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Expression of *in vitro*-tested ribozyme against cucumber mosaic virus RNA in tobacco plant

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Abstract : *In vitro*-tested ribozyme against synthesized cucumber mosaic virus (CMV) RNA (Agric. Chem. & Biotech. 37:56-63(1994)) was expressed in tobacco plant to develop virus resistant plants. The ribozyme sequence was linked to cauliflower mosaic virus 35S promoter and nopaline synthase(nos) terminator and this chimeric 35S-ribozyme-nos gene was sequenced. The sequenced chimeric gene was transferred to *Agrobacterium tumefaciens* LBA4404 using tri-parental mating system. The *E. coli* HB101 containing chimeric gene was incubated with *E. coli* HB101(pRK2073) as a helper and *Agrobacterium tumefaciens* LBA4404. Then *Agrobacterium* cells containing the ribozyme construct was cocultivated with tobacco leaf pieces. Ten different plants were regenerated from kanamycin containing MS medium. The presence of the ribozyme construct in the transgenic tobacco plants was confirmed by polymerase chain reaction (PCR). Seven different transgenic plants in ten different kanamycin resistant plants showed the expected size (570 base pairs) of 35S-ribozyme-nos gene fragment. Total RNAs were isolated from four different transgenic plants and separated on a 1% agarose gel containing formamide. Northern hybridization with 35S-ribozyme-nos gene fragment as a probe indicated that ribozyme transcripts may be degraded by nuclease. Therefore, nuclease-resistant ribozymes are needed for the development of virus-resistant transgenic plants using ribozymes.
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Introduction

Molecular biolgical techniques have demonstrated near phenomenal success in dissecting and understanding the organization and structure of genomes and coding of regulatory nucleic acid sequences, as well as the physiology of cellular processes. Developments in the use of sequence-specific anti-RNA technologies offer the possibility of furthering this evolution quite dramatically.

It is already well established that anti-RNA technology functions successfully in vitro, in the laboratory. Ultimately, this technology is being incorporated into medical, cellular, and molecular research programs focusing on in vivo applications. Potentially, these applications range from treatment for viral injections in plants or animals, down-regulation of endogenous genes to alter the stoichiometric balance of key enzymes in biochemical pathways. For these reasons it is imperative that anti-RNA technologies be developed and evaluated in *in vivo* eukaryotic systems.

Sequence-specific anti-RNA regulation of gene expression began with the use of anti-sense RNA and has been evolved to the use of ribozymes; enzymatic, autocatalytic oligoribonucleotides. The former interferes with translation by creation of a RNA:RNA duplex in a Watson-Crick manner to specific target RNAs. The actual mechanisms by which this inhibition occurs are much more complex than a single 'blockage' of translation.[1] The latter interferes by recognizing and cleaving the mRNA target, thus destroying the message.[2] Ribozymes differ in primary and proposed secondary structures and mechanisms of the reaction to cleave the phosphodiester bond of the target RNAs2). Five different forms of ribozymes, such as hammerhead, hairpin, group I intron, axhead, and RNase P, have been reported.[3]

A direct comparison of anti-sense RNA, anti-sense DNA, and a ribozyme was made *in vitro* using a nuclear extract from mammalian tissue culture cell.[4] In this study it was determined that a six-fold excess of anti-sense RNA was required to inhibit U7 small nuclear ribonucleoprotein-mediated histone pre-mRNA processing by 90%, while a 1000-fold excess of the U7-targeted ribozyme was required for complete inhibition. Similar level of ribozyme (1000-fold excess) was required to suppress chloramphenicol acetyl transferase (CAT) activity by 60% in electrophorated monkey cells.[5]

Key words: Hammerhead ribozyme, CMV, Transgenic plants

It is important to keep in mind however that inhibition by ribozymes is irreversible due to destruction of the target, whereas inhibition by anti-sense RNA is theoretically, reversible.

In addition, expression of a ribozyme gene in tobacco protoplasts resulted in cleavage of neomycin phosphotransferase (*npt*) gene and the reduction of NPT activity was explained by both hydrolytic cleavage of target gene and an antisense effect.[6] However, expression of the ribozyme to high molar excess over the β-glucuronidase (GUS) transcript did not lead to any significant decrease of GUS activity in the transformed *Arabidopsis* protoplasts.[7] These contradictory results were from transient expression of ribozyme genes in protoplast. Therefore, we expressed a hammerhead ribozyme, which has been shown to cleave synthesized CMV RNAs *in vitro*,[8] gene in tobacco plants to monitor ribozyme action in transgenic plants.

Materials and Methods

Agarose gel electrophoresis of DNAs, electroelution of DNA fragments, and cloning into appropriate of plasmid were carried out according to the published methods.[9] The sequences of clones were determined by the dideoxy chain-termination method[10] utilising Sequenase™. Sequencing gels contained 8% polyacrylamide, 7M urea and 0.5X TBE(45 mM Tris-borate, 1 mM EDTA). Following electrophoresis the gels were wraped with Clean Wrap® and subjected to autoradiography using X-ray film(Kodak).

Plasmid constructed in *E. coli* was transferred into *Agrobacterium tumefaciens* LBA4404 by the tri-parental mating method.[11] The *E. coli* HB101 containing an appropriate plasmid, helper of *E. coli* HB101:pRK2073 and recipient of *Agrobacterium* were mixed well on an MG/L[12] plate and incubated overnight at 28℃. After mating, a huge globe of the mating mix were streaked out on AB plate containing kanamycin and incubated 28℃ for 48 hrs. The selected colonies were restreaked for single colonies and incubated. The structure of the transferred plasmid was analyzed after mating back to *E. coli*.

Transformation of tobacco plant (*Nicotiana tabacum* cv. Xanthi) was carried out using co-cultivation method.[12] *Agrobacteium* cells carring both the helper Ti plasmid and the binary vector containing pRT-Ribo (See Results and Disscussion) were cocultivated with sterile leaf pieces for 2 days. The bacterial cells were washed away and transformed tobacco calli were selected on a Murashige-Skoog(MS) medium containing 3% sucrose, kanamycin(200 mg/*l*), cefotaxime(250 mg/*l*), and an appropriate amount of phytohormone (naphthaleneacetic acid at 2 mg/*l* and benzyladenine at 0.5 mg/*l* for callus induction: benzyladenine at 0.5 mg/*l* for shoot induction). Plants were regenerated on MS agar medium containing same concentration of sucrose and antibiotic but lacking the phytohormones. Regenerated plants were transferred to pots and grown in a greenhouse.

Tobacco leaf DNAs were isolated by the method of Doyle and Doyle.[13] Leaf tissue of 1.0 g was ground to a powder in liquid nitrogen in a chilled mortar and pestle. The powder was scraped into preheated extraction buffer (2%(w/v) CTAB(sigma), 1.4 M NaCl, 0.2%(v/v) 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl,(pH 8.0)) and incubated at 60℃ for 30 min with occasional swirling. After incubation, chloroform-isoamyl alcohol(24:1;v/v) were added and mixed thoroughly followed by centrifugation at 1,600×g. The supernatant was transfered to new tube and 2/3 volume of cold isopropanol was added in order to precipitate nucleic acids. The nucleic acids were collected with centrifugation and dried at room temperature. The pellet was resuspended in 1 m*l* TE(10 mM Tris-HCl(pH 7.4), 1 mM EDTA) and RNase was added to a final concentration of 10 ug/m*l* and incubated 30 min at 37℃. DNAs were precipitated with an addition of 2.5 M ammonium acetate(pH 7.7) and ethyl alcohol. The precipitated DNAs were dried on vacuum and resuspended in TE.

The DNA oligonucleotides were synthesized by DNA synthesizer(Applied Biosystem Inc., model 381A) using phosphoramidite chemistry. After deprotection the oligonucleotides were purified by oligonucleotide purification cartridge(ABI). Two different oligonucleotides were synthesized for the polymerase chain reaction(PCR) of the tobacco leaf DNAs: 5'-GTGAAGATAGTGGAAAAG-3'(18 mer), located at −209 to −226 relative to the transcription start site of cauliflower mosaic virus(CaMV) 35S promoter and 5'-TTATCCTAGTTTGCGCGC-3'(18 mer), located at the nopaline synthase(nos) terminator.[17] Both oligonucleotides were utilized for detection of 35S-ribozyme-nos construct in transgenic tobacco plants. The reaction conditions for PCR followed published methods.[14]

Isolation of RNAs from plant leaves and electrophoresis of RNAs on a formamide gel were carried out according to the published method.[16] Northern hybridization of the isolated RNAs with HindIII/EcoRI digest of the pBS-Ribo[11] was also carried out according to the published method.[16] Labelling of probe was carried out using a commercial kit from Amersham. Autoradiography was carried out with Kodak X-Omat films.

Results and Discussion

The endonucleolytic cleavage of synthesized cucumber mosaic virus RNA by hammerhead ribozyme has been shown *in vitro*.[8] This ribozyme sequence has been linked to 35S promoter and nos terminator. The

schematic diagram of cloning procedure for the chimeric gene is shown in the Fig. 1. The ribozyme sequence of plasmid, pBS-Ribo, was linked to BamHI/SacI digested-pBI221 to make pBI-Ribo. The chimeric 35S-ribozyme-nos gene of pBI-Ribo was sequenced using a primer, sequences located at −209 relative to transcription start site of 35S promoter. The autoradiogram of nucleotide sequence of the chimeric gene (Fig. 2) showed that the plasmid pBI-Ribo contains the right construct. Nucleotide sequences of HindIII/EcoRI fragment of the pBI-Ribo and transcripts of 35S-ribozyme-nos gene are shown in the Fig. 3. Size of transcript which will be synthesized in transgenic tobacco cells is 239 nucleotides (nt) including 38 nt, 22 nt, 18 nt, and 161 nt derived from ribozyme sequences, 35S promoter, restriction enzyme sites and nos terminator, respectively.

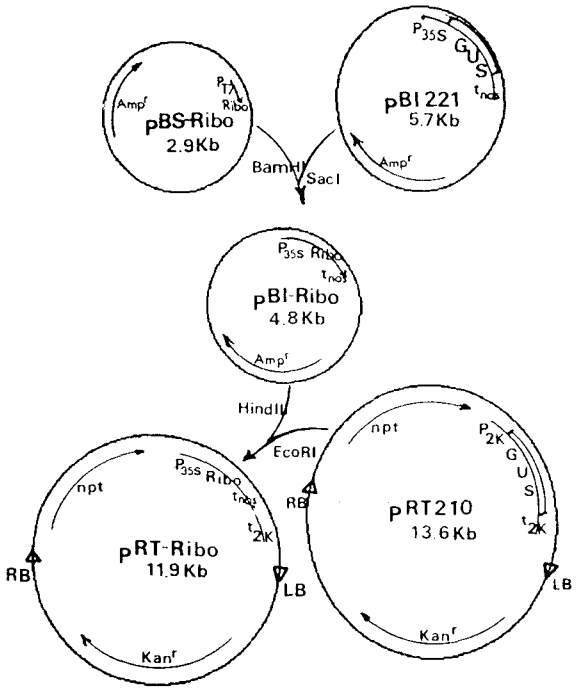

Fig. 1. Schematic diagram of cloning procedure for the chimeric gene. Plasmid pBS-Ribo was used for the *in vitro*-tested endonucleolytic cleaveage of synthesized CMV RNA.[8] P_{T7}: bacteriophage T7 promoter, P_{35S}: CaMV 35S promoter, t_{nos}: nos terminator, GUS: β-glucuronidase gene, Ribo: ribozyme sequence, P_{2K} and t_{2K}: potato proteinase inhibitor IIK promoter and terminator,[16] npt: neomycin phosphotransferase gene, RB and LB: right and left border of *Agrobacterium* Ti-plasmid.

The sequenced chimeric gene was transferred to *Agrobacterium tumefaciens* LBA4404 using tri-parental mating system. The *E. coli* HB101 containing chimeric gene was incubated with *E. coli* HB101(pRK2073) as a helper and *Agrobacterium tumefaciens* LBA4404. Then *Agrobacterium* cells containing the ribozyme construct was cocultivated with tobacco leaf. Ten different plants were regenerated from kanamycin (200 mg/*l*) containing MS medium. The presence of the ribozyme construct in the transgenic tobacco plants was confirmed by polymerase chain reaction (PCR). Two different primers were synthesized; 5'-GTGAAGATAGTGGAAAAG-3'(18 mer), located at −209 to −226 relative to the transcription start site of 35S promoter and 5'-TTATCCTAGTTTGCGCGC-3'(18 mer), located at the nopaline synthase(nos) terminator. Fig. 4 shows the agarose gel electrophoresis pattern of PCR products. Seven different transgenic plants in ten different kanamycin resistant plants showed the expected size (570 base pairs) of 35S-ribozyme-nos gene fragment and #2, #3, and #7 seems to contain more copies of 35S-ribozyme-nos chimeric DNA than other plants. Total RNAs were isolated from four different transgenic plants (#2, #3, #7, and #8) and Northern hybridizations were carried out with HindIII/EcoRI fragment of pBI-Ribo(See Fig. 1.) as a probe. The size of ribozyme RNA should be 239 nucleotide-long however level of transcribed RNAs for ribozyme was below than detection level(data not shown). This result indicates that ribozyme RNAs seems to be degraded by ribonuclease as soon as be synthesized.

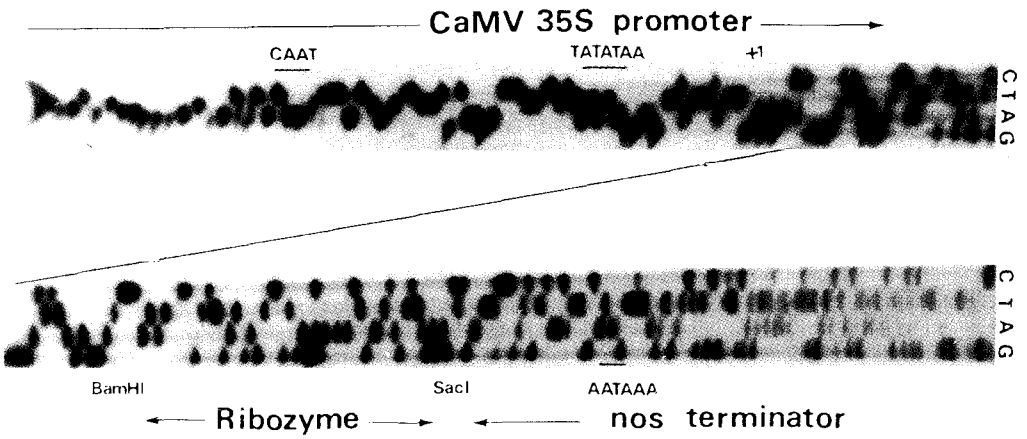

Fig. 2. Autoradiogram of nucleotide sequence of the 35S-ribozyme-nos chimeric gene. CAAT box, TATA box and poly A signal (AATAAA) are shown with capital letters. The cloning sites, BamHI and SacI, are also shown. +1 indicates the transcription intiation site.

(A)

HindIII
AAGCTTGCATGCCTGCAGTTAGAGAGGCTTACGCAGCAGGTCTCATCAAGACGATCTACCCGAGC

AATAATCTCCAGGAGATCAAATACCTTCCCAAGAAGGTTAAAGATGCAGTCAAAAGATTCAGGAC

TAACTGCATCAAGAACACAGAGAAAGATATATTTCTCAAGATCAGAAGTACTATTCCAGTATGGA

CGATTCAAGGCTTGCTTCACAAACCAAGGCAAGTAATAGAGATTGGAGTCTCTAAAAAGGTAGTT

CCCACTGAATCAAAGGCCATGGAGTCAAAGATTCAAATAGAGGACCTAACAGAACTCGCCGTAAA

GACTGGCGAACAGTTCATACAGAGTCTCTTACGACTCAATGACAAGAAGAAAATCTTCGTCAACA

TGGTGGAGCACGACACGCTTGTCTACTCCAAAAATATCAAAGATACAGTCTCAGAAGACCAAAGG

GCAATTGAGACTTTTCAACAAAGGGTAATATCCGGAAACCTCCTCGGATTCCATTGCCCAGCTAT

CTGTCACTTTATTGTGAAGATAGTGGAAAAGGAAGGTGGCTCCTACAAATGCCATCATTGCGATA

AAGGAAAGGCCATCGTTGAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACG

AGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATAT

CTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAG
+1 BamHI
GAAGTTCATTTCATTTGGAGAGAACACGGGGGACTCTAGAGGATCCCATATTCCGTCTGATGAGT

CaMV 35S promoter SacI Hammerhead-
CCGTGAGGACACTGAATCAGTCTAGAGAGCTCGAATTTGCCCGATCGTTCAAACATTTGGCAATA

Ribozyme nos terminator
AAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATT

ACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATGATT

AGAGTCCCGCAATTATACATTTAATACGCGATAGAAAAGAAAATATAGCGCGCAAACTAGGATAA
EcoRI
ATTATCGCGCGCGGTGTCATCTATGTTACTAGGAATTC

(B)

agagaacaaggggacucuagaggaucccAUAUUCCGUCUGAUGAGUCCGUGAGGACACUGAAUC

ACucuagagagcucgaauuugcccgaucguucaaacauuuggcaauaaaguuucuuaagauuguaa

uccuguugccggucuugcgaugauuaucauauaauuucuguugaauuacguuaagcaugUaaua

auuaacuguaauugcaugaacguuauuuauggagauggguuuuuuaug

Fig. 3. Nucleotide sequences of 35S-ribozyme-nos chimeric gene and transcript. (A) DNA sequence of HindIII and EcoRI fragment of pBI-Ribo is shown. TATA box and poly A signal (AATAAA) are underlined. +1 stands for the transcription initiation site. Double underlined sequence is not shown in the published nos sequence.[17] (B) The expected RNA sequence of 35S-ribozyme-nos gene is shown. The ribozyme sequence is in capital letters.

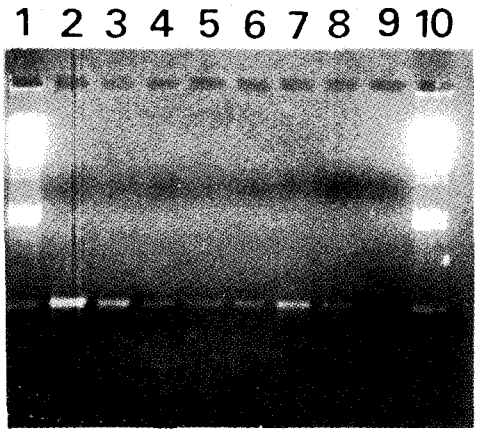

Fig. 4. Separation of PCR products on a agarose gel. An agarose gel (1%) electrophoresis was carried out on PCR products from DNAs from 7 different transgenic tobacco leaves (lanes #2-8)and untransformed tobacco leaf (lane #9) as a negative control. HindIII cut lambda DNA were run in lane #1 and #10 as molecular size markers. Marker sizes are 23, 9.4, 6.6, 4.4, 2.3, 2.0 kbp, and 0.56 kb.

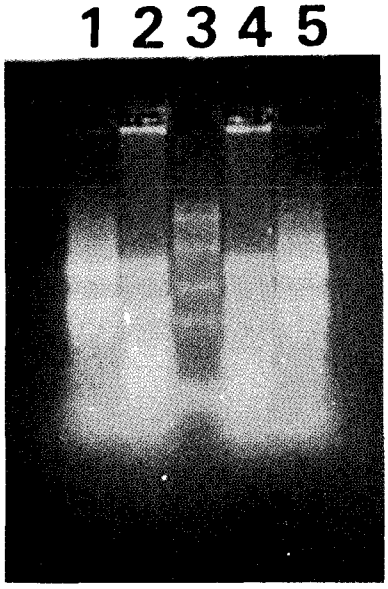

Fig. 5. Formamide gel electrophoresis of total RNAs isolated from transgenic plants. Total RNAs were isolated from transgenic plant #2, #3, #7, and #8 leasves and 20 μg of RNAs were separated on a 1% agarose containing 2.2 M of formamide. Lane #3 contains the RNA ladder(0.24, 1.35, 2.37, 4.40, 7.46, and 9.49 kb) from BRL.

Analysis of transgenic plants indicated that DNAs for ribozyme were in genomic DNAs but RNAs for ribozyme were not detected indicating that nuclease-resistant ribozymes are needed for development of virus-resistant transgnic plants. Nuclease-resistant ribozymes can be made utilizing modified (2'-O-allyl- and 2'-O-methyl-) ribonucleotides substitute for the corresponding ribonucleotides.[18,19] The use of ribozyme for virus-resistant transgenic plants was promising but the efficacy was probably not higher than use of viral coat protein genes and satellite RNAs.[20]

In addition to this, there is so far no published papers on virus-resistant transgenic plants utilizing ribozymes eventhough there are lots of papers on *in vitro* cleavage tests of plant viral RNAs by ribozymes.[21-24] There is only one paper on the expression of a ribozyme gene in tobacco protoplasts resulted in cleavage of neomycin phosphotransferase (*npt*) gene and the reduction of NPT activity was explained by both hydrolytic cleavage of target gene and an antisense effect.[6] However, expression of the ribozyme to high molar excess over the β-glucuronidase (GUS) transcript did not lead to any significant decrease of GUS activity in the transformed *Arabidopsis* protoplasts.[7]

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시험관내에서 합성한 오이모자이크 바이러스 RNA단편을 성공적으로 절단한 ribozyme의 식물체내의 발현

박상규 (대구대학교 농화학과)

초록 : 시험관내에서 합성한 오이모자이크 바이러스 RNA단편을 성공적으로 절단한 ribozyme (한국농화학회지 37: 56-63(1994))을 담배 식물체에 발현시켜 바이러스 저항성 식물체를 만들려고 하였다. 해당 ribozyme 염기서열을 함유한 DNA 단편을 꽃양배추 바이러스 35S promoter와 nopaline 합성효소 terminator에 연결시키고 연결부위 및 ribozyme의 염기서열을 확인하였다. 염기서열을 확인한 합성유전자를 *Agrobacterium tumefaciens* LBA 4404에 합성유전자를 함유한 *E. coli* HB101을 *E. coli* HB101(pRK2073)를 helper로 *Agrobacterium tumefaciens* LBA4404와 함께 배양하는tri-parental mating system을 이용하여 도입시켰다. Ribozyme 유전자를 함유한 *Agrobacterium* 세포를 담배잎 조각과 함께 배양한 후 항생제인 kanamycin을 함유한 MS 배지에서 자란 열개의 작은 식물체를 재분화하였다. 형질전환한 식물체내에 ribozyme 유전자가 존재하는지의 여부는 합성효소증폭반응(PCR)을 이용하였던바, 일곱개체가 예상된 570 염기쌍의 DNA 단편을 가지고 있었다. 이들 중 네 개체로부터 RNA을 분리하여 formamide를 함유한 agarose에서 전기영동한 후 35S-ribozyme-nos의 DNA 단편으로 Northern hybridization을 행하였던 바, 식물세포내의 nuclease에 의해 ribozyme RNA가 분해된 듯 감지 할 수 없었다. 따라서 ribozyme을 이용하여 바이러스 저항성 식물체를 얻으려면 nuclease에 의해 분해되지 않는 ribozyme이 필요할 것으로 사료된다.