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Construction of a Hammerhead Ribozyme that Cleaves Rice Black-Streaked Dwarf Virus RNA

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Abstract : To develop an antiviral agent for the rice black-streaked dwarf virus (RBSDV), a hammerhead type ribozyme, which has a potential target site on the genome segment 3, was designed. Oligonucleotides for the ribozyme and its substrate were synthesized, annealed, and cloned into a plasmid pBluescript II KS(+). Ribozyme and substrate RNAs were then synthesized by *in vitro* transcription with T_3 RNA polymerase, obtaining RNAs in expected size, 193 and 182 nucleotides, respectively. The substrate RNA was efficiently cleaved into two fragments when incubated with the ribozyme at 55℃, while the cleavage was not detected at 37℃. In addition, the segment 3 RNA of RBSDV was also cleaved into two fragments by the same ribozyme at 55℃. Taken together, our results demonstrated that the hammerhead ribozyme has an in vitro endonucleolytic activity and may be used as an antiviral agent in transgenic plants(Received September 22, 1995; accepted November 6, 1995).

Introduction

Rice black streaked dwarf virus (RBSDV) is a member of Fijivirus and consisted of 10 genome segments of dsRNA. This virus is transmitted by small brown planthopper (*Laodelphax striatellus* F.) and known to attack a wide range of monocotyledons such as rice, maize, wheat and barley. Plant breeding to obtain a RBSDV-resistant rice variety has not been successful yet. The nucleotide sequences of genome segment 7, 8, and 10 of RBSDV were reported.[1] We have previously characterized the nucleotide sequence of genome segment 3 of RBSDV, revealing that the segment 3 contains two partial open reading frames.[2]

Ribozyme is a RNA molecule which can act as an enzyme catalyzing cleavage of other RNAs. The RNA cleavage is normally an intramolecular reaction and a single RNA molecule can function as both enzyme and a substrate. These observations imply that if a specific ribozyme DNA is synthesized and introduced into living cells, it may inactivate a specific messenger RNA, providing a new way to control gene expression.[3-5]

Several types of ribozyme are defined. Four different ribozymes, hairpin, group I intron, axhead ribonuclease (RNase) P and hammerhead types have been reported.[6] Each ribozyme differs in primary and proposed secondary structures and reaction mechanisms to cleave the phosphodiester bond of the target RNAs.[7] Of the ribozymes the hammerhead type is the most attractive for use as an antiviral agent because it is the smallest in size and has potentially higher substrate specificity than other types and proven to be effective as antiviral agent *in vitro*.[8-12] There are some evidences of successful cleavage of target RNAs using hammerhead ribozyme *in vivo*.[13]

Based on sequences of hammerhead ribozymes, Keese and Symons[14] deduced a consensus secondary structure hybridized with target RNA. This secondary structure was composed of two single-strand regions containing 13 highly conserved nucleotides and 3 non-conserved stem-loop structures.[8,15] Haseloff and Gerlach[10] adapted the consensus structure to develop a sequence-specific endoribonuclease to cleave exogenous RNA molecule containing GUC triplets, and proposed a secondary structure of the hammerhead ribozyme of self-cleaving satellite RNA of TRV. This model consists of three elements: GUC sequences of substrate, highly conserved secondary structure of ribozyme and flanking regions between the substrate and ribozyme.

Hammerhead ribozymes are employed to cleave many different target RNAs *in vitro*. including sTobRV,[15] potato leafroll virus,[16] *E. coli* β-glucuronidase RNA,[17] cucumber mosaic virus[18] and AIDS virus.[5]

We report here the construction of a hammerhead

Key words : ribozyme, rice black-streaked dwarf virus, antiviral agent, *in vitro* transcription
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ribozyme to cleave an RNA coding for the second open reading frame of RBSDV genome segment 3 and the evaluation of the *in vitro* cleavage of viral RNA-specific transcripts.

Materials and Methods

Construction of a ribozyme and substrate

A hammerhead type ribozyme was designed complementary to the 23 bp (from 529 to 552 bp) of RBSDV genome segment 3 (pBRV3) RNA region containing a GUC triplet (from 539 bp to 541 bp). The oligodeoxyribonucleotides were synthesized by using DNA synthesizer. The synthesized oligodeoxyribonucleotides of substrate are 5′-aagcttagatctGATACAAATCGTCCCAATC -3′ (31 nt, +strand) and the complementary 5′-ctgcagAAGAATATTAGCATGA TTGGACGAT-3′ (31 nt, −strand). The synthesized oligodeoxyribonucleotides of a hammerhead ribozyme are 5′-aagcttagatctGCATGATTGGCTGATGAGTCCGTGA-3′ (37 nt, +strand) and the complementary 5′-ctgcagGATACAAATCGTTTCGTCCTCACGG ACTCAT-3′ (37 nt, - strand). Capital letters are sequences of the ribozyme and substrate and small letters are restriction sites. The synthesized substrate and hammerhead type ribozyme sequences were single-stranded oligodeoxyribonucleotides with the additional sequences containing restriction sites for *Hind*III (aagctt) and *Pst*I (ctgcag) at the 5′ and 3′ termini, respectively. These additional sequences in the oligonucleotide provided restriction sites for cloning into *Hind*III and *Pst*I sites of RNA expression vector pBluescript II KS(+) to form pBSub and pBHead. Another site (*Bgl*II) was used to select the clones containing a substrate or hammerhead type ribozyme. The synthesized oligodeoxyribonucleotides were annealed in the Klenow fragment buffer (100 mM Tris-HCl, 50 mM $MgCl_2$, 75 mM DTT) at 60℃ for 15 min. The annealed oligonucleotides were treated with Klenow fragment in the presence of 330 μM of dNTP. The electrophoresis-confirmed double-stranded oligodeoxyribonucleotides of substrate and hammerhead type ribozyme were digested with *Hind*III and *Pst*I, and then introduced into *Hind*III and *Pst*I sites of pBluescript II KS(+) vector to contain hammerhead type ribozyme (pBHead) and substrate (pBSub). The sequences of substrate and hammerhead type ribozyme in pBSub and pBHead plasmids were determined by the dideoxy chain-termination method.[19] After sequence analysis, 1 μg of plasmids were digested with BssHII at 50℃ for 3 hours for *in vitro* transcription.

In vitro transcription and purification of transcripts

In vitro transcription of the eluted DNA templates with T_3 RNA polymerase was performed according to the procedure of Mazzolini *et al.*[17]

To synthesize the substrate and hammerhead type ribozyme RNA from the DNA templates, T_3 RNA polymerase was added to the linearized DNAs in the presence of 5X transcription buffer, 0.4 mM of rNTP(-rUTP), 0.75 M of DTT, 10 unit of RNase inhibitor, 10 μCi of [α-32P]-UTP and incubated at 37℃ for 1 hour. To remove the DNA templates, 10 unit of RNase free DNase was added and incubated at 30℃ for 15 minutes.

In vitro cleavage of synthesized substrate RNAs

In vitro endonucleolytic cleavage of substrate RNA by a hammerhead type ribozyme was carried out according to the procedure of Mazzolini *et al.*[17] The substrate RNA, hammerhead type ribozyme RNA and 4X reaction buffer containing 100 mM Tris-HCl, pH 7.5, 80 mM $MgCl_2$ were transferred to a tube maintained on ice sequentially. Hammerhead type ribozyme RNA and substrate RNA labeled with [α-32P] UTP were combined to a total reaction volume of 12 μl in 25 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$. Two tubes of the mixtures were covered with RNase-free mineral oil (Sigma) and then incubated at 37℃ and 55℃ for 2 hours respectively. The cleavage reaction was initiated by placing the tubes in water baths maintaining 37℃ and 55℃, respectively. The reaction was terminated by adding equal volume of formamide loading buffer (75% formamide, 0.01% bromophenol blue, 0.005% xylene cyanol) containing 20 mM EDTA. The solutions were stored on ice and incubated at 65℃ for 3 min. prior to 6% polyacrylamide gel electrophoresis. The strategy for synthesis of substrate RNA and secondary structure of hammerhead type ribozyme RNA is shown in Fig. 1 A. This transcribed substrate RNA should be complementary on the both arm regions of ribozyme RNA. T_3 promoter of pBluescript II KS(+) lets T_3 RNA polymerase to transcribe RNAs of substrate and hammerhead type ribozyme. A synthetic oligodeoxyribonucleotide containing the complete ribozyme sequence was cloned in a plasmid vector at downstream of the T_3 promoter (pBHead). A plasmid coding for the corresponding substrate RNA (pBSub) was also obtained by cloning a synthetic oligodeoxyribonucleotide containing the potential ribozyme cleavage site at downstream of the T_3 promoter in the same RNA expression vector.

In vitro cleavage of RBSDV RNA

The insert containing RBSDV gene was obtained from pBRV3 plasmid digested with *Bss*HII. The cDNA of the RBSDV genome was inserted into pBluescript II KS(+) as described before. This 1,075 bp fragment containing RBSDV gene (939 bp) was transcribed *in vitro* by T_3 RNA polymerase to generate the corresponding RNA molecules. The strategy to synthesize full length of RB-

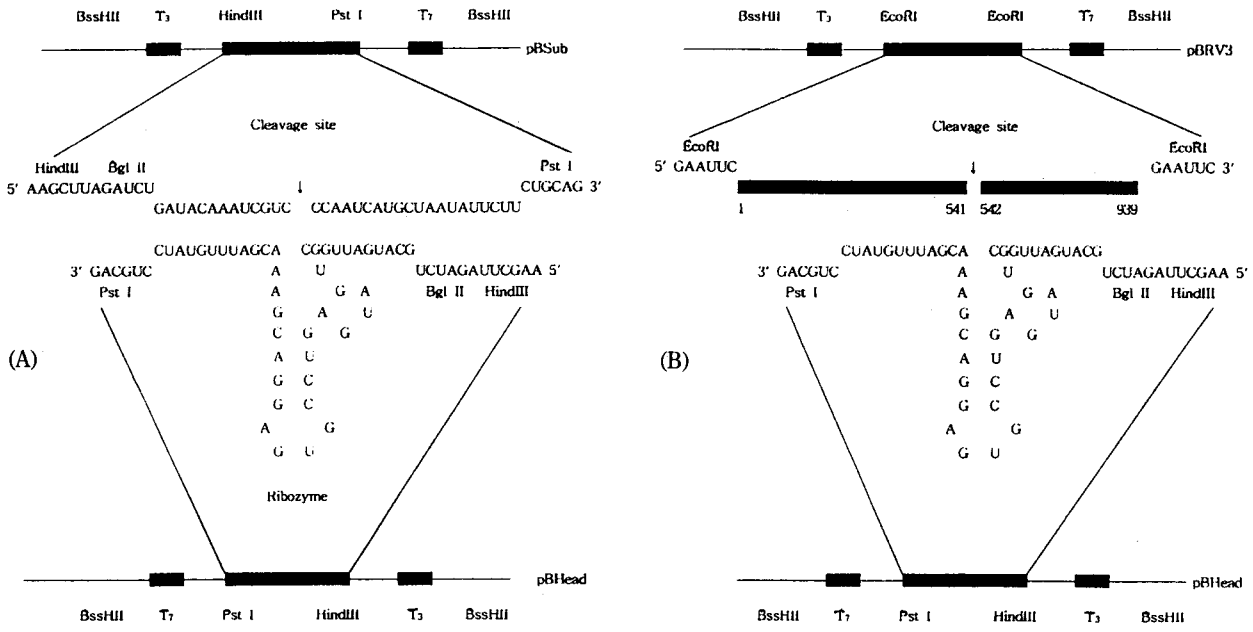

Fig. 1. A. Strategy for substrate and hammerhead type ribozyme RNA synthesis by *in vitro* transcription. The plasmids pBSub and pBHead were used to produce the corresponding RNAs by *in vitro* transcription. The possible hammerhead structure of the ribozyme is shown. The cleavage site of the substrate is indicated by a vertical arrow. B. Strategy for synthesis of RBSDV fragment 3 and hammerhead type ribozyme RNAs by *in vitro* transcription. RBSDV cDNA sequences were inserted to the pBluescript II KS(+) to make the pBRV3. The vectors (pBRV3 and pBHead) were transcribed *in vitro* to produce the corresponding RNAs. The cleavage site is indicated by a vertical arrow.

SDV fragment 3 RNA and hammerhead type ribozyme RNA *in vitro* is shown in Fig. 1 B. The RNA transcripts (1,095 nt) consist of 939 nt of RBSDV fragment 3 sequences, 12 nt of *Eco*RI site sequences and 144 nt of vector-derived sequences. The GUC triplet ranged from 539 to 541 nt in RBSDV fragment sequences was chosen to cleave the target site. RBSDV fragment 3 RNA was cleaved *in vitro* by the same ribozyme RNA as described before.

Analysis of *in vitro* cleavage reaction

Ribozyme RNA and cleavage products were analyzed on 6% PAGE (29 : 1 acrylamide bisacrylamide) - 7 M urea gels (16 cm×18 cm×3 mm). The cleaved RNA products from pBSub and pBRV3 were electrophoresed at 120 voltage for 4 and 16 hours, respectively. After the electrophoresis, the gels were transfered to a filter paper, dried and subjected to autoradiography using a X-ray film.

Results and Discussion

Construction of the vectors containing ribozyme and substrate DNAs

To develop an antiviral agent for use to protect rice from rice black-streaked dwarf virus (RBSDV), a hammerhead type ribozyme, which has a potential target site on the viral genome segment 3, was designed (Fig. 1). The ribozyme includes 20 nucleotides complementary to the target RNA sequence, which was believed to be long enough to find its specific target site. Oligonucleotides for the ribozyme and the substrate were synthesized, annealed, and cloned into the plasmid pBluescript KS (+), resulting in two plasmids named pBHead containing the ribozyme DNA and pBSub containing the substrate DNA. The sequences of the ribozyme and the substrate were confirmed by sequencing of the plasmids as shown in Fig. 2. The substrate DNA contains 30 bp including a GTC target site and the ribozyme DNA contains 10 bp of the complementary arms in both sides of the cleavage site and 24 bp including 11 bp of conserved sequences. Braun and Hemenway[20] reported that a single mutation in the ribozyme domain can have great effect on its activity. Park and Hwang[18] however found that the deletion of 3 nucleotides from the middle loop region did not cause any harmful effect on the cleavage reaction. These results suggested that 11 out of 19 nucleotides in the conserved region are essential for the cleavage reaction. The hybridizing arms of our synthesized ribozyme are 10 bases in length each located in both sides of the cleavage site. The length and G+C content of the ribozyme-substrate duplex is important for both duplex formation and cleavage product dissociation. Longer flanking sequences of a ribozyme hybridize readily with target RNAs, but slow down the dissociation rate of cleaved products from the ribozyme. Moreover, since

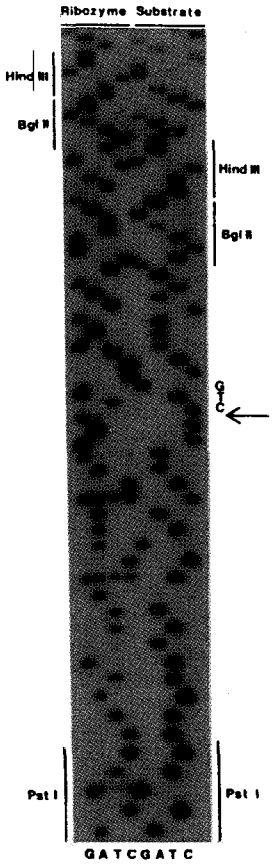

Fig. 2. Autoradiogram of nucleotide sequences of cloned ribozyme and substrate pBluescript II KS(+). The synthesized nucleotides were annealed, digested with *Hind*III and *Pst*I and cloned into *Hind*III-*Pst*I site of the vector. Double strand DNA sequencing was carried out with Sequenase™. Arrow indicates a proposed cleavage site of substrate. Restriction enzyme sites used for cloning are indicated.

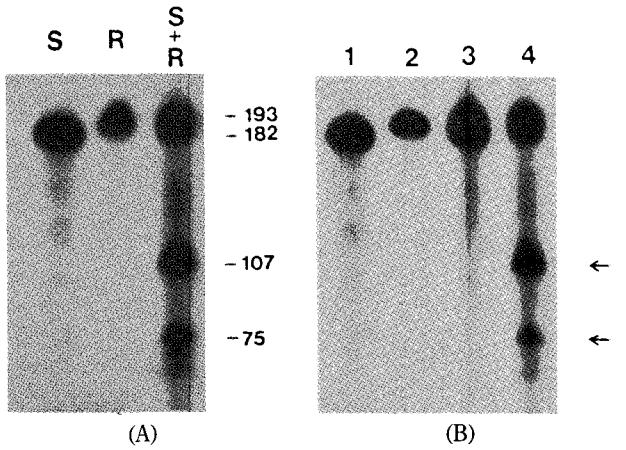

Fig. 3. A. Electrophoretic analysis of *in vitro* endonucleolytic cleavage of substrate by ribozyme. [α-^{32}P]UTP-labeled substrate and ribozyme RNAs were mixed and incubated at 55℃ for 2 hours in 25 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$. The reaction products were fractionated on 6% polyacrylamide-7M urea gel (S, substrate RNA; R, ribozyme RNA; S+R, substrate plus ribozyme). B. Effect of temperature on *in vitro* endonucleolytic cleavage reaction. Electrophoretic analysis of *in vitro* endonucleolytic cleavage products at two different temperatures of 37℃ (lane 3) and 55℃ (lane 4). Lane 1, substrate RNA; lane 2, ribozyme RNA.

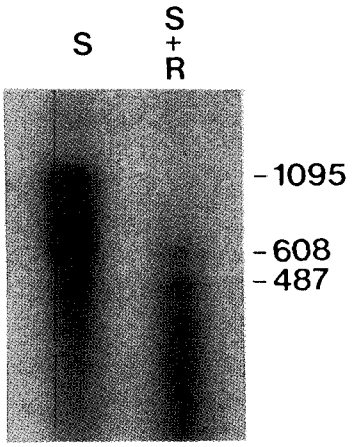

Fig. 4. *In vitro* cleavage of pBRV3 RNA by the action of the hammerhead ribozyme. The ribozyme RNA was incubated with [α-^{32}P]UTP-labeled pBRV3 RNA at 55℃ for 2 hours in the presence of 25 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$. The reaction products were fractionated on 6% polyacrylamide-7M urea gel. S, pBRV3 RNA; S+R, substrate plus ribozyme RNA.

longer flanking sequences can tolerate mismatches more readily than shorter ones, they may allow hybridization of the ribozyme to nontargeted substrates, which in turn could increase the probability of undesired cleavage reaction. Our flanking sequences of 10 bases in length are appropriate for association and dissociation of the substrate in cleavage reaction.

In vitro cleavage of synthesized substrate RNA

For the *in vitro* cleavage reaction, we prepared substrate and ribozyme RNAs from the plasmids pBSub and pBHead, respectively, by *in vitro* transcription using T_3 RNA polymerase as described in materials and methods. The transcription from the pBSub and the pBHead yielded 182 and 193 nucleotide RNAs, respectively. After synthesis, equal amounts of the ribozyme and the substrate RNAs was mixed in 25 mM Tris-HCl, pH 7.5 and 20 mM $MgCl_2$ and incubated at 55℃ for 2 hours. As shown in Fig. 3, the substrate RNA (182 nucleotide) was cleaved to two fragments of expected size (107 and 75 nucleotide) (lane S+R). When the substrate and ribozyme RNAs were incubated separately under the same condition, they were not degraded (Fig. 3A, lanes S and R), indicating clearly that the substrate RNA were cleaved by the ribozyme RNA. When the mixture of the ribozyme and the substrate RNAs were incubated at 37 ℃, no cleavage products were detected (Fig. 3B, lane 3). The optimum temperature of the hammerhead type ribozymes appears to be higher than those of the hairpin types.[9,18,21] Many hammerhead ribozymes seem to func-

tion optimally at temperatures above 50℃.[22] This is in part due to the effects of the higher temperature on the product dissociation and an increased rate of diffusion of ribozyme and target at the higher temperature.

In vitro cleavage of RBSDV segment 3 RNA by the ribozyme

To test whether our ribozyme can cleave RBSDV RNA *in vitro*, we prepared a ^{32}P-labeled substrate RNA from the plasmid pBRV3 containing RBSDV genome segment 3 cDNA as described in Materials and Methods.[2] The resulting pBRV3 transcript was cleaved specifically into two fragments when the transcript was incubated with unlabeled ribozyme (Fig. 4). Our results demonstrated that the hammerhead ribozyme can cleave RBSDV RNA genome segment 3, providing an antiviral agent which can be used for the development of the RBSDV-resistant transgenic plants. The *in vivo* expression of such ribozyme and cleavage of the specific transcripts would inhibit expression of the corresponding viral gene, thereby prevent viral infection.

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흑조위축병 바이러스 RNA를 절단하는 망치머리형 라이보자임의 제작

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초록 : 흑조위축병 바이러스(RBSDV)에 대한 antiviral agent를 개발하기위하여 RBSDV 게놈 조각 3번을 절단하는 망치머리형 구조의 라이보자임을 설계하였다. 라이보자임과 기질의 oligonucleotides는 DNA 합성기로 합성 후 서로 합치고, pBluescript KS(+)에 삽입하였다. 라이보자임과 기질 RNA는 T_3 RNA polymerase로 기내 전사하여 각각 193과 183 nucleotide의 RNA를 얻었다. 기질 RNA는 라이보자임 RNA에 의해 55℃에서 2개의 조각으로 절단되었으나 37℃에서는 절단되지 않았다. 또한 RBSDV의 3번 조각 RNA도 동일한 라이보자임에 의해 절단되었다. 이러한 결과로 합성된 헤머해드 라이보자임은 기내 절단 능력을 가지며 형질전환 식물체에서 antiviral agent로 이용될 수 있을 것이다.

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