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Molecular Cloning of a cDNA Encoding Ribosome Inactivating Protein from *Amaranthus viridis* and Its Expression in *E. coli*

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In order to isolate a cDNA clone of ribosome inactivating protein (RIP), a cDNA library was constructed in Uni-ZAP XL vector with poly(A) RNA purified from leaves of *Amaranthus viridis*. To get the probe for screening the library, PCR of phage DNA was conducted using the vector primer and degenerate primer designed from a conserved putative active site of the RIPs. Twenty-six cDNA clones from about 600,000 plaques were isolated, and one of these clones was fully sequenced. It was 1,047 bp and contained an open reading frame encoding 270 amino acids. The deduced amino acid sequence had a putative signal sequence of 17 amino acids and a putative active site (AIQMVAEAARFFKYIE) conserved in other RIPs. *E. coli* cells expressing *A. viridis* RIP cDNA did not grow well as compared to control cells, indicating that recombinant *A. viridis* RIP presumably inactivated *E. coli* ribosomes. In addition, recombinant *A. viridis* RIP cDNA produced by *E. coli* had translation inhibition activity *in vitro*.

Keywords: *Amaranthus viridis*; cDNA; Expression; Ribosome Inactivating Protein.

Introduction

Ribosome-inactivating proteins (RIPs) are widely distributed throughout the plant kingdom as stable, highly basic proteins ranging in pI 9 to 11. To date, all RIPs are classified as type 1, type 2, and type 3 (Mehta and Boston, 1988). Type 1 RIPs such as pokeweed antiviral protein (PAP) possess a single active polypeptide chain, with an approximate molecular mass of 30,000 Da. Type 2 RIPs such as ricin consist of two disulfide-bonded subunits, with an approximate molecular mass of 60,000 Da: an enzymatically active A chain (functional homologue of type 1) and a galactose-binding lectin (B chain). Type 3 RIPs, like type 1 RIPs, are single chain proteins that do not contain lectin-binding moiety, but differ from type 1 because they are synthesized as inactive forms that require proteolytic processing of internal regions to form active proteins. RIPs function as *N*-glycosidases to remove a specific adenine in a conserved loop of the large rRNA (Endo and Tsurugi, 1987; Endo *et al*., 1987). This irreversible modification renders the ribosome unable to bind the elongation factor, thereby leading to inhibition of translation.

RIPs have been shown to possess antiviral activity *in vitro*, presumably by inhibiting viral replication. There has also been considerable interest in RIPs due to their therapeutic potential as immunotoxin (Stirpe and Babieri, 1986). It has recently been reported that transgenic plants expressing PAP exhibited the broad-spectrum virus resistance and PR-genes in the plants were expressed in the absence of lesion formation (Lodge *et al*., 1993; Moon *et al*., 1997; Zoubenko *et al*., 1997). We previously reported purification of a ribosome-inactivating protein which has antiviral activity from *Amaranthus viridis* and which is named amaranthin (Kwon *et al*., 1997). In this report we describe the preparation of probes for RIP and the cloning of a new RIP gene from the leaves of *A. viridis*, which is of the amaranth family and which is used as edible vegetables in Korea.

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Abbreviations: MAP, *Mirabilis* antiviral protein; PAP, pokeweed antiviral protein; RIP, ribosome inactivating protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Materials and Methods

cDNA library construction The complete RNA was isolated from the leaves of *Amaranthus viridis* grown in the green house. Poly(A)$^+$ RNA was prepared from complete RNA using PolyA Track (Promega, USA). Double-stranded cDNA was synthesized, ligated to Uni-ZAP XL vector (Stratagene, USA), and *in vitro* packaged with packaging extract according to the instructions of the manufacturer. The cDNA library was constructed with 10^9 recombinant phages.

Polymerase chain reaction and cDNA library screening PCR was conducted to obtain the portion of RIP cDNAs needed for library screening, using SK primer (5′-TCTAGAACTAG-TGGATC-3′) of pBluescript and a degenerate primer [5′-CGIGCIGC(T/C)TCIGANACCAT-3′] designed with conserved active site amino acid sequence of RIPs and phage DNA of cDNA library as template (94°C, 1 min; 60°C, 1 min; 72°C, 1 min). The amplified fragment was cloned into T-vector prepared from pBluescript SK vector by the method of Papp *et al*. (1995), sequenced, and analyzed on agarose gel electrophoresis. The cDNA library was screened with the amplified fragment as a probe.

Expression of *A. viridis* RIP cDNA in *E. coli* For expression of RIP cDNA, *E. coli* expression vector, pQE30 (Qiagen, USA) was used. The fragment containing full-length or putative signal sequence deleted version of partial length ORF of RIP cDNA was generated by PCR. The sequences of 5′-primer were 5′-AAAGGATCCATAATGCTTATTATCATGA-3′ for full length clone and 5′-AAGGATCCCAACAATATCGTACGGTGG-3′ for partial length clone, and that of 3′-primer was 5′-AAAGAGCTCGCGTAAAATTTAGTACTTG-3′ for both clones. The amplified fragment was digested with *Bam*HI and *Sac*I (underlined parts of above primers), and subcloned into the corresponding sites of pQE30.

The expression vector was transformed into *E. coli* strain M15. A single colony harboring the recombinant plasmid was used for expression. The overnight culture was then inoculated into 50 ml of LB containing 100 µg/ml ampicillin and 25 µg/ml kanamycin. 0.1 M IPTG was added to the culture at 0.3 of A_{600}. *E. coli* growth was monitered by measuring A_{600}.

***In vitro* translation inhibition assay** Rabbit reticulocyte lysate (Promega, USA) was used for assaying the *in vitro* translation inhibition activity by recombinant RIP expressed in *E. coli*. Brome mosaic virus (BMV) RNA, [^{35}S] methionine (1,000 Ci/mmole), and an equal amount of protein from *E. coli* crude extract were used. *E. coli* crude extract was obtained by sonication of harvested cells and fractionated to soluble and insoluble protein by centrifugation at 5,000 rpm and 15,000 rpm, respectively. The synthesized peptides were collected by the method given by manufacturer, and the incorporation rate of [^{35}S] methionine to peptides was measured by scintillation counting (Beckman LL6600).

Results and Discussion

Isolation of partial RIP cDNAs To clone genes encoding RIP from *A. viridis*, PCR was performed from *A. viridis* leaf cDNA library, using the SK primer of pBluescript vector and the degenerate primer designed for the conserved active site of RIPs as described in Materials and Methods. 700 bp PCR fragments were obtained. The deduced amino acid sequence of the amplified fragments had low homology compared with other RIPs. This is expected because it is well known that there is low homology among the RIPs except at the active site (Stirpe, 1992). However, the amino acid sequence (AIQMVSEAAR) in the putative active site region is well conserved.

From this approach, it is possible to use the above primer set to isolate genes encoding the other RIPs in the other plants.

Screening of *A. viridis* leaf cDNA library using the amplified PCR fragment 26 cDNA clones were obtained from the leaf cDNA library by screening with the above amplified PCR fragment. One of the cDNA clones had 1,047 bp cDNA corresponding to the PCR product which contained an ORF encoding 270 amino acids (30,442 Da). The entire sequence and deduced amino acid sequence of *A. viridis* RIP cDNA are shown in Fig. 1. A

```
gaagaaggtt ttaggattat taggaacatg ggtttggtgg tgcATGATAA   50
                                               M  I
TGCTTATTAT CATGATCACT ACCGTAGTTA AGCAATCTGA AGCACAACAA  100
M  L  I  I   M  I  T   T  V  V   K  Q  S  E   A▲Q  Q
TATCGTACGG TGGGATTCGA ATTACATAAA GAGAATTCAC CAAATGGGTA  150
 Y  R  T   V  G  F  E   L  H  K   E  N  S   P  N  G  Y
CGCAAATTTC TTGAGAAGGT TGCGCAGTGC CAGAGAATAT GTCTACATAA  200
  A  N  F   L  R  R   L  R  S  A   V  S  G   P  T  R
GGCTACAATT TAGCGACACA CAATGGGTAG TACTAGGAAT AGTCTCAGGT  250
A  C  N  L   N  I  T   Q  S  N   P  P  I  D   R  E  Y
CCCACAAGAG CATGTAATTT AAACATTACA CAAAGTAATC CACCAATAGA  300
 V  Y  I   R  L  Q  F   S  D  T   Q  W  V   V  L  G  M
GGCTGCCAAA GATATGTATA TTTGGGGTTA TGTTGACAAT AGGCCAGGCT  350
  A  A  K   D  M  Y   I  W  G  Y   V  D  N   R  P  G
TCGGACCAGG CCAACCACCT GAGTCAAACT TCCTAATGGA TTCTCCGCCA  400
F  G  P  G   Q  P  P   E  S  N   F  L  M  D   S  P  P
GAAGCACGAC AACGTCTTTT CCCAGGTTCT AACAGAAGAA TAACAGATTA  450
 E  A  R   Q  R  L  F   P  G  S   N  R  R   I  T  D  Y
CGGAGGAAAT TACAACAGTC TTCAACAAAG AGCACAAAGG AATCGAGATA  500
  G  G  N   Y  N  S   L  Q  Q  R   A  Q  R   N  R  D
ATGTTCCATT GGGACTGACA AGCCTAGACG GCGCACTTAA AAGCGTGTAT  550
N  V  P  L   G  L  T   S  L  D   G  A  L  K   S  V  Y
GGAAAATCAA CTTCACAATT GAATGAAGGG AATGCAGAAG CAAGATTTTT  600
 G  K  S   T  S  Q  L   N  E  G   N  A  E   A  R  F  F
TCTTACGGCC ATCCAAATGG TTGCAGAAGC AGCACGTTTT AAGTACATCG  650
  L  T  A   I  Q  M   V  A  E  A   A  R  F   K  Y  I
AGAGAGGGAT ATCAGCACCA CCAGCTAATT TTAGACAAAA TATGATAGCT  700
E  R  G  I   S  A  P   P  A  N   F  R  Q  N   M  I  A
TTCCAAAATG GTTGGGCTAG AATCTCTACT CTTATACATA ATGCAGAAGG  750
 F  Q  N   G  W  A  R   I  S  T   L  I  H   N  A  E  G
GGCTACTCCT AAGTGTCAGG CTTTTCCTCA GCCTCTTCGT ATTGGTACCC  800
  A  T  P   K  C  Q   A  F  P  Q   P  L  R   I  G  T
TTACGTATGG AAATGTCAAT GAGATAAGGA ATGAGATTGG AATTATCAAG  850
L  T  Y  G   N  V  N   E  I  R   N  E  I  G   I  I  K
TACTAAattt tacgctatcc tagataatgc aaccagacat caaatgtata  900
 Y  *
cattaatgat cgtcgtttat gtatAATAAA tgttgtatgc atgcatgtgt  950

atcgttgttg atataaagat tagtatatgg ttacaataat aataataata 1000

ataataataa taactgaaat agtttaataa aaaaaaaaaa aaaaaaa    1047
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Fig. 1. Nucleotide and deduced amino acid sequence of *A. viridis* RIP cDNA clone. The polyadenylation signal (AATAAA) is found in the 3′ untranslated region. A potential cleavage site of signal sequence predicted by Prosite Search is indicated by an arrowhead. Highly conserved putative active site is written in bold letters. Used primer sequences are underlined. This sequence has been submitted to the GenBank/EMBL Data Bank with accession number U85225.

conserved active site (AIQMVAEAARFKYI) (Fig. 2), and other amino acids known to play key roles in the tertiary structure of RIPs (Tyr-69, Trp-224) were also found (Huang *et al*., 1995). The sequence also shows a potential cleavage site of signal sequence predicted by Prosite search. The amino acid sequence of *A. viridis* RIP cDNA was compared to RIPs from other plants by using Clustal W (Thompson *et al*., 1994). The MAP had the highest degree of identity (31%) with *A. viridis* RIP while a-PAP (Kataoka *et al*., 1992), PAP II (Poyet *et al*., 1994) and Dianthin30 (Legname *et al*., 1991) had 25% identity with *A. viridis* RIP. A dendrogram displaying phylogenetic relationship (Fig. 3) showed that *A. viridis* RIP and MAP can be grouped together.

Expression of *A. viridis* RIP cDNA in *E. coli* The total soluble protein and insoluble protein of *E. coli* producing *A. viridis* RIP was fractionated by centrifugation, and added to the *in vitro* translation system (Promega, USA). From the soluble fraction, the induced protein could not be detected as a visible band in SDS-PAGE of *E. coli* total soluble protein (data not shown), but the induced protein bands were clearly detected from the total insoluble protein fraction (Fig. 4A). The sizes of induced protein bands were

```
 (1)  ------------MIMLIIMITTVVK----QSEAQQYR------------TVGFELHKENSP
 (2)  MK--------RFTVL-------ILA----IFVAASTV---EA--------DVRFSLSGSSS-
 (3)  MI--------RFLVLSLL----ILT----LFLTTPAV---EG--------DVSFRLSGATS-
 (4)  MV--------KCLLLSFL----IIA----IFIGVPTA---KG--------DVNFDLSTATA-
 (5)  MK--------IYLVAAIA---WILF----Q-SSSWTT-DAAT-------AYTLNLANPSA-
 (6)  MK--------IYVVATIA---WILL----Q-FSAWTTTDAVT-------SITLDLVNPTA-
 (7)  MK--------SMLVVTIS--IWLIL----APTSTWAV----N-------TIIYNVGSTTI-
 (8)  MK--------MMVVVVVMMLSWLIL----KPPSTWAI----N-------TITFDVGNATI-
 (9)  MA--------AKMAKN---------------VDKPLF------------TATFNVQASS--
(10)  ------------MAKN---------------VDKPLF------------TATFNVQASS--
(11)  MK--------MKVLEVVG-LAISIW----LMLTPPAS----S-------NIVFDVENATP-
(12)  ---------------------------------MEDR------------PIKFSTEGATS-
(13)  -----------------------------MVPKQYP------------IINFTTAGATV-
(14)  MKGNMKVYWIKIAVATWFCCTTIVLGSTARIFSLPTNDEEETSKTLGLDTVSFSTKGATY-
(15)  -----------MLTTTKVFFLLLTTWITWYAIVNPQS--RAAPT--LETIASLDLNNPT--
(16)  ----------MAEITLEPSDLMAQT----NKRIVPKF------------TEIFPVEDAN--

      NGYANFLRRLRSAVSGP-TRACN---LNTQSNP--PIDREIYVYIRLQFSDTQWVVLGMAA
      TSYSKFIGDLRKALPSN-GTVYN---ITLLLSSA--SGASRYTLMTLSNYDGKAITVAVDV
      SSYGVFISNLRKALPNE-RKLYD---IPLLRSSL--PGSQRYALIHLTNYADETISVAIDV
      KTYTKFIEDFRATLPFS-HKVYD---IPLLYSTI--SDSRRFILLDLTSYAYETISVAIDV
      SQYSSFLDQIRNNVRDT-SLIYGGTDVEIGAPS--TT-DKVFLRLNFQGP-RGTVSLGLRR
      GQYSSFVDKIRNNVKDP-NLKYGGTDIAVIGPPS--K--EKFLRINFQSS-RGTVSLGLKR
      SKYATFLNDLRNEAKDP-SLKCYG--IPMLPNTN--TN-PKYVLVELQGSNKKTITLMLRR
      NKYATFMKSIHNQAKDP-TLKCYG--IPMLPNTN--LT-PKYLLVTLQDSSLKTITLMLKR
      ADYATFIAGIRNKLRNPAHFSHNRPVLPPVEPNV--PPSRWFHVVLKASPTSAGLTLAIRA
      ADYVTFINGIRNKLRNPGHSSHNRPVLPPIEPNV--PPSRWFHIVLKTSPASTGLTLATRA
      ETYSNFLTSLREAVKDK-KLTCHG---MIMATTL--TEQPKYVLVDLKFG-SGTFTLAIRR
      QSYKQFIEALRERLRGG--LIHDIP-VLPDPTTL--QERNRYITVELSNSDTESIEVGIDV
      QSYTNFIRAVRGRLTTGADVRHE---IPVLPNRVGLPINQRFILVELSNHAELSVTLALDV
      ITYVNFLNELRVKLKPE-GNSHG---IPLLRKKCDDPG-KCFVLVALSNDNGQLAEIAIDV
      -TYLSFITNIRTKVADK-TEQCT---IQKISKTFT----QRYSYIDLIVSSTQKITLAIDM
      YPYSAFIASVRKDVIKH-CTDHKGIFQPVLPPEK--KVPELWFYTELKTR-TSSITLAIRM

      KDMYIWGYVDNRPGFGPGQPPESNFLMDSPP--EARQ---RLFPGSN----------RRIT
      TNVYIMGYLVNSTS--------YFFNESDAK--LASQ---YVFKGST----------IVTL
      TNVYIMGYRAGDTS--------YFFNEASAT--EAAK---YVFKDAMR---------KVTL
      TNVYVVAYRTRDVS--------YFFKES-PP--EAYN---ILFKGTR----------KITL
      ENLYVVAYLAMDNAN---VNRAYYFKNQITS--AELT---ALFPEVVVANQKQ---LEYGE
      DNLYVVAYLAMDNTN---VNRAYYFRSEITS--AEST---ALFPEATTANQKA---LE YT
      NNLYVMGYSDPFETN---KCRYHIFNDISG---TERQ---DVETTLCPNANSR---VSKNI
      NNLYVMGYADTYNG----KCRYHIFKDISNT--TERN---DVMTTLCPNPSSR---VGKNI
      DNIYLEGFKSSDGT-------WWELTPGLIP--GATY---VGFGGTYR------------D
      DNLYWEGFKSSDGT--------WWELTPGLIP—GATH---VGFGGTYR------------D
      GNLYLEGYSDIYNG---KCRYRIFKDSESD--AQET---VCPGDKSKPG-------TQNNI
      TNAYVVAYRAGTQS---------YFLRDAPS--SASD---YLFTGTD----------QHSL
      TNAYVVGYRAGNSA--------YFFHPDNQEDAEAIT---HLFTDVQN---------RYTF
      TSVYVVGYQVRNRS--------YFFKDAPD---AAYE---GLFKNTIK---------TR-L
      ADLYVLGYSDIANN----KGRAFFFKDVTEA---VAN---NFFPGATGTN-------RIKL
      DNLYLVGFRTPGGV-------WWEFGKDGDTHLLGDNPRWLGFGGRYQDLIGNKGLETVTM

      DYGGNYNSLQQRAQ-RNRDNVPLGLTSLDGALKSVYGKSTSQLNEG-NAEARFFLTAIQMV
      PYSGNYEKLQTAAG-KIREKIPLGFPALDSAITTLF---HYDST----AAAAAFLVIIQTT
      PYSGNYERLQTAAG-KIRENIPLGLPALDSAITTLF---YYNAN----SAASALMVLIQST
      PYTGNYENLQTAAH-KIRENIDLGLPALSSAITTLF---YYNAQ----SAPSALLVLIQTT
      DYQAIEKNAKITTGDQSRKELGLGINLLITMIDGVN-KKVRVVK----DEARFLLIAIQMT
      EDYQSIEKNAQITQGDQSRKEGLGIDLLSTSMEAVN-KKARVVK----DEARFLLIAIQMT
      NFDSRYPTLESKAGVKSRSQVQLGIQILDSNIGKISGVMSFTEK----TEAEFLLVAIQMV
      NYDSSYPALEKKVG-RPRSQVQLGIQILNSGIGKIYGVDSFTEK----TEAEFLLVAIQMV
      LLGDTDKLTNVALG---RQQLADAVTALHGRTKADK-PSGPKQQ----QAREAVTTLLLMV
      LLGDTDKLTNVALG---RQQMADAVTALYGRTKADK-TSGPKQQ----QAREAVTTLLLMV
      PYEKSYKGMESKGG--ARTKLGLGKITLKSRMGKIYGKDATDQKQYQKNEAEFLLIAVQMV
      PFYGTYGDLERWAH-QSRQQIPLGLQALTHGISFFRSGGNDNEE-----KARTLIVIIQMV
      AFGGNYDRLEQLAG-NLRENIELGNGPLEEAISALYYYSTGGTQLP--TLARSFIICIQMI
      HFGGSYPSLEGEKA--YRETTDLGIEPLRIGIKKLDENAIDNYKP--TEIASSLLVVIQMV
      TFTGSYGDLEKNGG--LRKDNPLGIFRLENSIVNIYGKAGDVKK-----QAKFFLLAIQMV
      GRAEMTRAVNDLAKKKKMATLEEEEVKMQMQMPEAADLAAAAAADPQADTKSKLVKLVVMV

      AEAARFKYIERGISAPPAN---FRQN--MIAFQNG----WARISTLIHNAEG---ATPKCQ
      AEASRFKYIEGQIIERISKN--QVPSLATISLENE----WSALSKQIQLAQTNN-GTFKTP
      SEAARYKFIEQQIGKRVDKT--FLPSLAIISLENS----WSALSKQIQIASTNN-GQFETP
      AEAARFKYIERHVAKYVATN--FKPNLAIISLENQ----WSALSKQIFLAQNQG-GKFRNP
      AEAARFRYIQNLVTKNFPNK--FDSENKVIQFQVS----WSKISTAIFGDCKN--GVFNKD
      AEAARFRYIQNLVIKNFPNK--FNSENKVIQFEVN----WKKISTAIYGDAKN--GVFNKD
      SEAARFKYIENQVKTNFNRA--FNPNPKVLNLQET----WGKISTAIH-DAKN--GVLPKP
      SEAARFKYIENQVKTNFNRA--FYPNAKVLNLEES----WGKISTAIH-NAKN--GALTSP
      NEATRFQTVSGFVAGLLHPKAVEKKSGKIGNEMKAQVNGWQDLSAALLKTDVKP-PPGKSP
      HEATRFQTVSGFVAGVLHPK--EKKSGKIGNEMKAQVNGWQDLSEALLKTDANA-PPGKAP
      TEASRFKYIENKVKAKFDDANGYQPDPKAISLEKN----WDSVSKVIAKVGTSGDSTVTLP
      AEAARFRYISNRVRVSIQTGTAFQPDAAMISLENN----WDNLSRGVQESVQD---TFPNQ
      SEAARFQYIEGEMRTRIRYNRRSAPDPSVITLENS----WGRLSTAIQESNQ---GAFASP
      SEAARFTFIENQIRNNFQQR--IRPANNTISLENK----WGKLSFQIRTSGAN--GMFSEA
      SEAARFKYISDKIPSEKYEE--VTVDEYMTALENN----WAKLSTAVYNSKPSTTTATKCQ
      CEGLRFNTVSRTVDAGFNSQHGVTLTVTQGKQVQK----WDRISKAAFEWAD-------HP

      AFPQPLRIGTLTYGNVNEIRN-EIGIIKY--------------------------------
      VVITDDKGQRVEITNVTSKVVT-KNIQLLLNYKQNVAAFDEDVSAKH-------------
      VVLINAQNQRVMITNVDAGVVT-SNIALLLN-RNNMAAMDDDVPMTQSFGCGSYAI-----
      VDLIKPTGERFQVTNVDSDVVK-GNIKLLLN--SRASTADENFITTMTLLGESVVN-----
      YDFG-----FGKVRQAKDLQ---MGLLKYLG-RPKSSSIEANSTDDTADVL----------
      YDFG-----FGKVRQVKDLQ---MGLLMYLG-KPKSS-NEANS------------------
      LELVDASGAKWIVLRVDEIKPD-VALLNYVG-GSCQTTYNQNAMFPQLIMSTYYNYMVNLG
      LELKNANGSKWIVLRVDDIEPD-VGLLKYVN-GTCQATY-QSAMFPHL-------------
      AKFAP--IEKMGVRTAVQAANT-LGILLFVE-VPGGLTVAKALELFHASGGK---------
      AKFTP--IEKMGVRTAEQAAAT-LGILLFVQ-VPGGMTVAQALELFHKSGGK---------
      GDLKDENNKPWTTATMNDLKNDIMALLTHVTCKVKSSMFPEIMSYYYRTSISNLGEFE---
      VTLTNIRNEPVIVDSLSHPTVAVLALMLFVCNPPN--------------------------
      IQLQRRNGSKFSVYDVSILIP-IIALMVYRCAPPPSSQF---------------------
      VELERANGKKYYVTAVDQVKPK-IALLKFVDKDPKTSLAAELIIQNYESLVGFD-------
      LATSPVTISPWIFKTVEEIKLV-MGLLKSS-------------------------------
      TAVIP-DMQKLGIKDKNEAAR----IVALVKNQTTAAAATAASADNDDDEA----------

      ------ 270
      ------ 277
      ------ 289
      ------ 286
      ------ 293
      ------ 284
      DLFEGF 313
      ------ 294
      ------ 281
      ------ 275
      ------ 310
      ------ 252
      ------ 267
      ------ 316
      ------ 278
      ------ 301
```

Fig. 2. Comparison of the deduced amino acid sequence of *A. viridis* RIP with various RIPs. Sequence alignment was carried out using the program Clustal W. The shaded amino acids are identical in all sixteen RIPs, and there are identical amino acid sequences with *A. viridis* RIP within the conserved putative active site (Ala_{189}–Glu_{203}). (1) *A. viridis* RIP (this study); (2) α-luffin (Kataoka *et al*., 1992b); (3) TRHTCS, tricosanthin (Chow *et al*., 1990); (4) Momordin (Ortigao and Better, 1992); (5) Dianthin30 (Legname *et al*., 1991); (6) Saporin6 (Benatti *et al*., 1989); (7) PAPAP (Lin *et al*., 1991); (8) α-PAP (Kataoka *et al*., 1992a); (9) Barley (Leah *et al*., 1991); (10) Tritin (Habuka *et al*., 1993); (11) PAP2 (Poyet *et al*., 1994); (12) Abrin (Hung *et al*., 1994); (13) Ricin (Halling *et al*., 1985); (14) Gleonin (Nolan *et al*., 1993); (15) MAP (Kataoka *et al*., 1991); (16) Maize (Walsh *et al*., 1991).

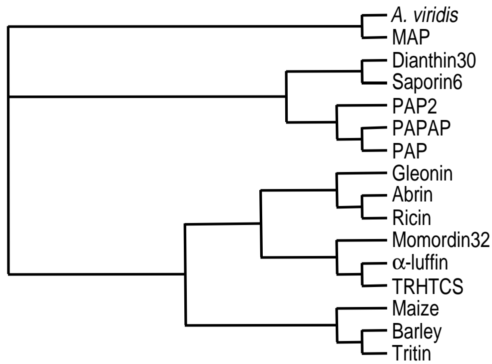

Fig. 3. Phylogenetic relationship of sixteen RIPs. Amino acid sequences in this figure are the same as in Fig. 2.

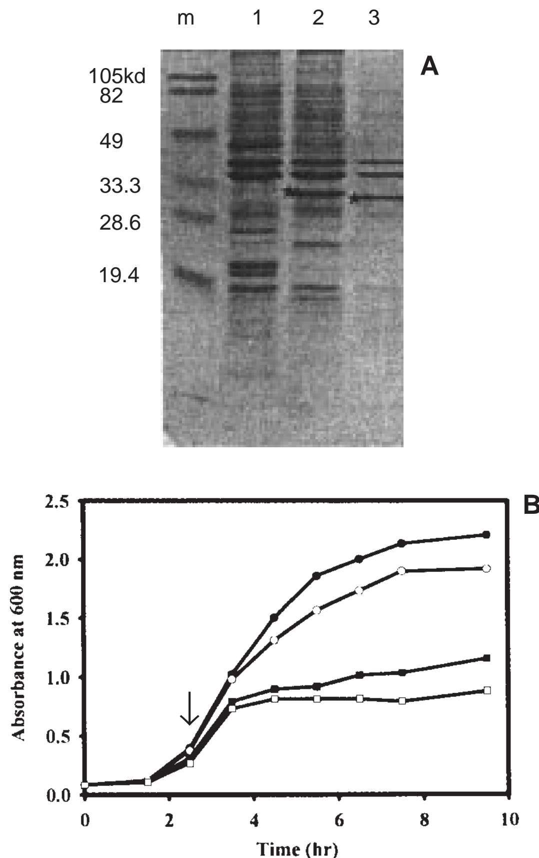

Fig. 4. Expression of *A. viridis* RIP cDNA in *E. coli* M15 using the pQE30 expression vector. **A.** SDS-PAGE analysis of *E. coli* M15 cell extract carrying pQE30, pQE30:full, and pQE:mat. m, molecular size marker; 1, *E. coli* M15 containing pQE30; 2, *E. coli* M15 containing pQE:full; 3, *E. coli* M15 containing pQE:mat. **B.** Growth curves of *E. coli* cells expressing the full-length or truncated *A. viridis* RIP cDNA. *E. coli* was grown in LB media containing ampicillin (100 μg/ml) and kanamycin (25 μg/ml) and optical density at 600 nm was measured. ●–● *E. coli* M15 containing pQE30; ○–○ pQE30 only, induced by IPTG; ■–■ pQE30: full, induced by IPTG; □–□ pQE30: mat (*A. viridis* RIP cDNA lacking putative signal sequence), induced by IPTG. Arrow indicates the time of induction.

about 30 kDa, and 17 amino acid differences between proteins from full length and truncated *A. viridis* RIP cDNA were noticed. The growth of *E. coli* that had full-length (pQE-full) or truncated (pQE-mat) *A. viridis* RIP cDNA lacking a putative signal peptide sequence was checked by measuring the A_{600} through the culture period. When the expression of RIPs was induced by IPTG, the growth of *E. coli* harboring the RIP cDNA was retarded by about 50% as compared to the control (harboring the pQE only) (Fig. 4B). This growth inhibition seems to be due to the result of partial depurination of bacterial ribosomes (Kataoka *et al*., 1993). In the case of pQE-full, the growth of *E. coli* was less inhibited than that of pQE-mat. The amino acid sequence analysis indicated that the *A. viridis* RIP had a putative signal sequence, as shown in Fig. 1. It seems that the expressed protein with putative signal sequence was targeted to periplasm, and could not depurinate the *E. coli* ribosomes very well. On the other hand, the truncated form of *A. viridis* RIP missing the signal peptide expressed in *E. coli* should have had more chances to depurinate host ribosomes than the full-length type, and resulted in more growth inhibition.

Recombinant *A. viridis* RIP had translation inhibition activity Experiments were carried out to find out whether the growth inhibition of *E. coli* was the result of translation inhibition activity of expressed *A. viridis* RIP. When the same amounts of total soluble protein (10 μg) of *E. coli* M15 expressing *A. viridis* RIP were added to the *in vitro* translation system, *in vitro* translation was inhibited by 66.9% (pQE-full) and 85.2% (pQE-mat), respectively (Table 1). But the insoluble protein had no inhibitory effects on *in vitro* translation. This result indicates that *A.*

Table 1. *In vitro* translation inhibition activity of *A. viridis* RIP expressed in *E. coli*.

	Protein	% Inhibition
Positive control		100.0
Soluble protein	pQE self[a]	48.9
	pQE:full[b]	66.9
	pQE:mat[c]	85.2
Insoluble protein	pQE self[a]	3.0
	pQE:full[b]	9.5
	pQE:mat[c]	2.5
In vitro translated protein[d]		91.7

Ten μg of proteins from soluble and insoluble fraction were added to reaction.
[a] pQE self, pQE30 was transformed to M15 cell.
[b] pQE: full, full length cDNA was translationally fused to pQE30.
[c] pQE: mat, putative mature protein was translationally fused to pQE30.
[d] *in vitro* translated protein, *in vitro* translation products of cDNA clone (1 μl of 50 μl reaction) was added to assay.

viridis RIP produced in *E. coli* M15 cells harboring each plasmid was correctly processed to an active form, and it had translation inhibition activity. In addition, the truncated form of *A. viridis* RIP was more active than its precursor form in inhibiting *in vitro* translation, indicating that the correct removal of the putative signal sequence of *A. viridis* RIP has an important role in the activities of *A. viridis* RIP.

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References

Benatti, L., Saccardo, M. B., Dani, M., Nitti, G. P., Sassano, M., Lorenzetti, R., Lappi D. A., and Soria, M. (1989) Nucleotide sequence of cDNA coding for saporin-6, a type I ribosome-inactivating protein from *Saponaria officinalis*. *Eur. J. Biochem.* **183**, 465–470.

Chow, T. P., Feldman, R. A., Lovett, M., and Piatak, M. (1990) Isolation and DNA sequence of a gene encoding α-trichosanthin, a type I ribosome-inactivating protein. *J. Biol. Chem.* **265**, 8670–8674.

Endo, Y. and Tsurugi, K. (1987) RNA *N*-glycosidase activity of ricin A-chain. Mechanism of action of the toxic lectin ricin on eukaryotic ribosomes. *J. Biol. Chem.* **262**, 8128–8130.

Endo, Y., Mitsui, K., Motizuki, M., and Tsurugi, K. (1987) The mechanism of action of ricin and related toxic lectins on eukaryotic ribosomes. The site and the characteristics of the modification in 28S ribosomal RNA caused by the toxins. *J. Biol. Chem.* **262**, 5908–5912.

Habuka, N., Kataoka, J., Miyano, M., Tsuge, H., Ago, H., and Noma, M. (1993) Nucleotide sequence of a genomic gene encoding tritin, a ribosome-inactivating protein from *Triticum aestivum*. *Plant Mol. Biol.* **22**, 171–176.

Halling, K. C., Halling, A. C., Murray, E. E., Ladin, B. F., Houston L. L., and Weaver, R. F. (1985) Genomic cloning and characterization of a ricin gene from *Ricinus communis*. *Nucleic Acid Res.* **13**, 8019–8033.

Huang, Q., Liu, S., Tang, Y., Jin, S., and Wang, Y. (1995) Studies on crystal structures, active-centre geometry and depurinating mechanism of two ribosome-inactivating proteins. *Biochem. J.* **309**, 285–298.

Hung, C. H., Lee, M. C., Chen, J. K., and Law J. Y. (1994) Cloning and expression of abrin A-chains and their mutants derived by site-specific mutagenesis in *Escherichia coli*. *Eur. J. Biochem.* **219**, 83–87.

Kataoka, J., Habuka, N., Furuno, M., Miyano, M., Takanami, Y., and Koiwai, A. J. (1991) DNA sequence of *Mirabilis* antiviral protein (MAP), a ribosome-inactivating protein with an antiviral property, from *Mirabilis jalapa* L. and its expression in *Escherichia coli*. *J. Biol. Chem.* **266**, 8426–8430.

Kataoka, J., Habuka, N., Masuta, C., Miyano, M., and Koiwai, A. (1992a) Isolation and analysis of a genomic clone encoding a pokeweed antiviral protein. *Plant Mol. Biol*. **20**, 879–886.

Kataoka, J., Habuka, N., Masuta, V., Miyano, M., Masuta, C., and Koiwai, A. (1992b) Nucleotide sequence of cDNA encoding α-luffin, a ribosome-inactivating protein from *Luffa cylindrica*. *Plant Mol. Biol*. **18**, 1199–1992.

Kataoka, J., Ago, H., Habuka, N., Furuno, M., Masuta, C., Miyano, M., and Koiwai, A. (1993) Expression of pokeweed antiviral protein in *Escherichia coli* and its characterization. *FEBS Lett.* **320**, 31–34.

Kwon, S.-Y., An, C. S., Liu, J. R., and Paek, K.-H. (1997) A ribosome-inactivating protein from *Amaranthus viridis*. *Biosci. Biotech. Biochem.* **61**, 1613–1614.

Leah, R., Tommerup, H., Svendsen, I., and Mundy, J. (1991) Biochemical and molecular characterization of three barley seed proteins with antifungal properties. *J. Biol. Chem.* **266**, 1564–1573.

Legname, G., Bellosta, P., Gromo, G., Modena, D., Keen, J. N., Roberts, L. M., and Lord, J. M. (1991) Nucleotide sequence of cDNA coding for dianthin 30, a ribosome inactivating protein from *Dianthus caryophyllus*. *Biochim. Biophys. Acta* **1090**, 119–122.

Lin, Q., Chen, Z. C., Antoniw, J. F., and White, R. F. (1991) Isolation and characterization of a cDNA clone encoding the anti-viral protein from *Phytolacca americana*. *Plant Mol. Biol.* **17**, 609–614.

Lodge, J. K., Kaniewski, W. K., and Tumer, N. E. (1993) Broad-spectrum virus resistance in the transgenic plants expressing pokeweed antiviral protein. *Proc. Natl. Acad. Sci. USA* **90**, 7089–7093.

Mehta, A. D. and Boston, R. S. (1988) Ribosome-inactivating proteins; in *A Look beyond Transcription: Mechanisms Determining mRNA Stability and Translation in Plants*, Bailey-Serres, J. and Gallie, D. R. (eds.), pp. 145–152, American Society of Plant Physiologists, Rockville, MD.

Moon, Y. H., Song, S. K., Choi, K. W., and Lee, J. S. (1997) Expression of a cDNA encoding *Phytolacca insularis* antiviral protein confers virus resistance on transgenic potato plants. *Mol. Cells* **7**, 807–815.

Nolan, P., Garrison D. A., and Better, M. (1993) Cloning and expression of a gene encoding gelonin, a ribosome-inactivating protein from *Gelonium multiflorum*. *Gene* **134**, 223–227.

Ortigao, M. and Better, M. (1992) Momordin II, a ribosome inactivating protein from *Momordica balsamina*, is homologous to other plant proteins. *Nucleic Acids Res.* **20**, 4662.

Papp, T., Kirchner, S., Diener, U., Jafari, M., Golka, A., and Schiffmann, D. (1995) Cloning of PCR fragments with modified M13mp18 T-vector. *Trends Genet.* **11**, 169.

Poyet, J.-L., Radom, J., and Hoeveler, A. (1994) Isolation and characterization of a cDNA clone encoding the pokeweed antiviral protein II from *Phytolacca americana* and its expression in *E. coli*. *FEBS Lett.* **347**, 268–272.

Stirpe, F. and Babieri, L. (1986) Ribosome-inactivating proteins up to date. *FEBS Lett*. **195**, 1–8.

Stirpe, F., Barbieri, L., Battelli, M. G., Soria, M., and Lappi, D. A. (1992) Ribosome-inactivating proteins from plants: present status and future prospects. *Bio/Technology* **10**, 405–412.

Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) Clustal w: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acid Res.* **22**, 4673–4680.

Walsh, T. A., Morgan, A. E., and Hey, T. D. (1991) Characterization and molecular cloning of a proenzyme form of a ribosome-inactivating protein from maize. *J. Biol. Chem.* **266**, 23422–23427.

Zoubenko, O., Uckun, F., Hur., Y., Chet, I., and Tumer, N. E. (1997) Plant resistance to fungal infection, induced by nontoxic pokeweed antiviral protein mutants. *Nat. Biotechnol*. **15**, 992–996.