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cDNA Clone of a Putative Peanut (*Arachis hypogaea* L.) Trypsin Inhibitor Has Homology with Peanut Allergens Ara h 3 and Ara h 4

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Trypsin inhibitors are pathogenesis-related (PR) proteins, which play an important role in the plant defense mechanism against insects and pathogens. Peanut trypsin inhibitors are low molecular mass seed storage proteins. Like peanut allergens, they are stable to acid and heat, resistant to digestion, and can have a negative impact on human health. In peanut, five Bowman−Birk trypsin inhibitors (BBTI) have been isolated and amino acid sequences published. However, to date, no peanut BBTI sequence is available at both the cDNA and the genomic levels. The objectives of this investigation were (i) to synthesize degenerate oligonucleotides based on conserved regions of published amino acid sequences of BBTI, BII, and BIII; (ii) to isolate, sequence, and analyze at least one positive peanut trypsin inhibitor cDNA clone using the synthesized ^{32}P-labeled oligonucleotides as probes; and (iii) to determine its trypsin inhibitory activity. Thirty-two degenerate oligonucleotides DNA primers of 24 nucleotides each were synthesized based on the published amino acid sequences of peanut BBTI, and two were selected as probes to screen a peanut Lambda gt 11 cDNA library. Three putative positive clones were isolated, purified, and subcloned, and one was sequenced. Sequence analysis revealed a partial cDNA clone of 643 bp with a start codon. This clone shares 93 and 96% nucleotide sequence homology with peanut allergens Ara h 3 and Ara h 4 cDNA clones, respectively. A trypsin inhibitor assay revealed that peanut allergen Ara h 3 has a trypsin inhibitory activity of 11 238 TIA/mg protein. We concluded that peanut allergen Ara h 3 may also function as a trypsin inhibitor.

KEYWORDS: Allergen; *Arachis hypogaea* L.; cDNA library; peanut; seed storage proteins; sequence homology; trypsin inhibitor

INTRODUCTION

Peanut insects are responsible for significant damages leading to an increased total production cost in many fields. For example, in 1996 in Georgia alone, over $30 million was spent due to losses and damage control of insect infestations (*1*). Insect management of peanut has traditionally been accomplished mostly through chemical approaches. However, increased concern over environmental impact of pesticides, increased public fear of pesticides, and the concern over food safety have led us to explore alternative approaches to control insect infestation in peanuts. Hilder et al. (*2*) demonstrated that trypsin inhibitors in cowpea and in transgenic tobacco function as natural protective compounds against herbivorous insects. Moreover, cowpea varieties with a naturally high level of trypsin inhibitors were more resistant to insect attacks (*3*). A first step toward producing a transgenic insect resistant peanut transformed with trypsin inhibitors is to isolate and characterize endogenous peanut trypsin inhibitor genes.

Proteinase inhibitors are abundant in seed and storage organs and constitute 1−10% of the total proteins (*4*). Proteinase inhibitors play a role in the plant defense mechanism and function as pathogenesis-related (PR) proteins (*4*−*6*). They are also dietary compounds with antinutritional and toxic factors, which inhibit digestive enzymes and depress the growth and performance of insects (*6*, *7*) and young animals (*8*, *9*). They do not inhibit endogenous plant proteases but are specific for animal and microbial enzymes.

Peanut trypsin inhibitors have been extensively studied. Their amino acid compositions, sequences (*10*, *11*), mechanisms of inhibition (*12*), crystalline structure (*13*) binding domains, participation of SS loops in inhibitory activity (*14*), and molecular evolution (*12*) have been determined. They are

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Table 1. Construction Strategy of Mixed Oligonucleotides Used as Probes to Screen a Peanut cDNA Library[a]

A

Partial amino acid sequence		ASP	THR	PHE	ASP	HIS	CYS	PRO	ALA	
mRNA sequence	5'-	GAU	ACU	UUU	GAU	CAU	UGU	CCU	GCU	-3'
		C	C	C	C	C	C	C	C	
			A					A	A	
			G					G	G	
oligonucleotide sequence	3'-	CTA	TGI	AAA	CTA	GTA	ACA	GGI	CGI	-5'
		G		G	G	G	G			

B

Partial amino acid sequences		CYS	CYS	ASN	GLY	CYS	LEU	CYS	ASP	
mRNA sequence	5'-	UGU	UGU	AAU	GGU	UGU	UUA	UGU	GAU	-3'
		C	C	C	C	C	C C	C	C	
					A		U			
					G		G			
oligonucleotide sequences	3' -	ACA	ACA	TTA	CCI	ACA	AAI	ACA	CTA	-5'
		G	G	G		G	G	G	G	

[a] The sequence of the oligonuclotide was deduced from partial mRNA sequence of peanut BBTI. Deoxyinosine was incorporated to reduce the level of degeneracy at the third codon position. (A) The first set of mixed oligonucleotides deduced from partial amino acid sequence (residues 31–39) of peanut BBTI-BII and represents all possible sequences for the selected 24 bp region. (B) Second set of mixed oligonucleotides deduced from partial amino acid sequence (residues 2–10) of peanut BBTI-BIII and represents all possible sequences for the selected 24 bp region.

classified as Bowman−Birk trypsin inhibitors (BBTI) in part because they have low molecular masses (6−12 kDa), are heat and acid stable, and are resistant to digestive enzymes (*15*).

Five trypsin inhibitors have been isolated from peanut seeds and sequenced at the amino acid level (*10*, *11*, *16*). A comparison of their amino acid sequences revealed that four of them (AI, AII, BI, and BIII) have the same amino acid sequences except for differences at their N-terminal regions, suggesting that they might be derived from the same gene or gene families (*11*). The fifth inhibitor (BII) had an extremely different amino acid sequence and was reported to be encoded by a different gene (*10*). To date, there is no sequence information available for a peanut trypsin inhibitor at both the cDNA and the genomic levels.

The objectives of this investigation were (i) to synthesize degenerate oligonucleotides based on conserved regions of published amino acid sequences of peanut trypsin inhibitors BII and BIII; (ii) to screen a peanut cDNA library using the synthesized ^{32}P-labeled oligonucleotides as probes, isolate, sequence, and analyze at least one resulting positive peanut trypsin inhibitor clone; and (iii) to determine its trypsin inhibitory activity.

MATERIAL AND METHODS

Construction of Synthetic Oligonucleotide Probes. Two sets of 16 degenerate oligonucleotides of 24 bp containing deoxyinosines (dITPs) were designed in our laboratory following the protocol of Takahashi et al. (*17*) and Tai et al. (*18*). The sequence was designed based on published amino acid sequences of peanut trypsin inhibitors BII and BIII (*10*, *11*) using a Pharmacia Plus DNA synthesizer.

Amino acid residue nos. 31−39 of peanut BBTI BII (ASP-THR-PHE-ASP-HIS-CYS-PRO-ALA) were selected to design the first set of 16 degenerate oligonucleotide fragments representing all possible coding sequences for the selected 24 bp region, and it was constructed as shown in **Table 1**A. The mRNA sequence was deduced from the selected partial amino acid sequences. Three dITPs bases were incorporated at the third codon position for threonine, proline, and alanine to reduce the level of degeneracy from 266 144 to 16 possible oligonucleotides.

The second set of degenerate oligonucleotides was designed as described above based on amino acid residue nos. 2−10 or CYS-CYS-ASN-GLY-CYS-LEU-CYS-ASP) (**Table 1B**) of peanut BBTI BII. Two dITPs bases were incorporated at the third codon position for glycine and leucine.

Screening of a Peanut cDNA Library. A peanut cDNA library constructed in a Lambda gt 11 cloning vector from seeds mRNA of the peanut line F78-1339 was screened as described by Viquez et al. (*19*). The following two synthetic oligonucleotides no. 10 (5′-XGCIGGGCAGTGITCAAAIGTATC-3′) and no. 17 (5′-XTCICCA-CAITGACATTCIAAATA-3′) used to screen the library were selected based on preliminary polymerase chain reaction (PCR) experiments conducted with 10 peanut varieties using the 32 synthesized oligonucleotides as primers. The PCR result revealed oligonucleotide nos. 10 and 17 to be the best markers at producing polymorphic bands among the peanut varieties tested (figure not shown).

The selected oligonucleotides were end-labeled with ^{32}P as previously described (*20*) and used as probes to hybridize to the nylon membranes replica of the peanut cDNA library as described by Viquez et al. (*19*).

Dot Blot Analysis of Putative Positive Clones. A dot blot experiment was performed on isolated clones to confirm positives using a Bio-Dot SF Microfiltration apparatus (Bio-Rad Laboratories, Inc., Hercules, CA). About 1 mg of each purified DNA clone was aliquoted and transferred by capillary action to a nylon membrane, prehybridized, hybridized as described by Viquez et al. (*19*) using labeled probe nos. 10 and 17, and exposed to Kodak X-OMAT AR X-ray film and developed after a week of exposure at −80 °C.

Subcloning in a pBluescript SK+ Vector. DNA from putative positive clones was purified and digested to completion overnight at 37 °C using 10 μL DNA + 2 μL 10 × BSA + 2 μL buffer E (10×) + 9.5 μL water + 1 μL *Hin*d III and electrophoresed on a 0.7% agarose gel. The released insert was subcloned into a pBluescript II SK(±) phagemid vector (Stratagene, La Jolla, CA) previously digested with *Hin*d III, after both DNAs were dephosphorilated (*21*), and ligated as described by Viquez et al. (*19*). Positive colonies were chosen by blue−white selection.

DNA Sequencing and Analysis. DNA of selected p-Bluescript clone 13 was purified and sequenced with an automated sequencer (ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit using AmpliTaq DNA Polymerase, FS) at ResGen, Inc. (Huntsville, AL) and the University of Alabama in Birmingham using T3 and T7 sequencing primers. Sequence analysis, sequence alignments, and homology searches were performed using the BLAST and BLAST 2 sequences tools (*22*, *23*).

Extraction and Purification of Peanut Protein Ara h 3. Ara h 3 protein was purified from crude peanut extracts (CPEs) by ammonium sulfate precipitation followed by ion exchange chromatography. Peanut was defatted for 6 h using petroleum ether. Ten grams of defatted CPE was solubilized in 500 mL of extraction buffer (50 mM Tris-Cl, pH 8.3, 10 mM DTT, 5 mM EDTA, and 1 mM PMSF) containing 200 mM NaCl. The solution was homogenized with ultrasound treatment on ice at 40% power using a Heat Systems Disrupter. The homogenate was cleared by centrifugation at 13 000 rpm for 30 min at 4 °C. Ara h 3 was precipitated from the cleared homogenate by addition of a saturated solution of ammonium sulfate while stirring, and stirring was continued for 30 min on ice. The ammonium sulfate precipitated proteins were collected by centrifugation at 13 000 rpm for 30 min at 4 °C and can be stored stably at 4 °C. The ammonium sulfate pellet was solubilized in extraction buffer and homogenized with ultrasound treatment on ice at 40% power using a Heat Systems Disrupter. Solubilized proteins were desalted into the same buffer and subjected to ion exchange chromatography. This protein was recognized by antibodies made to the recombinant Ara h 3 protein (figure not shown).

Trypsin Inhibitor Assay of Peanut Protein Ara h 3. Trypsin inhibitor activity (TIA) was determined for optimal trypsin inhibition using a modification of the protocol described by Dodo et al. (*24*). The enzyme used was bovine pancreas trypsin. The substrate was N-α-benzoyl-DL-arginine-*p*-nitroanilide. The reactions were performed in triplicate. TIA was determined by subtracting the absorbance reading of the trypsin enzyme from the absorbance reading of each peanut

Table 2. Degenerate Oligonucleotide Sequences of 24 bp Each, Synthesized Based on Conserved Regions of Amino Acid Sequences of Peanut Trypsin Inhibitors[a]

	Set A
1	5′ GC IGG ACA ATG ITC AAA IGT GTC 3′
2	5′ GC IGG ACA ATG ITC GAA IGT ATC 3′
3	5′ GC IGG ACA GTG ITC AAA IGT ATC 3′
4	5′ GC IGG GCA ATG ITC AAA IGT ATC 3′
5	5′ GC IGG GCA GTG ITC GAA IGT ATC 3′
6	5′ GC IGG GCA GTG ITC AAA IGT GTC 3′
7	5′ GC IGG GCA ATG ITC GAA IGT GTC 3′
8	5′ GC IGG ACA GTG ITC GAA IGT GTC 3′
9	5′ GC IGG ACA ATG ITC GAA IGT GTC 3′
10	**5′ GC IGG GCA GTG ITC AAA IGT ATC 3′**
11	5′ GC IGG ACA GTG ITC GAA IGT ATC 3′
12	5′ GC IGG GCA ATG ITC AAA IGT GTC 3′
13	5′ GC IGG ACA ATG ITC AAA IGT GTC 3′
14	5′ GC IGG GCA GTG ITC GAA IGT GTC 3′
15	5′ GC IGG ACA GTG ITC AAA IGT GTC 3′
16	5′ GC IGG GCA ATG ITC GAA IGT GTC 3′
	Set B
17	**5′ TC ICC ACA ITG ACA TTC IAA ATA 3′**
18	5′ TC ICC GCA ITG GCA CTC IAA GTA 3′
19	5′ TC ICC GCA ITG GCA CTC IAA ATA 3′
20	5′ TC ICC GCA ITG ACA TTC IAA GTA 3′
21	5′ TC ICC GCA ITG ACA CTC IAA GTA 3′
22	5′ TC ICC ACA ITG GCA CTC IAA GTA 3′
23	5′ TC ICC ACA ITG ACA TTC IAA GTA 3′
24	5′ TC ICC ACA ITG ACA CTC IAA ATA 3′
25	5′ TC ICC ACA ITG GCA TTC IAA ATA 3′
26	5′ TC ICC GCA ITG ACA TTC IAA ATA 3′
27	5′ TC ICC GCA ITG GCA TTC IAA ATA 3′
28	5′ TC ICC GCA ITG GCA TTC IAA GTA 3′
29	5′ TC ICC ACA ITG ACA CTC IAA GTA 3′
30	5′ TC ICC ACA ITG GCA CTC IAA ATA 3′
31	5′ TC ICC ACA ITG GCA CTC IAA ATA 3′
32	5′ TC ICC ACA ITG GCA TTC IAA GTA 3′

[a] (A) Oligonucleotides 1–16 were generated from peanut amino acid sequences of BBTIs AI, AII, BI, and BIII. (B) Oligonucleotides 17–32 were generated from amino acid sequences of peanut BBTI BII.

protein sample. TIA was expressed as TIA/mg of protein. One trypsin unit is defined as an increase of 0.01 absorbance units at 410 nm (*25*).

RESULTS

Construction of Synthetic Oligonucleotide Probes. Because of the degeneracy of the genetic code at the third codon position, the number of possible oligonucleotides that can encode the selected eight amino acids is 266 144. By incorporating deoxyinosine in some of the ambiguous codon positions, the degeneracy of the genetic code was reduced to 16 oligonucleotides for each set (**Table 1**).

Thirty-two degenerate oligonucleotides of 24 bp each were synthesized as listed in **Table 2**. The first set of 16 oligonucleotides was constructed based on eight amino acid sequences of peanut BBTI AI, AII, BI, and BIII, which are members of the same gene family. The second set of 16 oligonucleotides was constructed based on eight amino acid sequences of another peanut BBTI BII from a different gene family.

A preliminary PCR experiment conducted to determine the ability of the 32 oligonucleotides to detect polymorphisms in 10 different peanut genotypes (figure not shown) revealed that oligonucleotide nos. 10 and 17 (**Table 2**) were the most successful markers in generating polymorphic bands. They were therefore selected as probes to screen the peanut cDNA library for a trypsin inhibitor cDNA clone.

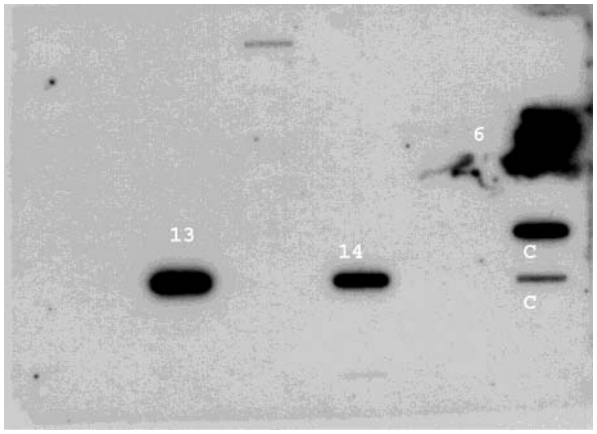

Figure 1. Dot blot analysis of 18 putative positive trypsin inhibitor clones isolated after the third round of screening of the peanut cDNA library using oligonucleotide nos. 10 and 17 as probes. Out of 18 clones initially blotted on the membrane, only cDNA clone 6, 13, and 14 hybridized to the ^{32}P-labeled probes and are shown as strong positives.

```
  1 atgctgcttgcgctttctgtttgcttttgctttctagtcctggga
    M  L  L  A  L  S  V  C  F  C  F  L  V  L  G
 46 gctagcagcatctccttcaggcagcagccggaggaaaatgcgtgc
    A  S  S  I  S  F  R  Q  Q  P  E  E  N  A  C
 91 cagttccagcgcctcaatgcgcaaaggcctgacaaccgcattgaa
    Q  F  Q  R  L  N  A  Q  R  P  D  N  R  I  E
136 tcggagggcggttacattgagacttggaacccaaacaaccaggag
    S  E  G  G  Y  I  E  T  W  N  P  N  N  Q  E
181 ttcgaatgcgccggcgtcgccctctcgcgcttagtcctccgccgc
    F  E  C  A  G  V  A  L  S  R  L  V  L  R  R
226 aacgcccttcggaggcctttctactccaatgctccccaggagatc
    N  A  L  R  R  P  F  Y  S  N  A  P  Q  E  I
271 ttcatccagcaaggaaggggatactttggtttgatattccctggt
    F  I  Q  Q  G  R  G  Y  F  G  L  I  F  P  G
316 tgtcctagcacatatgaagagcctgcacaacaaggacgccgacat
    C  P  S  T  Y  E  E  P  A  Q  Q  G  R  R  H
361 cagtcccaaagaccaccaagacgttttcaaggacaagaccaaagc
    Q  S  Q  R  P  P  R  R  F  Q  G  Q  D  Q  S
406 caacagcaacaggatagtcaccagaaggtgcaccgtttcgatgag
    Q  Q  Q  Q  D  S  H  Q  K  V  H  R  F  D  E
451 ggtgatctcattgcagttcccaccggtgttgctttctggatgtac
    G  D  L  I  A  V  P  T  G  V  A  F  W  M  Y
496 aacgaccatgacactgatgttgttgctgtttctcttactgacacc
    N  D  H  D  T  D  V  V  A  V  S  L  T  D  T
541 aacaacaacgacaaccagcttgatcagttccccaggagattcaat
    N  N  N  D  N  Q  L  D  Q  F  P  R  R  F  N
586 ttggctgggaaccacgagcaagagttcttaagataccagcaacaa
    L  A  G  N  H  E  Q  E  F  L  R  Y  Q  Q  Q
631 agcagacgaagaagc 645
    S  R  R  R  S
```

Figure 2. Partial nucleotide and deduced amino acid sequences of a putative peanut BBTI cDNA clone. Sequence starts with an AUG initiation codon (in bold). Lower case letters indicate nucleotide sequences. Upper case letters indicate amino acid sequences.

Library Screening and Dot Blot Analysis. Three rounds of peanut cDNA library screening resulted in the selection of 18 putative positive clones. Dot blot experiments revealed that clones 6, 13, and 14 were positive (**Figure 1**). Restriction digestion revealed that all three clones release the same size insert fragment.

Sequence Analysis. Sequence analysis of clone 13 revealed a truncated gene with a partial open reading frame of 643 nucleotides long and includes a start codon AUG (**Figure 2**). The deduced amino acid sequence is 228 residues long. Sequence homology searches revealed 96% homology with peanut Ara h 4 cDNA clone (**Figure 3**) (GenBank accession no. AF08684) and 93% homology with peanut Ara h 3 cDNA clone (GenBank accession no. AF093541).

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Peanut TI    M--LLALSVCFCFLVLGASSISFRQQPEENACQFQRLNAQRPDNRIESEG   48
Ara h4 cDNA MAKLLELSFCFCFLVLGASSISFRQQPEENACQFQRLNAQRPDNRIESEG   50
Ara h3 cDNA ----------------------RQQPEENACQFQRLNAQRPDNRIESEG   27

Peanut TI    GYIETWNPNNQEFECAGVALSRLVLRRNALRRPFYSNAPQEIFIQQGRGY   98
Ara h4 cDNA GYIETWNPNNQEFECAGVALSRLVLRRNALRRPFYSNAPQEIFIQQGRGY  100
Ara h3 cDNA GYIETWNPNNQEFECAGVALSRLVLRRNALRRPFYSNAPQEIFIQQGRGY   77

Peanut TI    FGLIFPGCPSTYEEPAQQGRRHQSQRPPRRFQGQDQSQQQQDSHQKVHRF  148
Ara h4 cDNA FGLIFPGCPSTYEEPAQQGRRYQSQRPPRRLQEEDQSQQQQDSHQKVHRF  150
Ara h3 cDNA FGLIFPGCPRHYEEPHTQGRRSQSQRPPRRLQGEDQSQQQRDSHQKVHRF  127

Peanut TI    DEGDLIAVPTGVAFWMYNDHDTDVVAVSLTDTNNNDNQLDQFPRRFNLAG  198
Ara h4 cDNA NEGDLIAVPTGVAFWLYNDHDTDVVAVSLTDTNNNDNQLDQFPRRFNLAG  200
Ara h3 cDNA DEGDLIAVPTGVAFWLYNDHDTDVVAVSLTDTNNNDNQLDQFPRRFNLAG  177

Peanut TI    NHEQEFLRYQQQSR---RRS                               215
Ara h4 cDNA NHEQEFLRYQQQSRQSRRRSLPYSPYSPHSRPRREEREFRPRGQHSRRER  250
Ara h3 cDNA NTEQEFLRYQQQSRQSRRRSLPYSPYSPQSQPRQEEREFSPRGQHSRRER  227

Ara h4 cDNA RAGQEEEDEGGNIFSGFTPEFLEQAFQVDDRQIVQNLWGENESEEEGAIV  300
Ara h3 cDNA RAGQEEENEGGNIFSGFTPEFLEQAFQVDDRQIVQNLRGETESEEEGAIV  277

Ara h4 cDNA TVRGGLRILSPDGTRGADEEEEYDEDQYEYHEQDGRRGRGSRGGGNGIEE  350
Ara h3 cDNA TVRGGLRILSPDRKRRADEEEEYDEDEYEYDEEDRRRGRGSRGRGNGIEE  327

Ara h4 cDNA TICTACVKKNIGGNRSPHIYDPQRWFTQNCHDLNLLILRWLGLSAEYGNL  400
Ara h3 cDNA TICTASAKKNIGRNRSPDIYNPQAGSLKTANDLNLLILRWLGPSAEYGNL  377

Ara h4 cDNA YRNALFVPHYNTNAHSIIYALRGRAHVQVVDSNGNRVYDEELQEGHVLVV  450
Ara h3 cDNA YRNALFVAHYNTNAHSIIYRLRGRAHVQVVDSNGNRVYDEELQEGHVLVV  427

Ara h4 cDNA PQNFAVAGKSQSENFEYVAFKTDSRPSIANFAGENSFIDNLPEEVVANSY  500
Ara h3 cDNA PQNFAVAGKSQSENFEYVAFKTDSRPSIANLAGENSVIDNLPEEVVANSY  477

Ara h4 cDNA GLPREQARQLKNNNPFKFFVPPFQQSPRAVA  530
Ara h3 cDNA GLQREQARQLKNNNPFKFFVPPSQQSPRAVA  507
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Figure 3. Amino acid sequence alignments of a putative peanut trypsin inhibitor (TI, GenBank accession no. AF487543) with amino acid sequences of peanut Ara h 4 (GenBank accession no. AF086821) and Ara h 3 (GenBank accession no. AF093541).

Table 3. Trypsin Inhibitor Assay Performed with Peanut Allergen Ara h 3[a]

sample	average ABS at 410 mm	TU	TIA/mL peanu protein	TIA/mg peanut protein
bovine pancreas trypsin	0.653	65.3		
Ara h3 protein	0.417	41.7	236	11 238

[a] Absorbance was measured at 410 nm. Trypsin inhibitory activity was expressed as TIA/mg peanut protein Ara h 3 (TIA/mg).

Trypsin Inhibitor Assay. The TI assay was performed on peanut allergenic protein Ara h 3 for three reasons: (i) the putative TI cDNA revealed high sequence similarity (93%) with Ara h 3, (ii) the isolated cDNA was truncated and was therefore not amenable for expression of the full TI protein, and (iii) peanut allergen Ara h 3 was available. The result revealed TIA to be 11 238 TIA/mg of peanut Ara h 3 protein sample (**Table 3**). Antibody made to a recombinant Ara h 3 recognized the Ara h 3 protein used in this assay (data not shown).

DISCUSSION

We have isolated the cDNA clone of a putative trypsin inhibitor from a Lambda gt 11 peanut cDNA library, which is a partial sequence of 643 bp long and includes a start codon AUG (**Figure 2**). The deduced amino acid sequence is 214 residues long (GenBank accession no. AF487543). A GenBank database similarity search revealed unexpected results of 96% homology with a peanut Ara h 4 cDNA clone and 93% homology with a peanut Ara h 3 cDNA clone (**Figure 3**) (*26*). In addition, two of the IgE binding domains on Ara h 3, R1 and R2 described by Rabjohn et al. (*27*), are present in the partial peanut TI sequence, strengthening the close similarity between the putative TI and the peanut allergen Ara h 3. This experiment was performed with the intention to isolate a peanut trypsin inhibitor clone from a peanut cDNA library using degenerate oligonucleotide probes constructed based on published amino acid sequences of peanut BBTIs (*11*, *12*).

Trypsin inhibitory assays performed confirmed for the first time that peanut allergen Ara h 3 has a trypsin inhibitory activity of 11 238 TI/mg peanut protein as shown in **Table 3** and provide the first evidence that a peanut allergen is a potential trypsin inhibitor. This finding suggests that in addition to being a storage protein, peanut allergen Ara h 3 may also function as a PR protein and play a role in the plant defense mechanism. Our data support previous reports on similarities between plant trypsin inhibitors and allergens. They are both seed storage proteins of low molecular masses (5−70 kDa), stable at low pH and to heat, resistant to digestion and proteolysis, and have a negative impact on human health. Breiteneder and Ebner (*28*) show that 33% of plant food allergens are PR proteins. Relationships based on sequence similarity between allergens and PR proteins were made, and plant allergens were grouped into six of the 14 families of PR proteins (*29*). These proteins

also share some of the characteristics relevant to allergens of plant sources. Moroz and Yang (*30*) also reported that a soybean trypsin inhibitor has allergenic properties and is involved in anaphylaxis reactions.

Sequence comparisons between Ara h 3 and Ara h 4 cDNA revealed 93.9% nucleotides homology and 91.3% amino acids identities (**Figure 3**). Except for few differences in the number of amino acid residues that can be accounted for by the difference in size of the two sequences, Ara h 3 and Ara h 4 are essentially the same gene and should therefore be classified as two isomers or two members of the same gene family and their nomenclature adjusted accordingly.

CONCLUSION

In addition to nucleotide and amino acids sequences similarities, our data revealed for the first time that peanut allergen Ara h 3 has TIA in vitro. This could explain why degenerate oligonucleotide probes designed specifically to anneal to a peanut BBTIs cDNA bind to an allergen. These data may suggest that peanut allergens might also have a defensive action for the plant. We also report that peanut allergens Ara h 3 and Ara h 4 are isomeric forms of the same gene and that their nomenclature should be adjusted accordingly. Results obtained in this experiment highlight concerns for cross-reactivity between trypsin inhibitors and allergens, especially in crops that are genetically modified for increased pest resistance using protease inhibitors. Further investigation in this area is warranted.

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