한국농화학회지 제37권 제1호(1994)
Agricultural Chemistry and Biotechnology
Vol. 37, No. 1, pp. 56~63(1994)

In vitro endonucleolytic cleavage of synthesized cucumber mosaic virus RNA by hammerhead ribozyme

Sang-Gyu Park[1]* and Young-Soo Hwang[2]

[1]Departarment of Agricultural Chemistry, Taegu University
[2]Biochemistry Division, Agricultural Biotechnology Institute, RDA

Abstract : Oligonucleotides for a conserved region of the coat protein gene of cucumber mosaic virus (CMV) and a hammerhead structure ribozyme against CMV RNA were synthesized using a DNA synthesizer. Both strands of oligonucleotides were annealed and restricted with BamHI/SacI, then cloned into a plasmid pBS SK (+). The cloned CMV substrate and ribozyme were sequenced to verify correct constuctions. *In vitro* transcriptions were carried out by using T7 RNA polymerase with BssHII or SspI digests of 1 μg of substrate and ribozyme clones. The size of substrate RNA was 176 nucleotides (nt) containing 50 nt of CMV RNA sequence, 6 nt of XbaI restriction site and 120 nt of vector-derived sequence in the case of BssHII digest. The size of ribozyme RNA was 164 nt containing 40 nt of ribozyme RNA sequence and same sequences of substrate. Substrate RNA was efficiently cleaved into two fragments (96 nt and 80 nt) by ribozyme RNA. This endonucleolytic cleavage occurred more efficiently at 55℃ than 37℃. SspI digest-derived substrate RNA (2234 nt) was also cleaved into two fragments by the same ribozyme. SspI digest-derived ribozyme RNA (2222 nt) cleaved the above substrate to two fragments. *In vitro*-tested ribozyme construct is being cloned into a plant transformation vector to develop virus-resistant plants
(Received November 25, 1993; accepted January 25, 1993).

Introduction

Conventional plant breeding and selection procedures have been used for many years to prevent the virus diseases. However a lengthy breeding period is needed to get virus-resistant plants. In addition to the selection procedure, cross-protection, preinfection of a plant with a virus causing mild symptoms prevents the development of symptoms caused by more virulent virus, has been used to control virus disease of tomato[1] and papaya.[2] Because inducing virus can be transformed into more virulent virus during replication or damage plants severely with other non-relating virus, the corss-protection has not been used widely in the field.[3]

Recently biotechnology offers a means by which foreign genes can be transferred and regulated in living organisms. This biotechnology would have an implication in virus disease-resistance as well as product quality. Approaches utilising viral genes and their anti-genes for the development of the virus-resistant plants were made in the several laboratories around the world. Coat protein genes, replicase genes, satellite RNAs, antisense RNAs and ribozymes (ribonucleic acid enzymes) were utilised for the virus-resistant transgenic plants.

Coat protein genes from 15 different viruses were transferred into tobacco, tomato, alfalfa, potato and sugarbeet and transformed plants showed the high level of resistancy against the related viruses.[4] However coat protein-mediated protection like classic cross-protection was overcome by inoculation

Key words : Ribozyme, hammerhead, CMV, *in vitro* transcription
*Corresponding author : S.-G. Park

with purified virus RNA at least as far as development of symptoms in inoculated leaves is conserved.[5,6]

In the case of the satellite RNA-mediated resistance expression of cucumber mosaic virus (CMV) satellite has been shown to be effective in glasshouse tests.[7] Infection by tobacco ring spot virus (TobRV) was inhibited in transgenic tobacco plants that expressed a naturally occuring TobRV satellite RNA.[8] But it is not clear how useful satellite RNA-mediated resistance will be in providing protection against infection with viruses already containing satellite RNA while it is effective in protecting against infection with satellite free virus.

Attempts to use antisense RNA molecules to inhibit virus infection of plants were not succeseful. Direct comparison showed that the efficacy of resistance obtained using antisense RNA complementary to virus coat protein genes was less than that obtained with coat protein-mediated protection.[9] Therfore antisense RNA-mediated protection can not be used in the field.

Viral replicase gene-mediated resistance was obtained by the expression of the sequence encoding 54-KDa protein of tobacco mosaic virus (TMV)[10] and a 94 base-pair deleted replicase gene of CMV in tobacco plants.[11] In addition, the expression of amino-terminal portions or full-length potato virus X (PVX) replicase genes in transgenic tobacco plants conferred resistance to PVX infection.[12] Therefore the replicase-mediated protection seems to a new approach for controlling plant viral infection.

Another promissing approach is recently developed utilising ribozyme. Ribozyme technology appears to offer an attractive expansion to the antisense strategies employed against plant viruses. So far however only a few example of ribozyme directed against plant viral RNAs have been described. Lam and Hay[13] demonstrated specific cleavage of potato leafroll luteovirus (PLRV) RNA *in vitro* by ribozymes designed against regions encoding the viral coat protein and RNA polymerase. Transgenic tobacco plants that expressed ribozymes against TMV showed some resistance to TMV infection.[14] While ribozymes directed against TMV RNA were also reported to cleave the viral RNA *in vitro* and in addition to inhibit virus replication a both in protoplast and transgenic plants.[15] Similar experiments are being carried out by Lee *et al.*[16] in Korea. They expressed hammerhead ribozyme E1 against TMV RNA but have not examined the resistancy against the TMV infection yet.

In this report we examined the endonucleolytic specific cleavage reactions of hammerhead ribozyme against synthesized CMV RNA *in vitro* in prior to the introdution of the ribozyme into plant cells to make virus-resistant transgenic plants.

Materials and Methods

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 catridge (ABI). Four different oligonucleotides were synthesized: (+) and (−) strands of CMV substrate, 5′-GGGGGATCC-TACCTGATTCAGTCACGGAATATGATAAGAAGC-TTGTTTCGCGCATTCAAATCTAGAGAGCTCCCC-3′(75 mer) and 5′-GGGGAGCTCTCTAGATTTGAA-TGCGCGAAACAAGCTTCTTATCATATTCCGTGA-CTGAATCAGGTAGGATCCCCC-3′(75 mer), respectively and (+) and (−) strands of ribozyme, 5′-GG-GGGATCCCATATTCCGTCTGATGAGTCCGTGA-GGACGAAACTGAATCAGTCTAGAGAGCTCCCC-3′ (66 mer) and 5′-GGGGAGCTCTCTAGACTGATT-CAGTTTCGTCCTCACGGACTCATCAGACGGAA-TATGGGATCCCCC-3′ (66 mer), respectively (restriction enzyme sites are underlined). Non-CMV substrate and non-ribozyme sequences in the oligonucleotides provide sites for the restriction enzyme BamHI and SacI, which were used for the cloning into pBluescript SK (+) and another restriction enzyme XbaI, which is located beside SacI, for the selection of substrate and ribozyme clones.

The substrate and ribozyme constructs were first selected by digesting with XbaI whose site should not be present in the self-ligated vector. Then the selected clones were digested with BssHII and run on a 1% agarose gel with a BssHII digest of pBlue-

script. The DNase-free RNase was added to the selected clones to a final concentration of 100 μg/ml to remove RNAs followed by precipitation with 2.5 volumes of ethanol in the presence of 0.3 M sodium acetate (pH 5.2). The sequences of the substrate and ribozyme clones were determined by the dideoxy chain-termination method[17] utilising Sequenase™. Sequencing gels contained 8% polyacrylamide, 7 M urea and 0.5×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).

In vitro transcription and purification of transcripts were performed according to the published method.[18] BssHII or SspI digests of 1 μg plasmids were incubated for 1 hour at 37℃ with 10 unit of T7 RNA polymerase (Stratagene) in 25 μl reaction mixture. The reaction mixture contains 5X transcription buffer (200 mM Tris-HCl pH 8.0, 250 mM NaCl, 40 mM $MgCl_2$, 10 mM spermidine), 30 mM DTT, 10 units of RNasin (Promega), 0.4 mM of each rNTP and 10 μCi of [^{32}P]-UTP (Amersham). Following transcription the DNA template was removed by adding 10 units of RNase-free DNase (BRL) to the reaction mixture and further incubating for 15 min at 37℃. The transcription mixture was then extracted twice with buffered phenol, phenol-chloroform-isoamyl alcohol (25 : 24 : 1), and chloroform-isoamyl alcohol (24 : 1). The RNA transcripts were precipitated with 2.5 volumes of ethanol in the presence of 2.5 M NH_4OAC. The radioactive RNA size marker (150 nt) was generated by T7 RNA polymerase-directed transcription of the BssHII digest of pBluescript.

In vitro endonucleolytic cleavage of substrate RNA by ribozyme was carried out according to the published procedure.[18] Substrate RNA, ribozyme RNA and 4X reaction buffer containing 100 mM Tris-HCl pH 7.5 and 80 mM $MgCl_2$ were sequentially added to a tube. The reaction mixture (final volume: 40 μl) was layered with RNase-free mineral oil (Sigma) and the reaction initiated by placing the tube in a water bath set at the desired temperature. The reaction was terminated by the addition of 80 μl of formamide loading buffer (75% formamide, 0.05% bromophenol blue, 0.005% xylene cyanol, 20 mM EDTA). The samples were heated to 65℃ for 3 min before electrophoresis. *In vitro* cleavage of the RNAs was assayed on 6% or 12% polyacrylamide-7M urea gels in 1X TBE (90 mM Tris-HCl pH 8.0, 90 mM boric acid, 2 mM EDTA). Following electrophoresis the gels were wraped with Clean Wrap™ and subjected to autoradiography using X-ray film (Kodak). The substrate and resulting products were quantified from autoradiograph using a laser densitometer (Cream Image Analyzer, Kenentec, Denmark).

Results

The nucleotide sequences of the CMV substrate and ribozyme constructs are shown in the Fig. 1(B). The cloning sites are indicated as restriction enzyme sites BamHI (5′-GGATCC-3′) and SacI (5′-GAGCTC-3′) which will be utilized to be cloned into the same enzyme sites of plant transformation vector pBI221.[19] The ribozyme sequences between these two sites composed of 10 nt of complementary arms in both sides, 19 nt of conserved sequence, 5′-TGATGAGTCCGTGACGAAA-3′, in which 3 nt (AGG) are deleted during the synthesis of oligonucleotudes. Without these three nucleotides, a stem structure might be maintained with two G nucleotudes which are in the middle loop region. The deleted 3 nucleotides which are in the middle loop region of the hammerhead ribozyme structure[20] did not make any harmful effect on the endonuclelytic cleavage reaction (See below the results of the *in vitro* cleavage reaction.). In case of the CMV substrate sequence, 50 nucleotides composed of the GTC triplet, 11 nt conserved CMV sequence for the coat protein gene in the left side of the triplet and 36 nt conserved CMV sequence in the right side of the triplet. This conserved sequence is from the published results[21-23] and spans from 1534 to 1584 according to the number of nucleotide from the Dr. Shintaku's result.[21] Fig. 1(A) shows the only complementary sequence of the conserved CMV RNA to arms of the hammerhead ribozyme. The complementary sequence from the 10 different strains of

CMV RNA is 10 nucleotides each in the both sides of ribozyme arms.

In vitro endonucleolytic cleavage of the CMV substrate RNA by ribozyme is shown in the Fig. 2. The molar ratio of the ribozyme to the substrate might be 1 : 1 because transcripts synthesized from 1 μg of each clone were added to microfuge as mentioned in 'Materials and Methods'. However the spot size of the ribozyme was much bigger than that of the substrate in the Fig. 2 but was similar each other in the Fig. 3. The size of the substrate RNA is 176 nt and consists of 50 nt of CMV conserved RNA sequence, 120 nt of vector-derived sequence and 6 nt of XbaI restriction site sequence. The size of ribozyme RNA is 164 nt and contains 38nt of ribozyme RNA sequence, 120 nt of vector-derived sequence and 6 nt of XbaI restriction site sequence. The substrate RNA was cleaved into two fragments of the expected sizes, 96 nt and 80 nt (lane 4 of Fig. 2) in the presence of Mg^{2+}. The marker (150 nt, lane 1) was derived from the T7 RNA polymerase-treated BssHII digest of pBluescript. Comparison of the marker RNA with ribozyme RNA, substrate RNA and products of the expected sizes is shown as nt in the figure.

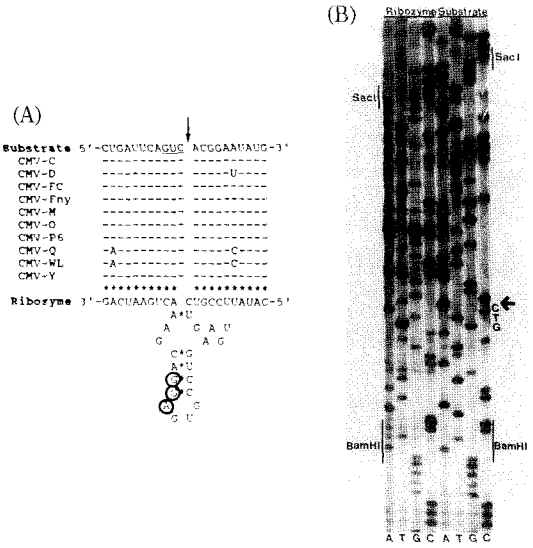

Fig. 1. Nucleotide sequences of CMV substrate and ribozyme.
(A) RNA sequences of a conserved region of coat protein (CP) gene and a hammerhead ribozyme. Sequences of ten different CMV strains are shown. Complementary sequences to ribozyme are shown. – indicates the same sequence to the conserved one and * indicates the complementary sequences between CMV CP and ribozyme. Circled nucleotides, A, G, and G, were deleted during the synthesis of oligonucleotides for the ribozyme. (B) Autoradiogram of DNA sequences of cloned substrate and ribozyme in pBS SK (+). Restriction sites used for cloning are indicated as BamHI and SacI. Arrow means a proposed cleavage site in the CMV substrate RNA.

In accordance with previously reported studies, the cleavage reaction rate was decreased by lowering the incubation temperature (Fig. 3). No cleavage products were detected upon incubation at 0℃ (data not shown) whereas substrate RNA cleavage still occurred at 37℃ but with a reduced rate compared to 55℃. Quantification of substrate RNAs and the cleavage products on the resulting autograph using a Kenentec's Cream Image Analyzer showed that the cleavage levels were 96% and 3% for 55℃ and 37℃ incubation, respectively. The temperature optimum for the hammerhead structure was 55℃ [24] while that for the hairpin structure was 37℃.[25]

The bigger substrate and ribozyme size did not make any difference in the specific cleavage reaction. Another substrate RNA (2234 nt) was synthe-

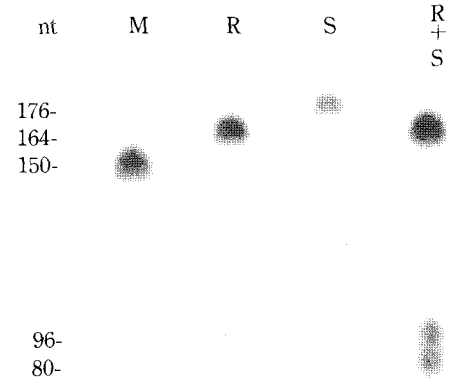

Fig. 2. *In vitro* endonucleolytic cleavage of substrate (S, CMV transcript) by ribozyme (R). BssHII digests of clones (1 μg) were subjected to *in vitro* transcription with T7 RNA polymerase. Both transcripts were incubated 2 hr at 55℃ in the presence of 20 mM $MgCl_2$. The reaction products were fractionated on a 6% polyacrylamide-7 M urea gel. M stands for marker derived from the vector. Sizes of transcripts were indicated with number of nucleotide (nt).

sized with SspI digest of the substrate clone by utilising *in vitro* transcription reaction. This transcript consists of 50 nt of CMV conserved sequence, 6 nt of XbaI sequence and 2178 nt of vector-derived sequence. The substrate contains 24 GUC triplets according to the sequences of pBluescript (23 trplets) and inserted CMV RNA (1 triplet). However the cleaved fragments consist of 96 nt product suggesting the specificity of ribozyme is high enough to find the expected site on the long substrate RNA (Fig. 4).

The bigger ribozyme RNA (2222 nt) was also synthesized with SspI digest of the ribozyme clone. This transcript contains 38 nt of ribozyme RNA, 6 nt of XbaI sequence and 2178 nt of vector derived sequence. Bigger substrate (2234 nt) was also cleaved by this ribozyme to make same size of the product (96 nt)(lane 3, Fig. 5). The cleavage rate was enhanced with the addition of 2X concentration of ribozyme RNA (lane 4, Fig. 5). However bigger substrate and ribozyme transcripts did not make distinct bands on autoradiographs suggesting synthesized RNAs might be degraded during cleavage reaction or electrophoresis. RNA inhibitor, RNasin, has been used in the *in vitro* transcription to prevent the degradation of transcripts but not in the cleavage reaction and electrophoresis.

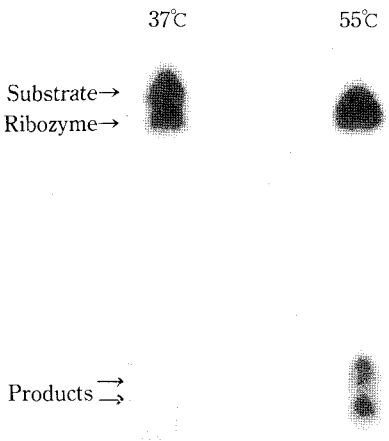

Fig. 3. Effect of the temperature for ribozyme action. Endonucleolytic cleavage reactions were carried out at 37℃ and 55℃ as described in the Fig. 2. A 6% polyacrylamide-7M urea gel electrophoresis was utilized to fractionate the reaction products. Sizes of transcript and endonucleolytic products are same as described in the Fig. 2.

Discussion

The catalytic properties of the hammerhead ribozyme were determined by Uhlenbeck.[24] The reaction rates are dependent upon pH and Mg^{2+} concentration. The optimum temperature for the hammerhead structure was 55℃ while that for the hairpin structure was 37℃.[25] However we have chosen the hammerhead ribozyme rather than the hairpin ribozyme because there are no published papers on *in planta* test using hairpin type ribozyme. On the other hand, there are several published papers on the endonucleolytic cleavage of the substrate RNA

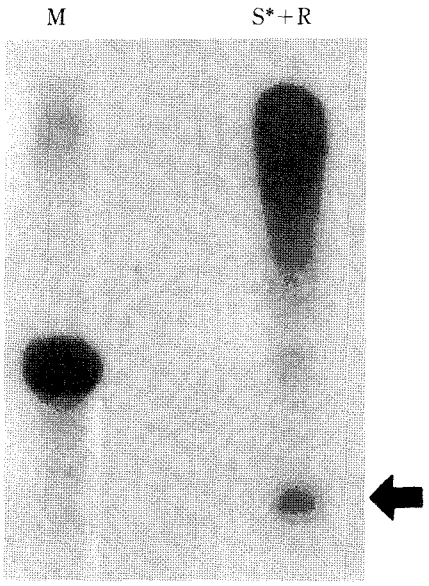

Fig. 4. Specificity of ribozyme action. Another substrate RNA (2234 nt) containing 50 nt of CMV transcript, 6 nt of XbaI restriction enzyme site and 2178 nt of vector derived transcript were synthesized with SspI digest of the substrate clone. Labeled substrate (S*) contains 24 sites of GUC triplet according to the sequences of the pBS SK (+) and the inserted CMV substrate. Unlabeled ribozyme (R) was used to differentiate with product (s). The reaction products were fractionated on a 12% polyacrylamide-7M urea gel. M stands for the size marker (150 nt) derived from the vector and arrow indicates the endonucleolytic product (96 nt).

by the hammerhead ribozymes. The expression of a hammerhead ribozyme gene driven by CaMV 35S promoter in tobacco protoplasts resulted in the endonucleolytic cleavage of transcript for the neomycin phosphotransferase gene (npt) and a concomitant reduction of the enzyme activity.[26] In addition hammerhead ribozymes directed against TMV RNA were also reported to inhibit virus replication both in protoplasts and transgenic plants.[15] The transgenic tobacco plants that expressed hammerhead ribozymes against TMV showed some resistance to TMV infection.[14]

Two different methods are known to clone the synthesized oligodeoxynucleotide into vectors. One is the method for cloning single stranded oligonucleotides in a plasmid vector[27] and the other is the method for cloning the complementary oligonucleotides in a vector.[26-30] We followed the latter because the method seems to be much simple and precise than the first. Following cloning procedures, putative clones were sequenced to confirm the constructions since we cloned relatively small synthetic

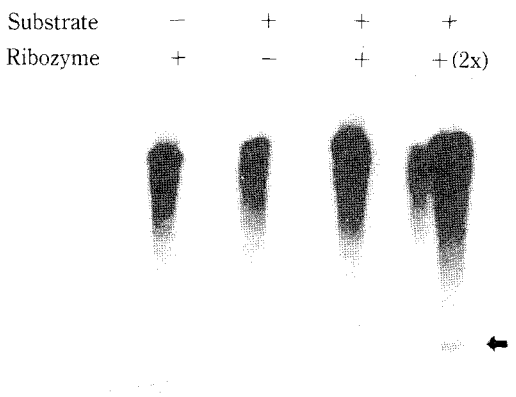

Fig. 5. Effect of ribozyme size for the cleavage of substrate.
Bigger ribozyme RNA (2222 nt) containing 38 nt of ribozyme sequence, 6 nt of XbaI restriction enzyme sequence and 2178 nt of vector derived transcript were synthesized with SspI digest of the ribozyme clone. Bigger substrate (2234 nt) was subjected to the ribozyme action as described in the legend of the Fig. 2. The reaction products were fractionated as described in the legend of Fig. 4. + and − indicate the presence and sbsence of substrate and ribozyme in reactions. Arrow indicates the endonucleolytic product (96 nt).

DNA fragments and one single mutation in the ribozyme domain can have a great effect on the ribozyme domain.[12] This strategy permits us not only to obtain in one step a clone to (1) check the sequence of the synthetic oligonucleotide; (2) obtain the sense transcript by T7 polymerase transcription that is necessary to check the cleavage capacity *in vitro*; (3) obtain anti-sense RNA probes by T3 driven transcription to access the steady-state level of the ribozyme in transgenic plants; but also permit us to clone conviniently ribozyme construct in a plant transformation vector.

The hammerhead ribozyme against CMV RNA was more active at 55℃ than 37℃ (Fig. 3) meaning that our hammerhead ribozyme acted like the other reported hammerhead ribozyme in *in vitro* experiments. The hammerhead ribozyme was actually developed from the positive strand of satellite tobacco ringspot virus (TobRV).[25] The typical catalytic regions is a secondary structure composed by two single-stranded regions, containing 13 highly conserved nucleotides and 3 non conserved stem-loop structures. Therefore the deletion of 3 nucleotides in the middle region of the 3 non conserved stem-loop structures would not have made any visible effect on the cleavage reaction.

In case of the hammerhead ribozyme against NPTII RNA,[26] three nucleotides, U, G, and G, were changed to other nucleotides, C, C, and A, respectively, and two more nucleotides (A and U) were inserted in the non conserved stem-loop regions compared with the typical hammerhead ribozyme structure.[28] However the expression of a ribozyme gene resulted in endonucleolytic cleavage of target mRNA and a concomitant reduction of gene expression *in vivo*. This result and our result support that other sequences beyond 13 conserved sequences of hammerhead ribozyme do not affect the ability of the target RNA cleavage. Indeed, the ability to make ribozyme cleave specifically target RNAs *in vitro* appeared to be applicable to virtually any RNA molecules.

Since almost no sequence requirements for efficient cleavage exist on the target RNA except the presence of a GUC triplet immediately proceding

the cleavage point,[28,29] the bigger substrate RNA derived from the SspI digest of the substrate clone was subjected to the ribozyme reaction. Even though the substrate RNA contains 24 GUC triplets, the cleavage was occurred specifically 3′ to the GUC triplet of the CMV sequence (Fig. 4). This result supports the above implication that hammerhead ribozyme needs only the 13 conserved sequences and the complementary sequences to the substrate RNA containing GUC triplet.

Because the cleavage activity of the ribozyme was determined by the secondary structure of the substrate involved[30] and the unknown internal secondary structures in target RNAs also influenced the accessibility of the ribozyme cleavage site[31] the analysis of RNA secondary structures should be performed using the computer with a sequence anaysis software package before the *in vivo* application of ribozyme.

In vitro-tested ribozyme clone will be transferred into the plant transformation vector to test whether our ribozyme can work *in vivo*: BamHI/SacI digests of the ribozyme clone is being cloned into BamHI/SacI digest of pBI221. The presence of the ribozyme in transgenic tobacco and cucumber plants will be confirmed by Southern and Northern hybridization. The resistancy of the transgenic plants to viral infections will be tested with several strains of cucumber mosaic virus.

Acknowledgement

본 연구는 농촌진흥청 농업특정연구개발사업의 일환으로 수행되었기에 이에 감사를 드립니다.

References

1. Broadbent, L.: Ann. Rev. Phytopath., 14 : 75-90 (1976)
2. Yeh, S.-D. and Gonsalves, D.: Phytophology, 74 : 1086-1091(1984)
3. Hamilton, R. I.: In 'Plant Diesease: An Advanced Treatis', Horsfall, J. G. and Cowling, E. B. (ed), Vol. 5, pp. 279-303, Academic Press, New York
4. Reavy, B. and Mayo, M. A.: In 'Plant Genetic Manipulation for Crop Protection', Gatehouse, A. M. R., Hilder, V. A. and Boulter, D. (ed) pp. 183-214, CAB International, Wallingford, UK(1993)
5. Nelson, R. S., Powell-Abel, P. and Beachy, R. N.: Virology, 158 : 126-132(1987)
6. Angenent, G. C., Van den Ouweland, J. M. W. and Bol, J. F.: ibid, 175 : 191-198(1990)
7. Harrison, D. D., Mayo, M. A. and Baulcombe, D. C.: Nature, 328 : 799-802(1987)
8. Gerlach, W. L., Llewellun, D. and Haseloff, J.: ibid, 328 : 802-805(1987)
9. Hemenway, C., Fang, R.-X., Kaniewski, W. K., Chua, N.-H., and Tumer, N. E.: EMBO J., 7 : 1273-1280 (1988)
10. Golemboski, D. B., Lomonossoff, G. P. and Zaitlin, M.: Pro. Natl. Acad. Sci. USA, 87 : 6311-6315(1990)
11. Anderson, J., Palukaitis, P. and Zaotlin, M.: ibid, 89 : 8759-8763(1992)
12. Braun, C. J. and Hemenway, C. L.: Plant Cell, 4 : 735-744(1992)
13. Lamb, J. W. and Hay, R. T.: J. Gen. Virol., 71 : 2257-2264(1990)
14. Gerlach, W. and Young, M.: In 'Antisense Nucleic Acids and Proteins. Fundamentals and Applications', Mol, J. N. M. and Van der Krol, A. (eds.), pp. 157-168, Marcel Dekker, New York(1991)
15. Edinton, B. V. and Nelson, R. S.: In 'Gene Regulation: Biology of antisense RNA and DNA', Erickson, R. P. and Izant, J. G. (eds), pp. 209-221, Raven Press, New York(1992)
16. Lee, Y. S., Kim, S. H., Park, H. S., Song, J. Y. and Park, Y. I.: '93 Fall Conference on Biochemistry and Molecular Biology, Abstract, p. 308(1993)
17. Sanger, F., Nicklen, S. and Coulson, A. R.: Proc. Natl. Acad. Sci. USA. 74 : 5463-5467(1977)
18. Mazzolini, L., Axelos, M., Lescure, N. and Yot, P.: Plant Mol. Biol. 20 : 715-731(1992)
19. Jefferson, R. A.: Plant Mol. Biol. Rep. 5 : 387-405 (1987)
20. Haseloff, J. and Gerlach, W.: Nature, 334 : 585-591 (1988)
21. Shintaku, M.: J. Gen. Virol., 72 : 2587-2589(1991)
22. Namba, S., Ling, K., Gonslaves, C., Gonslaves, D. and Slightom, J. L.: Gene, 107 : 181-188(1991)
23. Gould, A. R. and Symons, R. H.: Eur. J. Biochem., 126 : 217-236(1982)
24. Uhlenbeck, O.: Nature, 328 : 596-600(1987)
25. Hampel, A. and Tritz, R.: Biochemistry, 28 : 4929-4933(1989)
26. Steinecke, P., Herget, T. and Schreier, P.: EMBO J., 11 : 1525-1530(1992)

27. Mounts, P., Wu, T.-C., and Peden, K.: Biotechniques, 7 : 356-359(1989)
28. Foster, A and Symons, R.: Cell, 49 : 211-220(1987)
29. Ruffner, D., Dahm, S. and Uhlenbeck, O.: Gene, 82 : 31-41(1989)
30. Van der Vlugut, R. A. A., Prins, M. and Goldbach, R.: Virus Research, 27 : 185-200(1993)
31. Fedor, M. J. and Uhlenbeck, O. C.: Proc. Natl. Acad. Sci. USA. 87 : 1668-1672(1990)

인공적으로 합성한 오이모자이크 바이러스 RNA의 헤머헤드 ribozyme에 의한 시험관 내에서의 절단

박상규[1]* · 황영수[2]([1]대구대학교 농화학과, [2]농업유전공학연구소 생화학과)

Abstract : 오이모자이크 바이러스(CMV)의 외피단백질 유전자의 일정한 염기서열을 보유하는 부분과 CMV RNA에 대항한 헤머헤드(hammerhead) 구조의 ribozyme을 만드는 올리고뉴클레오타이드(oligonucleotide, nt)를 DNA 합성기를 이용하여 제조하였다. 올리고뉴클레오타이드의 양쪽가닥을 서로 합친 후 제한효소 BamHI과 SacI으로 처리하여 플라스미드 pBS SK(+)에 삽입하였고 CMV 기질과 ribozyme 클론의 염기서열을 결정하여 확인하였다. 기질과 ribozyme 클론 1 μg을 BssHII이나 SspI으로 처리한후 T7 RNA 합성효소를 이용하여 튜브내에서 전사반응을 실시하였다. 제한효소 BssHII를 처리한 경우 만들어진 기질 RNA의 크기는 176 nt였는데 50 nt의 CMV RNA 염기, 6 nt의 XbaI 제한효소 염기, 120 nt의 벡터에서 비롯된 염기를 포함한다. Ribozyme RNA의 크기는 164 nt인데 38 nt의 ribozyme 염기부분과 그외는 기질의 것과 같은 염기를 포함한다. CMV 기질 RNA는 ribozyme RNA에 의하여 특이적으로 절단되어 96 nt와 80 nt 두개의 조각을 만들었다. 이러한 특이적 절단은 37℃ 보다 55℃에서 더 빠르게 일어났다. SspI으로 처리한 경우 만들어진 기질 RNA(2234 nt)도 역시 위의 ribozyme에 의해 두조각으로 절단되었으며 SspI 처리 후 만들어진 ribozyme RNA(2222 nt)에 의해서도 특이적으로 절단되었다.