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Cloning of Gene Fragment having Homology with the Polypetide Chymotrypsin Inhibitor from the Potato Proteinase Inhibitor II Gene and Its Expression in *E. coli.*

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Abstract : The potato proteinase inhibitor II (PI-II) protein contains chymotrypsin and trypsin inhibitory site. Among several PI-II genes isolated from genomic library, amino acid sequence deduced from PI-IIT gene has 84% identity with that of the polypeptide chymotrypsin inhibitor (PCI). Therefore a gene fragment having homology with the PCI was cloned into a vector using polymerase chain reaction(PCR) from the potato proteinase inhibitor IIT gene. Two different primers were utilized for cloning; primer A contains NdeI restriction site and 30 nucleotides, which has AUG N-terminal methionine codon, primer B contains BclI restriction site and 28 nucleotides, which has TAG translation stop codon. After PCR, about 160 bp-long DNA fragment was cloned into pRT146, derivative of pUC118, and sequenced. The sequenced NdeI/BclI fragment was moved to pET3a, containing bacteriophage T7 promoter and terminator. The expressed proteins in *E. coli* BL21(DE3) were determined on a polyacrylamide gel containing sodium dodecyl sulfate. The expected size of protein deduced from the sequenced gene fragment is about 6,500 dalton whose size was similar to the IPTG-induced protein (6,000 dalton) on a gel. However the expression level was much lower than expected(Received August 25, 1995; accepted September 11, 1995).

Introduction

Proteinases and their inhibitors have been found in almost all organisms and are probably ubiquitous in nature and necessary for life. The interaction of proteinases with their inhibitors results in a specific inactivation of the proteinases leading to reduced proteolytic activity and decreased proteolytic degradation of tissue constituents.[1] Plants contain at least nine different families of proteinase inhibitors.[2]

Functional roles of these proteinase inhibitors have been a question of much debate. Two hypotheses have been advanced to explain the role of proteinase inhibitors in plant tissues: they are present to regulate endogenous proteinases,[3] or they function as allelochemical components in plant defense against insect herbivors[4] or microbes.[5] The resolution of this debate is based primarily upon the nature of the proteinase inhibitors themselves. The majority of plant proteinase inhibitors which have been described to date (well over 100 different inhibitors) are inhibitors of serine proteinase.[2] Although serine proteinases are the predominant type of proteinase in most eukaryotes and prokaryotes, only a few members of this class of proteinase have been decribed from plants. Thus, the principal function of these serine proteinase inhibitors is apparantly not to regulate endogenous plant proteinases. It is therefore likely that the true function of these proteinase inhibitors are herbivores or other insects which would destroy the plant tissues. Many microbes and most insects contain serine proteinases.[6,7]

One of the best characterized systems of proteinase inhibitors occurs in the tubers and foliage of solanaceous and leguminous plants, potato, tobacco, tomato, and alfalfa.[8] The proteinase inhibitors from potato have been purified by classical methods. The most abundant family of these inhibitors, proteinase inhibitor II (PI-II), consists of many different isoinhibitor forms, which differ only in a few amino acids. Inhibitor II proteins typically exist as dimers of approximately 24,000 daltons. However, the Inhibitor II family from potato also contains a series of small molecular weight inhibitors termed polypeptide trypsin inhibitors(PTI) and polypeptide chymotrypsin inhibitors(PCI).[9] These molecules have high homology with the central portion of the Inhibitor II monomer. They do not dimerize like PI-II and exist as single mo-

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nomeric subunits.

The genes for several members of the Inhibitor II family have been isolated and characterized. Initially, messenger RNA isolated from wounded tomato leaves was used to prepare cDNA clones coding for Inhibitor II.[10] After isolation and characterization, the cDNA was utilized as a probe to identify genes from potato and tomato gene libraries.[11,12] One particular potato Inhibitor II gene was shown to code for a wound-inducible foliage mRNA. This was confirmed by direct transformation of a chimeric Inhibitor II-CAT(chloramphenicol acetyl transferase) gene into tobacco plants and testing directely for wound-induction.[12] A second gene, Inhibitor IIT, has been isolated and characterized.[13] The nucleotide sequence of this gene makes it a better candidate for protein engineering studies because it shares 84% amino acid identity with PCI and 76% identity with PTI. Therefore, in this study, gene fragment having homology with the PCI was isolated from Inhibitor IIT gene using polymerase chain reaction and expressed in *E. coli*.

Materials and Methods

Genomic DNA was extracted from potato leaf by the method of Doyle *et. al.*[14] Leaf tissue of 1.0 g was ground to a powder in liquid nitrogen in a chilled mortar and pestle. The powder was scraped into preheated extraction buffer (2%(w/v) CTAB(sigma), 1.4 M NaCl, 0.2%(v/v) 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, (pH 8.0)) and incubated at 60℃ for 30 min with occasional swirling. After incubation, chloroform-isoamyl alcohol(24 : 1; v/v) were added and mixed thoroughly followed by centrifugation at 1,600x g. The supernatant was transferred to a new tube and 2/3 volume of cold isopropanol was added in order to precipitate nucleic acids. The DNA was collected by centrifugation and dried at room temperature. The pellet was resuspended in 1 m*l* TE (10 mM Tris-HCl(pH 7.4), 1 mM EDTA) and RNase was added to a final concentration of 10 μg/m*l* and incubated 30 min at 37℃. DNA was precipitated with an addition of 2.5 M ammonium acetate(pH 7.7) and ethyl alcohol. The precipitated DNAs were dried on vacuum and resuspended in TE.

Southern blot hybridization was carried out according to the published method.[15] The probe was derived from the tomato Inhibitor II cDNA($pT_2$47).[10] Labelling of probe was carried out using a commercial kit from Amersham. Autoradiography was carried out with Kodak X-Omat films.

The DNA oligonucleotides were synthesized by DNA synthesizer(Applied Biosystem Inc., model. 381A) using phosphoramidite chemistry. After deprotection, the oligonucleotides were purified by oligonucleotide purification cartridge(ABI). Two different oligonucleotides were synthesized: 5'-CCGACATATGCCCATCTGCATCAATTGTT-3'(30 mer) containing *Nde*I restriction site and 5'-TAGC-GATTTTCCTGATCAACTAGGACAT-3'(28 mer) containing BclI restriction site. Both oligonucleotides were utilized for cloning of gene fragment having homology with the PCI from the PI-IIT gene using PCR.

Reaction conditions for PCR followed published methods,[16] step 1: denaturation of DNA at 94℃ for 5 min, annealing of primers at 37℃ for 2 min, primer extension at 72℃ for 3 min (1 cycle), step 2: denaturation of DNA at 94℃ for 1 min, annealing of primers at 37℃ for 2 min, primer extension at 72℃ for 3 min (25 cycles), step 3: denaturation of DNA at 94℃ for 1 min, annealing of primers at 37℃ for 2 min, primer extension at 72℃ for 10 min (1 cycle). PCR products were separated on a 1% agarose gel. The electroelution of PCR product and cloning into appropriate plasmid were carried out according to the published methods.[16] The sequences of clones were determined by the dideoxy chain-termination method[17] utilizing Sequenase™. Sequencing gels contained 8% polyacrylamide, 7 M urea and 0.5X TBE(45 mM Tris-borate, 1 mM EDTA). Following electrophoresis the gels were wrapped with Clean Wrap® and subjected to autoradiography using X-ray film(Kodak).

The expression of protein in *E. coli*. was monitored according to the published method.[16] Host for clones was *E. coli*. BL21(DE3)[18] where T_7 RNA polymerase is supplied by induction and cultures have been grown in NZY medium(10 g N-Z amide A and 5 g NaCl/*l*) supplemented with 0.4% (w/v) glucose. The cells were induced with 0.4 mM IPTG and the culture reached an A_{600} of 1, corresponded to about 5×10^8 to 10^9 viable cells per m*l*. Cells were harvested 3 hr after induction. The cells(1 m*l* each) were collected by centrifugation and the proteins subjected to electrophoresis in the presence of sodium dodecyl sulfate (SDS) in a 15% polyacrylamide gel.[16] Molecular size standards are from 3 to 43 kilodaltons (Kd) obtained from GIBCO-BRL.

Results and Disscusion

The tomato proteinase inhibitor II cDNA($pT_2$47) was utilized as a probe to examine the size of the gene family in potato. By Southern hybridization (Fig. 1), it was determined that the Inhibitor II family consists of approximately 15 to 20 unique members. The same probe was also used to isolate genes from potato genomic library. The isolated potato proteinase inhibitor II(PI-II) genes were characterized.[12,13] Among these genes, the Inhibitor II-T (PI-IIT)gene was used to isolate a gene fragment coding for the polypetide chymotrypsin inhibitor (PCI) gene. The PI-IIT gene was identified on a

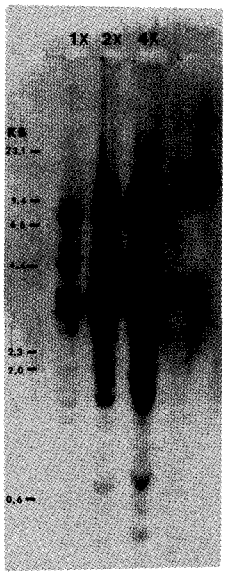

Fig. 1. Autoradiogram of Southern analysis of potato genomic DNA with the tomato proteinase inhibitor II cDNA as a probe. Lane 1, 2, and 3 contain 20, 40, and 80 ug of DNA, respectively. The isolated genomic DNAs were restricted with an appropriate amount of *Eco*RI overnight then separated on a 1% agarose gel.

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                1          10         20         30         40
PI-II T     MAVHKEVSFVAYLLIVLGMFLYVDALGCTKECGNLGFGIC

                     50         60         70         80
PI-II T     PRSEGSPTNPICINCCSGYKGCNYYSAFGRFICEGESDPK
                    *** *** ********** * ***** ****
PCI                PICTNCCAGYKGCNYYSANGAFICEGQSDPK
                    ************************** ****
PTI                RICTNCCAGYKGCNYYSANGAFICEGESDPK
                             10         20         30

                     90         100        110        120
PI-II T     NPKACPLNCDTNIAYSRCPRSEGKSLIYPTGCTTCCTGYK
                    *********  **** ****
PCI         KPKACPLNCDPHIAYSKCPRS
                    *  ** ***  ****** *
PTI         NPNVCPRNCDTNIAYSKCLR
                   40         50

                    130        140    147
PI-IIT      GCYYFGTNGKFVCEGESDEPKPYMSTA#
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Fig. 2. Identity between potato proteinase inhibitors. A comparison of the amino acid sequence derived from the proteinase inhibitor IIT gene with the data at each end of the polypeptide chymotrypsin inhibitor and polypeptide trypsin inhibitor. The single letter amino acid code is used. The active site specific for trypsin and chymotrypsin are indicated with a line. The identity between the two inhibitors is indicated by asterisks. The symbol, #, represents a stop codon in the PI-IIT.

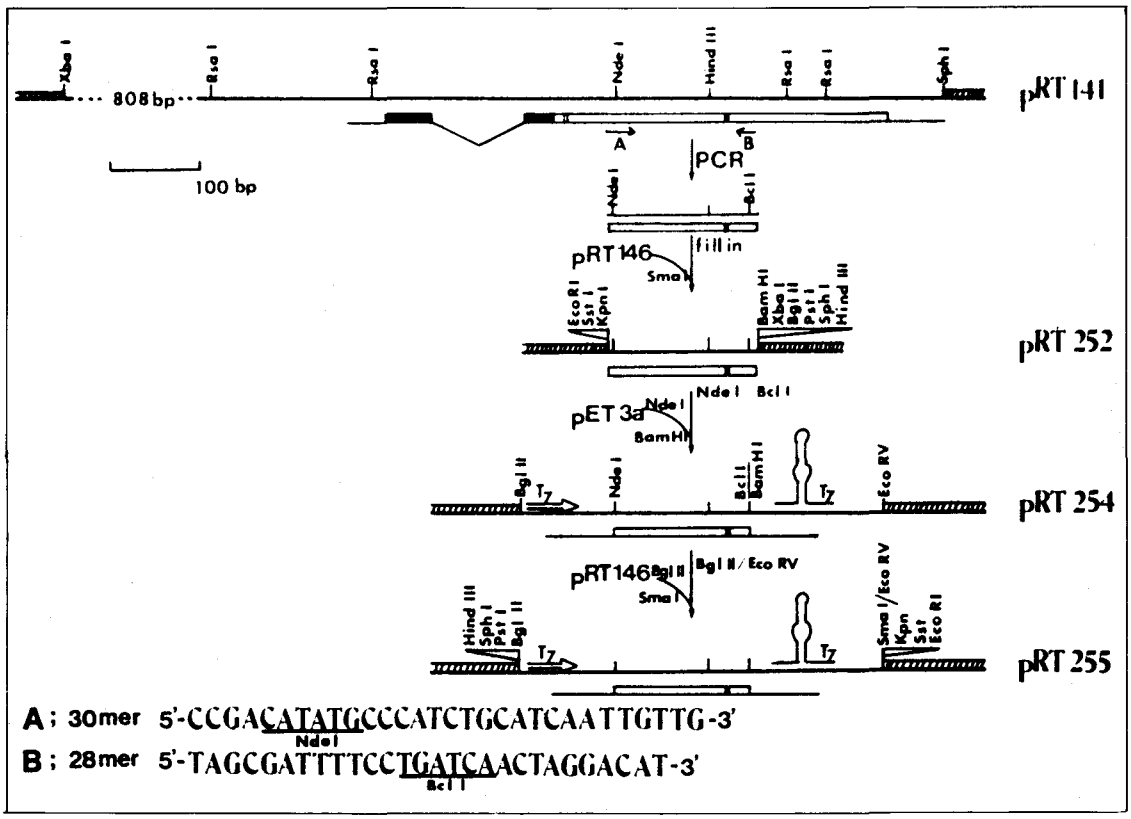

Fig. 3. Schematic diagram of the cloning of the gene fragment having homology with the PCI from the PI-IIT gene (pRT141). The sequences of two different primers, A and B, are shown as primers for cloning using a PCR technique. The bacteriophage T7 promoter is located upstream from the NdeI site and is indicated by open arrow. The bacteriophage T7 terminator is located downstream from the BclI/*Bam*HI hybrid site and is indicated by hairpin. The chymotrypsin inhibitory site is represented by a filled small box. Plasmid pRT146 is a derivative of pUC119 and contains BglII site instead of SmaI. Plasmid pET3a is a derivative of pBR322 and an *E. coli*. expression vector.

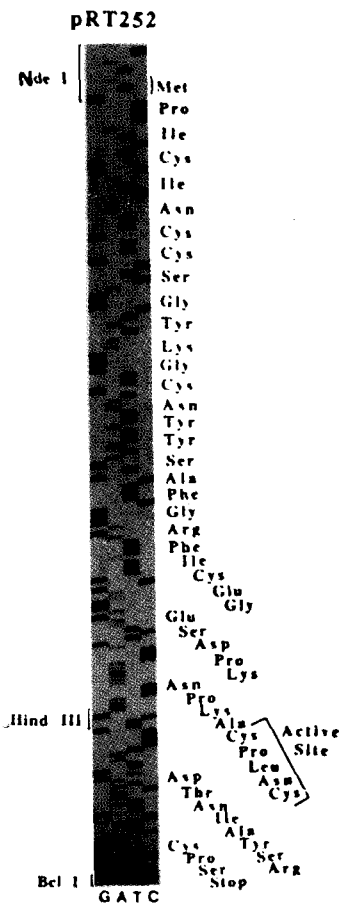

Fig. 4. Autoradiogram of sequence analysis of the cloned gene. The sequences of gene fragment having homology with the PCI obtained from the plasmid pRT252 are shown. The illustrated sequences contain cloning sites of NdeI and BclI restriction enzyme. The deduced amino acid sequences of the gene fragment are shown as three letters. The chymotrypsin inhibitory site is indicated by the active site.

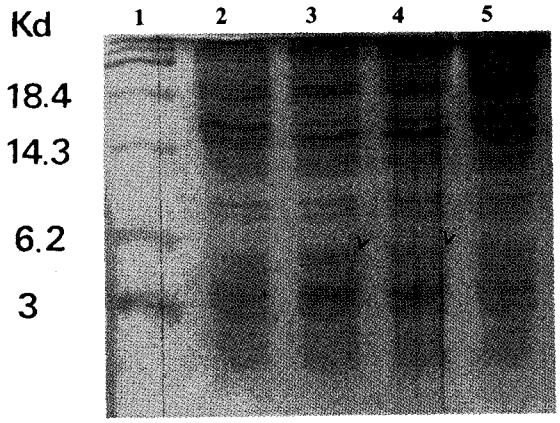

Fig. 5. Separation of the proteins on a polyacrylamide (15%) gel containing SDS. Lane 1 contains molecular weight standards from 3 to 43 Kd. Lanes 2 and 5 contain extracts from *E. coli.* BL21(DE3) uninduced and induced by IPTG, respectively. Lanes 3 and 4 contains extracts from transformed *E. coli.* with pRT254 and 255, respectively. The expressed protein for the gene fragment having homology with the PCI was indicated with 'v'.

17 kb *Eco*RI fragment. From this fragment a 1.6 kb *Xba*I/*Sph*I fragment containing the entire coding region of the PI-IIT gene have been subcloned to make pRT 141.[14] The protein encoded by the PI-IIT gene is 148 amino acids long (see Fig. 2). By analogy with previously cloned Inhibitor II genes,[10-13] the mature Inhibitor II protein begins at amino acid 27. The first 26 amino acids code for a signal peptide which are responsible for targeting the proteinase inhibitor into the central vacuole of the potato cell. The nucleotide sequence of this gene makes it a better candidate for protein engineering studies because it shares 84% amino acid identity with PCI and 76% identity with PTI. Fig. 2 shows a comparison of the amino acid sequence derived from the PI-IIT gene with the datas at each end of sequence of the PCI and PTI. Therefore PCR were subjected to the pRT141 using two different primers; one contains *Nde*I site (CATATG) which has ATG N-terminal methionine codon to the PCI coding region and the other contains *Bcl*I site (TGATCA) which has in frame TAG tanslation stop codon. The first primer changes the nucleotide sequence from GAAAT to CATATG (PI-II amino acids 49-50; see Fig. 2.) and the second primer changes from TTAGAA to TGATCA (PI-II amino acids 102-103).

The overall procedure for the cloning is in the Fig. 3. The PCR fragment was inserted in SmaI site of pRT 146[13] to make pRT252. The nucleotide sequence of pRT 252 is shown in the Fig. 4. The cloning sites of *Nde*I and *Bcl*I, deduced amino acid sequences, and the active site of proteinase inhibitor having homology with the PCI are shown in the Fig. 4. The active site, Cys-Pro-Leu-Asn-Cys, corresponds P'2-P'1-P1-P2-P3 according to the specificity of the inhibitor-proteinase interaction.[19] When the P1 site is occupied by an amino acid (tryptophan, phenylalanine, tyrosine, or leucine) recognized by chymotrypsin or chymotrypsin-like enzyme, the inhibitor is a chymotrypsin inhibitor.

The sequenced gene fragment having homology with the PCI was moved into *Nde*I/*Bcl*I site of pET3a,[20] *E. coli.* expression vector and a derivative of pBR322. It contains a bacteriophage T7 promoter and terminator flanking a 42 bp sequence which contains unique *Nde*I and *Bam*HI sites (*Bcl*I is compatible with BamHI) in the proper orientation. The insertion of the coding region having homology with the PCI into the pET3a vector produced a clone pRT254, which should express the potato inhibitor having homology with the PCI under control of the bacteriophage T7 promoter. The constructed vector contains the T7 promoter on a 78 bp fragment, the entire 160 bp Inhibitor II gene fragment containing the coding region, and a 145 bp transcription terminator. This pRT254 was transformed into the *E. coli.* strain

BL21(DE3).[18] This *E. coli.* strain contains the T7 RNA polymerase gene, inserted on the chromosome, under the inducible control of the lac-uv5 promoter. This vector and bacteria therefore provide an unique inducible expression system for the overproduction of engineered proteins. In addition to the pRT254, the *Bgl*II/*Eco*RV fragment of the pRT254 was cloned into *Bgl*II/*Sma*I sites of the pRT146 to produce pRT255 (Fig. 3), which will be used for the oligonucleotide directed mutagensis.

The expected size of protein is deduced from the sequence of gene fragment(see Fig. 4). The amino acid composition of protein is 8 Cys, 5 Asn, Pro and Ser, 4 Gly, Tyr, and Ile, 3 Lys and Ala, 2 Phe, Arg, Glu and Asp, 1 Leu, Thr and Met to make about 6,500 dalton. The expression patterns of proteins in *E. coli.* are shown in the Fig. 5. The size of the IPTG-induced protein is about 6,000 dalton(lanes 3 and 4 of Fig. 5). The size of the other IPTG-induced protein is about 4,000 dalton which also shown in IPTG-induced host cell (lane 5 of Fig. 5) but the expression level is lower than those of gene fragment-containing cells. The Western blot(data not shown) with a polyclonal antibody (gift of Dr. Thornburg in Iowa State University) of tomato proteinase inhibitor II showed very weak signal of 6,000 dalton indicating that the gene fragment having homology with the PCI was expressed in *E. coli.* but at low level. The reason for the low expression in *E. coli.* may be the harmful effect of the protein having homology with the PCI to the host cells because the chymotrypsin inhibitor from tubers was found to have inhibitory activity against proteinase from mammalian, bacterial and fungal origin but not from plant origin.[21] Therefore the fusion system, such as pMAL-P2, maltose binding protein gene containing vector, will be a candidate for the overproduction of protein having homology with the PCI.

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감자 단백질 분해효소 억제제-II 유전자로부터의 폴리펩타이드 카이모트립신 저해제와 homology가 있는 유전자단편의 클로닝 및 대장균에서의 발현

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초록 : 감자의 단백질 분해효소 억제제-II (PI-II) 단백질은 카이모트립신 저해부위와 트립신 저해부위로 나뉘어 있는데 PI-II 유전자중의 하나인 PI-IIT 유전자의 염기서열에서 비롯된 아미노산 서열이 폴리펩타이드 카이모트립신 저해제 (PCI) 단백질의 아미노산 서열과 84%의 높은 동질성을 가지고 있으므로 감자의 단백질 분해효소 억제제유전자 집단 (family) 중의 하나인 PCI와 homology가 있는 유전자단편을 클로닝하기 위하여 이미 클론된 PI-IIT 유전자로부터 PCR을 행하여 얻어진 DNA 단편을 벡터에 클로닝하고 염기서열을 결정하였다. 염기서열을 확인한 유전자 단편을 박테리오파아지 T7 promoter와 terminator를 갖고있는 플라스미드 pET3a에 옮겨 대장균 BL21(DE3)에서 발현시켰던바 IPTG의 부가에 따라 유기되는 것을 확인 하였으나 발현수준은 기대했던것에 미치지 못하였다.

*연락저자