferson, *G. microphylla* PI440956, and *G. microphylla* PI505188 were obtained from Dr. Randall Nelson, curator of the Northern Soybean Germplasm Collections, Urbana, IL. Soybeans were grown in a greenhouse in 25-cm plastic pots containing commercial garden soil. The photoperiod was 14-h day and 10-h night, and plants were fertilized every 15 days with Peter's soluble fertilizer (Allentown, PA, USA). Flowers were tagged at anthesis and seeds were harvested at 15 and 25 days after anthesis (DAA) and frozen in liquid nitrogen and stored at −80 °C until use.

Genomic DNA isolation

Soybean genomic DNA was isolated by a modified hexadecyltrimethylammonium bromide (CTAB) method (Saghai-Maroof et al. 1984). Freshly harvested soybean leaves (0.5 g) were ground into a fine powder under liquid nitrogen with a mortar and pestle. The powder was transferred into 15-ml plastic tubes to which 1.5 ml of prewarmed (65 °C) CTAB solution containing 30 µl of β-mercaptoethanol was added. The samples were mixed by gentle inversion and incubated for 10 min in a 65 °C oven. Following this step, the samples were subjected to centrifugation at 1,350 *g* for 10 min. The aqueous phase was extracted twice with chloroform/octanol (24:1, v/v). Genomic DNA from the aqueous phase was precipitated by adding an equal volume of isopropyl alcohol. Precipitated DNA was recovered by centrifugation at 5,000 *g* for 4 min. The DNA pellet resuspended in 150 µl of 5 mM EDTA (pH 8.0) was mixed with 1.5 ml of Plant DNAZOL (Invitrogen, Grand Island, NY, USA)–ethanol solution. The samples were stored for 5 min and centrifuged at 5,000 *g* for 4 min. The DNA was washed with 1.5 ml of 70% ethanol and the DNA pellet obtained after the centrifugation step was air-dried and dissolved in 100 µl of 8 mM NaOH (pH 7.5). The DNA was quantified by measuring the A_{260} and A_{280} ratio using a spectrophotometer.

Cloning of the Bowman–Birk protease inhibitor genes

The coding regions corresponding to the BBI and Soy C-II were obtained following polymerase chain reaction (PCR) amplification of genomic DNA using conserved gene-specific primer pairs. PCR reactions were carried out in a final volume of 50 µl containing 10 mM Tris–HCl (pH 8.0), 50 mM KCl, 1 mM $MgCl_2$, 200 µM of each of dNTP, 1 µM of each primer, 100 ng of genomic DNA, and 2 units of Ex Taq DNA polymerase (Pan Vera Corporation, Madison, WI, USA). The primer sequences were based on the published nucleotide sequences for BBI and Soy C-II (Baek et al. 1994). The N- and C-terminal specific primers for BBI were 5′-ATGGTGGTGCTAAAGGTGTG-3′and 5′-TTAGTTTTCCTTGTCATCCTC-3′, for Soy C-II were 5′-ATGGTGGTGTTAAAGGTGTG-3′ and 5′-CTAGTCATCATCTTCATCACTGG-3′, respectively. These primers were used to amplify approximately 330-bp (BBI) and 310-bp (Soy C-II) DNA fragments from *G. max* and *G. microphylla* genomic DNA. The PCR products were isolated and purified from a 1.2% agarose gel using Ultrafree-DA columns (Millipore Corporation, Bedford, MA, USA). The gel-purified PCR products were individually cloned into pGEM-T Easy vector (Promega, Madison, WI, USA). Plasmid DNA was prepared using the Wizard Plus SV Minipreps DNA Purification System (Promega). DNA sequences were determined at the University of Missouri DNA Core Facility using SP6 and T7 primers. The nucleotide sequences of BBI from *G. max* cv. Jefferson, *G. microphylla* PI440956, and *G. microphylla* PI505188 BBI have been submitted to the GenBank database under accession numbers AY233800, AY233801, and AY233802, respectively.

RNA isolation

To minimize ribonuclease (RNase) contamination, sterile, disposable polypropylene tubes were used, where appropriate, for RNA isolation. One gram of frozen soybean seeds was pulverized under liquid nitrogen and extracted with 10 ml of lysis buffer, which consisted of 100 mM Tris–HCl (pH 7.6), containing 1% Tris-isopropyl-naphthalenesulfonic acid, 6% *p*-aminosalicylic acid, 50 mM EGTA, 100 mM NaCl, 1% SDS and 50 mM 2-mercaptoethanol.

After phenol:chloroform extraction, nucleic acid was precipitated by adding 1/10 volume of 3 M sodium acetate (pH 4.8) and 2.5 volumes of ice-cold ethanol. Precipitated nucleic acids were recovered by centrifugation at 12,000 *g* for 25 min and dissolved in RNase-free water. An equal volume of 4 M LiCl was added and the suspension left on ice overnight. The precipitated RNA was recovered by centrifugation as before and subjected to an additional round of LiCl precipitation. The RNA pellet was briefly air-dried and dissolved in RNase-free water and stored at −70 °C until used. The RNA was quantified by measuring the A_{260} and A_{280} ratio using a spectrophotometer.

Reverse transcription–polymerase chain reaction (RT–PCR) analysis

Total RNA (1 μg) was treated with one unit of DNase I (Invitrogen) for 15 min at room temperature to remove residual DNA. The same primers that were used to clone the Bowman–Birk protease inhibitor genes were used in RT–PCR reactions. RT–PCR was carried out according to the procedures recommended by the manufacturer (Qiagen, Valencia, CA, USA). The RT–PCR products were electrophoresed in a 1.2% (w/v) agarose gel. The resolved products were purified using a commercial kit (Millipore). The gel-purified PCR products were cloned into the pGEM-T Easy vector (Promega) and then sequenced at the University of Missouri DNA Core Facility.

Results and discussion

Cloning and analysis of the BBI gene sequences

Based on the published nucleotide sequence of soybean BBI (Baek et al. 1994), primers were designed to PCR-amplify DNA fragments utilizing the genomic DNA from *G. max* cv. Jefferson, *G. microphylla* PI509486, and *G. microphylla* PI440956. BBI-specific primers amplified approximately 330-bp DNA fragments from the genomic DNA from all the *Glycine* species employed in this study (Fig. 1). Control PCR reactions from which template DNA was omitted resulted in the absence of any amplified DNA fragments (Fig. 1). Each of the amplified PCR products was individually cloned into pGEM-T Easy vector and their nucleotide sequences were determined.

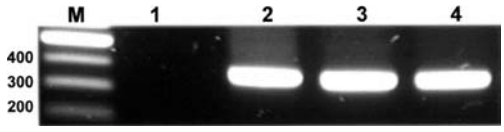

Fig. 1 PCR amplification of BBI coding region from the genomic DNA of *Glycine max* cv. Jefferson, *G. microphylla* PI505188, and *G. microphylla* PI440956. *Lanes*: *1*, control reaction performed in the absence of DNA template; *2*, *G. max* cv. Jefferson; *3*, *G. microphylla* PI505188; *4*, *G. microphylla* PI440956. Sizes of the molecular weight markers (in bp) are indicated on the *left* side of the figure

Sequence analysis revealed that the BBI coding regions from *G. max* cv. Jefferson, *G. microphylla* PI509486, and *G. microphylla* PI440956 consisted of 333 bp, 336 bp, and 330 bp, respectively (Fig. 2). The nucleotide sequences, when aligned with each other using the Clustal method with Weighted residue weight table (DNASTAR, Madison, WI, USA), revealed that they were highly similar to each other. The BBI nucleotide sequence of *G. max* cv. Jefferson shared 97% sequence identity to *G. microphylla* PI509486 BBI sequences and 88% identity with *G. microphylla* PI440956 BBI sequences. The BBI nucleotide sequences from *G. microphylla* PI509486 and *G. microphylla* PI440956 shared 88% identity. Computer-assisted analysis of the DNA sequence using the open-reading

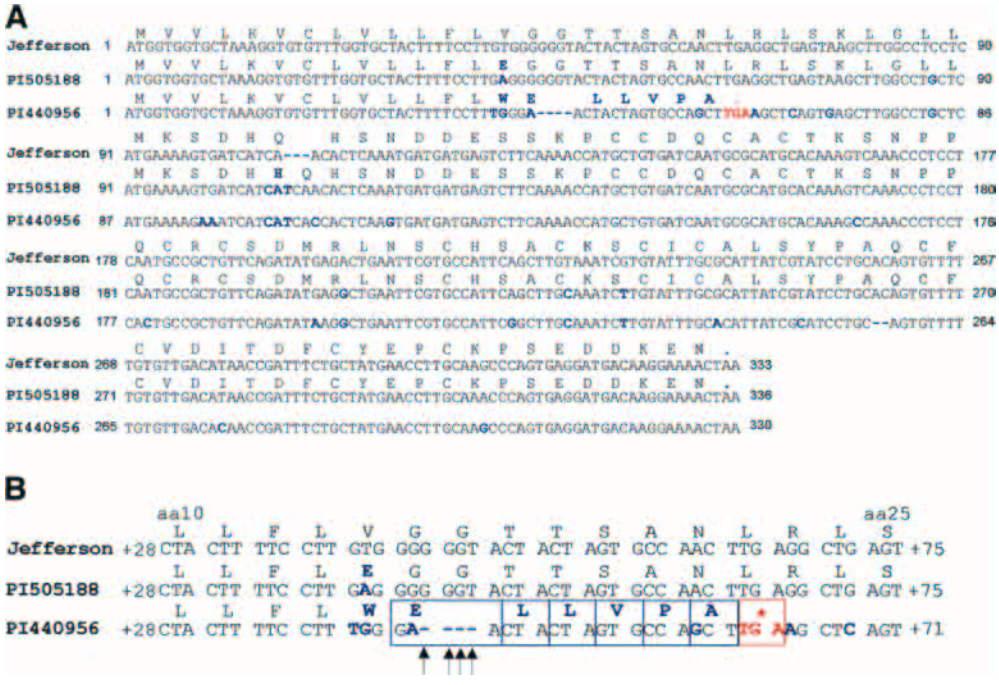

Fig. 2 **A** Nucleotide and the deduced amino acid sequence alignment of the BBI gene from *G. max* cv. Jefferson, *G. microphylla* PI505188, and *G. microphylla* PI440956. **B** Partial nucleotide and amino acid sequence comparison of BBI. The *arrows* point to the nucleotides that have been mutated in *G. microphylla* PI440956. These mutations introduce a premature stop codon, which is indicated by an *asterisk*. Differences in the nucleotide and amino acid sequences are shown with *blue letters*. The stop codon is shown with *red letters*

frame (ORF) finder program identified a 333-bp-long ORF in *G. max* cv. Jefferson and a 336-bp-long ORF in *G. microphylla* PI509486 (Fig. 2). The predicated ORFs encode proteins of 110 and 111 amino acids with molecular weights of 12,093 and 12,260, respectively. We submitted the amino acid sequences to a search of the SwissProt data bank, which indicated that the sequences shared significant identity with BBI sequences from other plants. The amino acid sequences of BBI from *G. max* cv. Jefferson and *G. microphylla* PI509486 are identical to each other except for an insertion of an additional histidine residue at position 36 from the amino terminal in *G. microphylla* PI509486 (Fig. 2). It therefore seems that the BBI genes have not diverged significantly among *Glycine* species.

The *G. microphylla* PI440956 BBI coding region contains an in-frame stop codon

Computer-assisted analysis of the BBI nucleotide sequence from *G. microphylla* PI440956 revealed no potential ORFs greater than 100 bp. Alignment of the BBI nucleotide sequences with BBI sequences from *G. max* cv. Jefferson and *G. microphylla* PI509486 revealed that there were 4 bp missing in the coding region from *G. microphylla* PI440956 (Fig. 2). This deletion results in the insertion of a stop codon near the amino terminus of the protein. As a consequence, the BBI ORF is truncated and can only code for the first 20 amino acids of the BBI (Fig. 2). A similar situation has been reported for KTi null mutants. The soybean genome contains at least 10 distinct KTi genes (Jofuku et al. 1989a). Three of them, KTi1, KTi2, and KTi3, have been cloned and characterized (Jofuku et al. 1989a). The majority of trypsin inhibitor activity in soybean seed is due to the activity of KTi3 gene product. Orf and Hymowitz (1979) identified two soybean accessions (PI196168 and PI157440) lacking the KTi3 from the USDA germplasm collection. The absence of KTi3 in these two soybean accessions resulted in substantial reduction in the trypsin inhibitor activity. The molecular basis for the reduced accumulation of KTi in PI196168 and PI157440 has been elucidated (Jofuku et al. 1989b; Krishnan 2001). The nucleotide sequence of the KTi3 genes from PI196168 and PI157440 reveal mutations in the coding region resulting in the introduction of in-frame stop codons (Jofuku et al. 1989b; Krishnan 2001).

BBI mRNA does not accumulate in developing seeds of *G. microphylla* PI440956

Based on immunoblot analysis using monoclonal antibodies raised against the BBI, it was established that *G. microphylla* PI440956 did not accumulate BBI in the seeds (Domagalski et al. 1992). We wanted to examine if the lack of accumulation of BBI was due to lack of mRNA encoding this protein. Using total RNA isolated from developing seeds, we performed RT–PCR analysis. An RT–PCR product of about 330 bp was detected from reactions that contained RNA from *G. max* cv. Jefferson and *G. microphylla* PI509486. However, under identical conditions, no RT–PCR products were generated using RNA from *G. microphylla* PI440956 (Fig. 3). However, when primers specific for the 18S ribosomal gene were used in the RT–PCR reaction, an RT–PCR product was detected from all three samples (Fig. 3). This observation indicates that mRNA encoding the BBI is absent in *G. microphylla* PI440956. Thus, the earlier report of the absence of BBI in *G. microphylla* PI440956 (Domagalski et al. 1992) appears to be related to the absence of the BBI mRNA in developing seeds. In the case of the KTi3 null mutant, it was shown that the mutation in the coding region results in KTi3 mRNA destabilization, resulting in dramatic reduction in KTi3 mRNA. It was proposed that reduced accumulation of KTi3 mRNA in *G. max* PI157440 is regulated by post-transcriptional events (Jofuku et al. 1989b). It still remains to be seen if the apparent lack of BBI mRNA in *G. microphylla* PI440956 is regulated at the transcriptional or post-transcriptional level.

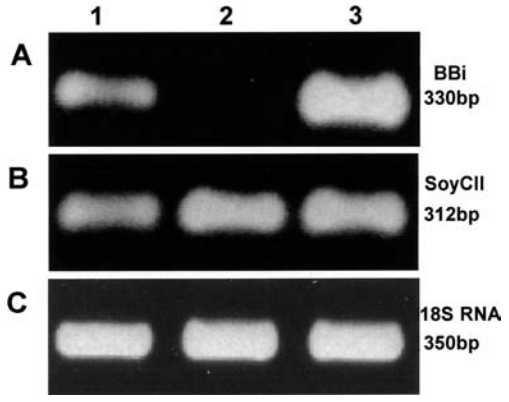

Fig. 3 A, B RT–PCR analysis of BBI (**A**) and BBI-related isoinhibitor Soy C-II expression (**B**) in developing *Glycine* seeds. **C** The 18S ribosomal mRNA was used as loading control. *Lanes*: *1*, *G. max* cv. Jefferson; *2*, *G. microphylla* PI440956; *3*, *G. microphylla* PI505188. Sizes of the RT–PCR products are indicated on the *right* side of the figure

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