Resistance to Viral Infection in Transgenic Plants Expressing Ribozymes Designed against Cucumber Mosaic Virus RNA3

Shigeo NAKAMURA*, Ryoso HONKURA*, Masashi UGAKI**, Masahiro OHSHIMA** and Yuko OHASHI**

Key words: ribozyme, cucumber mosaic virus, transgenic plant, virus resistance.

Ribozymes are RNA molecules that catalyze an intramolecular self-cleavage reaction. Hammerhead-type ribozyme has been engineered to work in trans by Haseloff and Gerlach1). The molecule possesses a 22-mer catalytic domain embedded within flanking sequences that are complementary to target RNA sequences. The only sequence required for its cleavage activity in the target RNA sequence is GUX trinucleotides (X=A, U or C), which is common in RNAs. Subsequently, it was shown that the first G can be replaced by any other bases10). Because of its portability and flexibility, hammerhead ribozyme has been successfully used as a regulator of gene expression in mouse9), Drosophila13), tobacco12) and human immunodeficiency virus11).

Although plant virus RNAs can also be cleaved by ribozymes in vitro5), few studies have reported in vivo expression of ribozymes for controlling plant virus infection1,2). We report here the construction of hammerhead ribozymes designed to cleave RNA encoding the viral movement protein (3a) of cucumber mosaic virus (CMV) and its activity in vitro and demonstrate the anti-CMV-infection activity of the ribozymes in vivo, by comparing transgenic plants expressing the antisense RNA containing the ribozymes (catalytic antisense RNA) with the plants expressing simple (non-catalytic) antisense RNA or coat protein.

We constructed three catalytic antisense fragments, which include one or two catalytic domains (Fig. 1A). Two triplets within 3a gene, GUC at nucleic acid position 159-161 and GUU at 570-572, were chosen for the target sites because they are located within highly conserved region among several strains of CMV. Insertion of catalytic sequence (5'-CTGATGAGTCCGTGAGGACGAA-3') originating from the genome of the satellite RNA from tobacco ringspot virus, into the antisense cDNA of 3a gene was performed by recombinant polymerase chain reaction (PCR) using plasmid pCM314) carrying full-length cDNA of CMV-Y RNA3 as a template. Amplified catalytic antisense (860bp) fragment was inserted into the BamHI-SacI sites of the RNA expression vector pT7/T3α-19(BRL). Resulting plasmid, designated pRz2, pRz3 and pRz4, were linearized with EcoRI and the ribozymes were transcribed by T7 RNA polymerase according to the Stratagene protocol (RNA Transcription Kit) for the purpose of confirming their catalytic activity in vitro. Ten µg of total RNA prepared from purified CMV virion was mixed with an excess of in vitro transcribed catalytic antisense RNA and incubated in 25 µl of 50 mM Tris-HCl (pH 8.0) and 20 mM MgCl2 for 30 min at 30°C. Reaction products were fractionated on 1.5% agarose gel under denaturing conditions (Fig. 1B). As expected, all ribozymes with heat denaturation prior to the incubation together with the substrate cleaved CMV-RNA3 in the predicted positions. Without heat denaturation, however, reaction rate was significantly reduced (data not shown), indicating that the ribozyme and/or CMV-RNA3 might fold in stable, inactive structures under the in vitro cleavage conditions.

To determine whether the ribozyme expressed in plant cells could inhibit viral infection in vivo, we introduced the ribozyme sequence into tobacco plants by Agrobacterium-mediated gene transfer. The ribozyme fragments were excised from pRz2, pRz3 and pRz4, and inserted into the BamHI-SacI sites of binary vector pBE2113) to give plasmids pBE2113-Rz2, pBE2113-Rz3 and pBE2113-Rz4, respectively. Non-catalytic antisense cDNA of 3a gene was employed as a control and its expression vector pBE2113-As5 was constructed in the same manner. These four plasmids were mobilized into Agrobacterium tumefaciens LBA4404 and used to transform Nicotiana tabacum cv. Samsun NN as described previously7). Nine to seventeen transformants were obtained for each ribozyme or the non-catalytic antisense construct. They were self-pollinated, and kanamycin-resistant R1 seedlings were used to examine antiviral activity together with transgenic line CP7118), transformed with the CMV coat protein gene. Test plants were grown in a growth room at 28°C. When the plants with 4-5 leaves were inoculated with CMV at a concentration of 10 µg/ml, typical mosaic symptoms developed in upper leaves of all of control plants, transformed with firefly luciferase gene7), within four days after inoculation, whereas delay of symptom development was obser-
Fig. 1. Diagrammatic representation of ribozyme constructions (A) and in vitro cleavage of the CMV RNA3 (B). A. Triangles indicate the ribozyme target sites. Four antisense or ribozyme constructs contain whole 3a-coding region. B. Electrophoretic analysis of cleavage products. Total CMV RNAs prepared from virion were incubated with in vitro transcripts from pRz2 (lane 2), pRz3 (lane 3), pRz4 (lane 4) and pAs5 (lane 5) at 30°C for 30 min and fractionated on 1.5% agarose gel under denaturing conditions. Each transcript was detected just below RNA4 in lanes 2-5. Lane 1, total CMV RNA alone. The positions corresponding to RNA1-5 are indicated to the left.

Fig. 2. Effect of ribozyme and antisense constructs on symptom development in transgenic tobacco plants after inoculation with CMV. A. R₁ progeny with 4-5 leaves of Rz204 (ribozyme construct), As508 (antisense construct) and CP711 (coat protein construct) were inoculated with purified CMV at a concentration of 10 μg/ml. Each ten to twelve plants were inoculated and grown at 28°C all day. Systemic mosaic symptoms in upper leaves were observed. B. R₁ progeny with 5-6 leaves were used and grown at 26°C (day)/22°C (night). Symptom development in the progeny of CP711 were significantly delayed (Fig. 2B). As Okuno et al.⁹ pointed out, increasing temperature may reduce the resistance of CP-transgenic plants. In other cases with different concentrations of inocula (2 and 50 μg/ml), the percentages of plants showing symptoms in progeny of Rz204 after inoculation with CMV were always lower than those of As508. Northern blot analysis showed that they expressed the predicted sizes of RNAs and the expression level of transgene in Rz204 was equal to As508 or less (Fig. 3). Therefore, the antiviral activity observed in Rz204 might be due to the catalytic activity of ribozyme to some extent.

The ribozymes constructed in this study were flanked by the long complementary arms (860 bases) for the reason of increasing the stability of the ribozyme-target RNA hybrids, so that the three ribozyme constructs can be considered to be catalytic antisense molecules. Therefore the plants expressing simple antisense (As508) also showed significant resistance against CMV infection. While the longer complementary arms have shown to increase the reaction rate³, they prevent its turnover. That may be the reason why, in the cleavage experiment in vitro, the longer incubation time (up to 5 hr) did not significantly increase the cleavage efficiency (data not
Fig. 3. Northern blot analysis of total RNA extracted from transgenic plants. One gram each leaf tissues from five R1 plants were combined and subjected to RNA extraction. RNAs fixed on membrane were hybridized with CMV RNA3 cDNA probe. Lane 1, total RNA from Rz204; lane 2, As508; lane 3, non-transformant; lane 4, CP711; lane 5, standard CMV RNA. The positions of CMV RNA3 and RNA4 are indicated to the right.

The determination of the optimum length of complementary arms as well as selection of target sequence is required for providing resistance to viral pathogen in plants.

We are grateful to Dr. Yoshio Ehara, Tohoku University, for the kind gift of CMV Y strain.

Literature cited

和文摘要
中村茂雄・本藏良三・宇垣正志・大島正弘・大橋裕子：キュウリモザイクウイルス RNA3 をターゲットとしたリポザイムを発現する形質転換タバスのウイルス抵抗性
遺伝子組換えによるウイルス抵抗性植物作製を目的として、リポザイムの実用性を検討した。キュウリモザイクウイルス（CMV）RNA3 の 3a 遺伝子内を 1 か所または 2 か所切断するようにデザインしたハンマーヘッド型リポザイムを作製し、その in vitro 転写 RNA を CMV 全 RNA と混合してインキュベートしたところ、予想された RNA3 の切断片が確認された。このリポザイムをバイナリーベクターに挿入し、アグロバクテリアムを用いてタバスを形質転換した。得られた形質転換後代を用いて、CMV に対する抵抗性検定を行ったところ、リポザイムを発現する植物は対照としたアンチセンスを発現する植物より強い抵抗性を示した。

(Received August 1, 1994)