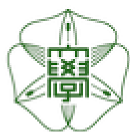

HOKKAIDO UNIVERSITY

Title	MOLECULAR CHARACTERIZATION OF A KUNITZ-TYPE PROTEINASE INHIBITOR EXPRESSING SPECIFICALLY IN POTATO TUBERS
Author(s)	YAMAGISHI, Kazutoshi; NAGATANI, Koh; FUKASE, Takako; MITSUMORI, Cristina; KIKUTA, Yoshio
Citation	Journal of the Faculty of Agriculture, Hokkaido University, 66(1), 1-12
Issue Date	1994-03
Doc URL	http://hdl.handle.net/2115/13125
Type	bulletin (article)
File Information	66(1)_p1-12.pdf

Instructions for use

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

J. Fac. Agr. Hokkaido Univ., Vol. 66, Pt. 1 : 1~12 (1994)

MOLECULAR CHARACTERIZATION OF A KUNITZ-TYPE PROTEINASE INHIBITOR EXPRESSING SPECIFICALLY IN POTATO TUBERS

Kazutoshi YAMAGISHI, Koh NAGATANI, Takako FUKASE, Cristina MITSUMORI and Yoshio KIKUTA
Laboratory of Crop Physiology, Faculty of Agriculture,
Hokkaido University, Sapporo 060, Japan.
(Received December 13, 1993)

Introduction

Potato tubers arise from differentiation of apical tips of underground stems, called stolons. The subapical regions of meristem cells were initially found swelling due to radial cell expansion, followed by enlargement by cell divisions with concomitant accumulation of reserve starch and protein[2,7,11,23]. The process of tuber formation was phenologically stimulated by short day and chilling temperature, and also by many other factors including genotype, shortage of nitrogen nutrition and physiological aging of mother tubers[4,5,8,14,17,20]. By the grafting experiments, tuber-inducing stimulus was postulated to be produced in leaves, then to be transmitted to the stolon tips[9]. Recently tuberonic acid, a derivative of jasmonic acid that was isolated from potato leaves, has been found to induce tuberization by an assay system using single-node stem segments of etiolated shoots[15,38].

For the study of the molecular mechanism of tuberization, several kinds of tuber-specific cDNAs have been isolated by the differential screening technique. Among those cDNA clones, amino acid sequences deduced from pT1[36] and p34021[31] were reported to have characteristics of Kunitz-type proteinase inhibitors. The corresponding proteins (PKPI) were isolated from tubers and demonstrated to compose the 22 kDa protein family (molecular mass of 20.5, 22, 23 and 24 kDa). Some differences found among these proteins were not only in molecular mass but also in the charge of protein, amino acid composition and inhibitory property[32,33,35]. In potato tubers, major proteins consist of patatin together with PKPI and proteinase inhibitor II[3,26,32,35], and are expected to accumulate as reserve protein. The other role of those proteins is probably involved in defense responses against herbivorous insects and pathogens. Particularly, PKI-2, a 20.5 kDa protein of the PKPI family, was demonstrated to be one of the relatively few examples of a microbial proteinase inhibitor in potato tubers[35]. On the other hand, the developmental accumulation of PKPI mRNA and protein

has been investigated by using axillary bud tubers on stem segments[11,13,33]. This paper reports the PKPI mRNA accumulation by using an intact plant system, *in vitro*, which more closely resembles the process of tuberization in the field. Discussed as well are some of the molecular characteristics of the PKPI gene.

Materials and Methods

Plant materials

Stem apices of aseptic potato plants (*Solanum tuberosum* L. cv. Irish Cobbler) were serially subcultured in 300 ml culture flasks (Iwaki) containing 30 ml of shoot culture medium at 23 °C under fluorescent tubes (Toshiba, FL40S BRN) for a 16 h photoperiod (photon flux density of 37.6 μmol/m^2/s). The shoot culture medium consisted of half strength MS medium salts[21], supplemented with 0.5 g/l $CaCl_2$, 1 g/l casamino acids (Difco), 40 g/l sucrose, 8 g/l Bacto-agar (Difco), vitamin components (100 mg/l *myo*-inositol, 20 mg/l adenosine, 5 mg/l nicotinic acid, 2 mg/l glycine, 0.5 mg/l thiamine-HCl, 0.5 mg/l pyridoxine-HCl, 0.5 mg/l folic acid and 0.03 mg/l biotin), and was adjusted to pH 5.8. For tuberization, axillary buds of single-node stem segments were cultured on 30 ml of agarless shoot culture medium (20 ml of which was added after two weeks) under the above conditions for 3 weeks. Subsequently, the shoot culture medium was replaced by a tuberization medium, on which decapitated plants were cultured at 18 °C in the dark. The tuberization medium consisted of half strength MS salts and vitamin components, supplemented with 0.05 g/l casamino acids and 100 g/l sucrose.

RNA isolation

Total RNA was isolated according to the method of Shirras and Northcote[30] with some modification. Tissues excised from *in vitro* potato plants were immediately frozen in liquid nitrogen and stored at −85 °C. The frozen tissues were ground to a fine powder with a mortar and pestle. For each gram of frozen powder, 3 ml phenol mixture (phenol : chloroform : isoamyl alcohol, 25 : 24 : 1, v/v) and 3 ml RNA extraction buffer (0.1 M Tris-HCl pH 9.0, 0.15 M LiCl, 5 mM EDTA, 2 %(w/v) SDS and 0.1 M 2-mercaptoethanol) were added. This mixture was further ground in a Potter-Elvehjem homogenizer and centrifuged. The aqueous phase was again extracted with an equal volume of the phenol mixture. The final aqueous phase was adjusted to 0.2 M LiCl. The total RNA was precipitated with 2.5 volumes of ethanol at −85 °C for 1 h and collected by centrifugation. The dried pellet was dissolved in water and precipitated in 2 M LiCl on ice overnight. The pellet was again centrifuged, dissolved in water, and stored at −85 °C for later use.

Poly(A)$^+$ RNA was prepared by two cycles of affinity chromatography of oligo(dT) cellulose column[29].

Construction of cDNA library

Poly(A)$^+$ RNA was isolated from *in vitro* tubers harvested 3 weeks after tuber induction. Double-stranded cDNA was prepared from 5 μg of poly(A)$^+$ RNA as described by Gubler and Hoffman[10]. After second-strand synthesis, the cDNA was ligated to Eco RI adaptors (Amersham), and then inserted into the Eco RI site of the bacteriophage vector λgt11 (Amersham). The bacteriophage was *in vitro* packaged and used for infecting *Escherichia coli* Y1090 (Amersham). Eventually, ca. 1.0×10^6 recombinants were obtained per microgram of poly(A)$^+$ RNA.

Differential screening

Potato tuber cDNA clones were screened by differential hybridization method[29]. Single-stranded cDNA probes labeled with ^{32}P were prepared from 3 week-old tubers and 9 day-old leaves, and then hybridized respectively to duplicated membranes. Eight recombinants only hybridized to tuber cDNA were further screened with probes from 10 day-old tubers and 9 day-old leaves. The cDNA insert of the most strongly hybridizing recombinant was subcloned in phagemid vector, pBluescript SK(+) (Stratagene), and named pT1.

Northern blot analysis

Total RNA (20 μg) was separated by electrophoresis in a formaldehyde/formamide agarose gel (1.2 %) as described in Sambrook *et al.*[29] except that the concentration of formaldehyde in the gel was 0.66 M. The separated RNAs were transferred to nylon membranes (NY 13N, Schleicher & Schuell). The membranes were prehybridized at 65 °C for about 2 h in a solution containing 6×SSC (1×SSC consisted of 0.15 M NaCl, 15 mM trisodium citrate, pH 7.0), 5× Denhardt's solution [1×Denhardt's solution consists of BSA, PVP (molecular weight of 360,000) and Ficoll 400 (Pharmacia) at concentration of 0.02 % each], 0.1 % SDS and 0.1 mg/ml heat denatured herring sperm DNA, and then hybridized with ^{32}P-labeled pT1 insert at 65 °C overnight in the same solution. Blots were washed with 6×SSC at room temperature for 10 min and then washed twice with 6×SSC at 65 °C for 30 min. Hybridized probes were detected by exposure of the membranes to X-ray films (RX, Fuji Photo Film).

Southern blot analysis

Genomic DNA of potatoes was extracted from etiolated shoot tips by using the method of Rogers and Bendich[27]. Ten microgram DNA was digested with restriction enzyme, Eco RI or Hind III, and separated by electrophoresis in a 0.7 % agarose gel. Blotting and hybridization were performed in accordance with the procedure of the northern analysis except that the membrane was prehybridized overnight. After hybridization, the membrane was washed three times with 6×SSC, 0.5 % SDS at room temperature for 15 min, 2×SSC, 0.5 % SDS

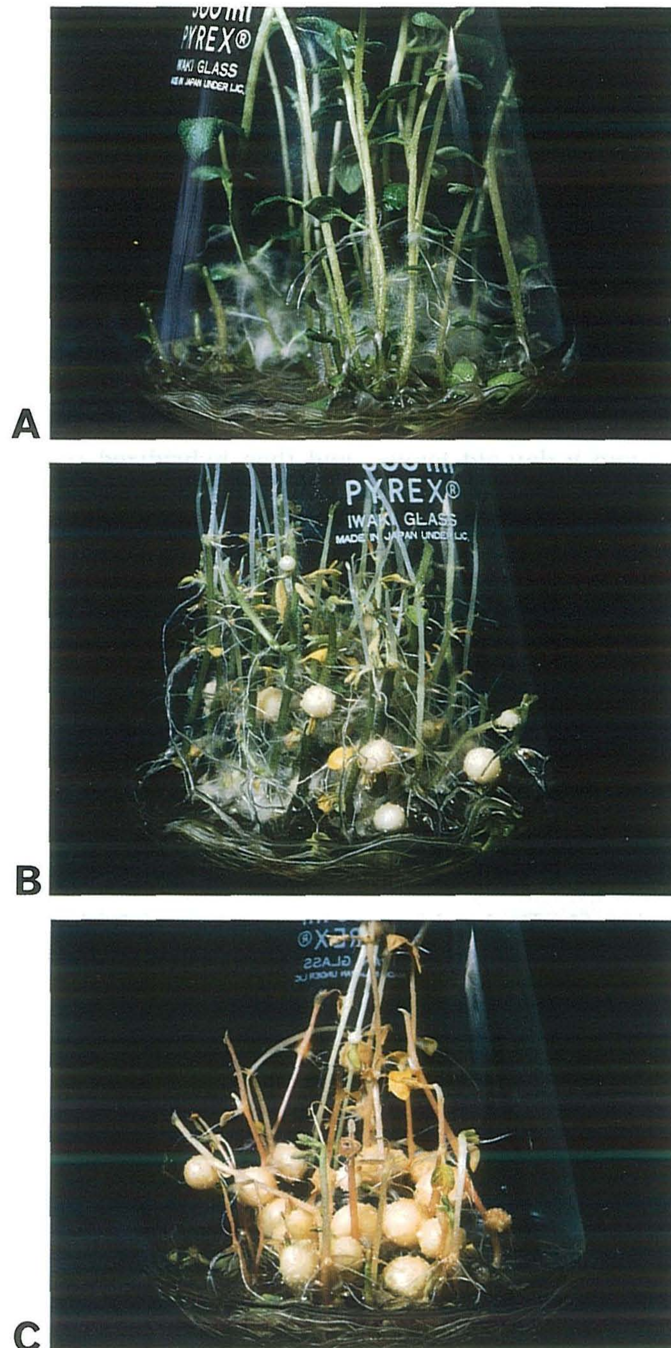

Fig. 1. Tuberization of *in vitro* potato plants. Potato plants were cultured for 2 weeks (A). Tubers were induced for 2 (B) and 5 weeks (C). Details are described in text.

at 42 °C for 30 min, 1×SSC, 0.5 % SDS at 67 °C for 15 min, and then 0.1×SSC, 0.5 % SDS at 67 °C for 30 min. A hybridized probe was detected by exposure of the membrane to X-ray film.

Results

Accumulation of PKPI mRNA during tuber development

The technique of differential screening was employed to isolate the tuber-specific cDNA clone, pT1. The nucleotide sequence of pT1 was previously reported to encode a Kunitz-type proteinase inhibitor (PKPI)[36]. Organ specificity of PKPI mRNA was first confirmed by the northern blot analysis. Potato plants were grown on a liquid medium (Fig. 1A), then cultured on tuberization medium. Sessile tubers appeared in the basal axillary buds followed by development of tubers with concomitant senescence of shoots (Fig. 1B and C). Tubers developed synchronously were used for RNA isolation.

The total RNAs isolated from roots, stems and leaves of 3 week-old plants on the liquid medium, and from tubers of 3 weeks after tuber induction were hybridized with PKPI cDNA probe (pT1 insert). Figure 2 shows that the PKPI mRNA was about 0.9 kb, and accumulated in tubers specifically, while only slightly in stems. Little mRNA accumulation was detected in the total RNA from roots or leaves.

The temporal accumulation of PKPI mRNA was examined with the total RNA from *in vitro* tubers of 3, 5, 7, 14, 21 and 28 days after tuber induction (Fig. 3). The PKPI mRNA appeared at the same position as field-grown tubers. The mRNA accumulation could be detected from 5th day after tuber induction and increased with tuber development. By longer exposure of hybridized membrane to X-ray film, a trace amount of the mRNA could be detected as early as the 3rd day.

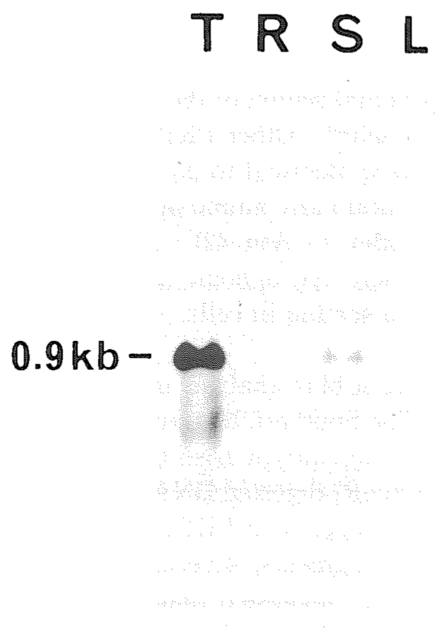

Fig. 2. Accumulation of PKPI mRNA in tubers (T), roots (R), stems (S) and leaves (L) of potato plants cultured *in vitro*. Total RNAs (20 μg each) were analyzed by northern blot hybridization. Size of the mRNA is indicated in kilobases (kb).

Comparison of amino acid sequence data deduced from pT1 with other PKPI

The previously reported nucleotide sequence (EMBL accession number

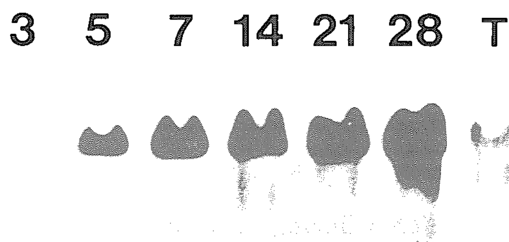

Fig. 3. Accumulation of PKPI mRNA during *in vitro* potato tuberization. Total RNAs (20 μg each) were analyzed by northern blot hybridization. The numbers indicate days after tuber induction. First visible swelling at the subapical meristem was detectable on the 3rd day. Tuberization was obvious from 5th day. Lane T corresponds to field-grown tubers stored at 4 °C for 2 months after harvest.

X56874) and deduced amino acid sequence of pT1[36] are again shown in Fig. 4 in addition to comparisons of amino terminal region with other PKPI. An amino acid sequence (Leu 43—Pro 67) deduced from pT1 was completely identical to the N-terminal region of the tuber protein, PKI-2, which was reported by Walsh and Twitchell[35]. Other PKPI proteins were different from pT1 in two amino acids, but were identical to p34021[31,32]. In addition to the facts, all those proteins did not contain any amino acid sequences corresponding to the N-terminal region of pT1 (Met 1—Asn 42). A hydropathic profile revealed that this region was extremely hydrophobic (Fig. 5), suggesting the possibility of a signal peptide for protein sorting in cells.

Southern blot analysis of PKPI genes

The Southern blot analysis was performed with genomic DNA isolated from tetraploid cultivar Irish Cobbler (Fig. 6). The restriction enzyme polymorphism with EcoRI-digested DNA seemed to contain a few more fragments which hybridized stronger with PKPI cDNA, than with fragments by Hind III. Besides these major fragments, several weak signs were found in the latter autoradiogram. This data suggested that PKPI genes might compose a small multigene family. However, the precise number of PKPI genes could not be estimated due to uncertainties of homology to the probe.

Discussion

The process of potato tuberization is accompanied by *de novo* synthesis of specific proteins[11,13,23]. Parts of those mRNAs have been isolated by a differential screening technique and a possible role in the molecular mechanism of tuberization was studied[19,28,34]. In a similar way, we isolated PKPI cDNA from

```
                                                          G          -1

ATGAAGTCGATTAATATTTTGAGTTTCCTCTTGCTTTCAAGTACCCTCTCTTTGGTTGCC          60
 M  K  S  I  N  I  L  S  F  L  L  L  S  S  T  L  S  L  V  A

TTTGCTAGATCTTTCAGTTCTGAGAATCCAATTGTCCTCCCCTCAACTTGTCATGATGAT         120
 F  A  R  S  F  S  S  E  N  P  I  V  L  P  S  T  C  H  D  D

GATAATCTTGTACTCCCTGAAGTTTATGACCAAGACGGCCATCCGCTGAGGATTGGTCAG         180
 D  N  L  V  L  P  E  V  Y  D  Q  D  G  H  P  L  R  I  G  Q

AGGTACATTATTAACAATCCTCTCATCGGGGCCGGAGCCGTATACTTGTACAATATTGGA         240
 R  Y  I  I  N  N  P  L  I  G  A  G  A  V  Y  L  Y  N  I  G

AACCTTCAATGCCCAAACGCCGTGTTGCAGCACATGTCCATTCCCCAATTTTTGGGAGAA         300
 N  L  Q  C  P  N  A  V  L  Q  H  M  S  I  P  Q  F  L  G  E

GGCACGCCCGTCGTGTTCGTTCGTAAGTCGGAGTCGGATTATGGTGATGTGGTGCGTGTA         360
 G  T  P  V  V  F  V  R  K  S  E  S  D  Y  G  D  V  V  R  V

ATGACTGGTGTTTATATCAAATTCTTTGTTAAAACAACAAAGTTGTGTGTTGACCAAACT         420
 M  T  G  V  Y  I  K  F  F  V  K  T  T  K  L  C  V  D  Q  T

GTTTGGAAAGTTAATCATGAAGGGTTGGTGGTAACTGGTGGTCAGGTAGGAAATGAAAAC         480
 V  W  K  V  N  H  E  G  L  V  V  T  G  G  Q  V  G  N  E  N

GACATCTTCAAGATTAGGAAAACTGACTTGGTGACACCAGAAGGTTCCAAATTTGTATAC         540
 D  I  F  K  I  R  K  T  D  L  V  T  P  E  G  S  K  F  V  Y

AAGTTACTGCATTGTCCCTCTCATCTTCAGTGCAAAAATATCGGCGGCAACTTTAAAAAT         600
 K  L  L  H  C  P  S  H  L  Q  C  K  N  I  G  G  N  F  K  N

GGATATCCTCGTCTGGTGACTGTCGATGATGATAAGGACTTTCTTCCATTTGTGTTCATC         660
 G  Y  P  R  L  V  T  V  D  D  D  K  D  F  L  P  F  V  F  I

AAGGCGTAGAATGCTAATTAGCTGGCTAGTTTGCAGCTTTCTAAATAAAGTGGGGGATAT         720
 K  A  ★

ATCCTTCTATCGCTCCATGTAATTTAATGTATGCTTATCAATAAATAAACAAGCTAGCAA         780

TTAGCCTATTACCTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA              835
```

```
pT1 (20.2 kDa)        1 MKSINILSFLLLSSTLSLVAFARSFSSENPIVLPSTCHDDDN 42

p34021 (20.2 kDa)     1   SINILSFLLLSSTLSLVAFARSFTSENPIVLPTTCHDDDN 40
 Coincidence              *********************** ******** *******

pT1                  43 LVLPEVYDQDGHPLRIGQRYIINNPLIG···        [36]
p34021               41 LVLPEVYDQDGNPLRIGERYIINNPLLG···        [31,32]
PKI-2 (20.5 kDa)      1 LVLPEVYDQDGHPLRIGQRYIINNP 25           [35]
22 kDa                1 LVLPEVYDQDGNPLRIGERYI 21               [32]
23 kDa                1 LVLPEVYDQDGNP 13                       [32]
24 kDa                1 LVLPEV 6                               [32]
 Coincidence            *********** ***** ******** *
```

Fig. 4. Comparison of N-terminal amino acid sequence deduced from nucleotide sequence of pT1 cDNA with other PKPI. Top : nucleotide and deduced amino acid sequences of the cDNA clone pT1[36]. Bottom : N-terminal sequences of pT1 and p34021 were deduced from the nucleotide sequences. Two amino acids were missing in p34021 because of incomplete clone. Potato Kunitz inhibitor-2 (PKI-2), and 22 kDa, 23 kDa and 24 kDa PKPI were isolated from tubers and analyzed by protein sequencing. Identical residues in all sequences are marked by asterisks. Also shown are molecular masses of those proteins and of corresponding parts deduced from nucleotide sequences. Numbers of references are indicated in brackets.

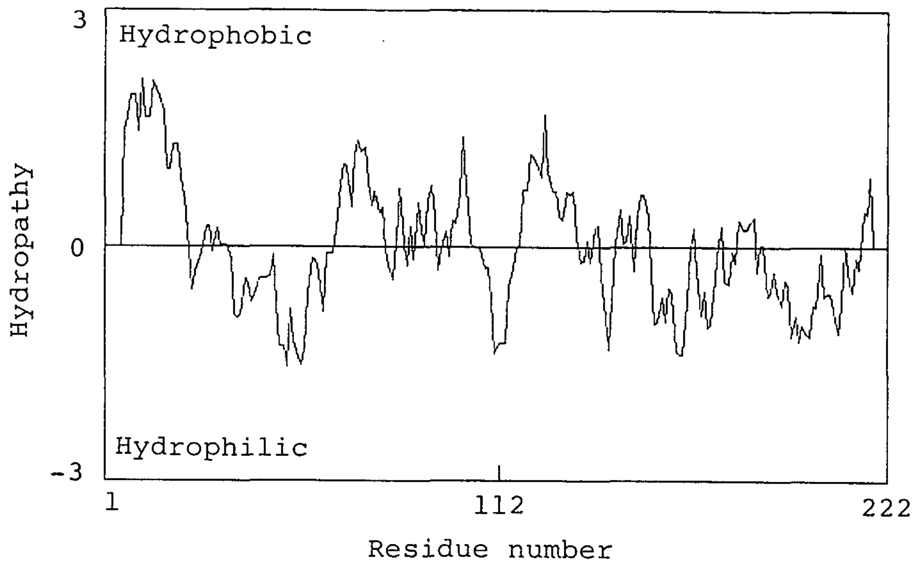

Fig. 5. Hydropathy plots of amino acid sequence deduced from pT1 cDNA. The horizontal line represents a hydrophilicity value of zero. Hydrophilic regions are represented by negative values and hydrophobic regions by positive. Numbers along the X-axis refer to the number of amino acid residues from the N-terminal of the deduced polypeptide. Plots were generated by the method of Kyte and Doolittle[18] using 11 amino acid intervals.

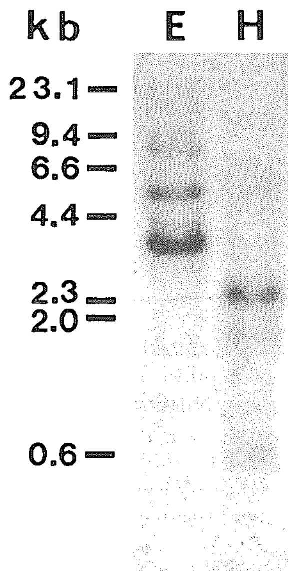

Fig. 6. Southern blot hybridization analysis of genomic DNA from potatoes. The genomic DNAs (10 μg each) were digested with Eco RI (E) or Hind III (H). After separation by electrophoresis, the DNA was transferred to a nylon membrane and hybridized with ^{32}P-labeled PKPI cDNA. Molecular weight markers are shown on the left.

in vitro tubers. The N-terminal amino acid sequence deduced from the cDNA was extremely hydrophobic and was proposed to bear the propeptide necessary for vacuolar targeting[1,6]. However, intracellular location of PKPI was not investigated. To the best of our knowledge, four members of the PKPI proteins and two cDNAs have been reported to be isolated from four potato cultivars[31,32,35,36]. Although they could be classified into two groups on the basis of the amino acid sequences, their sizes were somewhat different. In addition, another member was also isolated in our laboratory (Mitsumori *et al.*, in submission). These diversities might be arising from not only genetic variations among the cultivars but also genomic duplications, because the PKPI gene of each cultivar seemed to compose a small multigene family (Fig. 6 and Ref. 12, 31).

On the assumption that the PKPI gene consisted of several members, the gene expression might be collectively measured by northern analysis. Accumulation of PKPI mRNA in tubers was detectable from the early days of the development, followed by a gradual increase that resulted in specific accumulation in tubers. The similar patterns of accumulation have been reported by using the system of axillary bud tubers on stem segments[11,13,33]. Suh *et al.* reported that PKPI cDNA could hybridize with two mRNA fragments which showed major and minor signs[33], but the latter could not be detected in our experiment. Nevertheless, accumulation of PKPI mRNA could be used as a molecular marker of tuber development.

As reviewed by Park[24], the process of potato tuberization has been expected to be controlled by the available amount of photosynthate and by the tuber-inducing stimulus transported from leaves to stolons. Promotion of *in vitro* tuber formation by jasmonates and sucrose was consistent with this expectation[9,15,16,22]. On the other hand, those tuber-inducing compounds could also stimulate the mRNA accumulation of PKPI, patatin and proteinase inhibitor II in potato explants, except that patatin mRNA was not affected by jasmonates[12,25,37]. This coincidence implies that a common regulatory mechanism could be involved in both the tuber formation and the gene expression. Therefore, we are exceedingly interested in investigating the mechanism of gene transcription and factors which cause PKPI gene expression specifically in tubers of potato plants.

Summary

In potato tubers, Kunitz-type proteinase inhibitors are part of major storage proteins, and consist of several members of the 22 kDa protein family. Southern blot analysis suggested that PKPI genes might compose a small multigene family. By using *in vitro* tuberization system, accumulation of PKPI mRNA was specifically detected in tubers from early days of the development, and thus expected to be used as a molecular marker of potato tuber development.

Acknowledgements

This work was conducted in the Research Center for Molecular Genetics, Hokkaido University, Sapporo, Japan, and was in part supported by Grant-in-Aid for General Scientific Research (B) 05454042 from the Ministry of Education, Science and Culture, Japan.

Literature Cited

1. BEDNAREK, S. Y. and N. V. RAIKHEL : Intracellular trafficking of secretory proteins. *Plant Mol. Biol.* **20** : 133-150. 1992
2. BOOTH, A. : The role of growth substances in the development of stolons. In Ivins, J. D. and F. L. Milthorpe eds., The Growth of the Potato, pp. 99-113, Butterworth, London. 1963
3. BRYANT, J., T. R. GREEN, T. GURUSADDAIAH and C. A. RYAN : Proteinase inhibitor II from potatoes : isolation and characterization of its protomer components. *Biochemistry* **15** : 3418-3424. 1976
4. BURT, R. L. : Influence of short periods of low temperature on tuber initiation in the potato. *Eur. Potato J.* **7** : 197-208. 1964
5. CHAPMAN, H. W. : Tuberization in the potato plant. *Physiol. Plant.* **11** : 215-224. 1958
6. CHRISPEELS, M. J. and N. V. RAIKHEL : Short peptide domains target proteins to plant vacuoles. *Cell* **68** : 613-616. 1992
7. CUTTER, E. G. : Structure and development of the potato plant. In Harris, P. M. ed., The Potato Crop, pp. 70-152, Chapman and Hall, London. 1978
8. EWING, E. E. : Heat stress and the tuberization stimulus. *Am. Potato J.* **58** : 31-49. 1981
9. GREGORY, L. E. : Some factors for tuberization in the potato plant. *Am. J. Bot.* **43** : 281-288. 1956
10. GUBLER, U. and B. J. HOFFMAN : A simple and very efficient method for generating cDNA libraries. *Gene* **25** : 263-269. 1983
11. HANNAPEL, D. J. : Characterization of the early events of potato tuber development. *Physiol. Plant.* **83** : 568-573. 1991
12. HANSEN, J. D. and D. J. HANNAPEL : A wound-inducible potato proteinase inhibitor gene expressed in non-tuber-bearing species is not sucrose inducible. *Plant Physiol.* **100** : 164-169. 1992
13. HENDRIKS, T., D. VREUGDENHIL and W. J. STIEKEMA : Patatin and four serine proteinase inhibitor genes are differentially expressed during potato tuber development. *Plant Mol. Biol.* **17** : 385-394. 1991
14. IRITANI, W. M. : Factors affecting physiological aging (degeneration) of potato tubers used as seed. *Am. Potato J.* **45** : 111-116. 1968
15. KODA, Y., E. S. A. OMER, T. YOSHIHARA, H. SHIBATA, S. SAKAMURA and Y. OKAZAWA : Isolation of a specific potato tuber-inducing substance from potato leaves. *Plant Cell Physiol.* **29** : 1047-1051. 1988
16. KODA, Y., Y. KIKUTA, H. TAZAKI, Y. TSUJINO, S. SAKAMURA and T. YOSHIHARA : Potato tuber-inducing activities of jasmonic acid and related compounds. *Phytochemistry* **30** : 1435-1438. 1991
17. KRAUSS, A. : Tuberization and abscisic acid content in *Solanum tuberosum* as affected by

nitrogen nutrition. *Potato Res.* **21** : 183-193. 1978

18. KYTE, J. and R. F. DOOLITTLE : A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157** : 105-132. 1982
19. MIGNERY, G. A., C. S. PIKAARD, D. J. HANNAPEL and W. D. PARK : Isolation and sequence analysis of cDNAs for the major potato tuber protein, patatin. *Nucl. Acids Res.* **12** : 7987-8000. 1984
20. MONTALDI, E. R. and F. K. CLAVER : Tuberization of the potato plant under non-inducing conditions. *Eur. Potato J.* **6** : 223-226. 1963
21. MURASHIGE, T. and F. SKOOG : A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.* **15** : 473-497. 1962
22. OKAZAWA, Y. : Physiological studies on the mechanism of tuberization of potato plants. *Proc. Crop Sci. Soc. Japan* **23** : 247-248. 1955 (in Japanese with English summary)
23. PAIVA, E., R. M. LISTER and W. D. PARK : Comparison of the protein in axillary bud tubers and underground stolon tubers in potato. *Am. Potato J.* **59** : 425-433. 1982
24. PARK, W. D. : Tuber proteins of potato —a new and surprising molecular system. *Plant Mol. Biol. Rep.* **1** : 61-66. 1983
25. PEÑA-CORTÉS, H., X. LIU, J. SANCHEZ-SERRANO, R. SCHMID and L. WILLMITZER : Factors affecting gene expression of patatin and proteinase-inhibitor-II gene families in detached potato leaves: implications for their co-expression in developing tubers. *Planta* **186** : 495-502. 1992
26. RACUSEN, D. and M. FOOTE : A major soluble glycoprotein of potato tubers. *J. Food Biochem.* **4** : 43-52. 1980
27. ROGERS, S. O. and A. J. BENDICH : Extraction of DNA from plant tissues. In Gelvin, S. B., R. A. Schilperoort and D. P. S. Verma eds., Plant Molecular Biology Manual, pp. A6/1-A6/11, Kluwer Academic Publishers, Dordrecht, 1988
28. ROSAHL, S., P. ECKES, J. SCHELL and L. WILLMITZER : Organ-specific gene expression in potato: isolation and characterization of tuber-specific cDNA sequences. *Mol. Gen. Genet.* **202** : 368-373. 1986
29. SAMBROOK, J., E. F. FRITSCH and T. MANIATIS : Molecular Cloning : A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 1989
30. SHIRRAS, A. D. and D. H. NORTHCOTE : Molecular cloning and characterisation of DNAs complementary to mRNAs from wounded potato (*Solanum tuberosum*) tuber tissue. *Planta* **162** : 353-360. 1984
31. STIEKEMA, W. J., F. HEIDEKAMP, W. G. DIRKSE, J. VAN BECKUM, P. DE HAAN, C. TEN BOSCH and J. D. LOUWERSE : Molecular cloning and analysis of four potato tuber mRNAs. *Plant Mol. Biol.* **11** : 255-269. 1988
32. SUH, S. G., J. E. PETERSON, W. J. STIEKEMA and D. J. HANNAPEL : Purification and characterization of the 22-kilodalton potato tuber proteins. *Plant Physiol.* **94** : 40-45. 1990
33. SUH, S. G., W. J. STIEKEMA and D.J. HANNAPEL : Proteinase-inhibitor activity and wound-inducible gene expression of the 22-kDa potato-tuber proteins. *Planta* **184** : 423-430. 1991
34. TAYLOR, M. A., S. A. M. ARIF, A. KUMAR, H. V. DAVIES, L. A. SCOBIE, S. R. PEARCE and A. J. FLAVELL : Expression and sequence analysis of cDNAs induced during the early stages of tuberisation in different organs of the potato plant (*Solanum tuberosum* L.). *Plant Mol. Biol.* **20** : 641-651. 1992
35. WALSH, T. A. and W. P. TWITCHELL : Two Kunitz-type proteinase inhibitors from potato

tubers. *Plant Physiol.* **97** : 15-18. 1991

36. YAMAGISHI, K., C. MITSUMORI and Y. KIKUTA : Nucleotide sequence of a cDNA encoding the putative trypsin inhibitor in potato tuber. *Plant Mol. Biol.* **17** : 287-288. 1991
37. YAMAGISHI, K., C. MITSUMORI, K. TAKAHASHI, K. FUJINO, Y. KODA and Y. KIKUTA : Jasmonic acid-inducible gene expression of a Kunitz-type proteinase inhibitor in potato tuber disks. *Plant Mol. Biol.* **21** : 539-541. 1993
38. YOSHIHARA, T., E. S. A. OMER, H. KOSHINO, S. SAKAMURA, Y. KIKUTA and Y. KODA : Structure of a tuber-inducing stimulus from potato leaves (*Solanum tuberosum* L.). *Agric. Biol. Chem.* **53** : 2835-2837. 1989