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Precursor of the Inactive 2S Seed Storage Protein from the Indian Mustard *Brassica juncea* Is a Novel Trypsin Inhibitor

CHARACTERIZATION, POST-TRANSLATIONAL PROCESSING STUDIES, AND TRANSGENIC EXPRESSION TO DEVELOP INSECT-RESISTANT PLANTS*[S]

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A number of trypsin inhibitor (TI) genes have been used to generate insect-resistant plants. Here we report a novel trypsin inhibitor from Indian mustard *Brassica juncea* (BjTI) that is unique in being the precursor of a 2S seed storage protein. The inhibitory activity is lost upon processing. The predicted amino acid sequence of the precursor based on the *B. juncea* 2S albumin (Bj2S) gene cloned and sequenced in this laboratory (Bj2Sc; GenBank™ accession number X65972) showed a soybean-TI active site-like motif GPFRI at the expected processing site. The BjTI was found to be a thermostable Kunitz type TI that inhibits trypsin at a molar ratio of 1:1. The 20-kDa BjTI was purified from midmature seeds and found to be processed *in vitro* to 9- and 4-kDa subunits upon incubation with seed extract. The Bj2Sc sequence was expressed in *Escherichia coli* pET systems as the inhibitor precursor. The radiolabeled gene product was expressed *in vitro* in a coupled transcription-translation system and showed the expected processing into subunits. Two *in vitro* expressed pre-2S proteins, mutated at Gly and Asp residues, were processed normally to mature subunits, showing thereby no absolute requirement of Gly and Asp residues for processing. Finally, the 2S gene was introduced into tobacco and tomato plants. Third generation transgenics expressing BjTI at 0.28–0.83% of soluble leaf proteins showed remarkable resistance against the tobacco cutworm, *Spodoptera litura*. This novel TI can be used in transforming seed crops for protection to their vegetative parts and early seed stages, when insect damage is maximal; as the seeds mature, the TI will be naturally processed to the inactive storage protein that is safe for consumption.

Seed proteins play important roles in the survival of plants (*e.g.* maintaining viability of seeds, providing nutrition during the early seedling stage, and protecting the seeds from microbes and insects) (1). Seed proteins also play an important role in human and animal nutrition by providing the major share of dietary protein. These proteins may be classified as storage, structural, and biologically active proteins (2). The major biologically active proteins include lectins, enzymes, and enzyme inhibitors (*e.g.* trypsin inhibitor (TI)[1]). Many plant storage organs such as seeds and tubers contain from 1 to >10% of their soluble proteins as TIs. Besides their storage function, TIs have been ascribed other functional roles, such as regulating endogenous plant proteinases to prevent precocious germination, inhibiting trypsin during passage through an animal's gut, thus helping in seed dispersal, and protecting plants against pests and diseases (3–6). Their presence in major grain crops like cereals, legumes, and oil seeds, has nutritional and clinical implications and importance of their structure-function relationships has made TIs the subject of extensive studies (3, 7).

Despite wide diversity, some TIs share sequence homology with seed storage proteins (8). Proteinase inhibitors and many seed storage proteins are encoded by families of polymorphic genes that probably arose by a complicated process of gene duplication, DNA exchange, and exon reshuffling (9). Like many other seed proteins, TIs are synthesized as larger precursor proteins. After cotranslational cleavage of the signal (pre)peptide in the endoplasmic reticulum, they undergo limited proteolytic processing in vacuoles. Here short propeptides (N-terminal, internal, and C-terminal sequences) are cleaved off. Final assembly and deposition of the mature inhibitor take place in compartmentalized storage organelles along with inactive storage proteins (10, 11). Some TIs, like that from *Acacia confusa*, are equally active both in the single polypeptide precursor form and the two-subunit mature form (12). A more interesting variation is the proteolytic processing of a large precursor to several different mature proteins, as occurs with both TIs and storage proteins (13, 14). In previously reported cases, it is always the processed protein that is active as TI, whereas the precursors are either inactive or weakly active.

There is controversy about the processing proteases and recognized amino acid sequence motif in seed proteins. The propeptide cleavage site is reported to have an Asn (or Asp) residue and a GP motif at or near the cleavage site (10, 15, 16). However, our sequence analysis showed that although GP is present in the 2S protein precursors of many plants, it is not present in all processing sites (*e.g. Brassica juncea*, *Brassica carinata*, and Brazil nut) (17, 18).

A number of different low molecular weight, single polypeptide TIs have been isolated and characterized from plants belonging to the Cruciferae (Brassicaceae) family (19–21). Interestingly, Ruoppolo *et al.* (22) isolated five low molecular TIs with additional chymotrypsin inhibitor activity from *Sinapis*

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[S] The on-line version of this article (available at http://www.jbc.org) contains three additional tables and three additional figures.

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[1] The abbreviations used are: TI, trypsin inhibitor; TAME, *N*-α-*p*-tosyl-L-arginine methyl ester; BjTI, *B. juncea* trypsin inhibitor; Bj2S, *B. juncea* 2S albumin; SBTI, soybean trypsin inhibitor; DAF, days after flowering.

alba. On the other hand, TIs from *Spergula arvensis* (23) and *Brassica nigra* (24) are larger proteins composed of 9- and 4-kDa subunits, lack chymotrypsin inhibitor activity, and are similar to the napin family 2S albumin in amino acid sequence. In all of these crucifers, the processed protein is the inhibitor.

We have previously cloned and sequenced the 2S seed storage protein genes and their promoters from several *Brassica* species (17, 25). Comparing the amino acid sequences predicted by most of these cloned genes showed the sequence GPFRI, homologous to the TI active site motifs SPFRI and SPYRI in soybean Kunitz TIs (26). Here we report a novel TI from the Indian mustard *B. juncea* (BjTI), which is the precursor of a seed storage protein. BjTI is active in the precursor form and after being processed is converted to the inactive 2S seed storage albumin with simultaneous loss of TI activity. In the mustard plant, the protein is produced in the precursor form during the midmaturation stage of seed development. This inhibitor imparts resistance to the developing seeds in the field. As the seeds mature, the inhibitor is naturally processed to the inactive seed storage protein, which is safe for human and animal consumption. This unique feature not only opens up a new field of interesting study but also presents several potential applications in crop improvement. The *B. juncea* seeds, which normally express the TI, are naturally resistant to caterpillars and other insects. As the precursor of a seed storage protein, BjTI is expressed only in the seeds, not in any other plant part. Thus, it does not provide protection to the whole plant against insects, which feed on the vegetative parts. The coding sequence of BjTI was therefore introduced under a constitutive promoter for transgenic expression in tobacco and tomato plants, so that TI was expressed in all of the vegetative parts as well as the seeds. This gave highly significant protection against the major insect pest *Spodoptera litura*.

EXPERIMENTAL PROCEDURES

Purification and Characterization of the Trypsin Inhibitor BjTI from B. juncea Seeds—One g of *B. juncea* midmaturation stage seeds (28 DAF) was pulverized in the presence of liquid nitrogen, mixed with 20 ml of phosphate buffer (10 mM, pH 7.2), and centrifuged at 30,000 × *g* for 15 min. The clear light green extract was heat-treated at 70 °C for 5 min, recentrifuged to collect supernatant, and kept at 4 °C for 4 h. Part of the globulin protein precipitated here and was discarded after centrifugation. The final solution was vacuum-concentrated at low temperature to 2 ml and fractionated on a Biogel P-60 column (bed volume 70 ml; Bio-Rad), previously equilibrated with 10 mM phosphate buffer, pH 7.2, containing 50 mM NaCl. A 0.8-ml aliquot of the concentrated extract was loaded onto the column and eluted with the same buffer at 15 ml/h. Fractions (75 × 1 ml) were collected, and the protein was estimated using Lowry's method and OD at 280 nm. The peaks were assayed for TI activity (see below); the second small protein peak showed activity and was pooled and further purified on a carboxymethyl (CM)-cellulose column. Protein was bound to the column at low salt and eluted in a gradient of increasing (0–0.55 M NaCl) salt concentration. Fractions showing positive TI activity and a 20-kDa band on SDS-PAGE were pooled.

Assay of Trypsin Inhibitor Activity Using Azocasein as Substrate—A reaction mixture of 400 μl containing 1 μg of trypsin (bovine trypsin; Sigma), varying amounts of BjTI (0.25–1.0 μg; volume adjusted to 100 μl with water), and 3 mg of azocasein (Sigma) in reaction buffer (50 mM Tris, 5 mM $CaCl_2$, pH 8.0) was incubated at 37 °C for 15 min. The reaction was stopped with 400 μl of cold 10% trichloroacetic acid, incubated in ice for 10 min, and centrifuged at 30,000 × *g* for 10 min. To the supernatant, an equal volume of 0.5 M NaOH was added, and the A_{428} was taken (27). One unit of trypsin was arbitrarily defined as the amount that increased A_{428} by 0.01 under the assay conditions. One unit of TI was defined as the amount that inhibited one unit of trypsin.

Assay of Trypsin Inhibitor Activity Using the Synthetic Substrate N-α-p-Tosyl-L-Arginine Methyl Ester (TAME)—To measure the inhibition of amidolytic activity of trypsin by BjTI, 1 μg of trypsin and TAME (2.5–10 mM) were incubated with 0, 200, and 300 ng of BjTI, and the rate of reaction was measured by recording A_{247} (28). One unit of trypsin was again arbitrarily defined as the amount that increased absorbance by 0.01/min. Trypsin inhibitory units were calculated from the number of trypsin units inhibited under similar conditions.

In Vitro Post-translational Processing Assay—Purified BjTI (10 μg) was incubated with seed extract of different developmental stages containing 12 μg of protein in 150 mM NaCl and 10 mM sodium phosphate buffer (pH 7.2) in a total reaction volume of 20 μl at 37 °C for different durations. The reaction mix was analyzed by SDS-PAGE or native PAGE.

Cloning of Bj2Sc Sequence in pET28a+: Expression and Purification of BjTI from Escherichia coli—The *Xho*I-*Not*I fragment from pBSKS+:2S was cloned into the *E. coli* expression vector pET 28a+ (Novagen) cut at the *Sal*I and *Not*I sites. The resulting clones showed in-frame expression of the Bj2S sequence with His tag and T7 tag, confirmed by *in vitro* transcription and translation. The clones were transferred into a Bl21DE3pLysS *E. coli* host for overexpression. Overexpression and purification were carried out according to the manufacturer's protocol (pET system manual; Novagen).

In Vitro Site-directed Mutagenesis of Bj2Sc Sequence—The Bj2Sc coding sequence from plasmid Bluescript KS+:2S was digested with *Kpn*I and *Xba*I and cloned into the same sites of pGEM3Zf$^-$ vector. The resulting clone (pGEM3Zf$^-$:2S) was used for *in vitro* mutagenesis using the Stratagene QuikChange site-directed mutagenesis kit, following the manufacturer's protocol. The Mn1 mutant (Asp^{37} → Glu and Gly^{40} → Ser) was generated using sense (5′-acagaatcagccagcccatttaggatt-3′) and antisense (5′-tgtcttagtcggtcgggtaaatcctaa-3′) primer sets in which the nucleotides C^{111} and G^{118} were changed to A^{111} and A^{118}. The Mn2 (Pro^{41} → Cys and Phe^{42} → Pro) mutant was generated using sense (5′-acagactcagccggctgcccaaggatt-3′) and antisense (5′-tgtctgagtcggccgacgggttcctaa-3′) primer sets in which nucleotides C^{121}, C^{122}, A^{123}, T^{124}, T^{125}, and T^{126} were changed to TGCCCA. All mutations were confirmed by sequencing.

In Vitro Transcription-Translation and in Vitro Processing of Radiolabeled Pre-2S Protein—About 1 μg of purified DNA was used in an *in vitro* coupled transcription-translation reaction using TNT-T7 Quick-Coupled rabbit reticulocyte system (Promega). To 25 μl of reaction volume 20 μl of lysate mix and 10 μCi of [^{35}S]methionine were added. Two μl of this reaction mix was used in subsequent processing experiments. The labeled pre-2S was incubated with midmature *B. juncea* seed extract containing 10 μg of protein in a reaction volume of 20 μl containing 10 mM phosphate buffer, pH 7.2, and 150 mM NaCl. Incubation was carried out at 37 °C for 2 h, and the whole reaction mix was analyzed by SDS-PAGE, followed by autoradiography.

Cloning of Bj2Sc Sequence into pKYLX7.1 Vector and Plant Transformation—The Bj2Sc sequence, initially cloned between the *Xho*I and *Xba*I sites in plasmid Bluescript KS+ (pBSKS+), was subcloned into the same sites in pKYLX7.1 vector (29) having CaMV ^{35}S promoter with duplicated enhancer (30). The pKYLX7.1:2S construct was mobilized into *Agrobacterium tumifaciens* (LBA4404) by triparental mating. Seeds of tobacco (*Nicotiana tabacum* var. SR1), initially obtained from the Department of Agronomy, University of Kentucky (Lexington, KY), and tomato (*Lycopersicon esculentum* var. Pusa Ruby) were surface-sterilized and germinated on Murashige and Skoog basal medium. Mature leaf discs of tobacco and cotyledonary explants of tomato were transformed by the gene constructs essentially according to the methods of Schardl *et al.* (29) and Fillati *et al.* (31), respectively. After treatment with agrobacterium, explants were transferred onto Murashige and Skoog medium with 3 mg/liter 6-benzylaminopurine and 1 mg/liter indole acetic acid. The transformed plantlets were selected in presence of 500 mg/liter cefotaxime and kanamycin (300 mg/liter for tobacco and 50 mg/liter for tomato). The transformed regenerated shoots were rooted on hormone-free Murashige and Skoog medium containing cefotaxime and kanamycin. The plants were grown in tissue culture rooms at 25 °C and 16-h light/8-h dark cycles. After several cycles of selection in kanamycin medium, the plants were potted and self-pollinated for successive generations.

Molecular Analysis of Transgenic Plants—Total plant DNA was isolated from leaves by the method of Draper and Scott (32). PCR was done using 30-nucleotide primers corresponding to the 5′- and 3′-ends of the BJ2Sc gene (5′-atggcggacaagctcttcctcgtctcggca-3′) and (5′-ctagtaggagggcccaggcatggtcttctg-3′). Total plant RNA was isolated from leaf tissue by the acid guanidium thiocyanate-phenol/chloroform method. The radiolabeled probe was prepared by random-primed oligolabeling of DNA. For Western blot analysis, total leaf protein was prepared in phosphate-buffered saline. Forty μg of the leaf protein was separated on 12% SDS-PAGE and transferred onto nitrocellulose membrane. Polyclonal antiserum raised in rabbit against purified 2S protein was used as the primary antibody. The secondary antibody was goat anti-rabbit IgG-horseradish peroxidase conjugate (Sigma).

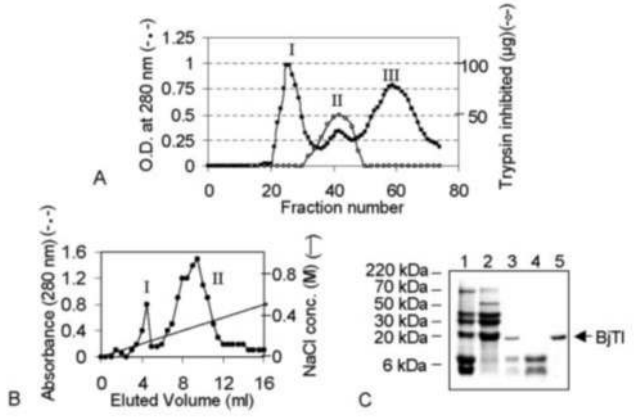

FIG. 1. **Purification of BjTI.** *A*, elution profile of *B. juncea* midmaturation seed extract from a 70-ml Biogel P-60 column; peaks I and III are the 12S globulin and 2S albumin fractions, respectively; peak II is a mixture of pre-2S protein showing TI activity, along with globulin and albumin contaminants. *B*, further purification of pooled peak II fractions from *A* by CM-cellulose chromatography. Here peak I is the purified BjTI. *C*, SDS-PAGE of different column fractions. *Lane 1*, crude extract of 28 DAF *B. juncea* seeds; *lanes 2–4*, peaks I, II, and III of biogel P-60 column; *lane 5*, CM cellulose-purified pre-2S (BjTI).

Assay of Expressed BjTI in Transgenic Tobacco and Tomato Using Azocasein—The assay was done as described above. The total protein of the leaf extracts was quantified using the Bradford method. Then approximately similar amounts of leaf protein (120–150 μg) had their volumes adjusted to 100 μl with water. The percentage of total soluble leaf protein as BjTI was calculated for each sample from a standard curve of SBTI, assuming SBTI (181 amino acids) and BjTI (178 amino acids) to be of the same molecular mass. A ΔA_{428} value of 0.226 was found to be equivalent to 1.0 μg of SBTI from the standard curve.

Insect Bioassay of Transgenic Leaves—Rearing of *S. litura* larvae in the laboratory was started with an egg mass to get a synchronous culture that was maintained for three generations (33). The optimum temperature for the feeding experiments was found to be 22 °C, which gave a sufficiently slow growth rate to measure weight at intervals (34). The leaves of transgenic plants were given as food to the first, second, and third instar larvae. For each sample, 30 insects (three sets of 10 insects each) were placed separately and fed with leaves of transgenic lines to be tested, and several sets were kept as control. The larvae were weighed on alternate days, and the number of deaths and any abnormalities in development were recorded.

RESULTS

Isolation and Characterization of the New Trypsin Inhibitor

The trypsin inhibitor was purified from midmature (28 DAF) *B. juncea* seeds by a combination of gel filtration and ion exchange chromatography. The elution profile of seed extract from the Biogel P-60 column (Fig. 1*A*) showed three peaks (I, II, and III). SDS-PAGE analysis showed that peak I consisted of 12S globulins (cruciferins) giving five major bands (Fig. 1*C*, *lane 2*); peak II consisted of a 20-kDa major band contaminated with 12S and 2S protein (Fig. 1*C*, *lane 3*); and peak III consisted of 9- and 4-kDa subunits of 2S protein (Fig. 1*C*, *lane 4*). The trypsin-inhibitory activity of each of these fractions was assayed by azocasein hydrolysis. The middle peak (II) showed TI activity, whereas peaks I and III did not, even when up to 30-fold more protein was added compared with peak II. To further purify this 20-kDa TI, the fractions of peak II were pooled and subjected to CM-cellulose ion exchange column chromatography. In this elution profile, the purified TI was found in the first peak (Fig. 1*B*, *peak I*) eluted with 0.1 M NaCl, whereas the second peak (II) had the contaminants. This novel 20-kDa TI was named *B. juncea* TI or BjTI (Fig. 1*C*, *lane 5*).

The TI activity of BjTI was assayed using both azocasein and the synthetic substrate TAME. Trypsin (5 μg) was incubated

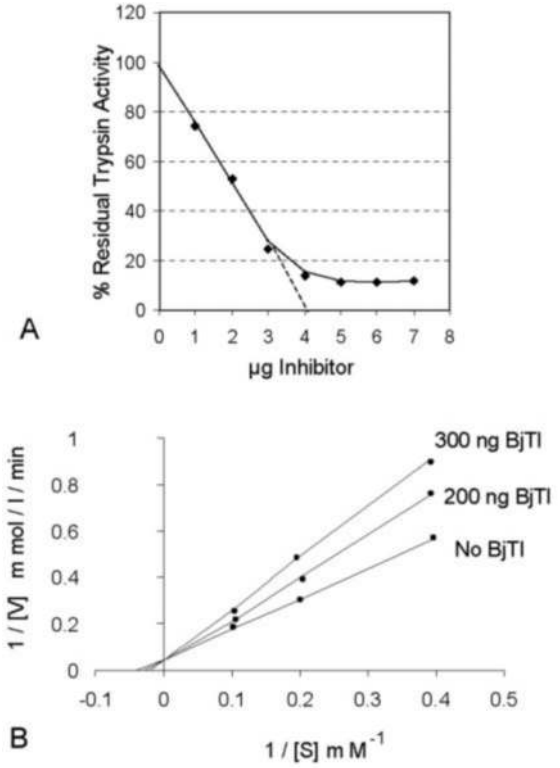

FIG. 2. *A*, effect of increasing concentration of inhibitor on amidolytic activity of trypsin on TAME. Trypsin (5 μg) was incubated with varying amounts of BjTI at 37 °C for 2 min and assayed for the residual trypsin activity. The stoichiometry was calculated by extrapolating the linear part of the curve to 0% residual activity of trypsin. *B*, Lineweaver-Burk plot of the inhibition of amidolytic activity of trypsin by BjTI. One μg of trypsin and TAME solutions of different concentrations (2.5–10 mM) were incubated with 0, 200, and 300 ng of BjTI, and the rate of reaction was measured by recording the A_{247}.

with varying amounts of BjTI according to protocol and assayed for residual TI activity. By plotting the residual activity against the amount of inhibitor used and extrapolating the linear part of the curve to 0% residual trypsin activity, it was found that 4.2 μg of BjTI was needed to completely inhibit the enzyme activity of 5 μg of trypsin (Fig. 2*A*). Since the crystalline bovine trypsin used was >98% pure and considering the M_r of bovine trypsin to be 24 kDa and that of BjTI to be 20 kDa, the values correspond to a molar ratio of 1:1 for the trypsin and inhibitor complex.

The stability of BjTI was studied by exposing it to altered conditions of pH, temperature, and protein-denaturing agents like urea and SDS and measuring the residual inhibitor activity. The results (presented as supplementary data; supplemental Tables II, III, and IV) showed BjTI to be stable over a wide pH range (pH 3.0–12.0; supplemental Table II) and to retain at least 70% activity after being heated to 80 °C for 10 min, losing significant activity only at 90 °C (supplemental Table III). Similarly, it retained 90% activity after treatment with 8 M urea or 1% SDS for 1 h (supplemental Table IV). It had no free SH groups, as assayed by 5,5′-dithio-bis-2-nitrobenzoic acid, but it contained four pairs of Cys residues, forming disulfide (-S-S-) linkages. It also lacks chymotrypsin or amylase inhibitory activity. From the molecular weight and the absence of chymotrypsin inhibitor activity, BjTI may be included in the Kunitz family of TIs. The kinetics of BjTI were studied by the extent of inhibition of amydolitic activity of trypsin in the presence of two different inhibitor concentrations and varying concentrations of the synthetic substrate, TAME. The Lineweaver-Burk plot of these data showed that the K_m changed with increasing

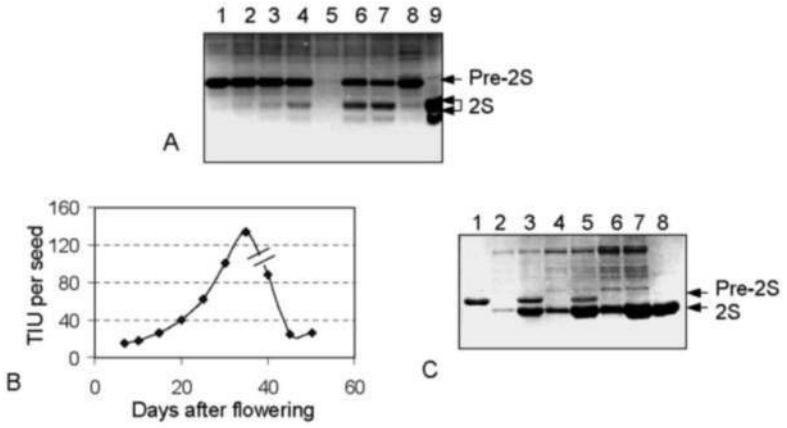

FIG. 3. *A*, SDS-PAGE showing *in vitro* processing of BjTI. 10 μg of BjTI was incubated with extract from 21 DAF mustard seeds having 12 μg of protein. *Lane 1*, 15 min; *lane 2*, 30 min; *lane 3*, 45 min; *lane 4*, 60 min; *lane 6*, 90 min; *lane 7*, 120 min; *lane 5*, seed extract only; *lane 8*, BjTI incubated with boiled seed extract; *lane 9*, partially purified *B. juncea* 2S protein. *B*, changes in BjTI activity in mustard seeds with developmental age. Protein extract from 10 seeds collected on different DAF was assayed using TAME as trypsin substrate and expressed as total trypsin inhibitory units per seed. For mustard seeds, 18–25 DAF is the green stage, 25–30 DAF is the orange midmature stage, 30–40 DAF is the brown stage, and 50 DAF is the harvest stage. *C*, nondenaturing PAGE showing increase in BjTI processing enzyme activity with developmental age of mustard seed. Eight μg of BjTI was incubated with seed extract having 12 μg of protein from different stages. *Lane 3*, 15 DAF; *lane 5*, 21 DAF; *lane 7*, 28 DAF; the respective seed extracts alone are shown in *lanes 2*, *4*, and *6*. *Lane 1*, purified BjTI alone; *lane 8*, purified mature 2S protein from *B. juncea*.

amount of the inhibitor with a K_i value of 6.4×10^{-10} M, as determined from Fig. 2*B*.

BjTI Is the Precursor of B. juncea 2S Seed Storage Protein

Several experiments were carried out with the purified BjTI and the 2S seed storage protein gene (Bj2Sc sequence), some of which indicated, while others conclusively proved, that BjTI is not only a novel TI but is also the precursor of the inactive 2S seed storage protein.

BjTI Isolated from Seeds Is Processed in Vitro to Yield 9- and 4-kDa Subunits

The purified BjTI from *B. juncea* seeds was subjected to proteolytic processing *in vitro* by incubation with maturing mustard seed extract, which contains the processing enzyme(s). With increasing time of incubation, more and more BjTI was processed to the mature 2S subunits (Fig. 3*A*, *lanes 3–7*). The processing activity was lost when the seed extract was heated in boiling water bath for 5 min (Fig. 3*A*, *lane 8*). This *in vitro* study indicates that BjTI is the precursor of 2S protein. The loss of TI activity upon processing indicates that the TI active site amino acid residues reside at or near the peptide processing site.

Temporal Correlation between Biosynthesis of BjTI and Its Processing Activity in Developing Seeds

The levels of BjTI and processing activity changed with the developmental age (days after flowering) of mustard seeds. Since mustard flowers are of indeterminate type, they were tagged on the day of complete opening, and seeds were harvested on the 7th and 10th DAF and at 5-day intervals thereafter, and the mature seeds were finally harvested on the 50th day. Crude protein extracts from these seeds were assayed for TI activity, and results were expressed as arbitrary TI units. TI activity could be detected as early as 7 DAF and increased up to 35 DAF, rapidly declining thereafter (Fig. 3*B*). However, a low level, about one-fifth of the highest activity, persisted in the harvested dry seeds.

To check the levels of pre-2S (or BjTI) processing enzyme(s) during the course of seed development, fixed amounts of BjTI were incubated with protein extract made from seeds collected at 15, 21, and 28 DAF, and the products were separated by nondenaturing PAGE. The results showed that 15 DAF seeds already contained an appreciable amount of processing enzyme(s) (Fig. 3*C*, *lane 3*). The level increased with the developmental age of the seeds (*lanes 4–6*) and was highest in the 28 DAF extract (*lane 7*). A low but detectable amount of processing enzyme(s) was present in the harvested dry seed extract. Thus, the biosynthesis of BjTI and its processing enzyme(s) occur in parallel. This pattern matches the synthesis and accumulation of 2S storage proteins, well studied in other *Brassica* seeds in this (35) and other laboratories. These *in vivo* studies indicated that BjTI is the precursor of 2S seed storage protein.

Expression of the Coding Sequence of B. juncea 2S Seed Storage Protein Gene in E. coli Yielded Active BjTI

We have previously cloned the *B. juncea* 2S protein gene (Bj2Sc; GenBank™ accession number X65972). To prove conclusively that BjTI is the precursor of *B. juncea* 2S albumin, we cloned the 2S coding sequence (Bj2Sc) into the expression vector pET 28a+. The clones were tested by *in vitro* coupled transcription and translation with [^{35}S]methionine, and the product of the expected molecular weight was obtained (supplemental Fig. 7). The bacterially expressed precursor of 2S was purified as an ~24-kDa tagged protein (supplemental Fig. 8). The purified precursor and the induced cell extract were assayed *in vitro* for TI activity using azocasein as substrate for trypsin. The expressed precursor was found to inhibit trypsin at a 1:1 molar ratio, like the native BjTI purified from *B. juncea* seeds. This conclusively proved that BjTI is the precursor of *B. juncea* 2S seed storage protein.

In Vitro Processing of in Vitro Translated Bj2Sc Sequence

For *in vitro* processing experiments with *in vitro* translated precursor of 2S, the Bj2Sc sequence was cloned into the pGEM3Zf$^-$ vector. *In vitro* transcription and translation in rabbit reticulocyte lysate of pGEM3Zf$^-$:2S in the presence of [^{35}S]Met yielded the expected 20-kDa product. For processing studies, the radiolabeled precursor of 2S was incubated for 120 min in PBS alone as control and also in 28 DAF *B. juncea* seed extract for 30 and 120 min. Whereas control incubation gave no processing (Fig. 4*A*, *lane 1*), some processing was observed after 30-min incubation with seed extract (*lane 2*), and processing was almost complete by 120 min (*lane 3*).

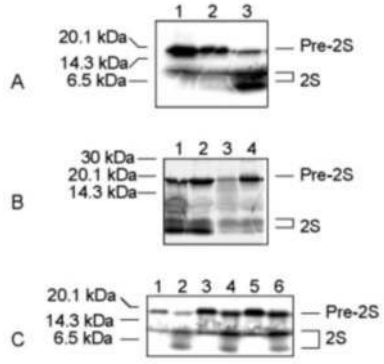

FIG. 4. *A*, autoradiogram showing processing of *in vitro* translated [^{35}S]Met-labeled Bj2Sc product on incubation with midmaturated (28 DAF) mustard seed extract. *Lane 1*, control (incubated in PBS for 120 min); *lane 2*, incubated with seed extract for 30 min; *lane 3*, incubated with seed extract for 120 min, showing almost complete processing to 9- and 4-kDa mature 2S subunits. *B*, autoradiogram showing thermostability of *in vitro* translated Bj2Sc product processing activity in mustard and pea seed extracts. *Lanes 1* and *2*, processing on incubation with control and heat-treated (65 °C, 5 min) mustard seed extract; *lanes 3* and *4*, incubation with control and heat-treated pea seed extract, respectively. *C*, autoradiogram of SDS-PAGE showing *in vitro* processing of *in vitro* translated radio-labeled products of control and mutated Bj2Sc sequences with 28 DAF *B juncea* seed extract. *Lanes 1*, *3*, and *5*, incubation of control Bj2Sc product, Mn1, and Mn2 in PBS for 2 h; *lanes 2*, *4*, and *6*, incubation of control Bj2Sc product, Mn1, and Mn2, respectively, in seed extract.

In Vitro Processing by Pea Seed Extract and Thermostability of Processing Activity

From earlier studies, it is known that processing enzymes for seed storage protein precursors are seed tissue-specific but not species-specific (36). In the present study, the precursor of 2S protein purified as BjTI from *B. juncea* seeds was processed *in vitro* by *B. juncea* seed extract (Fig. 4*A*) but not by *B. juncea* leaf extract (results not presented). We next tested the processing of *in vitro* synthesized radiolabeled BjTI protein with pea (*Pisum sativum*) seed extract and compared it with that with mustard seed extract. The results showed that precursor protein was processed *in vitro* into mature 2S subunits by extracts from pea seeds (Fig. 4*B*, *lane 3*) similar to that by mustard seed (*lane 1*). Furthermore, the processing enzymes showed partial thermostability toward heating at 65 °C for 5 min (*lanes 2* and *4*).

In Vitro Processing of Two Mutant Forms of BjTI

Earlier publications reported the involvement of an Asn (or Asp) residue for recognition of the processing enzyme(s) and the presence of a GP motif near the processing site. However, our previous alignment studies did not support this notion (17, 18). To investigate it further, along with the control wild type sequence, we made two mutant forms of the precursor of 2S, as described under "Experimental Procedures." These mutant proteins were translated *in vitro* with [^{35}S]methionine and subjected to *in vitro* processing. Both of these mutants were processed to 9- and 4-kDa subunits (Fig. 4*C*, *lanes 4* and *6*) like the control BjTI (*lane 2*). These results indicate that there is no absolute necessity for the presence of Asp (or Asn) residue or a GP motif near the processing site of the precursor of *B. juncea* 2S.

Small Peptide Spanning the TI Active Site Does Not Have Inhibitor Activity

Since other studies indicate that often small peptides spanning the TI active site give TI activity *in vitro*, we tested two synthetic 15-residue oligopeptides: TDSAGPFRIPKCRKE and TVSASPFRIPKCRKE, spanning the amino acid residues 36–50 of the *B. juncea* 2S coding sequence including the TI active site (GPFRI). The former has the original TI active site sequence of BjTI, whereas the latter has the TI active site region (SPFRI) found in soybean Kunitz inhibitor. However, when assayed by azocasein, neither peptide had TI activity even when up to 100-fold more peptide was used.

Plant Transformation and Expression of BjTI

Transformation of Tobacco and Tomato—The BjTI gene was introduced under a constitutive promoter into Tobacco (*Nicotiana tabacum*) and tomato (*Lycopersicon esculentum*) plants. The T_0 plants were self-pollinated and maintained through successive generations via seed germination in selection medium (supplemental Fig. 9). Finally, six tobacco lines and two tomato lines stably inheriting the transgene were used for further study. The presence of the transgene was confirmed by PCR amplification of the insert using 5′- and 3′-end primers designed to amplify the entire sequence with genomic DNA of transformed plants as templates. As expected, a single band of about 0.5 kb was synthesized with DNA from all confirmed transgenic T_2 generation plants (Fig. 5*A*). Upon Southern transfer, these amplified bands hybridized with labeled 2S gene probe. To determine the number of copies of the inserted gene, we performed genomic Southern blot hybridization after digestion of the total DNA with *Eco*RI and agarose gel electrophoresis. Two tobacco lines, SR1-2b and SR1-19a, revealed the integration of two copies of the 2S gene, whereas the rest of the tobacco lines (SR1-3c, -5a, -11, and -15) and two tomato lines (PR-9 and PR-14) each had one copy of the 2S gene (Fig. 5*B*).

Expression of Bj2Sc Gene in Transgenic Plants—We tested transformed T_2 generation tobacco and tomato plants for transcription of the 2S protein gene by Northern blot analysis. In transformed tobacco and tomato lines, the 2S gene was transcribed into mRNA of the expected size (~500 nucleotides), which was absent in the control untransformed plants (Fig. 5*C*). Transformed plants were also screened for the expressed 2S protein by Western slot blot assay. The total leaf protein from the six transformed tobacco and two transformed tomato lines gave strong positive signals (Fig. 5*D*). When tested by regular Western blots, several of these plants revealed a protein band with the expected M_r of 20 kDa, thus showing the expression of the introduced gene to yield BjTI (Fig. 5*E*). TI activity of Bj2Sc gene product assayed in the total soluble leaf protein extract showed expression levels ranging from 0.42 to 0.83% in tobacco lines and from 0.28 to 0.70% in tomato lines (Table I).

Plants Expressing Pre-2S Protein Are Insect-resistant—We next tested the ability of transgenic tobacco and tomato plants to resist insect attack. The polyphagous lepidopteron tobacco cutworm *S. litura* was chosen as the target organism for this bioassay, since it is a common pest to many crop plants. In feeding experiments, tobacco lines SR1-2b and SR1-15 showed 100% mortality to the insects within the first 4–5 days, starting with 4-day-old first instar larvae (Fig. 6*A*). The experiment was repeated with 7-day-old second instar larvae, which showed a longer period of survival, but ultimately 100% death occurred. Other tobacco lines showed significant mortality and retarded growth (Fig. 6*C*). The two transgenic tomato lines PR-9 and PR-14 also showed significant mortality (Fig. 6*B*) and growth retardation (Fig. 6*D*) of larvae compared with the control. The revertant larvae (*i.e.* those initially fed with transformed SR1-5a leaves and then reverted to control leaf diet) again resumed gaining body weight, showing that the inhibitor is present in the transformed plants and not in the controls (Fig. 6*E*). In insect feeding experiments, whole potted trans-

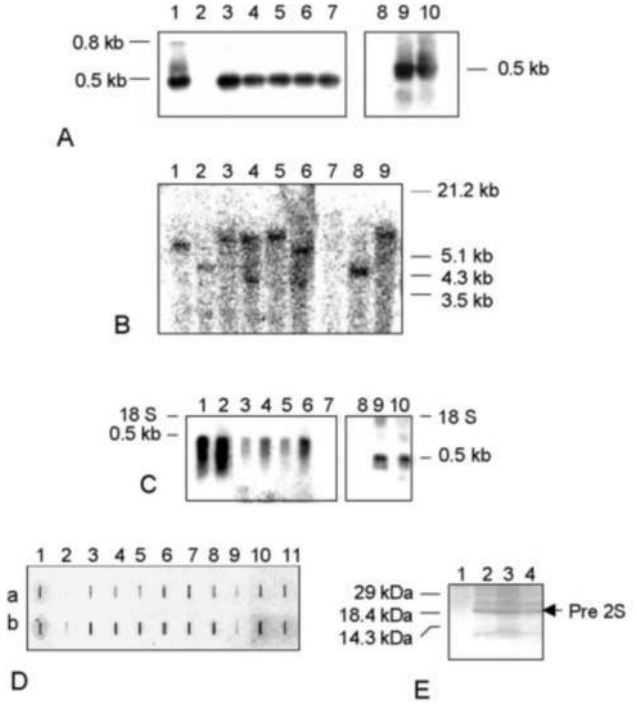

FIG. 5. **Molecular analysis of T_2 generation transgenic tobacco and tomato plant lines expressing BjTI.** *A*, Southern hybridization of PCR product of total plant DNA using primers corresponding to 5′- and 3′-end sequences of the 2S gene, with radiolabeled 2S fragment as probe. *Lane 1*, *B. juncea*; *lane 2*, control tobacco SR1; *lane 3*, SR1-2b; *lane 4*, SR1-3c; *lane 5*, SR1-11; *lane 6*, SR1-15; *lane 7*, SR1-19a; *lane 8*, control tomato PR; *lane 9*, PR-9; *lane 10*, PR-14. *B*, Southern blot hybridization of *Eco*RI-digested total leaf DNA using radiolabeled 2S gene as probe. *Lane 1*, SR1-5a; *lane 2*, SR1-15; *lane 3*, SR1-11; *lane 4*, SR1-19a; *lane 5*, SR1-3c; *lane 6*, SR1-2b; *lane 7*, control PR; *lane 8*, PR-9; *lane 9*, PR-14. *C*, Northern blot analysis of transgenic tobacco and tomato lines showing a 0.5-kb mRNA band. Total leaf RNA was separated on a formaldehyde gel, blotted, and probed with radiolabeled 2S gene. *Lane 1*, SR1-2b; *lane 2*, SR1-3c; *lane 3*, SR1-5a; *lane 4*, SR1-11; *lane 5*, SR1-15; *lane 6*, SR1-19a; *lane 7*, control SR1; *lane 8*, control PR; *lane 9*, PR-9; *lane 10*, PR-14. *D*, Western slot blot of total leaf proteins using antiserum raised in rabbit against purified Bj2S protein. *Row a*, 15 μg of protein; *row b*, 30 μg of protein. For *B. juncea* seeds, 5 and 10 μg of protein were used in *a* and *b*, respectively. *Lane 1*, *B. juncea* seed protein as positive control; *lane 2*, untransformed SR1; *lane 3*, SR1-2b; *lane 4*, SR1-3c; *lane 5*, SR1-5a; *lane 6*, SR1-11; *lane 7*, SR1-15; *lane 8*, SR1-19a; *lane 9*, untransformed PR; *lane 10*, PR-9; *lane 11*, PR-14. *E*, Western blot of total leaf protein separated on SDS-PAGE. *Lane 1*, untransformed SR1; *lane 2*, SR1-2b; *lane 3*, SR1-15; *lane 4*, PR-9.

formed plants showed remarkable resistance to insect damage and better growth than control plants (supplemental Fig. 10). Thus, the expression of BjTI protein in both the tobacco and tomato plants imparted a high level of protection against a major insect pest.

DISCUSSION

Trypsin inhibitors have long existed in plants for protection against pests and diseases, regulation of endogenous proteinases, and also inhibition of trypsin, whereas ingested seeds are passing through the animal gut, thus helping in seed dispersal. The TIs are then used like other ordinary storage proteins during seed germination. Our work shows that BjTI is not only a novel TI but also the precursor of the 2S seed storage albumin, which lacks TI activity.

We isolated BjTI as a 20-kDa protein from midmaturation seeds, which can be processed *in vitro* into 9- and 4-kDa 2S subunits by *B. juncea* seed extract. The TI inhibited trypsin in a 1:1 molar ratio, and antibody raised against Bj2S inhibited its activity. The Bj2S coding sequence, originally cloned in our laboratory, was expressed in *E. coli* cells, where it is produced as the precursor TI. This expressed TI is also processed *in vitro* like the native TI in presence of seed extract. These results conclusively establish the identity of the TI as the precursor of the Bj2S.

We observed that BjTI is stable in denaturing agents (up to 10 M urea and 1% SDS) and also over a wide range of pH and temperature. In a separate work, we have also studied the structure of mature Bj2S protein by CD, fluorescence spectroscopy, and protein modeling, which revealed a compact thermostable structure having two interchain and two intrachain cysteine disulfide bonds and other interesting features (Ref. 37).[2] This confirms the earlier results obtained by NMR (38), matrix-assisted laser desorption-ionization mass spectroscopy, and microcalorimetry (39) for a homologous napin Bn1b from *Brassica napus*. Limited proteolysis with several endopeptidases and CD spectroscopy indicated that the cleavable propeptide-peptide junctions of the napins are exposed to the exterior of the mature napin. Moreover, the conformation of the mature napin is not significantly different from that of the pronapin (15). This explains our observation that BjTI is stable in denaturing agents and over a wide range of pH and temperature.

The derived amino acid sequence of the Bj2Sc gene shows the presence of soybean Kunitz TI active site-like motif GPFRI at or near the reported processing site of similar 2S albumins. Our modeling of the mature Bj2S suggests that the location of the TI-active GPFRI motif is in a surface loop near the junction of the prosequence and the small subunit. This may well explain why the precursor loses TI activity upon processing.

Both mature and precursor napin storage proteins from *B. napus* share sequence homology with BjTI and its processed form. On the other hand, the two-subunit TIs from *S. arvensis* (23) and *B. nigra* (24) have sequence homology with mature napin. The allergen protein from *S. alba* (40) also has sequence homology with BjTI. Taken together, these observations suggest that BjTI, 2S seed storage proteins, some large molecular weight TIs, and some allergenic seed proteins are products of distinct members of a multigene family evolved from a common ancestral gene.

A synthetic 15-residue oligopeptide spanning the GPFRI motif was inactive as TI, even at a 100-fold excess over trypsin. This indicates that not only the active site motif region but also a longer sequence, giving rise to a local secondary structure around the active site, may be necessary for the TI activity. We have also tested the yellow mustard allergen (40) provided by R. Rodriguez (Universidad Complutense, Madrid, Spain). It has a GPFR motif just two residues away from its N-terminal end but otherwise has >90% amino acid sequence homology with mature Bj2S protein and also had no TI activity even at a 100-fold molar excess. Similarly, studies on the smallest TIs belonging to the squash family inhibitor having 27–35 amino acid residues show that all of the amino acids are necessary for TI activity, although amino acid replacement without altering the overall conformation is tolerated (41). Recently, a number of detailed studies have indicated the presence of a binding loop in some Kunitz and Bowmann-Birk type TIs; the alteration of the amino acid sequence in this reactive site affects the binding with trypsin (42–44).

Although earlier published reports propose the involvement of Asn (or Asp) residue for recognition of the processing enzyme(s) and the requirement for a GP motif near the processing sites, our previous sequence alignment studies did not support this notion (17, 18). In the present study, we expressed two

[2] G. Basu, M. Ghose, D. Roy, S. Mandal, and R. K. Mandal, unpublished results; Protein Data Bank entry 1KWL, Dt 01.30.02.

TABLE I

Trypsin inhibitor assay of leaf extracts of transgenic tobacco and tomato plants

Approximately identical amounts of leaf protein (120–150 μg) were taken, and volume was made up to 100 μl with water. The BjTI as a percentage of total soluble leaf protein for each sample was calculated with reference to the standard curve of soybean TI.

Plant no.	Total protein/ assay	ΔA_{428}[a]	TI (Pre-2S)[b]	Pre-2S protein as a percentage of total soluble protein
	μg		μg	%
Tobacco				
SR1–2b	135	0.224	0.991	0.70
SR1–3c	144	0.272	1.203	0.83
SR1–5a	144	0.130	0.575	0.40
SR1–11	149	0.204	0.903	0.61
SR1–15	138	0.249	1.102	0.80
SR1–19a	142	0.212	0.938	0.66
Tomato				
PR-9	128	0.202	0.894	0.70
PR-14	153	0.097	0.429	0.28

[a] ΔA_{428} of 0.226 is equivalent to 1.0 μg of soybean TI from standard curve.
[b] Soybean TI (181 amino acids) and BjTI (178 amino acids) are assumed to be of the same molecular mass.

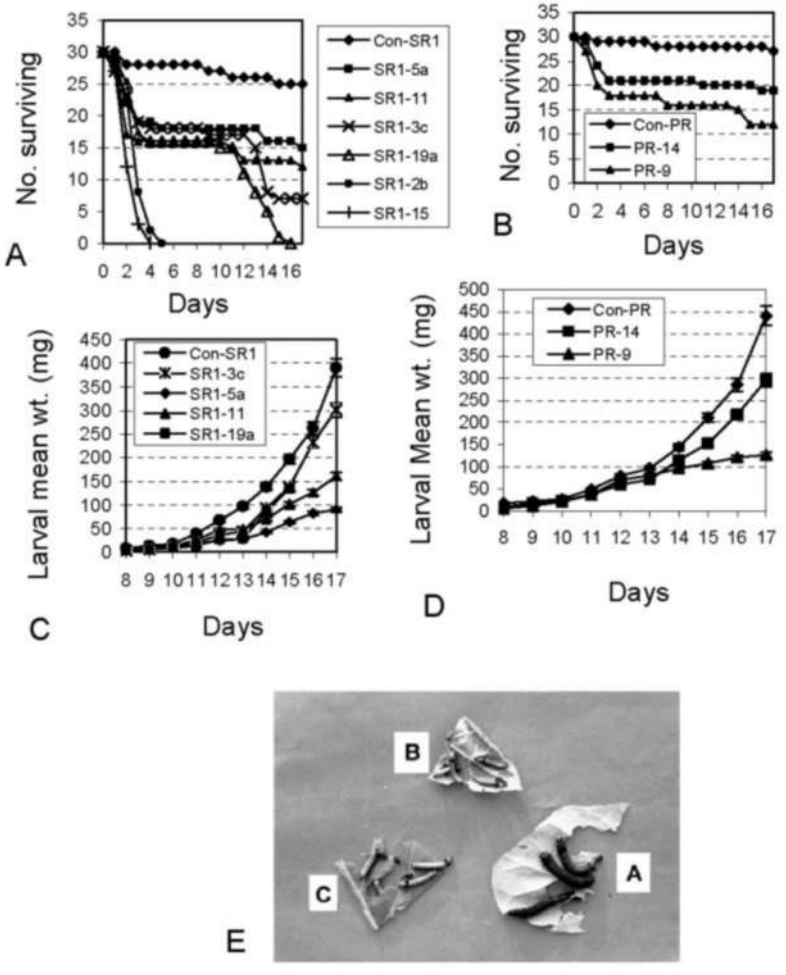

FIG. 6. **Insect bioassay of transgenic plants expressing BjTI.** *A*, graph showing survival (number of larvae surviving) of *S. litura* first instar larvae feeding on T_2 generation transgenic tobacco plants, starting with three replicates of 10 insects each on day 0. *B*, graph showing mortality of first instar larvae feeding on T_2 generation transgenic tomato plants. *C*, graph showing growth (mean weight ± S.E.) of surviving larvae feeding on T_2 generation transgenic tobacco plants, starting with three replicates of 10 insects each. *D*, graph showing growth of surviving larvae on T_2 generation tomato plants. *E*, feeding experiment on SR1-5a leaves shows significantly retarded growth of larvae (*B*) compared with the normal growth on control tobacco leaves (*A*), whereas the revertant larvae (*C*; *i.e.* those initially fed with SR1–5a leaves and then later fed with control leaves) show regaining of weight.

mutated BjTI genes by site-directed mutagenesis, the first mutant having D37E and G40S, and the second having P41C and F42P. Both were normally processed *in vitro*, showing that there is no absolute requirement for Asp or a GP motif near the processing site.

We introduced this BjTI gene under a constitutive promoter into tobacco as a model plant and tomato as a major vegetable crop. Transgenic plants showing stable expression were maintained up to the third generation and had BjTI levels of 0.28–0.83% of total soluble leaf protein. The levels of expression are comparable with those reported for transgenic expression of soybean Kunitz TI (45), cowpea TI (46), and barley TI Cme (47). The insect bioassay gave 100% mortality within the first few days starting with neonate larvae in case of two transgenic lines expressing TI at 0.7–0.8% of leaf protein. Thus, this novel TI gene can be used as a highly effective insect control protein.

That BjTI is processed into inactive storage protein also has evolutionary ramifications. Plants and herbivores have co-evolved over a long period, and natural selection must have played a major role. From an animal nutrition point of view, Bell and Rakow (48) analyzed seed meals from 124 different cultivars of oil seed *Brassica* belonging to several species and

found *B. juncea* strains to have lowest levels of TIs. *B. juncea* appears to have evolved somewhere in central Asia, and the Indian subcontinent has been the home for diversification of many *B. juncea* genotypes (49). It is still the most widely cultivated oil seed *Brassica* species there. Thus, humans may have unknowingly influenced the selection of the lowest TI containing rapeseed, the Indian mustard, in this part of the world. This is perhaps a case of positive selection for an otherwise insect-resistant plant in the field, but with lesser amounts of antinutritional factors in the harvested crop, which is favorable for human and animal consumption. On the other hand, the deliberate selection of winter rapeseed *B. napus* var. Brownski for low glucosinolates in recent years is an example of negative selection for higher TI content (48). Again, deliberate human selection for double "zero" (low glucosinolate and low erucic acid content) *B. napus* led to not only higher TI but also a more thermostable TI subtype (50).

Finally, BjTI possesses some special attributes over other TIs (51) such as that from soybean, cowpea, sweet potato, or other mustard TIs and α-amylase inhibitors from cereals or potato inhibitor II. First, it can be expressed in seed crops at high levels in the nonseed parts and in seeds up to late maturation stage when insect damage is maximal, thus providing field protection. In seeds during the dehydration stage, the TI would get naturally processed to inactive storage protein to yield a final low level safer for human and animal consumption. The residual low level remaining in the harvested seeds still may be enough to protect the dehydrated seeds for short term storage. Second, for long term storage of other seeds not meant for consumption, we could mutate the processing sites of the TI gene such that the protein retains TI activity, and processing to inactive 2S protein is prevented. Third, one could regulate the level of TI in any tissue at a particular stage of development not only by introducing the antisense of the TI under a suitable promoter (52) but also by using other means like manipulating the level or activity of the processing enzyme(s) or expressing the processing enzyme(s), at the desired time, in tissues where it is normally absent. Thus, the BjTI gene may be potentially a superior target gene for producing insect-resistant crops.

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