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Screening of a Peanut (*Arachis hypogaea* L.) cDNA Library To Isolate a Bowman−Birk Trypsin Inhibitor Clone

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Peanut crop losses due to insect and pest infestation cost peanut farmers nearly 20% of their annual yields. The conventional use of chemicals to combat this problem is costly and toxic to humans and livestock and leads to the development of resistance by target insects. Transgenic plants expressing a trypsin inhibitor gene in tobacco and cowpea have proven to be efficient for resistance against insects. Therefore, a transgenic peanut overexpressing a trypsin inhibitor gene could be an alternative solution to the use of toxic chemicals. Five Bowman−Birk trypsin inhibitor (BBTI) proteins were previously isolated from peanut. However, to date, neither cDNA nor genomic DNA sequences are available. The objective of this research was to screen a peanut cDNA library to isolate and sequence at least one full-length peanut BBTI cDNA clone. Two heterologous oligonucleotides were constructed on the basis of a garden pea (*Pisum sativa*) trypsin inhibitor nucleotide sequence and used as probes to screen a peanut lambda gt-11 cDNA library. Two positive and identical cDNA clones were isolated, subcloned into a pBluescript vector, and sequenced. Sequence analysis revealed a full-length BBTI cDNA of about 243 bp, with a start codon ATG at position +1 and a stop codon TGA at position +243. In the 3′ end, two poly adenylation signals (AATAAA) were identified at positions +261 and +269. The isolated cDNA clone encodes a protein of 80 amino acid residues including a leader sequence of 11 amino acids. The deduced amino acid sequence is 100% identical to published sequences of peanut BBTI AI, AII, BI, and BIII and 81% identical to BII.

KEYWORDS: Peanut; Bowman−Birk trypsin inhibitor; insects; transgenics; cDNA library; heterologous probes; plant defense

INTRODUCTION

Peanut (*Arachis hypogaea* L.) crop losses due to insect and pest infestation cost the peanut industry nearly 20% of its total annual yield. To combat this problem, peanut farmers have traditionally used chemical compounds. Although this has created a boom in the agrochemical industry with astonishing gains in agricultural production (*1*), there has been a growing concern about the detrimental effects of these chemicals on the environment and animal and human health. In addition, chemical insecticides are costly, and the majority of their primary targets have developed a resistance against them (*2*).

Plants high in trypsin inhibitors were shown to confer resistance to several insects (*3*). Boulter et al. (*4*) demonstrated that cowpea plants with high levels of trypsin inhibitors and transgenic tobacco engineered with a cowpea trypsin inhibitor were more resistant to lepidopter insect damages. Thus, an alternative solution of combating peanut insect problems in the field and during storage could be the production of transgenic peanut plants overexpressing the trypsin inhibitor gene.

Trypsin inhibitors are ubiquitous polypeptides in legumes (*5*, *6*) shown to act as pathogenesis-related (PR) proteins (*4*, *7*) and, thus, play a role in the plant defense mechanism.

Five Bowman−Birk trypsin inhibitor (BBTI) proteins were previously isolated in peanut. Studies revealed their amino acid composition and sequence, mechanism of inhibition, binding domain, and molecular evolution (*8*−*11*). However, to date, there is no information available for the genes encoding these proteins. The objectives of this research project were to isolate, sequence, and molecularly characterize at least one full-length peanut trypsin inhibitor cDNA clone. The long-term goal is to use this cDNA clone to produce transgenic peanut plant overexpressing the trypsin inhibitor protein in its vegetative tissues.

MATERIALS AND METHODS

Synthesis of Heterologous Oligonucleotides. Two 46 bp oligonucleotides TI46A and TI46B were designed and used as probes to screen a lambda gt-11 cDNA library. The oligonucleotides were

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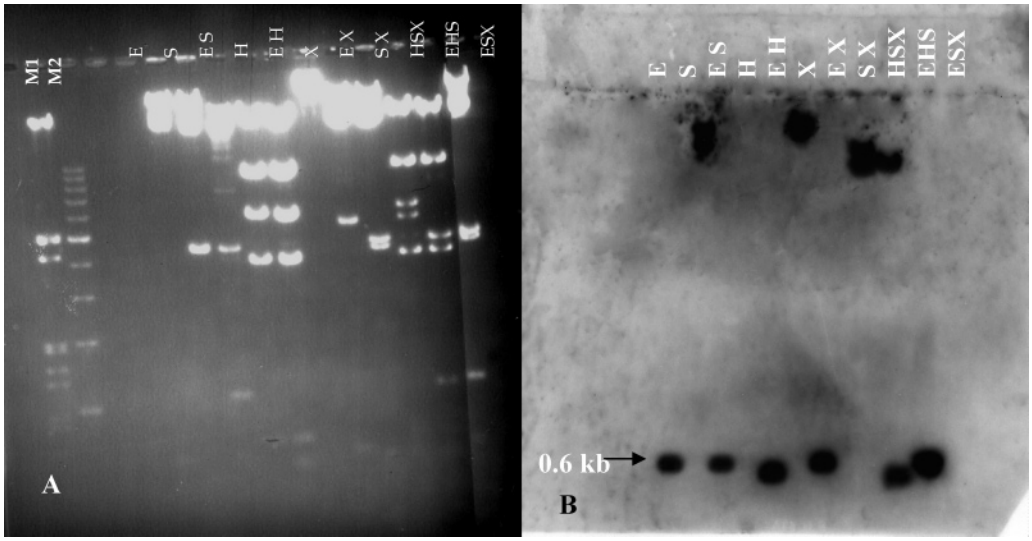

Figure 1. (**A**) 1.2% agarose gel electrophoresis of DNA fragments resulting from a restriction enzyme digestion of a putative positive peanut BBTI clone: (lane M1) lambda *Hin*dIII DNA marker; (lane M2) 1 kb marker; (lane E) *Eco*R1; (lane S) *Sac*I; (lane ES) *Eco*RI + *Sac*I; (lane H) *Hin*dIII; (lane EH) *Eco*R1 + *Hin*dIII; (lane X) *Xba*I; (lane EX) *Eco*RI + *Xba*I; (lane SX) *Sac*I + *Xba*I; (lane HSX) *Hin*dIII + *Sac*I + *Xba*I; (lane EHS) *Eco*RI + *Hin*dIII + *Sac*I; (lane ESX) *Eco*RI + *Sac*I + *Xba*I. (**B**) Autoradiograph of the membrane replica of the agarose gel in (**A**) after Southern hybridization. ^{32}P-Labeled probes TI46A and TI46B hybridized to several fragments including a unique 0.6-kb fragment released with *Eco*RI.

designed on the basis of regions of amino acid sequence identity between garden pea (*Pisum sativa*) trypsin inhibitor (GPTI) (GenBank Accession No. AJ296169) and peanut BBTI AII isolated by Norioka and Ikenaka (*8*). Nucleotide sequences were deduced from amino acid sequences using the DNASTAR program and submitted to ResGen Inc., an Invitrogen Corp. (Huntsville, AL), for synthesis.

Screening of a Lambda gt-11 Peanut cDNA Library. A peanut cDNA library was constructed from polyA+ RNA extracted from seeds of peanut line F78-1339 and cloned into a lambda gt-11 vector (Stratagene Inc., La Jolla, CA) (*12*). The library was screened using the two synthesized 46-bp oligonucleotide probes **TI46A** 5′TTG-CATTTGCACAAGGTCTCTTCCACCACAGTGTCGTTGCATTG-AT′3 and **TI46B** 5′TTGCACTTGCACAAGGTCTCTTCCACCA-CAGTGTCGTTGCACTGAT′3. About 10 pmol of each purified oligonucleotide was ^{32}P end-labeled as described by Fermentas Inc., (Hanover, MD). Prehybridization and hybridization were carried out as previously described (*13*). Briefly, prehybridization (42 °C for 3 h) was performed in a solution containing 20× SSPE (30%), 10% SDS (5%), 100× Denhardt's solution (5%), 5% sodium pyrophosphate ($Na_4P_2O_4 \cdot 10H_2O$) (1%), and (1%) denatured salmon sperm DNA. During hybridization, individually labeled probes were added to a solution containing 20× SSPE (30%)/10% SDS (5%) + 100× Denhardt's solution (1%) + 5% sodium pyrophosphate ($Na_4P_2O_4 \cdot 10H_2O$) (1%). After hybridization, the membranes were washed once with 6× SSPE/0.1% SDS at 42 °C for 15 min, once with 2× SSPE/0.1% SDS at 42 °C, and once with 0.2× SSPE/0.1% SDS at room temperature for 15 min each time and exposed to X-ray film (Kodak Biomax MS).

Southern Hybridization. Putative positive clones were identified after three rounds of plaque hybridization and stored at 4 °C in 1 mL of SM medium containing two drops of chloroform. Phage DNA was purified using a Qiagen Lambda kit (Valencia, CA) and digested with single and multiple restriction endonuclease digestions (*Eco*RI, *Sac*I, *Eco*RI + *Sac*I, *Hin*dIII, *Eco*RI + *Hin*dIII, *Xba*I, *Eco*RI + *Xba*I, *Sac*I + *Xba*I, *Hin*dIII + *Sac*I + *Xba*I, *Eco* RI + *Hin*dIII + *Sac*I, and *Eco*RI + *Sac*I + *Xba*I). DNA fragments were electrophoresed in 1% agarose gel, denatured (0.4 M NaOH), neutralized (0.25 M HCl) for 30 min, and transferred to a nylon membrane by capillary action for 24 h as described in ref *14*. The DNA was cross-linked on the membrane, and a Southern hybridization was performed using separately each of the ^{32}P-labeled probes at high-stringency conditions at 65 °C in HyperHyb buffer as described in ref *13*.

Subcloning into a pBluescript II SK$^+$ Vector. Positive DNA fragments were digested with *Eco*RI, and a 0.6 kb *Eco*RI fragment was cut from the gel, purified, and subcloned into pBluescript II SK$^+$ (Stratagene Inc., La Jolla, CA). Ratios of insert to vector DNA of 1:1, 2:1, and 3:1 were tested. The ligation reactions were performed at 4 °C overnight. About 20 μL of One Shot Top 10 Chemically Competent *Escherichia coli* cells (Invitrogen Corp., Carlsbad, CA) was mixed with 3 μL of ligation reaction and incubated on ice for 35 min. Vials were then incubated at 37 °C with shaking at 242 rpm for 1 h, spread onto plates, and incubated overnight at 37 °C until colonies formed. Plates were placed at 4 °C to enhance the formation of blue white colonies as described in ref *14*. Positive white colonies containing a plasmid with the peanut DNA insert were picked from plates, placed into 4 mL of LB medium containing ampicillin (25 mg/mL), and shaken vigorously at 246 rpm at 37 °C for 12 h. Plasmid DNA was extracted from the pellet following Qiagen Plasmid DNA extraction protocol and digested overnight with *Eco*RI to confirm the presence of the 0.6 kb peanut DNA insert.

Sequencing and Sequence Analysis. cDNA fragments subcloned into pBluescript II SK$^+$ were shipped to MWG Biotech (High Point, NC) for sequencing.

DNA sequence analysis, comparison, and homology searches were performed using BLAST algorithm tools (*15*). BLASTP and BLASTN were used to identify homologues of the peanut BBTI proteins (http://wanww.ncbi.nih.gov) (*15*). Comparative sequence analyses were performed using the computer software DNASTAR. The Clustal PAM250 method was used to conduct multiple sequence alignments. The deduced peanut BBTI protein signal peptide was predicted using Signal 1P software (*16*).

RESULTS

Library Screening. A total of 30 putative positive clones were isolated after three rounds of library screening using the two synthetic oligonucleotides as probes. Twelve putative positive clones were isolated from the first screening, 10 from the second, and 8 from the third. A clone was positive when it annealed simultaneously to both probes TI46A and TI46B. Two putative positive clones were randomly selected from the third screening for lambda DNA extraction and purification.

Southern Hybridization. Southern hybridization analysis of peanut BBTI clone digested with single, double, and triple restriction enzymes (*Eco*RI, *Sac*I, *Eco*RI + *Sac*I, *Hin*dIII, *Eco*RI + *Hin*dIII, *Xba*I, *Eco*RI + *Xba*I, *Sac*I + *Xba*I, *Hin*dIII + *Sac*I + *Xba*I, *Eco*RI + *Hin*dIII + *Sac*I, and *Eco*RI + *Sac*I + *Xba*I)

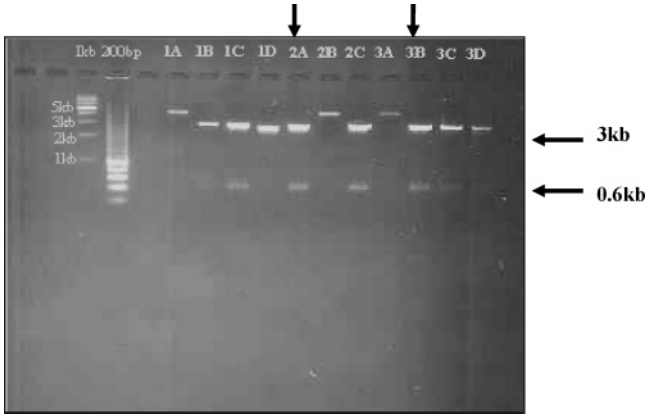

Figure 2. Restriction enzyme digestion of the positive *Eco*RI fragment subcloned into p-Bluescript II SK (±) vector. The 0.6-kb fragment represents the released insert and the 3 kb fragment the plasmid vector. Arrows indicate clones selected for sequence.

revealed that both probes hybridized simultaneously to a DNA fragment with a molecular weight of ~0.6 kb (**Figure 1**). *Eco*RI released a single positive fragment of ~0.6 kb.

Subcloning of a 0.6-kb Fragment into a pBluescript II SK± Plasmid Vector. The 0.6-kb peanut DNA insert fragment released by *Eco*RI from a putative positive clone was successfully subcloned into pBluescript vector. Digestion of 14 randomly selected white colonies with *Eco*RI generated two fragments (**Figure 2**). The upper fragment of 3 kb represents the size of the pBluescript plasmid vector, and the lower fragment of 0.6 kb represents the released putative peanut BBTI. Sample 2A and 3B were randomly selected for sequencing and further analysis.

Sequence Analysis. The nucleotide sequences of samples 2A and 3B were identical and ~430 bp in length. Analysis of the fragment revealed a full-length gene with an open reading frame (ORF) of 243 bp (**Figure 3**). The isolated peanut gene encodes a protein of 80 amino acids. When compared with data in the GenBank using the BLAST algorithm, the gene was identified as a BBTI with 100% amino acid sequence identity to peanut BBTI AI, AII, BI, and BIII and 81% identity with BII isolated by Norioka et al. (*9*) (**Figure 4**).

DISCUSSION

In this experiment, a peanut cDNA library cloned into a lambda gt-11 vector was screened using two heterologous probes designed from a garden pea BBTI. Garden pea trypsin inhibitor was selected because (1) it was the only available legume with comparative amino acid sequences for the BBTI and (2) it was the only BBTI cDNA sequence available. Despite the heterogeneity between peanut and garden pea BBTI, the probes were successful in annealing to a complementary sequence of a peanut BBTI. The result reveals for the first time a full-length cDNA clone of a peanut BBTI (**Figure 3**). The gene is 243 bp (GenBank Accession No. AY330200) with an initiation codon (ATG) at position +1 and a termination codon (TGA) at position +243. Two polyadenylation signals (AATAAA) were identified downstream of the termination codon at positions +261 and +269, respectively. In addition, a putative leader sequence also called a signal peptide of 18 amino acids (bold and underlined) is present at the 5′ end of the gene. A leader sequence is a chain of polypeptides upstream of the ORF in the 5′ but downstream of the promoter, which directs the transport of the protein to a definite location. The leader sequence is present in the nascent protein but cleaved off in the mature protein (*17*). The gene, as in most legume BBTIs, is relatively small and encodes a protein of 80 amino acid residues. The BLAST analysis of the amino acid sequence revealed that the peanut BBTI isolated in this experiment is 100% identical (except for the leader sequence) to AI, AII, BI, and BIII protein sequences belonging to the peanut BBTI AI family isolated by Norioka et al. (*9*) (**Figure 4**). This shows evidence that the four proteins of the AI family are in fact several samples of the same proteins with some lacking a few amino acids either at the N terminus or at the C terminus. The results also show that these four proteins are not variant expression products of different members of the same gene family as stated by Norioka et al. (*9*). These findings also

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 -81                                                    ggccgcgccaaggtagcactgttg
 -58  cttttccttgtgggactttcagccaccgttgaagctgtccgccttgacccaagcttg
   1  atgctctcacaggtgataaacaatattggcgaagcatcatcatcttcagacgacaat
       M  L  S  Q  V  I  N  N  I  G  E  A  S  S  S  S  D  D  N
  58  gtttgctgcaatggctgtctatgcgaccgtagggccccaccatatttcgagtgtgtt
       V  C  C  N  G  C  L  C  D  R  R  A  P  P  Y  F  E  C  V
 114  tgtgttgacacgttcgatcattgccctgcgtcttgcaactcctgcgtttgcacaagg
       C  V  D  T  F  D  H  C  P  A  S  C  N  S  C  V  C  T  R
 171  tctaatcctccacagtgccgttgcaccgacaaaactcaaggccgttgccctgtaaca
       S  N  P  P  Q  C  R  C  T  D  K  T  Q  G  R  C  P  V  T
 228  gaatgtcgttcttgaagcaattaagttcccttaataaatataataaattgcatatg
       E  C  R  S  *
```

Figure 3. Nucleotide and deduced amino acid sequences of peanut BBTI cDNA. The gene has an (ATG) initiation codon at position +1 and a stop codon (TGA) at position +243. Two poly-A signals are also identified in the 3′ end (underlined) at positions +261 and +269, respectively. The deduced amino acid sequence is below the nucleotide sequence. A leader sequence of 18 amino acids (bold and underlined) at position 5′ is also identified in the 5′ end of the gene or amino-terminal region of the deduced protein.

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PTI2A  MLSQVINNIGEASSSSDDNVCCNGCLCDRRAPPYFECVCVDTFDHCPASCNSCVCTRSNPPQCRCTDKTQGRCPVTECRS
 AI    ------------SSSDDNVCCNGCLCDRRAPPYFECVCVDTFDHCPASCNSCVCTRSNPPQCRCTDKTQGRCPVTECRS
 AII   ----------EASSSSDDNVCCNGCLCDRRAPPYFECVCVDTFDHCPASCNSCVCTRSNPPQCRCTDKTQGRCPVTECRS
 BI    ----------------DNVCCNGCLCDRRAPPYFECVCVDTFDHCPASCNSCVCTRSNPPQCRCTDKTQGRCPVTECRS
 BIII  ------------------VCCNGCLCDRRAPPYFECVCVDTFDHCPASCNSCVCTRSNPPQCRCTDKTQGRCPVTECRS
 BII   ------------AA-SD---CCSACICDRRAPPYFECTCGDTFDHCPAACNKCVCTRSIPPQCRCTDRTQGRCPLTPC-A
 GPTI  ------------------------------------------------------CKTC CTRSLPPQCRCIDITDF-C-YEKC-N
```

Figure 4. Sequence alignment of the deduced amino acid of peanut BBTI cDNA and peanut BBTI amino acid sequences AI, AII, BI, BII, and BIII isolated by Norioka et al. (*9*, *10*). The region of amino acid identity with garden pea protein used to design the oligonucleotides probes is included and underlined.

show that our isolated cDNA clone has 81% amino acid sequence identity to peanut BBTI BII, confirming that the BII protein isolated by Norioka et al. (*9*) is the expression product of a different gene. All of the proteins isolated by Norioka et al. (*9*) did not have a complete leader sequence; thus, one can assume that the leader sequence would have been spliced from the mature protein.

The cDNA sequence isolated in this experiment can be made available for the genetic transformation of transgenic peanut or other important agronomic crops for increased resistance to insects. In addition, the protein encoded by this gene can be expressed and evaluated for its therapeutic properties in human.

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