Ribozymes that cleave potato leafroll virus RNA within the coat protein and polymerase genes

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Two ribozymes were synthesized which were designed to cleave potato leafroll virus (PLRV) positive strand RNA within the regions known to encode the viral coat protein and the predicted RNA polymerase gene. DNA sequences encoding the ribozymes were inserted into the *Escherichia coli* plasmids pTz18R and pTz19R under the control of the bacteriophage T7 promoter and enzymically active RNA molecules generated by transcription by T7 RNA polymerase in vitro. Each ribozyme cleaved its cognate site in RNA derived from either cDNA or PLRV particles. Ribozyme cleavage sites within the polymerase gene and coat protein gene were determined and shown to be at the predicted sequence immediately downstream from a GUC motif. An altered version of the ribozyme which recognized the sequence in the coat protein gene was isolated in which a single adenosine residue in the enzymic loop of the ribozyme was deleted. This mutated ribozyme was unable to cleave RNA molecules containing the coat protein ribozyme target site.

Introduction

A number of small, circular RNAs of plants and animals have been found to self-cleave in the absence of protein at specific sites in vitro (reviewed by Symons, 1989). Self-cleavage of these viroid or satellite RNAs is thought to play a key role in their rolling circle mechanism of replication by cleaving concatemers into monomeric RNA molecules (Hutchins *et al.*, 1986; Forster & Symons, 1987). This self-cleavage reaction is catalysed by an RNA molecule containing a structure which is highly conserved between different self-cleaving molecules and is the smallest enzymic RNA structure known (Forster & Symons, 1987). Haseloff & Gerlach (1988) have adapted the consensus structure to develop a sequence-specific endoribonuclease which can be tailored to cleave any exogenous RNA molecule containing the sequence GUC and, less efficiently, molecules containing GUU or GUA but not GUG. Recently a second catalytic structure from a self-cleaving satellite RNA of tobacco ringspot virus has been described (Haseloff & Gerlach, 1989). It is clear that RNA enzymes with highly specific endoribonuclease activities are of considerable interest in the control of cellular gene expression and of infection by pathogens.

Of particular interest is the expression of ribozymes in plants to provide resistance to viral pathogens. Since techniques and expression vectors already exist to construct and regenerate transgenic plants from single transformed cells, all the steps are in place towards isolating transgenic plants expressing ribozymes able to cleave the genomic RNA of viral pathogens.

Potato leafroll virus (PLRV) is the causative agent of an economically important disease of potatoes and is a member of the luteovirus group. PLRV is aphid-transmitted and is normally confined to phloem tissue (Harrison, 1984). The genome is a single, positive-sense, strand of RNA which has been cloned as cDNA by several groups (Prill *et al.*, 1988; Kawchuk *et al.*, 1989) and has now been completely sequenced, and shown to contain six major open reading frames (ORFs) (Mayo *et al.*, 1989; van der Wilk *et al.*, 1989).

In this paper we report the construction of two ribozymes designed to cleave RNA encoding the viral polymerase and coat protein of PLRV and demonstrate their activity in vitro against PLRV RNA.

Methods

*Plasmid construction and purification.* Double-stranded synthetic oligodeoxyribonucleotides encoding the ribozyme recognizing the polymerase gene (Fig. 2) were inserted into the *EcoRI–HindIII* sites of the RNA expression vector pTz18R (Mead *et al.*, 1986) to form plasmid pSAB18, and those specifying the ribozyme recognizing the coat protein gene were inserted into the *XhoI–BamHI* sites of pTz19R to form pSAB30 and pSAB31 (Fig. 2). The 453 bp *EcoRI* fragment containing part of the polymerase gene from positions 2812 to 3265 was inserted into *EcoRI*-cleaved pTz18R and a plasmid with the insert in the desired orientation was designated pSAB20. A 999 bp cDNA
fragment spanning the coat protein gene region from positions 3582 to 4581 was inserted into the Smal site of the plasmid pUC19, from which an EcoRI-SaiI fragment containing the ribozyme target was subcloned into BlueScript M13 + (Stratagene Corporation); the resulting plasmid was designated pSCR103 (B. Reavy, personal communication). Double-stranded synthetic oligonucleotides specifying the 41-mer coat protein target RNA (Fig. 5) were inserted into the HindIII-SaiI sites of pTZ19R to generate plasmid pSAB36.

Plasmid DNA was prepared by SDS/alkali lysis of E. coli cells followed by centrifugation to equilibrium in CsCl-ethidium bromide gradients (Maniatis et al., 1982). Recombinant plasmids were linearized downstream of the T7 promoter and insert (either a cDNA fragment or oligonucleotide). Plasmid pSAB18 was linearized with HindIII, pSAB30 with BamHI, pSAB20 with HindIII, pSCR103 with XhoI and pSAB36 with XbaI. DNA was isolated after proteinase K digestion (50 μg/ml at 55 °C for 1 h), by phenol/chloroform extraction and ethanol precipitation and was redissolved in deionized water (autoclaved with 0.1% diethyl pyrocarbonate) at a concentration of 0.5 μg/μl.

Synthesis of RNA. Plasmids containing ribozyme sequences were transcribed by T7 RNA polymerase in a 25 μl reaction volume containing, 2.5 μg DNA, 5 units T7 RNA polymerase in 50 mM-Tris- HCl pH 7.5, 10 mM- NaCl, 6 mM-MgCl2, 2 mM-spermidine, 25 units RNasin and 500 μM-ATP, -CTP, -GTP and -UTP for 30 min at 37 °C. Plasmids containing target sequences were the same except that the UTP concentration was reduced to 50 μM and contained 80 μCi [α-32P]UTP (800 Ci/mmol).

Cleavage reactions. One quarter of the labelled target RNA (6 μl) was mixed with an excess of unlabelled ribozyme RNA (25 μl) and incubated for 1 h at 40 °C and 0 °C. Reaction products were fractionated on 7.5% (polymerase gene cleavage) or 4% (coat protein gene cleavage) polyacrylamide gels and autoradiographed.

Reverse transcription reactions on viral RNA. PLRV viral RNA (0.5 μg) purified as described by Mayo et al., (1982, 1989) was mixed with an excess (25 μl) of the T7 polymerase reaction mix containing ribozyme RNA synthesized as described above and incubated for 1 h at 40 °C. Ten μg of carrier yeast tRNA and 6 μl 3 m-sodium acetate pH 5.2 were added to each reaction and the products isolated by phenol/chloroform extraction and ethanol precipitation. RNA was redissolved in 65 μl 10 mM-Tris- HCl pH 7.5, 1 mM-EDTA, followed by the addition of 2:5 μl 1 M-KCl, and 1 μl (5 ng) 5'-32P-labelled oligodeoxyribonucleotide primer to analyse the cleavage; in the polymerase gene the primer 5'-TTTGTACACCCGGAACA 3' (complementary to positions 3075 to 3092) was used and for the coat protein gene the oligonucleotide 5'-GTGATCTTATATCATGG 3' (complementary to positions 4012 to 4029) was used. Primers were annealed to cleaved and uncleaved PLRV RNA for 60 min at 60 °C then 25 μl 20 mM-Tris- HCl pH 8.3, 10 mM-MgCl2, 5 mM-DTT and 250 μM each of dATP, dCTP, dGTP and dTTP and 2 units of avian myeloblastosis virus (AMV) reverse transcriptase were added and the samples were incubated for 1 h at 42 °C ethanol-precipitated and fractionated on a 6% sequencing gel alongside a DNA sequence generated by the chain termination method (Sanger et al., 1977) from the same primers annealed to M13 cDNA clones spanning the cleavage sites. Clone V21 H5/1 is M13mp10 containing a HindII-HaeIII PLRV cDNA insert from positions 4136 to 3469; clone 15L1 is M13mp10 containing an EcoRI insert from positions 3265 to 2812.

Sequence of ribozyme-specific plasmids. Plasmids pSAB30 and pSAB31 (specifying the coat protein ribozyme with a deletion) were cut with SpHl-EcoRI and the EcoRI site was end-labelled by Klenow polymerase in the presence of [α-32P]dATP. The products were fractionated on a 10% polyacrylamide gel and the 86 bp (wild-type), and 85 bp (deletion mutant) fragments of each plasmid were isolated from the gel, partially degraded by the method of Maxam & Gilbert (1977, 1980), and fractionated on a 6% polyacrylamide sequencing gel containing 7 M-urea.

Oligonucleotide synthesis. Oligodeoxyribonucleotides were synthesized on an Applied Biosystems model 381A synthesizer and purified on 10% polyacrylamide gels containing 7 M-urea. Purified oligonucleotides were desalted by passage through a Bio-Gel P-6DG column and their concentration was determined by absorbance at 260 nm.

Results

Synthesis of ribozymes with targets in the polymerase and coat protein genes of PLRV

Haseloff & Gerlach (1988) demonstrated that to cleave a specific RNA sequence with the ribozymes they described required that the target RNA contained the sequence 5' GUC 3', and that the ribozyme had arms complementary to at least six bases either side of the chosen GUC target. PLRV has a genome of nearly 6 kb of single-stranded RNA encoding six major ORFs. Of these, sequences encoding a putative RNA-dependent RNA polymerase (P2b, Fig. 1) and the identified coat protein gene (P3) are likely to be required for virus infectivity. Thus we chose to construct two ribozymes which were complementary to sequences within the coat protein and putative polymerase genes of PLRV RNA and should cleave at positions 3968 and 3039 respectively (Fig. 1).

Fig. 1. Diagram representing the genome of PLRV. The numbers above the central line are nucleotides. The six major ORFs are shown (hatched boxes; Mayo et al., 1989). The regions of RNA selected and synthesized from cDNA clones containing part of the P2b (polymerase gene, positions 2812 to 3265) and the P3 (coat protein gene, positions 3582 to 4581) as targets for ribozymes are shown as open boxes. The sequence of the regions recognized by the ribozymes are shown with the GUC motif underlined. Predicted ribozyme cleavage sites are represented by arrowheads.
Ribozyme cleavage of PLRV genes

Fig. 2. The oligonucleotides synthesized to encode ribozymes specific for the polymerase gene (a) and coat protein gene (b) are shown. Those for the polymerase gene ribozyme were inserted into the EcoRI and HindIII sites of pTz18R (designated pSAB18), and those encoding the coat protein gene ribozyme into the XbaI and BamHI sites of pTz19R (designated pSAB30). The boxed regions denote the sequences encoding the complementary arms of the ribozyme. The arrows denote the direction of RNA synthesis by T7 RNA polymerase. Upper RNA sequence: PLRV ribozyme target sequences with the GUC motif underlined. Lower RNA sequence: transcripts made from linearized recombinant plasmids (a) pSAB18 linearized with HindIII and (b) pSAB30 linearized with BamHI. The underlined RNA indicates the complementary ribozyme arms. The mutant coat protein ribozyme (b) expressed by pSAB31 had one of the three As (indicated by a vertical line) deleted. (c) [32P]UMP-labelled transcription products made from the recombinant plasmids were fractionated on an 8% polyacrylamide gel containing 7 M-urea. The polymerase ribozyme (pSAB18, lane 1) is a 53-mer RNA molecule, and the coat protein ribozyme (pSAB30, lane 2) is a 79-mer.

To express ribozymes, oligodeoxyribonucleotides specifying the ribozyme enzymic loop and adjacent complementary sequences were synthesized and inserted into the plasmids pTz18R and pTz19R downstream from the T7 RNA polymerase promoter. In vitro transcription of the linearized plasmids pSAB18 and pSAB30 generated ribozymes that were complementary to PLRV RNA sequences at positions 3029 to 3048 and 3957 to 3977 and should cleave at position 3039 (in the polymerase gene) and position 3968 (coat protein gene) of PLRV respectively (Fig. 2).

Endoribonucleolytic activity of synthesized ribozymes

To generate RNA molecules recognized by their respective ribozymes, cDNA copies of regions of the PLRV genome were inserted into T7 promoter plasmids as described above. Linear plasmids containing either a 453 bp fragment of the polymerase gene (pSAB20), or a 999 bp fragment of the coat protein gene (pSCR103) were used to generate the corresponding RNA molecules by in vitro transcription with T7 RNA polymerase.

Each of the ribozyme RNAs was incubated for 1 h at 40 °C separately with labelled RNA from the polymerase and coat protein genes. The products of these reactions were analysed by electrophoresis in denaturing polyacrylamide gels. Fig. 3 demonstrates that ribozymes to both the polymerase and coat protein gene sequences cleave their respective RNA targets expressed from cDNA clones of these regions to give products of the predicted size. Although cleavage was detected after incubation at 40 °C, no cleavage was detected at 0 °C.
Determination of ribozyme cleavage sites

It was not clear whether an RNA viral genome which is likely to have a native form containing extensive secondary structure would be susceptible to cleavage by ribozymes. To establish that the ribozymes synthesized in vitro could cleave the entire genomic RNA of PLRV and to map the sites of cleavage, PLRV genomic RNA was extracted from purified viral particles isolated from infected potato plants. This RNA (0.5 µg for each reaction) was cleaved separately with an excess of both the polymerase and coat protein ribozymes and the cleavage point determined by primer extension analysis. Oligodeoxyribonucleotides complementary to sequences downstream of the cleavage sites of the ribozymes were used to prime DNA synthesis by AMV reverse transcriptase across ribozyme-cleaved PLRV genomic RNA templates. To generate a sequence ladder the same oligodeoxyribonucleotides were used to prime DNA synthesis on M13 clones spanning the expected ribozyme cleavage sites at positions 3039 and 3968.

The 32P-labelled products were fractionated on a 6% polyacrylamide sequencing gel and autoradiographed. Fig. 4 shows that both the polymerase- and coat protein-targeted ribozymes cleaved genomic PLRV RNA at the expected sites immediately downstream of the GUC motif.

Isolation of ribozymes with sequence alterations in the enzymic loop

After ligation of oligonucleotides specifying coat protein gene ribozymes into plasmid pTzl9R and transformation of *E. coli* DH5x, the presence of small, poorly growing colonies was noticed. Plasmids from these small colonies were isolated and the DNA sequence of the insert specifying the ribozyme was determined (Maxam & Gilbert, 1977, 1980). As shown in Fig. 5(a), the plasmid pSAB31 isolated from slow growing bacteria has a deletion of one A residue of the three adjacent As in the region corresponding to the enzymic loop of the ribozyme (see Fig. 2b) when compared with pSAB30 which specifies the wild-type coat protein ribozyme.

To assess the endoribonucleolytic activity of the altered ribozyme, a small target RNA 41 bases long was synthesized which contained the nucleotide sequence of the coat protein gene flanking position 3968 (Fig. 5b).
Ribozyme cleavage of PLRV genes

The T7 RNA polymerase transcripts which constitute the 41 base target RNA and wild-type and altered ribozymes are shown in Fig. 5(c). Wild-type and mutant ribozymes were incubated separately with the radioactively labelled 41-mer target transcript as previously described. The products of these reactions were fractionated on a 20% polyacrylamide sequencing gel and autoradiographed as shown in Fig. 5(d). The wild-type ribozyme cleaves the 41-mer transcript into two fragments of 20 and 21 bases (lane 3) whereas the mutant ribozyme does not cleave the transcript but appears to form a complex with a higher $M_r$. The difference in intensity of the 21- and 20-mer cleavage products is due to the different $^{32}$P-labelled UMP residue content; the 20-mer contains six U residues but the 21-mer contains only three U residues.
Discussion

We have shown here that ribozymes designed to cleave the genomic RNA of PLRV within two important viral genes work efficiently in vitro. In the experiments described, the synthesized ribozymes have been shown to cleave PLRV RNA at the sites to which the ribozymes were targeted immediately downstream of the sequence GUC. Furthermore we have shown that these ribozymes cleave RNA macromolecules of various sizes, including the entire 6 kb viral genome of PLRV. This is a significant finding because one concern was that a large, folded RNA structure may not be accessible to an exogenous ribozyme designed, and shown to cleave at, a specific sequence present in a small target RNA molecule. Thus the endoribonuclease structure described
by Forster & Symons (1987), and Haseloff & Gerlach (1988) consisting of three parts, i.e. the conserved loop and stem enzymic structure, the homologous arms and the GUC sequence on the target RNA, successfully cleaved every potential RNA target molecule tested in this study. The mechanism by which these ribozymes cleave RNA is by facilitating a nucleophilic attack by an exogenous base upon the phosphodiester bond immediately downstream of the cytosine in the GUC target (reviewed by Cech, 1987). This is likely to involve a torsional strain being exerted upon this bond by the annealing of the ribozyme and target RNA. The divalent cation Mg$^{2+}$ is also required for activity. In this study the cleavage reactions were performed in T7 reaction buffer at pH 7.5 and Mg$^{2+}$ concentration of 6 mM, conditions that were somewhat less favourable than those used by Haseloff & Gerlach (1988), yet cleavage was still observed.

From the more than one-hundred GUC target sites available for cleavage on the PLRV genome two sites were chosen. One was in the polymerase gene, near the consensus sequence for RNA-dependent RNA polymerases (positions 3181 to 3189) identified by Mayo et al. (1989) and Kamer & Argos (1984), which is motif III described by Hodgman (1988). The second was near the middle of the coat protein gene in a region of highly conserved amino acid sequence when compared with the coat protein sequences of the related viruses beet western yellows virus and barley yellow dwarf virus (Mayo et al., 1989). While cleavage at any of the GUC sequences in the viral genome would destroy infectivity, targeting ribozymes to highly conserved regions of the genome should diminish the possibility of PLRV escaping cleavage by mutation of sequences recognized by ribozymes. In addition these important sites ought to be conserved in field isolates of PLRV, which is a significant consideration when choosing viral ribozyme cleavage sites as targets for ribozymes expressed in crops of transgenic potato plants. The optimization of ribozyme structure, expression within different plant tissues and expression within cellular compartments are key considerations towards achieving effective protection from viral pathogens in plants by ribozymes.

The ribozymes synthesized in this study had arms complementary to the target sequence of nine and 10 bases (polymerase gene ribozyme) and nine and 11 bases (coat protein gene ribozyme) and the cleavage reactions were performed at 40 °C. Higher temperatures gave more rapid cleavage (Haseloff & Gerlach, 1988) while longer complementary arms increased the reaction rate (J. Haseloff & W. L. Gerlach, personal communication). Therefore transgenic potato plants expressing ribozymes in field conditions of perhaps 10 to 20 °C may require ribozymes with somewhat longer arms than were used in this study. However ribozymes with long complementary arms may not be able to dissociate readily from the target molecule and therefore will be unable to catalyse further cleavage reactions. The optimum length for ribozyme complementary arms in vivo will have to be determined empirically.

We have shown here that deletion of an A residue within the consensus ribozyme sequence abolishes enzymic cleavage activity on PLRV RNA. This provides further evidence, in an assay system in which the ribozyme is cleaving an exogenous target sequence, that the consensus structure described by Forster & Symons (1987) and Haseloff & Gerlach (1988) is absolutely required for activity. It is interesting to note that the altered ribozyme appears to form some as yet unexplained structure with the 41-mer target RNA. The band observed does not migrate to the same position as the substrate RNA molecules put into the reaction and appears to migrate at a position that indicates that it is too small for a complex between these two molecules unless it is adopting an unusual, folded structure. It may be possible using altered ribozymes incubated with their target molecules in various physiological conditions to observe cleavage intermediates which offer insights into the detailed mechanism of ribozyme cleavage.

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References


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