식물학회지 제32권 제2호
Korean J. Botany 32(2): 79-87(1989)

Isolation of Proteinase Inhibitor II Genes from Potato

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감자로부터 단백질분해효소 억제제 II 유전자의 분리

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ABSTRACT

Southern hybridization of genomic DNAs with radioactively labeled cDNA of tomato proteinase inhibitor II revealed that proteinase inhibitor II proteins in potato plants are encoded by a family of about 10 related sequences. Screening of potato EcoRI genomic library with the cDNA resulted in isolation of 13 recombinant phage clones which carry 3 different genomic regions. Of these clones, clones 8, 18, and 39 were subjected to restriction mapping and subcloning. Further characterization of the subclones of clones 8, 18 and 39 indicated that two inhibitor II genes are present on a 8.0 kb EcoRI fragment of clone 8, one on 3.3 and 0.8 kb EcoRI fragments of clone 18 and two genes on a 13.5 kb EcoRI fragment of clone 39.

INTRODUCTION

Proteinase inhibitors are usually present in seeds and tubers of plants, particularly in those of the Gramineae, Leguminosae and Solanaceae families (Laskowski and Kato, 1980). Plant inhibitors are powerful inhibitors of serine endopeptidases such as trypsin and chymotrypsin that are found in both animals and microorganisms but only rarely in plants (Ryan, 1981).

In potato tubers, as many as thirteen different species of inhibitors were identified and studied in soluble proteins. Two nonhomologous inhibitors, inhibitors I and II, appear early during tuberization and continue to accumulate until the tubers are mature. Furthermore, these inhibitors accumulate in leaves of tomato and potato plants in response to mechanical wounding (Green and Ryan, 1972; Plunkett *et al.*, 1981). The synthesis of these two inhibitors in leaves of wounded tomato and potato plants is regulated systemically by a wound signal called the proteinase inhibitor-inducing factor (PIIF) (Green and Ryan, 1972). These proteins are considered to be antinutrients produced as a defense response against attacking pests (Ryan,

The present investigation was supported by the GE program (#62), MOE-ROK, 1987.

1981).

Potato inhibitor Ⅱ, an inhibitor of trypsin and chymotrypsin, is a heat-stable dimeric protein of 21,000 daltons. Four monomeric isoinhibitor species of 10,500 daltons comprise inhibitor Ⅱ in Russet Burbank potato (Bryant *et al.*, 1976). Their differences in amino acid composition, ionic properties and isoelectric points suggest that inhibitor Ⅱ proteins might be encoded by a family of related sequences. Inhibitor Ⅱ accumulates in tubers coordinately with inhibitor I, representing about 4% of soluble proteins in Russet Burbank potato (Ryan *et al.*, 1976). It is synthesized as a preprotein and is believed to be compartmentalized as a mature protein finally in the central vacuole (Shumway *et al.*, 1976; Nelson *et al.*, 1980). The amino acid sequence of inhibitor Ⅱ revealed that inhibitor Ⅱ genes evolved from a much smaller gene by gene duplication and elongation (Graham *et al.*, 1985; Sanchez-Serrano *et al.*, 1986). Inhibitor Ⅱ also exhibits amino acid homology with two much smaller trypsin and chymotrypsin inhibitors called PCI-I and PTI-I present in potato tubers (Hass *et al.*, 1982) and with a small trypsin inhibitor from egg plant (Richardson, 1979). It has been speculated that the small inhibitors might represent processed products of inhibitor Ⅱ proteins (Pearce *et al.*, 1982; Graham *et al.*, 1985).

Therefore, inhibitor Ⅱ genes provide a useful system for studies on differential regulation of gene expression and protein processing in plants. In this study, determination of the number of inhibitor II genes in the genomes of diploid as well as tetraploid potatoes and isolation of the genes from a genomic library were carried out.

MATERIALS AND METHODS

Materials. Tetraploid potatoes, *Solanum tuberosum* subsp. *tuberosum* cv. Russet Burbank and *S. tuberosum* subsp. *andigena* and diploid potato, *S. phureja* were obtained from the Experimental Station of Horticulture, Korea and were grown in the green house. *E. coli* strain K802 was used as the host of bacteriophage λ and JM101 was used for transformation with plasmids.

Restriction enymes and nick-translation system were purchased from Promega and used as indicated by the manufacturer. Radioisotopes and GeneScreen *Plus* were purchased from New England Nuclear and nitrocellulose filter was from Fischer Scientific Co. Other chemicals were from Sigma Chemical Co.

DNA isolation. Genomic DNAs were isolated from leaves of potato plants as described by Dellaporta *et al.* (1984). Plasmid DNA was isolated from *E. coli* as described by Brush *et al.* (1985). Phage DNA was isolated by the methods of Blattner *et al.* (1977) and Maniatis *et al.* (1982).

Screening of a potato genomic library. About 5×10^5 recombinant bacteriophage were screened as described by Woo (1979) from an EcoRI-partial genomic library of Russet Burbank potato which was a gift of D.M. Anderson of Phytogen Corporation, Pasadena, CA. Tomato

inhibitor II cDNA clone, pT2-47 (Graham *et al.*, 1985) was used as the probe after labeling radioactively by nick-translation (Maniatis *et al.*, 1982).

Southern hybridization. DNAs digested with various restriction enzymes were electrophoresed through agarose gels and transferred onto GeneScreen *Plus* by the method of Southern (1975). Pretreatment, hybridization and washing of filters were carried out as described by Wahl *et al.* (1979).

Molecular cloning. EcoRI restriction fragments of genomic clones were subcloned into pUC19 (Yanisch-Perron *et al.*, 1985) as described by Maniatis *et al.* (1982).

RESULTS

Inhibitor II genes as a multigene family. The presence of four monomers of inhibitor II in Russet Burbank potato tubers suggests that inhibitor II proteins may be encoded by a multigene family (Bryant *et al.*, 1976). In order to determine the number of the inhibitor II genes in potato plants, Southern hybridizations with genomic DNAs isolated from tetraploid *S. tuberosum* subsp. *tuberosum* cv. Russet Burbank and subsp. *andigena* and diploid tuber-bearing *S. phureja* were carried out after digestion with various restriction enzymes. As shown in Fig. 1, each lane revealed the presence of about 10 genomic fragments hybridizing with inserts of tomato inhibitor II cDNA clone, pT2-47 (Graham *et al.*, 1985), indicating that inhibitor II genes comprise a multigene family.

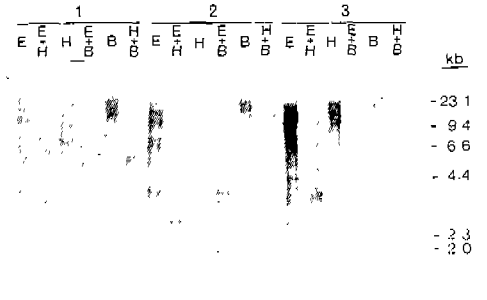

Fig. 1. Hybridizations with nick-translated inserts of tomato inhibitor II cDNA clone, pT2-47, of genomic DNAs from *S. phureja* (1) and *S. tuberosum* subsp. *andigena* (2) and subsp. *tuberosum* cv. Russet Burbank (3) after digestion with various restriction enzymes as indicated above each lane. Symbols: E, EcoRI; H, HindIII; B, BamHI.

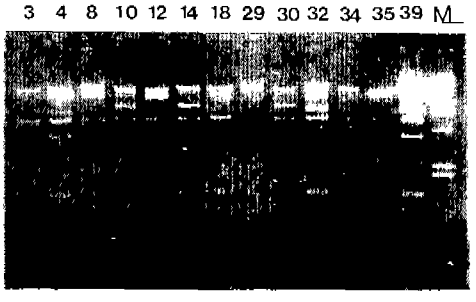

Fig. 2. Electrophoresis in a 0.8% agarose gel of recombinant λ clones isolated independently from a potato EcoRI-partial genomic library. Numbers indicate the name of the genomic clones. M, HindIII-digested λ DNA as size markers.

Isolation of genomic clones. In order to isolate inhibitor Ⅱ genes, screening of a EcoRI-partial genomic library of Russet Burbank potato DNA comstructed in bacteriophage vector Charon 4 was carried out with the tomato inhibitor II cDNA as the probe. It resulted in the independent isolation of 13 genomic clones carrying inhibitor II sequences. BamHI digestion patterns of the DNAs isolated from the 13 clones revealed that they indeed consist of 6 different groups of clones; clone 3 (34), clone 4 (18, 32 and 35), clone 8 (12), clone 10 (14 and 30), clone 29 and clone 39 (Fig. 2).

Of these, 9 clones were digested with EcoRI, separated in an agarose gel (Fig. 3A), followed by Southern hybridization (Fig. 3B), It revealed that they carry 13.5, 8.0, 3.3, and 0.8 kb EcoRI fragments containing inhibitor Ⅱ sequences. These are indeed the genomic fragments containing inhibitor Ⅱ genes as compared with the size of EcoRI fragments observed in Fig. 1.

Cloning of inhibitor II genes. Phage DNAs of clones 8, 18 and 39 were subjected to subcloning into pUC19 after digestion with EcoRI as shown in Fig. 4. Subclones carrying EcoRI fragments that were identified to contain inhibitor Ⅱ sequences in Fig. 3 were further characterized by Southern hybridization (Figs. 5 and 6). As shown in Fig. 5, three HindⅢ fragments of 1.9, 1.6 and 1.0 kb in size, respectively, in pJSL179 carrying the 8.0 kb EcoRI fragment of clone 8 were hybridized with the probe. Since the size of inhibitor Ⅱ cDNA is about 500 bp in tomato and potato (Graham *et al.*, 1985; Sanchez-Serrano *et al.*, 1986), it

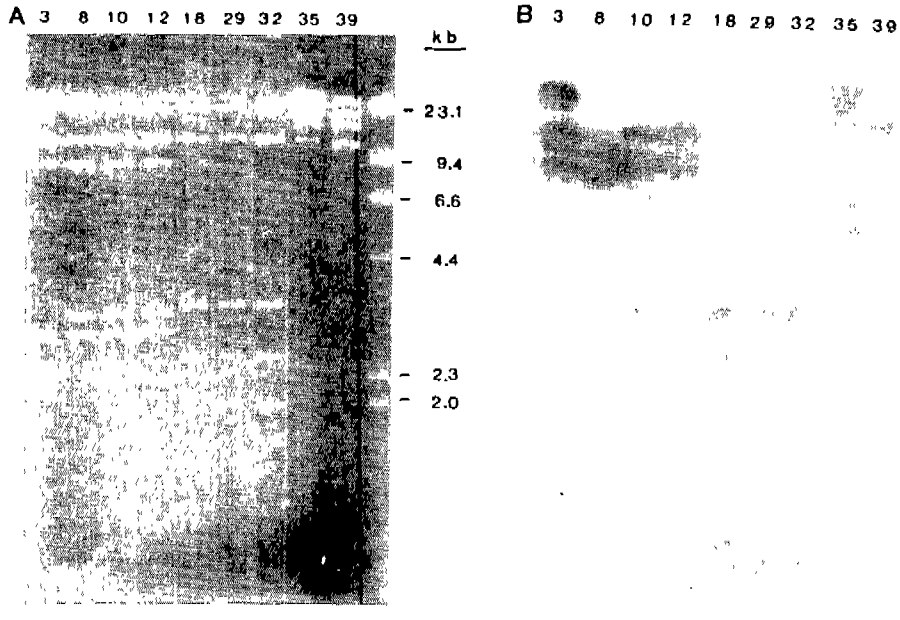

Fig. 3. Electrophoresis in a 0.8% agarose gel of λ DNAs digested with EcoRI (A) and Southern hybridization with radioactively labeled tomato inhibitor II cDNA after transfer onto GeneScreen *Plus* membrane (B). Numbers indicate the name of the genomic clones.

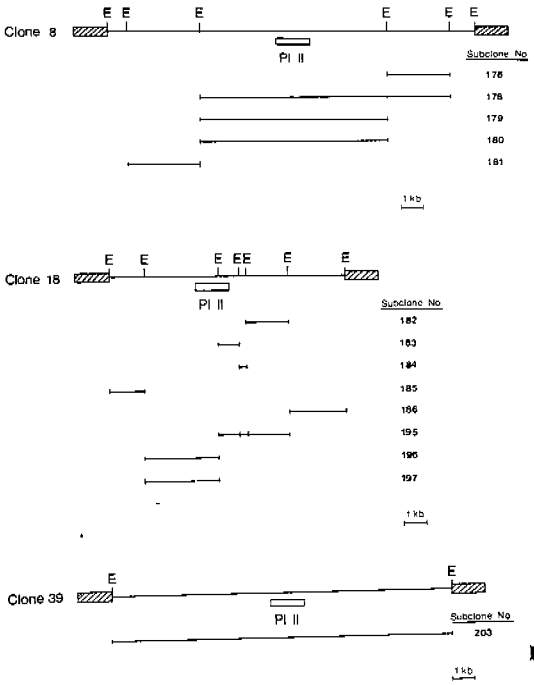

Fig. 4. Restriction map of genomic clones 8, 18, and 39 carrying potato inhibitor II genes and their subclones in pUC19. Only genomic DNA fragments cloned in pUC19 are shown.

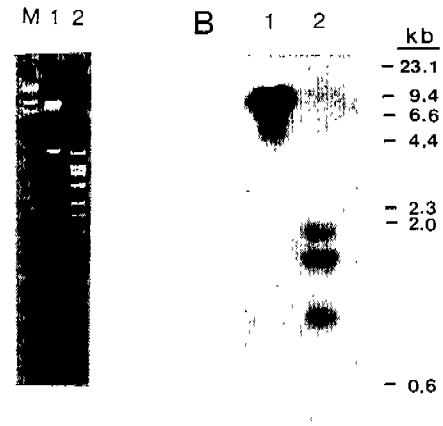

Fig. 5. Electrophoresis in a 1% agarose gel of a subclone pJSL179 carrying 8.0 kb EcoRI fragment of clone 8 after digestion with restriction enzymes (A) and hybridization with the probe (B). 1, EcoRI; 2, EcoRI and Hind III; M, HindIII-digested λ DNA as size markers.

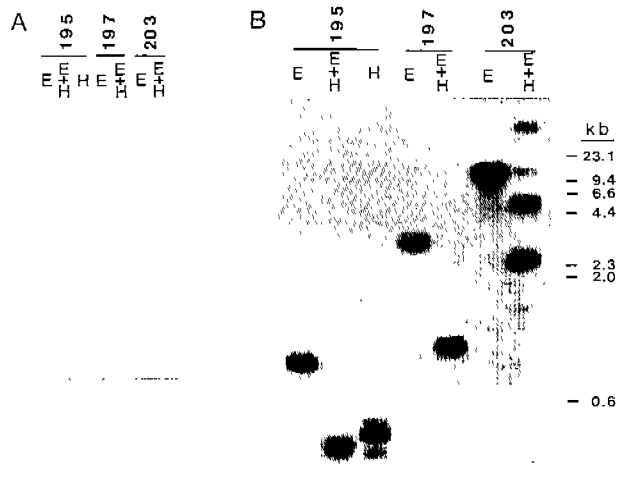

Fig. 6. Electrophoresis in a 1% agarose gel of subclones pJSL195, 197 and 203 shown in Fig. 4 after digestion with restriction enzymes (A) and hybridization with the probe (B). Symbols: E, EcoRI; H, HindIII; M, HindIII-digested λ DNA as size markers.

suggests that two inhibitor II genes may be present on the 8.0 kb EcoRI genomic frament of clone 8. Fig. 6 also suggests the presence of one inhibitor II gene on the 3.3 and 0.8 kb fragments of clone 18 and at least two on the 13.5 kb fragment of clone 39, respectively.

DISCUSSION

Inhibitor II is synthesized as a defensive chemical against attacking pests in tomato and potato plants. It is not only synthesized as tubers develop but also induced to accumulate in leaves of wounded plants. In potato tubers, inhibitor II is post-translationally modified to small inhibitor molecules. As an initial step toward understanding mechanisms of differential regulations of its expression and post-translational processing, determination of the copy number in the genome and isolation of inhibitor II genes in potato were carried out in this study.

Hybridizations with genomic DNAs showed that inhibitor II genes are encoded by a family of related sequences in diploid as well as tetraploid potatoes (Fig. 1). Since inhibitor II genes exhibit complex patterns of expression, the presence of multiple genes encoding inhibitor II in the potato genome may reflect their differential regulations by developmental signals in tubers and by environmental signals in wounded leaves. The presence of the almost same number of restriction fragments containing inhibitor II genes in the genomes of diploid *S. phureja* as well as tetraploid potatoes was rather unexpected. Since tetraploid *S. tuberosum* subsp. *antigena* is believed to be an ancester of cultivated potatoes (*S. tuberosum* subsp. *tuberosum*), it suggests that tetraploid potato may have been generated by autotetraploidization of a diploid tuber-bearing potato such as *S. phureja* or allotetraploidization between two species which were highly related from each other. Isolation of all the inhibitor II genes present in the genome of a tetraploid potato and their chromosomal localizations will light up the origin of tetraploid cultivated potatoes. It is quite common in plants that proteins synthesized at high level are encoded by a multigene family. In tomato and potato, inhibitor I proteins are known to be encoded by a family of related sequences (Lee, 1988). Leghemoglobins in soybean (Lee *et al.*, 1983), the small subunits of ribulose-1,5-bisphosphate carboxylase in pea (Coruzzi *et al.*, 1984) are also known to be encoded by multigene families.

Screening of a EcoRI genomic library of Russet Burbank potato resulted in the isolation of 13 recominant phage clones carrying inhibitor II sequences (Fig. 2). Analyses of these clones by restriction mapping and Southern hybridizations revealed that they carry 3 different genomic regions (Figs. 3 and 4). However, it is not clear yet whether they are located on the same chromosome or not. The presence of one or two genes in a region indicated that inhibitor II genes are dispersed on the genome of potato (Figs. 5 and 6). Partial nucleotide sequence of the three HindIII fragments in the 8.0 kb EcoRI fragment of clone 8 indicated that two inhibitor II genes are in the same transcriptional orientation with the intergenic region of

about 2.0 kb (unpublished data). A clone carrying a 5.2 kb EcoRI fragment with an inhibitor II gene has been reported (Keil *et al.*, 1986; Thornburg *et al.*, 1987). The inhibitor II gene was shown to be wound-inducible in transgenic tobacco plants (Sanchez-Serrano *et al.*, 1987; Thornburg *et al.*, 1987). So far, it is not clear whether the inhibitor II genes isolated in this study are wound-inducible or not. Further characterization of these inhibitor II genes at the molecular level is in progress.

적 요

감자에서 단백질분해효소 억제제 II 유전자의 구조와 발현조절 메카니즘을 규명하기 위하여 억제제 II 유전자의 수를 결정하고 이를 유전자은행에서 분리해냈다.

감자의 잎에서 DNA를 추출하여 혼성화 반응을 수행한 결과 억제제 II 유전자는 10여개가 존재하고 있음을 확인했다. 감자의 유전자은행을 screening하여 13개의 재조합phage clone을 얻었는데 이들을 분자수준에서 분석한 결과 염색체상 세 부위에 위치하고 있었다. 이들 clone중에서 clone 8과 18 그리고 39를 대상으로 제한효소지도 작성과 subcloning을 수행하였다. 이들의 subclone을 분석한 결과 clone 8에 존재하는 8.0 kb EcoRI절편에는 두 개의 억제제 II 유전자가, clone 18의 3.3kb와 0.8 kb EcoRI절편에는 하나의 유전가가, 그리고 clone 39의 13.5kb EcoRI절편에는 두 개의 유전자가 존재하고 있음을 확인하였다.

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(Received February, 17, 1989)