Proteomic and genomic characterization of Kunitz trypsin inhibitors in wild and cultivated soybean genotypes ☆

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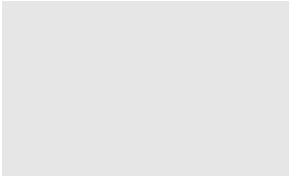
Summary

In this study, we investigated protein and genetic profiles of Kunitz trypsin inhibitors (KTIs) in seeds of 16 different soybean genotypes that included four groups consisting of wild soybean (*Glycine soja*), the cultivated soybean (*G. max*) ancestors of modern N. American soybean cultivars (old), modern N. American soybean (elite), and Asian cultivated soybean landraces that were the immediate results of domestication from the wild soybean. Proteins were well separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and stained protein cut from a 2D-PAGE indicated that KTI exists as multiple isoforms (spots) in soybean. Protein spots of KTI were identified and characterized using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Although overall distribution patterns of the KTI protein spots appeared similar, the number and intensity of the protein spots between wild and cultivated genotypes varied. Three KTI peptides were identified in three of the wild genotypes, PI 393551, PI 407027 and PI 407282, in which KTI3 peptide showed highest intensity. The remaining wild genotype, PI 366120, showed four protein spots. In contrast, the ancestors, modern and Asian landrace genotypes showed only two protein spots corresponding to KTI. On the basis of DNA blot analysis, there is one copy of the KTI3 gene in all 16 genotypes. Polymorphism was detected in one of the wild genotypes (PI 366120) both in proteomic and genomic

analyses. Our data suggest that the major variation of protein profiles were between wild and cultivated soybean genotypes rather than among genotypes in the same group. Genetic variation of KTI1, KTI2 and KTI3-related genes were detected within and between groups.

Introduction

Soybean ranks among the eight most significant food allergens and its wide usage for the production of protein in the food industry makes it difficult to eliminate from the diet. Kunitz trypsin inhibitor (KTI) is an antinutritional/allergen protein found in soybean that acts as a proteinase inhibitor. KTI belongs to the family of all antiparallel proteins that are highly resistant to thermal and chemical denaturation (Roychaudhuri et al., 2004). KTIs are the major trypsin inhibitors found in soybean. They are involved in respiratory hypersensitivity reactions, and can reduce the growth and development of herbivorous insects (Besler et al., 2000; Jansen et al., 1986). The total seed protein of soybean contains about 6% proteinase inhibitors (Wang et al., 2004). KTIs have been characterized as food allergens in humans (Burks et al., 1994; Quirce et al., 2002) and have 32% sequence homology with a rye grass pollen allergen (Ree van et al., 1995). In soybean, three KTI genes (KTI1, KTI2 and KTI3) have been reported, and both transcriptional and post-transcriptional processes regulate KTI gene expression (Jofuku and Goldberg, 1989). The KTI3 transcript was detected only in the soybean seed, while KTI1 and KTI2 transcripts are expressed in soybean leaf, root and stem (Jofuku and Goldberg, 1989). The genes encoding KTI1 and KTI2 have nearly identical nucleotide sequences, while the KTI3 gene has diverged (20%) from the other two genes (Jofuku and Goldberg, 1989).

A comprehensive and comparative analysis of KTI proteins and its genetic variation among different soybeans is important for a better understanding of allergenicity. In addition, variation in KTIs has been used as an index of genetic diversity, and for determining phylogenetic relationships among species of soybean (Wang et al., 1998; Yu and Kiang, 1993). We have conducted a detailed comparative analysis of KTI protein profiles and their genetic structure in a variety of soybeans including wild soybean *Glycine soja*, and cultivated soybean, *G. max*, as part of an effort to identify and eliminate KTI proteins from soybean seed. Such an analysis of the soybean allergen proteins could also prove useful for comparative analysis of the allergen proteins of other legume species. The objective of this investigation was to determine the variation of antinutritional/allergen proteins and genes of KTIs in a wide range of soybean genotypes. Therefore, in this study, 16 genotypes of soybean including cultivated (*G. max*) and wild (*G. soja*) were selected. The 16 genotypes were selected from four groups including wild (*G. soja*), Asian landraces of *G. max*, ancestral cultivars (old), as well as a set of modern (elite) cultivars. Each group was represented by four genotypes. We observed differences in protein profiles between wild and cultivated genotypes but not within the same group except in one wild genotype. However, genetic variation was detected both within and between groups.

Materials and methods

Plant materials

Soybean [*G. max* (L.) Merr.] seeds of wild and cultivated genotypes were obtained from the USDA soybean germplasm collection, Urbana, IL. Seeds were stored at $-80\,°C$ until used.

Chemicals

Chemicals for electrophoresis including acrylamide, bis-acrylamide, SDS, TEMED, ammonium persulfate, thiourea, dithiothreitol (DTT), CHAPS and immobilized pH gradient (IPG) strips were purchased from GE Healthcare (Piscataway, NJ). Urea and ampholytes (pH 4.0–7.0) were purchased from Bio-Rad Laboratories (Hercules, CA). Tris-HCl (pH 8.8), 2-mercaptoethanol, trichloroacetic acid (TCA) and glycerol were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). α-Cyanohydroxycinnamic acid (CHCA) matrix was purchased from Bruker Daltonics, Billerica, MA. Water from a Millipore Milli-RO4 reverse osmosis system was used for making all solutions.

Extraction and 2D-PAGE analysis

Protein extraction from seed and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) separation protocols were performed according to Natarajan et al. (2005). The concentration of proteins extracted by the modified TCA/acetone method was determined by the Bradford method (Bradford, 1976). The first-dimension isoelectric focusing (IEF) was performed using 13 cm pH 4.0–7.0 linear IPG strips in a IPGphor apparatus (GE Healthcare, Piscataway, NJ), according to the manufacturer's recommendations. A protein molecular weight standard was used for the second dimension in each gel. For the second dimension, the IPG strips were incubated with 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, and 1% DTT for 15 min, acetylated with iodoacetamide, and subsequently placed on to 12% polyacrylamide gels prepared as described by Laemmli (1970). Electrophoresis was performed using a Hoefer SE 600 Ruby electrophoresis unit (GE Healthcare, Piscataway, NJ) according to the manufacturer's recommendations. The 2D-PAGE gels were visualized by staining with Colloidal Coomassie Blue G-250 as described by Newsholme et al. (2000). The gels were stored in 20% ammonium sulfate solution and scanned using laser densitometry (GE Healthcare, Piscataway, NJ). Triplicate samples were used for soybean seed protein extraction and 2D-PAGE analysis.

In-gel digestion of protein spots

Protein spots were excised from the stained gel and washed first with distilled water to remove ammonium sulfate and then with 50% acetonitrile containing 25 mM ammonium bicarbonate to destain the gel plug. The gel plug was dehydrated with 100% acetonitrile, dried under vacuum, and then re-swollen with 20 μL of 10 μg/mL trypsin (modified porcine trypsin, sequencing grade, Promega, Madison, WI) in 25 mM ammonium bicarbonate. Digestion was performed overnight at 37 °C. The resulting tryptic fragments were extracted with 50% acetonitrile and 5% trifluoroacetic acid with sonication. The extract was dried to completeness and dissolved in 50% acetonitrile and 0.1% trifluoroacetic acid.

Protein identification

A Voyager DE-STR matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Applied Biosystems, Framingham, MA) operated in positive ion reflector mode was used to analyze tryptic peptides. Samples were co-crystallized with CHCA matrix, and spectra were acquired with 50 shots of a 337 nm nitrogen laser operating at 20 Hz. Spectra were calibrated using the trypsin autolysis peaks as internal standards at m/z 842.51 and 2211.10. The raw data were processed by Sequest to generate DTA files for database searching. The *merge.pl* script from Matrix Science (Boston, MA) was used to convert multiple Sequest DTA files into a single Mascot generic file suitable for searching in Mascot. For MALDI data to qualify as a positive identification, a protein score had to equal or exceed the minimum significant score of 64.

DNA blot analysis

DNA was extracted from the leaf tissues of 16 different soybean varieties using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA). The extracted DNA was quantified using a spectrophotometer and integrity was verified on 0.8% agarose gels. PCR primers were designed to amplify a 602-bp fragment of KTI3 based on sequence information reported in the GenBank (Accession No. AF233296). The forward primer sequence was 5′-CACTAAATTAATGGTGAATC-3′ and the reverse primer sequence was 5′-GCAAAGATGGTGCTC TTCAT-3′ (Fig. 2A). DNA from *G. max* (PI 423954) was used to amplify the KTI3 fragments used as probes. KTI3 fragments were amplified using the following conditions: initial activation of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) at 94 °C for 5 min, denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C, extension for 1 min at 72 °C for 30 cycles, and an additional 10 min extension at 72 °C. The PCR product from each reaction was fractionated by electrophoresis, purified using QIAEX II Gel Extraction System (Qiagen, Valencia, CA), and cloned into pCR2.1-TOPO vector (Invitrogen, Calsbad, CA) for sequencing to confirm its identity.

For DNA blot analysis, 2.5 μg of the genomic DNA was digested with restriction enzymes *Hind*III and *Hae*III at 37 °C overnight, separated in a 0.8% agarose gel, blotted onto a nylon membrane, and hybridized with ^{32}P-labeled, PCR-amplified 602-bp KTI3 fragments. Blots were washed and imaged using a Typhoon 8600 Variable Mode Imager (Molecular Dynamics/Amersham-Pharmacia Biotech, Sunnyvale, CA). Probe hybridization and washing blots were performed under medium stringency condition according to Keates et al.

(2003). DNA sequence comparisons for KTI1/2/3 were performed with Biology WorkBench (http://workbench.sdsc.edu/).

Results and discussion

In this study, we have compared and characterized protein and genetic profiles of KTIs in 16 different genotypes using 2D-PAGE separation, MALDI-TOF-MS analysis, and Southern hybridization. The 16 genotypes include four wild (PI 366120, PI 393551, PI 407027, PI 407282), four Asian landraces (PI 423954, PI 89138, PI 594777, PI 59845), four ancestors (PI 548298, PI 548445, PI 548318, PI 548362), and four modern (PI 536635, PI 525453, PI 513382, PI 533655).

Proteomic analysis of KTIs

Our results showed that although the overall distribution patterns of KTI protein spots are quite similar in all 16 genotypes we have tested, the number of protein spots and their intensities varied among these genotypes. A representative 2D-PAGE image from each group is shown in Fig. 1. Three protein spots (#1, 2, and 3) of KTI were identified by their MALDI-TOF-MS and database searches in three wild varieties, PI 393551, PI 407027, and PI 407282. However, wild genotype PI 366120 showed four protein spots (# 1, 2, 3, and 4) in this region of the gel (Fig. 1A), which were identified by MALDI-TOF MS as KTI except one protein spot (#4). The ancestor, modern and Asian landraces genotypes showed only two protein spots (#1 and 2) in this region of the gel (Fig. 1B–D). The four KTI spots electrophoresed to isoelectric points (pI) of between 4.6 and 5.0, and had an estimated molecular mass of between 20 and 23 kDa, similar to previous reports (Ashida et al., 2000; Baur et al., 1996). Our results matched the previous reports, in which polymorphism had been observed among the KTIs of wild soybeans by Western blotting and PAGE analysis (Hymowitz, 1973; Ritt et al., 2004; Stahlhut and Hymowitz, 1983; Wang et al., 2005). The KTI proteins observed in the current study were identified by MALDI-TOF-MS and NCBI non-redundant database searches and are listed in Table 1. An assigned protein spot number, theoretical pI, and molecular weight (Mr), protein identity, number of peptides matched, percent sequence coverage, molecular weight search (MOWSE) score, expected
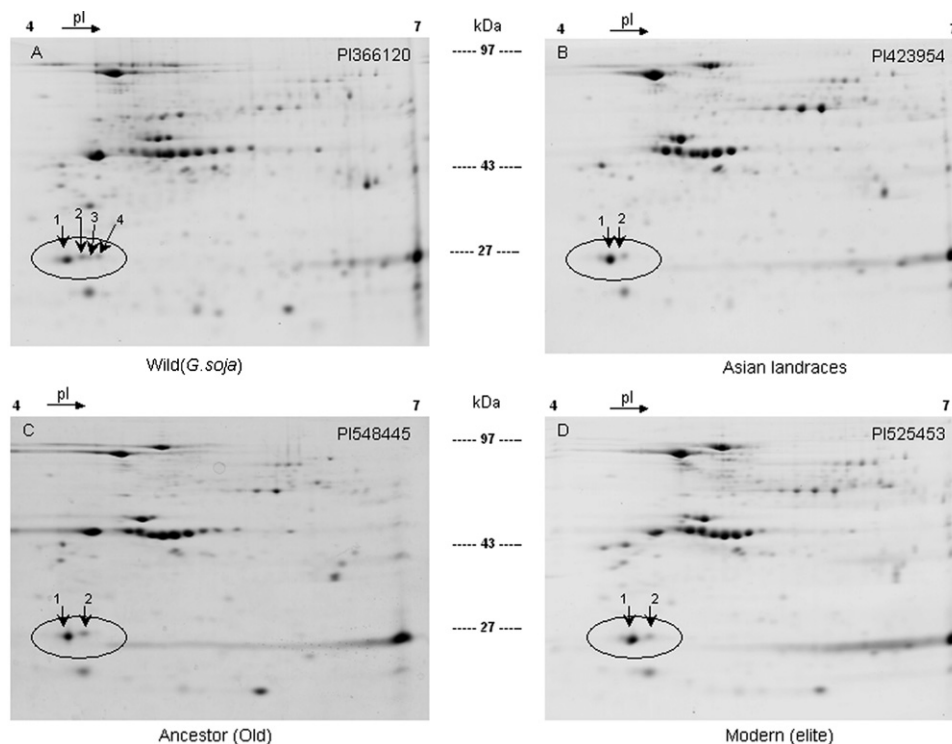

Figure 1. A proteomic comparison of the KTI proteins of wild, *G. soja* (A), Asian landraces (B), ancestor (C), and modern (D). The first dimension was run using a pH gradient from 4.0 to 7.0 and the second dimension was a 12% SDS-polyacrylamide gel.

Table 1. Proteins identified by MALDI-TOF-MS in wild and cultivated genotypes

SP #	Theoretical PI/Mr	Protein identity	Peptides matched	Sequence coverage (%)	MOWSE score	Expect value	NCBI accession #
1	4.61/20310	Soybean Kunitz trypsin inhibitor (KTI3)	15	53	147	3.00E-10	gi│3318877
2	4.97/22817	Kunitz trypsin inhibitor (KTI2)	8	42	72	1.10E-02	gi│125722
3	4.61/20310	Soybean Kunitz trypsin inhibitor (KTI3)	9	44	60	1.40E-01	gi│3318877

value, and the National Center for Biotechnology Information (NCBI) database accession number of the best match.

The increased number of KTI protein spots in wild compared to the cultivated genotypes may result in increasing the wild plant's defense mechanism by acting as a protective agent against soil pathogens and insects (Marchetti et al., 2000). Our 2D-PAGE results of all 16 varieties showed that spot #1 was the most abundant KTI, encoded by the KTI 3 gene (gi│3318877). Spot #2, encoded by KTI2 gene (gi│125722), was weaker than spot # 1 (KTI3) but was present in all 16 varieties. Jofuku and Goldberg (1989) reported the presence of KTI3 transcripts only in the seed and KTI1 and KTI2 transcripts only in leaf, root, and stem of cultivated soybean genotypes. Spot #3, which matched the KTI3 gene with low MOWSE score, was found only in the four wild genotypes. Spot #1 and 3 were encoded by KTI3 (gi│3318877) but they showed two different pI and Mr. This could be due to co-existence of precursor and mature protein or alternatively, spot #3 could be encoded by an as yet unidentified gene that has homology with KTI3. One of the wild genotypes, PI366120 showed an additional protein spot (spot #4) in the same region of the gel with a different pI and the same Mr as spot #3. We were unable to identify this spot (spot #4) because of low MOWSE score and low protein intensity. This spot might be the product of post-translational modification of one of the other KTI proteins, an unidentified KTI or an unrelated protein. Post-translational modification of KTI has been observed in chickpea (Srinivasan et al., 2005).

Genetic variation of KTI genes

DNA blot analysis of the 16 genotypes was conducted in order to determine the genomic characteristics of KTI3 and its closely related genes (e.g., KTI1 and KTI2, Fig. 2A). In DNA blot analysis, the third exon of KTI3 was amplified and hybridized to HindIII- and HaeIII-digested soybean genomic DNA. According to sequence information (Fig. 2A), the 602-bp PCR products aligns with the KTI3 gene (Accession No. AF233296). While the coding sequences of KTI1 (Accession No. S45035) and KTI2 (Accession No. S45035) are nearly identical in DNA sequence (97% identity) and encode minor Kunitz trypsin inhibitor genes, KTI3 encodes the predominant Kunitz trypsin inhibitor in cultivated soybean genotypes (Jofuku, 1987; Jofuku and Goldberg, 1989). The KTI3 coding sequence has 75% and 77% identity with KTI1 and KTI2, respectively, at the DNA sequence level. Sequence alignment of the 602-bp probe with the corresponding regions of KTI1 and KTI2 showed 78% and 79% identity, respectively (Fig. 2B).

Figure 2. DNA blot analyses of soybean KTI genes in 16 different soybean genotypes. (A) Map of relevant restriction enzyme sites and the location of probe within KTI3 region (Accession No. AF233296). The coding sequence of KTI3 is located from 2089 to 2739. (B) Comparison of DNA sequences of the 602-bp probe generated from KTI3 (AF233296) gene (indicated by arrow) with homologous regions of KTI1 and KTI2 (S45035). Alignment of the DNA sequences was performed using ClustalW and identities were highlighted with BOXSHADE (http://seqtool.sdsc.edu/CGI/BW.cgi#!). Dashes indicate gaps that were introduced to optimize the alignment. (C) Soybean seed DNAs (2.5 μg) were digested with restriction enzyme, HindIII or HaeIII, then separated in a 0.8% agrose gel, and blotted onto a nylon membrane. The blot was hybridized with the 602-bp KTI3 probe, washed under medium stringency conditions and imaged. The bands were assigned numbers (left side of each autoradiogram) and thin arrows indicate the KTI3 gene band (1467 bp for HindIII and 1752 bp for HaeIII). The asterisk indicates the approximately 2-kb hybridizing band observed in G. soja (PI 366120 in lane 9) HaeIII-digested DNA.

When the 602-bp PCR product of KTI3 was used as a probe, one dominant band was detected along with multiple less intense bands (Fig. 2C). On the basis of the restriction map of the gene region in Fig. 2A, the dominant band in the blot is KTI3, with the expected sizes of 1467 bp for HindIII digest and 1752 bp for the HaeIII digest, as indicated by the arrow in Fig. 2C. However, the size of the dominant hybridizing band observed in the HaeIII-digested G. soja PI 366120 DNA, was approximately 2 kb
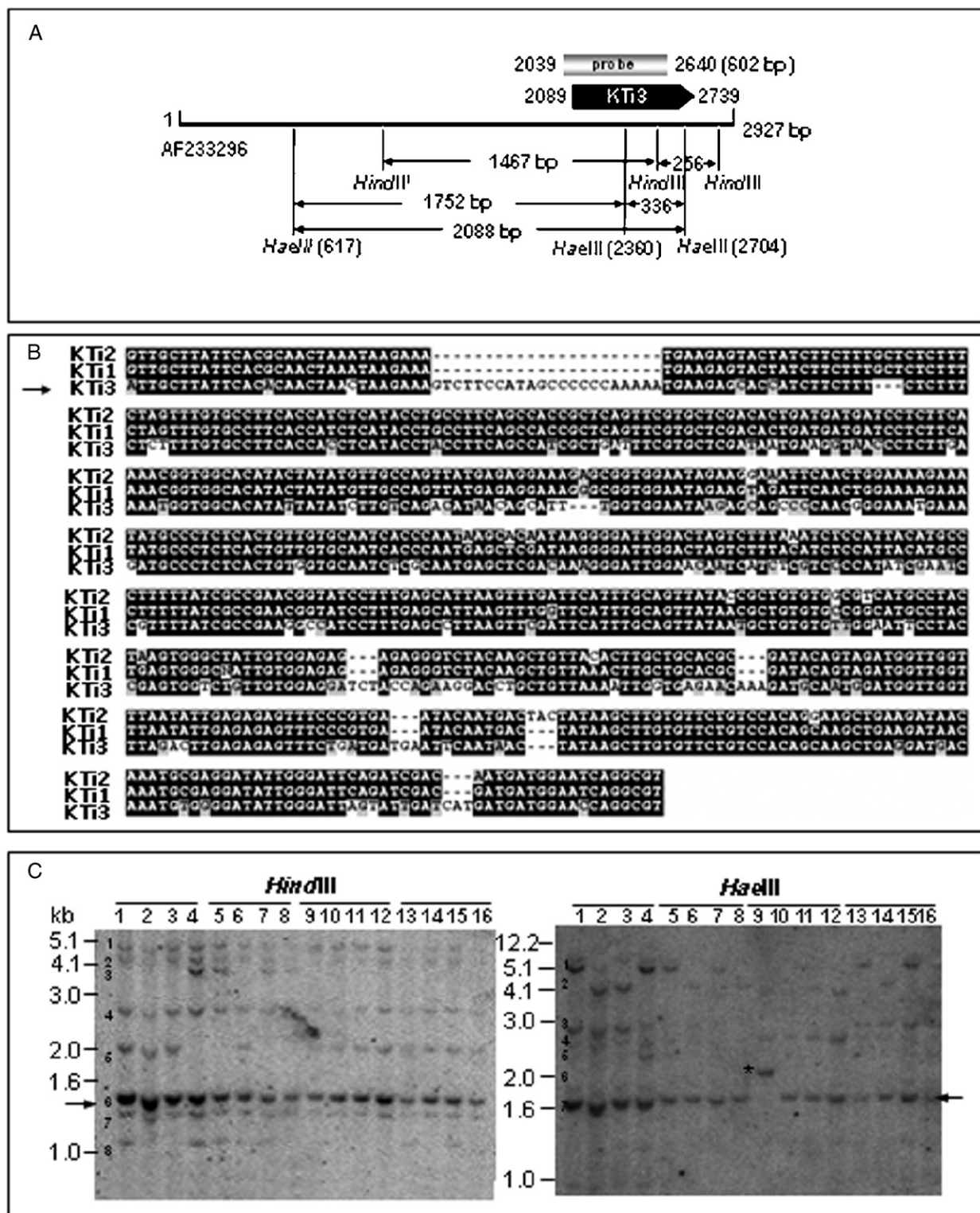
Table 2. Patterns of bands hybridizing to a 602-bp fragment of KTI3 in wild and cultivated soybean genotypes

Lane	Group	Accession no.	Band patterns for HindIII-digested DNA[a]								Band patterns for HaeIII-digested DNA[a]						
			1	2	3	4	5	6	7	8	1	2	3	4	5	6	7
1	Ancestor (Old)	PI 548298	+	+	−	+	+	+	+	+	+	−	+	+	+	−	+
2		PI 548445	+	+	−	+	+	+	+	−	+	+	+	+	+	−	+
3		PI 548318	+	+	−	+	+	+	+	−	+	+	+	+	+	−	+
4		PI 548362	+	+	+	+	−	+	+	+	+	−	+	+	+	−	+
5	Modern (Elite)	PI 533655	+	+	+	+	−	+	+	+	+	−	+	+	+	−	+
6		PI 525453	+	+	−	+	+	+	+	+	−	+	+	+	+	−	+
7		PI 513382	+	+	+	+	−	+	+	+	+	−	+	+	+	−	+
8		PI 536635	+	+	+	+	−	+	+	−	−	+	+	+	+	−	+
9	Wild (G. soja)	PI 366120	+	+	−	+	+	+	+	−	−	+	−	+	+	+	−
10		PI 393551	+	+	−	+	+	+	+	−	−	+	−	+	−	−	+
11		PI 407027	+	+	−	+	+	+	+	−	+	+	−	+	−	−	+
12		PI 407282	+	+	−	+	+	+	+	−	−	+	−	+	−	−	+
13	Asian landraces	PI 423954	+	+	−	+	+	+	+	+	+	−	+	+	−	−	+
14		PI 89138	+	+	−	+	+	+	+	−	−	+	+	+	−	−	+
15		PI 594777	+	+	−	+	+	+	+	+	+	−	+	+	+	−	+
16		PI 59845	+	+	−	+	+	+	+	−	+	+	+	−	−	−	+

[a]Presence/absence of band is indicated by "+" and "−". Column numbers indicate different size bands in Fig. 2C.

(asterisk in lane 9 in Fig. 2C). The increase in band size is most probably due to the loss of the HaeIII restriction enzyme site at position 2360 (Fig. 2A) in PI 366120 because the expected fragment size would be 2088 kb in this case. An alternative possibility is an insertion within the HaeIII fragment (between 617 and 2360 in Fig. 2A).

Genetic variation in the KTI3 and KTI3-related genes was detected between and within groups. When the soybean DNAs were digested with HindIII, eight different bands were detected in all 16 genotypes, while seven different bands were detected when DNA was digested with HaeIII (Fig. 2C and Table 2), indicating that there is more than one copy of KTI1/2 and/or there are other sequences that have homology with KTI3 gene in the soybean. Bands 1, 2, 4, 6, and 7 were consistent with HindIII digestion, while no consistent band was observed with HaeIII digestion. Band 3 in HindIII digestion was only found in some of the ancestor and modern groups. Band 8 HindIII digestion was not detected in the wild group. In HaeIII-digested DNA, band 3 was not detected in the wild group while band 6 was only observed in the wild group. When a KTI3-specific fragment (5′ untranslated region) was used as a probe, only a single band was detected in all 16 genotypes (data not shown), which is consistent with the results of the previous study (Jofuku and Goldberg, 1989). The earlier studies have shown that there are at least 10 distinct KTI genes in the soybean genome of a cultivated genotype (Dare). Among them, the KTI1, KTI2 and KTI3 genes are up-regulated during embryogenesis (Jofuku, 1987; Jofuku and Goldberg, 1989). We could also detect genetic variation within and between groups. On the basis of the band numbers and patterns, the copy numbers of the KTI3 and KTI3-related genes appear to be different within and between groups. On the basis of the protein profiles, the KTIs are monomorphic within groups of ancestor, modern and Asian landraces genotypes while an additional protein spot was observed in one wild genotype G. soja PI 366120, compared to other three genotypes (PI 393551, PI 407027, and PI 407282). The same wild genotype, PI 366120, showed a higher molecular weight for the KTI3 gene (lane # 9 on the HaeIII-digested DNA blot) than the other wild genotypes. Our combined analyses of proteomics and DNA band patterns revealed that there is a limited genetic variation in KTI genes between groups. This might be due to the limited gene pool of US soybean cultivars and wild types.

Acknowledgements

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