Pergamon

Phytochemistry, Vol. 44, No. 6, pp. 1001–1006, 1997
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0031–9422/97 $17.00+0.00

PII: S0031–9422(96)00668–1

JASMONIC ACID INDUCIBLE ASPARTIC PROTEINASE INHIBITORS FROM POTATO

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(*Received in revised form* 5 *September* 1996)

Key Word Index—*Solanum tuberosum*; Solanaceae; potato; cDNA cloning; defence mechanism; proteinase inhibitors; jasmonic acid.

Abstract—A new cDNA clone coding for an aspartic proteinase inhibitor homologue was isolated from a potato tuber cDNA library. Southern blot analysis was used to study the structural diversity of the aspartic proteinase inhibitor gene family in several species of the Solanaceae. The existence of sequence-homologous genes was confirmed in the genomic DNA of different potato cultivars (*Solanum tuberosum* L. cv. Désirée, Pentland Squire and Igor), tomato (*Lycopersicon esculentum* Mill.), aubergine (*S. melongena* L.) and a wild type of bittersweet (*S. dulcamara* L.). Northern blot hybridization of total RNA, isolated from leaves under non-stress conditions, of different solanaceous species and of potato tubers showed that the gene transcripts encoding aspartic proteinase inhibitors occur mainly in potato tubers. The presence of several cathepsin D inhibitor isoforms has been detected at the protein level. At least four isoforms were isolated by affinity chromatography on cathepsin D–Sepharose and characterized. Additionally, exogenous treatment of potato plantlets by jasmonic acid (JA) over a wide range of concentrations (0–100 μM) was performed in a stem node culture *in vitro*. We demonstrated that the expression of aspartic proteinase inhibitor mRNA was drastically induced in potato shoots at concentrations of 50–100 μM JA. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Protein inhibitors of proteases are widely distributed in the plant kingdom. They are usually concentrated in seeds and tubers, particularly in the Fabaceae and Solanaceae [1]. Potato tubers represent an important source of serine [2–4] and cysteine [5, 6] proteinase inhibitors. From the same tissue, a few aspartic proteinase inhibitor homologues have been also detected and isolated in the last few years. Two sequentially related iso-inhibitors, PDI [7] and NID [8], were isolated from potato tubers and their primary structures were determined. Subsequent reports described the cDNA sequence of a potato inhibitor homologue of aspartic proteinases (API-8) with 99 and 94% identity to the protein sequences of PDI and NID, respectively [9], and of p749 with 94% identity to the deduced amino acid sequence of API-8 [10]. Additionally, the primary structures of two aspartic proteinase inhibitor genes, PIG [11] and CathInh gene [12] from different cultivars of potato were determined. Comparison of the deduced protein sequence of the PIG with the amino acid sequence of PDI and NID shows *ca* 75% identity, while deduced protein sequence of CathInh gene shows 99 and 92% identity to PID and NID, respectively. The existence of a potato aspartic proteinase inhibitor gene family was confirmed by a Southern blot analysis [13].

In response to wounding or severe mechanical damage, many serine proteinase inhibitors are synthesized in both wounded and unwounded leaves [14]. Moreover, oligouronides [15], auxins [16], abscisic acid [17] and methyl jasmonate (MeJA) [18] were shown to influence the expression of plant serine proteinase inhibitors. On this basis, it can be concluded that proteinase inhibitors are involved in the defence mechanism of plants [19].

In this study, we used the potato stem node tissue culture in order to observe the accumulation of aspartic proteinase inhibitor mRNA due to exogenous application of jasmonic acid (JA) into the growth medium. We determined the cDNA and deduced the amino acid sequence of a new aspartic proteinase inhibitor homologue. Additionally, we show the structural diversity of aspartic proteinase inhibitor genes in different potato cultivars and some other solanaceous species. The expression of examined gene transcripts

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  1     G ATG ATG AAG TGT TTA TTT TTG TTA TGT TTG TGT TTG CTT CCC ATT TTG GTG       52
  1        M   M   K   C   L   F   L   L   C   L   C   L   L   P   I   L   V        17

 53   TTT TCA TCA ACT TTC ACT TCC CAA AAT CCC ATC AAC CTA CCC AGT GAA TCT CCT      106
 18    F   S   S   T   F   T   S   Q   N   P   I   N   L   P   S   E   S   P       35

107   GTA CCT AAG CCG GTA CTT GAC ACA AAT GGT AAA GAG CTC AAT CCT AAT TCG AGT      160
 36    V   P   K   P   V   L   D   T   N   G   K   E   L   N   P   N   S   S       53

161   TAT CGC ATT ATT TCC ATT GGT AGG GGT GCC TTA GGT GGT GAT GTA TAC CTA GGA      214
 54    Y   R   I   I   S   I   G   R   G   A   L   G   G   D   V   Y   L   G       71

215   AAG TCC CCA AAT TCA GAT GCC CCT TGT CCA GAT GGC GTA TTC CGT TAC AAT TCC      268
 72    K   S   P   N   S   D   A   P   C   P   D   G   V   F   R   Y   N   S       89

269   GAT GTT GGA CCT AGC GGT ACA CCC GTT AGA TTC ATT CCT TTA TCT ACA AAT ATC      322
 90    D   V   G   P   S   G   T   P   V   R   F   I   P   L   S   T   N   I      107

323   TTT GAA GAT CAA CTA CTT AAC ATA CAA TTC AAT ATT CCA ACA GTG AAA TTG TGT      376
108    F   E   D   Q   L   L   N   I   Q   F   N   I   P   T   V   K   L   C      125

377   GTT AGT TAT AGA AAT TGG AAA GTC GGA AAT CTA AAT GCA CAT CTA TGG ACG ATG      430
126    V   S   Y   R   N   W   K   V   G   N   L   N   A   H   L   W   T   M      143

431   TTG TTG GAG ACG GGA GGA ACC ATA GGG CAA GCA GAT AGC AGC TAT TTC AAG ATT      484
144    L   L   E   T   G   G   T   I   G   Q   A   D   S   S   Y   F   K   I      161

485   GTT AAA TCA TCA AAA TTT GGT TAC AAC TTA TTG TAT TGC CCT ATT ACT CGC CAT      538
162    V   K   S   S   K   F   G   Y   N   L   L   Y   C   P   I   T   R   H      179

539   TTT CTT TGT CCA TTT TGT CGT GAT GAT AAC TTC TGT GCA AAA GTG GGT GTA GAT      592
180    F   L   C   P   F   C   R   D   D   N   F   C   A   K   V   G   V   D      197

593   ATT CAA AAT GGA AAA AGG CGT TTG GCT CTT GTC AGC GAA AAT CCT CTT GAT GTC      646
198    I   Q   N   G   K   R   R   L   A   L   V   S   E   N   P   L   D   V      215

647   TTA TTC CAG GAA GTC TAG TAA CAA ATA ACT GCA GCA GCT AGA CTA TAC TAT GTT      700
216    L   F   Q   E   V   *   *                                                   221

701   TTA GCC TGC CTG CTG GCT AGC TAC TAT GTT ATG TTT TTT GTA AAA TAA ACA CCT      754

755   GCT AAG GTA TAT CTA TAT ATA TTT TAG CAT GGC TTT CTT AAA AAA AAA AAA AA       807
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Fig. 1. Nucleotide and deduced amino acid sequence of aspartic proteinase inhibitor homologue API-13. Polyadenylation signal is underlined. Putative vacuolar targeting signal is double underlined. These sequence data are available from EMBL/GenBank/DDBJ under accession number X62095.

was assayed by Northern blot hybridization. Moreover, the aspartic porteinase inhibitor isoforms have been isolated from potato tubers of different cultivars and partially identified.

RESULTS AND DISCUSSION

Screening of a potato tuber cDNA λgt11 library of 6×10^4 independent recombinants yielded 14 positive clones. Three clones with the largest inserts were subjected to sequence analysis. The nucleotide sequences of two cDNA clones were identical and were denoted as API-8 [9], while the third clone (API-13) was found to represent a new aspartic proteinase inhibitor homologue. The nucleotide sequence and the primary structure of the API-13 deduced from its cDNA sequence are shown in Fig. 1. The deduced amino acid sequence contains an open reading frame of 220 amino acid residues. The 3′-non-coding region (including two stop codons) consists of 132 nucleotides followed by a poly-(A+) tail. A putative polyadenylation signal (AATAAA) was located 44 nucleotides upstream from the poly-(A+) sequence. The first 19 amino acid residues reveal typical hydrophobic character similar to other eukaryotic signal sequences [20].

The deduced protein sequence of API-13 reveals an overall structure very similar to that described for other potato aspartic proteinase inhibitors (NID [8] and PDI [7]), their cDNAs (API-8 [9], p749 [10], CathInh [12] and clone 4 [21]) or gene (PIG [11]). It was previously shown that both PDI and NID inhibit cathepsin D, which is aspartic protease and trypsin, which belongs to the family of serine proteinases [7, 8]. The presumed active site responsible for trypsin inhibitory activity (Arg-67–Phe-68) is conserved in API-13 and in all other compared amino acid sequences. Besides other potato aspartic proteinase inhibitors, API-13 also shares a moderate similarity to the double-headed Kunitz soybean trypsin inhibitor family (Fig. 2). The binding site responsible for the inhibition of cathepsin D is not yet determined. Comparison of the amino acid sequences of API-13, API-8, PIG, NID, PDI, p749 and clone 4 (data not shown) with the protein sequence of soybean trypsin inhibitor shows that all known aspartic proteinase inhibitors possess an extra region of eight amino acid residues

```
                                                   -1
API-13         MMKC-LFLLCL-CLLPIL----VFSSTFTSQNPINLPS
STI             ..STI.F.F.F.AFTTSYLPSAIA-------------

             1       10        20        30        40
API-13       ESPVPKPVLDTNGKELNPNSSYRIISIGRGALGGDVYLGK
API-3                           .....................
API-6                           .....................
NID          ...L....................................
STI          -----DF...NE.NP.ENGGT.Y.L.DIT-.F..---IRA

                     50          60        70
API-13       SPNSDAPCPDGVFR-YN-SDVGPSGTPVRFIPLST-NIFE
API-3        ..............-..-.................-....
API-6        ..............-..-................G-G...
NID          ..............-..-................G-G...
STI          A.TGNER..LT.VQSR.EL.K.-I..-IISS.YRIRF.A.

              80         90        100       110
API-13       DQLLNIQFN-IPTVKLCVSY-RNWKVGNLNAHLWTMLLET
API-3        .........-..........-TI........YFR......
API-6        .........-..........-TI........YFR......
NID          .........-..........-TI........YFR......
STI          GHP.SLK.DSFAVIM...GIPTE.S.--VEDLPEGPAVKI

               120       130       140       150
API-13       GGTIGQADSSYFKIVKSSKFGYNLLYCPITRHFLCPFCRD
API-3        ........N..........I.....S..F.-SII.LR.PE
API-6        ........N..........I.....S..F.-SII.LR.PE
NID          ........N.......L.N......S..F.-SII.LR.PE
STI          .ENKDAM.G-W.RLERV.DDEF.NYKL-----VF..QQAE

               160         170       180
API-13       DNFCAKVGVDIQ--NGKRRLALVSENPLDVLFQEV
API-3        .Q.......V..--.........N...........
API-6        .Q.......V..--.........N...........
NID          .Q.......V..--.........N...........
STI          .DK.GDI.IS.DHDD.T...VVSKNK..V.Q..KLDKESL
```

Fig. 2. Comparison of the deduced amino acid sequences of three new aspartic proteinase inhibitors (API-13, API-3 and API-6) with amino acid sequences of NID and STI [33]. Dots indicate the amino acids identical to those in API-13. For optimal alignment, gaps were introduced that are marked by dashes. First amino acid of mature proteins is numbered by 1.

at the C-terminal part of the molecule which might be involved in cathepsin D inhibition.

Interestingly, API-13 as well as other compared aspartic proteinase inhibitors contain in their *N*-terminal propeptides a region of six amino acid residues very similar to a vacuolar targeting signal (N–P–I–R–L–P) found in sporamin propeptide [22], which allows sporamin to enter vacuoles. Subcellular immunohistochemical localization showed that aspartic proteinase inhibitors are also secreted into vacuoles [23].

To determine the existence and complexity of the aspartic proteinase inhibitor genes in some solanaceous species, chromosomal DNA from different potato cultivars (*Solanum tuberosum* L. cv. Pentland Squire, Désirée and Igor), tomato (*Lycopersicon esculentum* Mill.), aubergine (*S. melongena* L.), wild-type of bittersweet (*S. dulcamara* L.), black nightshade (*S. nigrum* L.) were analysed. Hybridization of EcoRI-digested potato genomic DNA with an API-13 cDNA probe revealed a complex pattern of hybridizing bands in all cultivars, whereas two bands were observed in a sample of EcoRI-cleaved tomato genomic DNA (Fig. 3). Surprisingly, weak single signals were obtained after hybridization of a EcoRI-cleaved genomic DNA of bittersweet and aubergine. Positive signals indicate that aspartic proteinase inhibitor sequence-related genes could be present in bittersweet and aubergine as well.

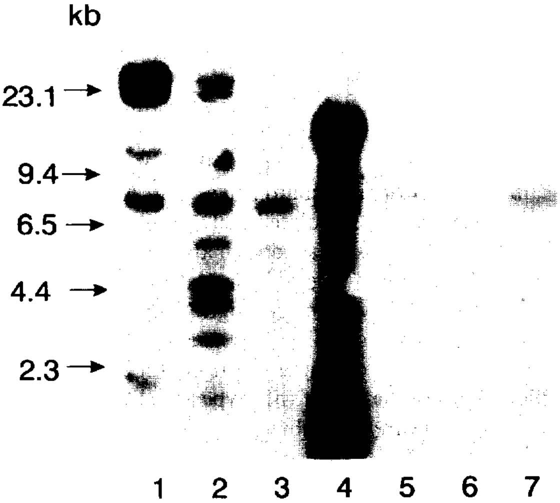

Fig. 3. Southern blot hybridization analysis. Lanes 1–7 contain EcoRI-restricted genomic DNA from potato cv. Désirée, cv. Pentland, cv. Igor, tomato, aubergine, nightshade and bittersweet, respectively.

The finding that aspartic proteinase inhibitor genes exist not only in potato, but also in other solanaceous species, led us to examine whether corresponding mRNA can be found in the examined plants. Northern blot analysis of total RNA, isolated from cultivated (cv. Désirée) potato tubers and potato, tomato, aubergine and bittersweet leaves was performed (data not shown). Aspartic proteinase inhibitor mRNA was detected only in potato tubers, which clearly indicates that, under non-stress conditions, the expression of aspartic proteinase inhibitors takes place mainly in potato tubers.

From tubers of different cultivars (Désirée, Ulster Sceptre, Igor and Pentland Squire) several inhibitors of cathepsin D were isolated. The isoinhibitors with pI 6.55, 8.05 and 8.65 were present in equal quantities in all examined cultivars, whereas the inhibitors with pI 8.15 were determined only in cultivated Désirée and Pentland Squire potato tubers. An extra band with pI 9.3 was observed in analytical isoelectric focusing of the supernatants from cultivars Ulster Sceptre and Pentland Squire. The first 14 amino acid residues of all four isolated isoinhibitors were identical, with the exception of the fourth amino acid residue in the sequence of iso-inhibitor with pI 8.05 where Leu is replaced with Val (data not shown). The differences in isoelectric points of other iso-inhibitors could result from different post-translational modifications or from amino acid sequence heterogeneity in another part of the molecules. All four isolated inhibitors bind tightly and rapidly to cathepsin D ($K_i = 1.2-6.8 \times 10^{-9}$), while the complexes of APIs with trypsin are *ca* 10-fold weaker ($K_i = 2.0-5.3 \times 10^{-8}$). The number of expressed inhibitors in potato tubers of different cultivars only partially corresponds to the number of presumed genes. These results indicate that unexpressed inhibitor genes could be activated during plant growth and development or exist as non-functional pseudogenes.

MeJA induces the accumulation of serine proteinase inhibitors in tomato leaves [18]. Additionally, Hildmann *et al.* [21] isolated a cDNA clone (clone 4) from potato plants sprayed with abscisic acid (ABA). Clone 4 has a high degree of similarity to API-8 and API-13, which by analogy might demonstrate that aspartic proteinase inhibitors are ABA inducible. They used clone 4 as a hybridization probe in an RNA blot analysis of leaves of wild-type and ABA-deficient plants of *S. phureja*, incubated in MeJA solutions. The accumulation of sequence related aspartic proteinase inhibitors in leaves of *S. phureja* was observed. In this study, JA added to the culture media at concentrations of up to 1 μM stimulated the growth of axillary shoots and the development of roots. Concentrations higher than 10 μM caused stunted growth in the plants and inhibition of adventitious root system development (Fig. 4). These results correspond to previously reported effects of JA [24].

Interestingly, a large increase in aspartic proteinase inhibitor mRNA was observed in the shoots grown in media supplemented with higher concentrations of JA (50–100 μM) (Fig. 4). At such concentrations exogenous JA simulates a typical stress response in developed shoots.

The strong similarity between the API-13 nucleotide sequence and the API-8 and clone 4 [21] allows a very strong cross-hybridization. In an attempt to establish whether API-13 is involved in the JA induction mechanism, poly-(A+) RNA isolated from control tissue culture potato plantlets and that from JA-treated plantlets at a 100 μM concentration were reverse transcribed and PCR was performed. The sequences of amplimers corresponded to the *N*- and *C*-terminal regions of deduced amino acid sequences of mature API-13. An intense band of *ca* 600 bp of JA-treated samples was visualized on an ethyl bromide stained agarose gel after 23 PCR cycles, which indicates that a large amount of specific mRNA is present in total RNA, whereas a very weak band was detected in the control lane. The PCR products of JA-induced samples were subcloned and sequenced. From 10 sequenced clones, four corresponded to the nucleotide sequence of API-13, three clones were identical to the deduced amino acid sequence of NID, and three represent two new inhibitors (named API-3 and API-6). They differ from all known potato aspartic proteinase inhibitor sequences for at least eight amino acid residues (Fig. 2). Their deduced amino acid sequence is related to the amino acid sequence of NID, contrary to the API-13 which shares a higher degree of similarity with API-8. Based on the extensive nucleotide and deduced amino acid sequence similarity we assume that API-13 as well as API-3 and API-6 represent novel structural homologues of aspartic proteinase inhibitor family.

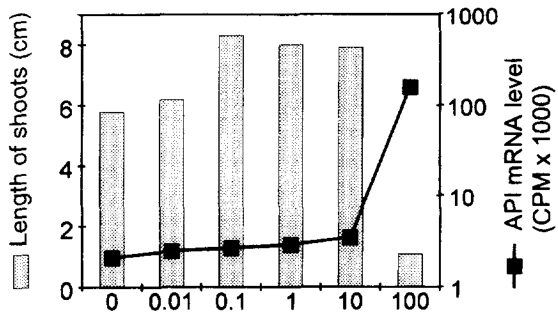

Fig. 4. The effect of increasing JA concentration on the length of shoots (columns) and on the expression of aspartic proteinase inhibitor mRNA (line).

EXPERIMENTAL

Plant material. Virus-free mature tubers and plants of potato (*S. tuberosum* L. cv. Pentland Squire, Désirée, Ulster Sceptre and Igor) were obtained from the Laboratory for Potato Physiology and Viral Diseases, Kranj. Potato, tomato (*L. esculentum* Mill.) and aubergine (*S. melongena* L.) plants were grown under greenhouse conditions with a photoperiod of 16 hr light and a temp. of 23 ± 2°. Wild-type of black nightshade (*S. nigrum* L.) and bittersweet (*S. dulcamara* L.) were collected in their natural habitat near Ljubljana. All plant materials were frozen under liquid N_2 immediately after harvesting and stored at −70°. Specimens have been deposited at the Department of Biochemistry and Molecular Biology, Jožef Stefan Institute, Ljubljana, Slovenia.

Induction experiments. Stem cuttings with one leaf node were segmented from *in vitro* propagated healthy plants (*S. tuberosum* L. cv. Désirée). A Murashige–Skoog medium was supplemented with 3% sucrose and 0.7% Difco Bacto agar and (±)-JA in concns of 0.1–100 μM. A medium without JA was used as control medium. All media were adjusted to pH 5.7–5.8 before autoclaving. Cultures were kept at 25 ± 2°, with a photoperiod of 16 h at 50 μmol m^{-2} s^{-1}. After 4 weeks the axillary shoots (6–8 cm in length) and adventitious roots were harvested.

Protein isolation, purification and characterization. Supernatants of heated extract of potato cultivars Désirée, Ulster Sceptre, Igor and Pentland Squire were applied to cathepsin D–Sepharose. After elution of unbound material, bound proteins were first eluted with NH_4OAc buffer (pH 6.1); *ca* 80% of bound proteins were eluted in this step. These proteins showed inhibitory activity only against trypsin. With Tris–HCl buffer (pH 8.4), proteins, which showed inhibition against cathepsin D and trypsin, were eluted. Further, the iso-inhibitors were purified to homogeneity and characterized according to the procedure of ref. [25]. *N*-Terminal sequences were determined by automated gas phase Edman degradation in the

Applied Biosystems 470A protein sequencer, followed by on-line detection of released amino acid phenylthiohydantoin derivatives. Inhibitory activity against cathepsin D was determined according to a modification of the method in ref. [26]. Inhibition of trypsin was assayed with Bz-DL-Arg–Nan as substrate following the modified method of ref. [27].

Isolation and sequencing a cDNA clone (API-13). A mixed 15-mer oligonucleotide corresponding to the region Ile-75 to Leu-80 of the known sequence of NID was synthesized, labelled with [γ-^{32}P]ATP by T4 polynucleotide kinase and used as probe. The potato tubers λgt11 cDNA library [13] was screened following standard procedures [28]. cDNA inserts were excised from positive clones and inserted into pUC19 plasmid. Both DNA strands of the insert were completely sequenced by the dideoxy chain termination method [29] using T7 DNA polymerase (Pharmacia).

Southern blot analysis. Total genomic DNA was isolated from young leaves of examined solanaceous species using the procedure described in ref. [30]. Samples (15 μg) of DNA were digested to completion by EcoRI restriction endonuclease. The fragments were sepd by 0.7% agarose gel electrophoresis in TAE, transferred to Hybond-N nylon membrane by capillary blotting and UV-crosslinked.

Northern blot analysis. Total cellular RNA was isolated from samples of potato tubers, from leaves of some solanaceous plants, from untreated potato leaves and from JA-treated potato leaves as described in ref. [31]. Poly(A)$^+$ RNA was prepd from JA-treated potato leaves following standard procedures [28]. Glyoxylated RNA samples [10 μg total RNA or 2 μg poly(A)$^+$ RNA] were sepd by electrophoresis on 1.2% agarose gels [28]. RNA was transferred to Hybond-N nylon membranes by capillary transfer, UV-crosslinked and hybridized as described below.

Nucleic acid hybridization. A 0.6 kb EcoRI/PstI fragment of API-13 cDNA probe was radiolabelled with [α-^{32}P]dCTP using random oligonucleotides as primers according to the manufacturer's instructions. Southern blots and Northern blots were hybridized at 42° in 5 × SSPE, 5 × Denhardt's soln, 50% deionized $HCONH_2$, 0.1% SDS and 50 μg ml^{-1} sonicated salmon sperm DNA. Washes of Southern blots were performed at 42°, 55° and 62° in 0.1 × SSC, 0.1% SDS. A stringent wash at 62° was omitted for Northern blot hybridization. Washed filters were exposed to X-ray film. Pieces of nylon membranes corresponding to bands on X-ray film were excised and their radioactivity measured with a scintillation counter.

PCR analysis. Poly(A)$^+$ RNA samples (1.5 μg) from potato control and JA-treated (100 μM JA) leaves were reverse transcribed in a reaction mixt. containing 1 × PCR buffer, 1 mM of each dNTP, 1 U μl^{-1} RNasin, 0.2 μg oligo-dT$^{(12-18)}$ and 200 U MoMuLV reverse transcriptase (Perkin-Elmer Cetus). The mixt. was incubated for 10 min at 23° and 45 min at 42° and then underwent heat treatment for 10 min at 95°. Subsequently, 1 × PCR buffer in a total vol. of 100 μl, 25 pmol of the 5′-amplimer, 5′-d(GGGAATTCGAATCTCCTGTACCT), 25 pmol of the 3′-amplimer, 5′-d(CTGGGATCCCTAGACTTCCTGGAA) and 2.5 U AmpliTaq DNA polymerase (Perkin-Elmer Cetus) were added. After being overlaid with 100 μl mineral oil, PCR reaction was performed for 23 cycles on a thermal cycler (Perkin-Elmer Cetus). After fractionation on 1.2% agarose gel electrophoresis, the excised bands of interest were purified using glass milk according to ref. [32], inserted into pUC19 and sequenced as previously described.

Acknowledgements—We wish to thank Drs Sembdner and Miersch, Institute of Plant Biochemistry Halle/Saale, Germany, for the gift of JA, and the Laboratory for Potato Physiology and Virus Diseases, Kranj, Slovenia, for the starting plant material. This work was supported by the Ministry of Science and Technology of the Republic of Slovenia.

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