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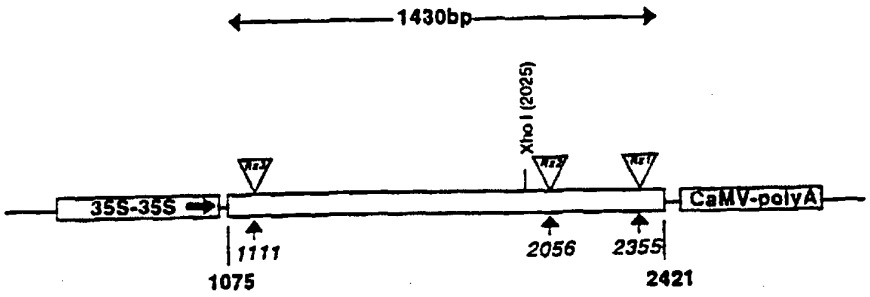

(57) Abstract

The invention relates to a ribozyme comprising a hybridising region and a catalytic region wherein the hybridising region is capable of hybridising to at least part of a target mRNA sequence transcribed from a DNA molecule associated with a DNA virus capable of infecting plant cells, wherein said catalytic region is capable of cleaving said target mRNA sequence thereby substantially reducing replication, infection and/or assembly of said DNA virus.

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DNA VIRUS RIBOZYMES

The present invention is directed generally to synthetic ribozymes and their mutants and derivatives capable of catalysing cleavage of virus specified mRNA and, more particulary, DNA virus specified mRNA. The present invention further contemplates the use of these ribozymes in the generation of transgenic cells and, in particular, transgenic plant cells and plants capable of inhibiting infection by the viruses.

Viruses are the causative agents of a large number of serious and potentially serious diseases in humans, animals and plant. Plant viruses in particular have the potential to destroy or reduce crop yield and to otherwise have a deleterious effect on agricultural and horticultural industries to economically significant levels.

Particularly important viruses in this regard are the DNA viruses, including the Caulimo- and the Geminiviruses and two unclassified viruses, the subterranean Clover Stunt Virus (SCSV, Shu and helms, Virology, 1986, 167, 38-49) and Coconut Foliar Decay Virus (CFDV, Rohde et al, Virology, 1990, 176, 648-51). The geminiviruses are a group of small viruses which infect plant cells. They contain a single stand of circular DNA referred to as "virion-sense" or "positive-sense" DNA of less than about 2900 base pairs. Upon infection of a host cell by a geminivirus, the viral coat protein is removed and a double stranded replicative form of DNA is synthesised comprising the virion-sense strand and a "complementary-sense" strand. Transcription occurs from both the virion-sense strand and from the

"complementary sense" strand, giving rise to (+) and (-) sense RNA transcripts respectively.

Examples of geminiviruses include Maize Streak Virus (MSV), Bean Golden Mosaic Virus (BGMV), African Cassava Mosaïc Virus (ACMV), Chloris Striate Mosaic Virus (CSMV), Tomato Yellow Leaf Curl Virus (TYLCV), Tomato Golden Mosaic Virus (TGMV), Beet Curly Top Virus (BCTV) and Tomato Leaf Curl Virus (TLCV) amongst many others (see also the viruses mentioned in table 1 below). These viruses cause economically important diseases in cultivated plants including leaf curling and other leaf distortion. TLCV in particular causes severe crop damage in northern parts of Australia and infected plants show symptoms similar to those caused by TYLCV in the Middle East and Europe. These tomato viruses are transmitted by the white-fly Bemisia tabaci and constitute a major disease problem world-wide. For convenience, and unless otherwise specified, TLCV and TYLCV are used interchangeably and reference herein to Israeli and Sardinian isolates of TLCV should be taken as TYLCV isolates. In general, "TLCV" refers to the Australian isolate.

Geminiviruses replicate in the nucleus of the infected cell and are thought to employ a rolling-circle mechanism similar to one used by the single-stranded DNA containing coliphages (e.g. QX174) and certain Staphylococcus aureus and Bacillus subtilis plasmids. The other known plant viruses, including other plant DNA viruses, replicate via RNA intermediates.

Geminivirus particles accumulate in the nuclei of infected cells where DNA replication and virus assembly probably take place (Davies et al., J. Cell. Sci (Suppl) (1987) 7 : 95-107). Their putative replicative forms are double-stranded covalently

closed circular DNA Ca. 2,7 Kb in chromatine-like structures and are likely to be the transcriptionally active forms of the virus (Abouzid et al., Mol. Gen. Genet. (1988) 212 : 252-258). Based on host range and insect vectors, they may be conveniently divided into three subgroups : the viruses which infect dicotyledonous hosts, have bipartite genomes and are transmitted by white-fly vectors Bemisia tabaci (Genn.) (ACMV, African Cassava Mosaic Virus (Stanley and Gay, Nature (1983) 301 : 260) ; TGMV, Tomato Golden Mosaic Virus (Hamilton et al, EMBO J. (1984) 3 : 2197) ; BGMV, Bean Golden Mosaic Virus (Horwarth et al, PNAS (1985) 82 : 3512) ; those which have monopartite genomes infect graminaceous hosts, and are transmitted by leafhopper vectors (MSV, Maize Streak Virus (Nigeria strain, Mullinaux et al., EMBO J. (1984) 3 : 3063 ; Kenyan strain, Havel NAR (1984) 12 : 7359) ; DSV, Digitarian Streak Virus (Donson et al., Virology (1987) 161 : 160) ; WDV Wheat Dwarf Virus (Mac Dowell et al., EMBO J. (1985) 4 : 2173)) and those which possess monopartite genomes and have members which can be transmitted by leafhoppers (BCTV, Beet Curly Top Virus (Stanley et al., EMBO J. (1986) 5 : 1761) ; ToYDV, Tobacco Yellow Dwarf Virus (Maris et al., Virology (1992) 187 : 633)) or by whiteflies (TYLCV, Tomato Yellow Leaf Curl Virus (Israeli isolate, Navet et al., Virology (1991) 185 : 151 ; Sardinian isolate, Kheyr-Pour et al. NAT (1991) 19 : 6763), TLCV, Tomato Leaf Curl Virus (Australian isolate, Dry et al., J. Gen. Virol. (1993) 74 : 147)).

Due to economic importance of plant DNA viruses and, in particular, geminiviruses, there is a need for disease resistance strategy to be developed.

One approach is to artificially suppress gene expression by antisense RNA which targets

complementary RNA (Helene, C. & Tolume, J. J. Biochim. Biophys. Acta 1049:99-125, 1990 ; Vander Kro et al Biotechniques 6:958-976, 1988). Although antisense RNA has been successfully used in suppressing viral infection in bacteria and animal tissue (Helene, C & Tolume, J. J. Supra), its use in plants has met with only limited success (Van Del Elzen, P. J. M. et al Plant Mol. Biol. 13:337-346, 1989 ; Powell, P A et al Proc. Nad. Acad. Sci. USA 86:6949-6952, 1989).

Indeed, transgenic plants expressing Cucumber Mosaic Virus (CMV) antisense RNA to different regions were generally not resistant to CMV infection, except one line, which paradoxically produced only very low levels of antisense RNA (Rezaian et al., Plant Mol. Biol. (1988) 11 : 463). There was no proof that antisense was the cause of this resistance. In potato, antisense to PVX coat protein RNA sequence in transgenic plants protected against PVX only at low levels of inoculum (Hemenway et al., EMBO J. (1988) 7 : 1273).

Two major problems appear to be the quantity of antisense needed to cope with a replicating system that can rapidly produce large amounts of viral nucleic acid, and viral RNA is largely in the cytoplasm whereas antisense transcripts are produced in the nucleus.

With respect to geminiviruses, antisense technology has been applied to tobacco plants expressing an antisense DNA sequence of virally encoded AL1 gene of TGMV transcriptionally fused to a drug resistance gene (Day, A. G. et al Proc. Nad. Acad. Sci. USA 88:6721-6725, 1991). Although transgenic plant lines expressing the antisense transcript showed reduced symptom development, it is possible that the transgenic plants were infertile

which would prevent large scale production of virus resistant plants.

In work leading up to the present invention, the inventors sought to overcome the disadvantages of the antisense approach to generating plant DNA virus-resistant plants by employing the strategy of ribozymes.

Ribozymes are synthetic RNA molecules which possess highly specific endoribonuclease activity. In particular, they comprise a hybridising region which is complementary in nucleotide sequence to at least part of a target RNA and a catalytic region which is adapted to cleave the target RNA. Ribozymes are well described by Haseloff J. and Gerlach L. Nature 334:586-591, 1988 and in International Patent Application No. WO89/05852.

Howewer, despite the existence of ribozyme technology, heretofore, it has not been used on plant DNA viruses and in particular plant single stranded DNA viruses like geminiviruses. In fact, the success of the use of ribozymes on DNA viruses was unpredictable in view of their extremely complex replication and transcription mechanism, and the lack of information presently available concerning the compartmentalization of these different operations and the precise level of intervention of the different target sequences in the virus life cycle. For example, it is postulated that the replicase C_1 intervenes in the "rolling circle" replication step but not in the preceding step of the formation of the double-stranded replicative form of the virus (Elmer et al, Nucl. Acid. Res., 1988, 16, p 7043-7060).

In accordance with the present invention, the inventors have successfully developed ribozymes capable of targeting plant DNA viruses thereby

providing an economically viable and efficient means of controlling such viruses.

Accordingly, one aspect of the present invention contemplates a ribozyme comprising a hybridising region and a catalytic region wherein the hybridising region is capable of hybridising to at least part of a target RNA transcribed from a DNA molecule associated with a DNA virus, wherein said catalytic region is capable of cleaving said target mRNA sequence thereby substantially reducing replication, infection and/or assembly of said DNA virus.

More particulary, the present invention is directed to a ribozyme comprising a hybridising region and a catalytic region wherein the hybridising region is capable of hybridising to at least part of a target mRNA sequence transcribed from a virion-sense DNA strand or a complementary-sense DNA strand of a plant single stranded DNA virus, wherein said catalytic region is capable of cleaving said target mRNA sequence thereby substantially reducing replication, infection and/or assembly of said DNA virus.

For convenience, reference to a plant single stranded DNA virus, includes reference to its double stranded replicative form.

In a preferred embodiment, the plant single stranded DNA virus is a geminivirus and the target mRNA encodes a protein essential for replication. By "essential" is meant a protein specified by the viral genome (or a complementary form thereof) and required or substantially required for a particular function such as replication, infection and/or viral assembly. A decrease in the level of the essential protein results in a reduction in the level of that function. The decrease in the level of the particular function. Preferably, the level of essential protein (or level

of particular function) is 20 % less, more preferably at least 30 % less, even more preferably at least 45 % less, still more preferably at least 55 % less and still even more preferably 65-7 5% less and yet more preferably more than 85-90 % less relative to the level of essential protein (or level of particular function) in the absence of the ribozyme.

Conveniently, the level of essential protein (or level of particular function) can be tested in vitro within the limits of biological variation in vivo. Such a test, for example, can be by analysis of mRNA degradation products in vitro.

According to one preferred aspect of the present invention, there is provided a ribozyme comprising a hybridising region and a catalytic region wherein the hybridising region is capable of hybridising to at least part of a target mRNA sequence transcribed from a virion-sense DNA molecule of a geminivirus or a DNA molecule complementary to said virion-sense DNA molecule, said mRNA encoding a protein essential for replication of said geminivirus in a plant cell wherein said catalytic region is capable of cleaving said target mRNA sequence thereby substantially reducing replication of said geminivirus.

In an even more preferred embodiment, the target mRNA sequence is present in low abundance within the cell. An example of such a mRNA molecule is the transcript of the C1 gene or its equivalent in geminiviruses, such as in TLCV or TYLCV and its mutants, derivatives and variants. The single stranded DNA of TLCV contains six open reading frames (ORFs), two on the virion-sense strand and four on the complementary-sense strand of the replicative form DNA produced in leaf curl infected plants. Analysis of the viral mRNAs in infected plant cells reveal that the

mRNA encoding the C1 gene, which is essential for replication, occurs at lower abundance than the other mRNA transcripts specified by the TLCV genome. Equivalent forms of the C1 gene include the genes L1, AL1 and AC1 and reference herein to the "C1 gene" is taken as including equivalents thereof.

Other targets include the C2, C3 and C4 transcripts which play roles in transactivation of virion-sense gene expression and spread of the virus, expression of the disease phenotype, and development of the disease respectively.

According to another preferred embodiment, the present invention is directed to a ribozyme comprising a hybridising region and a catalytic region wherein the hybridising region is capable of hybridising to at least part of a target mRNA sequence encoding the C1 gene of geminivirus and in particular TLCV and wherein said catalytic region is capable of cleaving said target mRNA sequence thereby substantialy reducing replication of said geminivirus (e, g, TLCV).

In a preferred variant, the ribozymes of the present invention can be defined as nucleic acid compounds having endoribonuclease activity and being capable of inhibiting the replication, infection or assembly of a DNA virus, said compound comprising at least one unit, "R", having the general formula :

5' (X-Y-X') 3'

in which :

- X and X' each represent, independently, a nucleotide sequence having at least 4 bases and being complementary to at least a part of either the viral genome, or the (-)sense RNA transcript of a DNA virus ;

- Y represents the catalytic region of a ribozyme.

According to this variant, the target RNA is preferably the (-)sense RNA transcript, i.e. the mRNA originating from the transcription of the complementary sense strand of the double-stranded replicative form of the DNA virus genome, particularly a geminivirus. The sequences X and X' are therefore complementary to at least a part of the (-)sense RNA transcript (which is equivalent to being complementary to at least a part of the single stranded genomic DNA).

The sequences X and X', hereinafter referred to as "hybridising arms" are sufficiently long to allow both stable hybridisation of the ribozyme to the substrate RNA, and efficient cleavage of the substrate. Normally, X and X' will have a length of at least 4 nucleotides each, and preferably at least 6. The upper limit of the lengths of the hybridising arms is generally determined by the length of the targetted substrate and can be as long as 800 or 1000 bases or more. Preferred lengths are between 6 and 300 bases, for example 10 to 150. The complementarity of the hybridising arm to the substrate or to a part of the substrate, normally extends along the entire length of the hybridisisng arm.

The catalytic region of the ribozyme, "Y" is derived from any type of suitable ribozyme, for example "hammerhead" or "hairpin". These ribozymes are described in patent applications EP-A-321201 and EP-A-360257 respectively. Particularly preferred are "hammerhead" catalytic regions (see for example, figure 7), and which will be described in detail hereafter.

The ribozymes may be either "monoribozymes" possessing one single "R" unit of the formula [X-Y-X'], and having only one catalytic region.

Alternatively, the ribozymes may be "polyribozymes" comprising a plurality of "R" units and having the general formula : R_1-R_2-----R_n, wherein n > 1, and may have a value as high as 50 or 100. In practice, a value for "n" of about 5 to 10 is frequent. The polyribozymes are thus constituted by a head-to-tail series of ribozymes, the monoribozyme "R" or [X-Y-X'] unit, being the unit motif. In other words, it is a series of catalytic regions connected together by hybridising arms. Hereinafter, unless otherwise indicated, the aggregate of the X and X' motifs contributed by all the "R" units of a polyribozyme, will be designated collectively as the "complementary" or "hybridising" sequence. The polyribozyme normally acts as a "uni-molecule" against a single transcript, i.e. the cleavage sites of each of the catalytic regions are located on the same transcript.

The precise structure of the polyribozyme is in fact primarily determined by the nature of the substrate it is designed to cleave. The number of catalytic regions, their position in the complementary sequence and the distance between two consecutive catalytic regions are dependent to a large degree on the position of the "three-base target" sites in the substrate.

The "hammerhead" ribozymes cleave the substrate immediately downstream from a "target" site XXX, preferably XUX, in which X represents one of the 4 bases A, C, G, U and U represents uracil. One particularly preferred target sequence is X^0UY in which X^0 is any ribonucleotide, U is uracil and Y

represents adenine, cytosine or uracil. X^0UY forms part of a base pair flanking region and Y is not base paired. Preferred triplets defining X+0UY include, but are not limited to GUC, GUA, GUU, UUC and CUC. Other target sites are possible , but less efficient, for example CAC, UAC and AAC. Perriman et al (Gene, 1992, 113:157) have studied extended target sites for hammerhead ribozymes. It has been found that GUC, UUC, CUC, GUA and GUU targets show equivalent rates of studies. For GUG, the normal ribozyme cannot induce cleavage, but an alteration of the stem-loop in the catalytic domain leads to the formation of a weakly active ribozyme. There are some targets with nucleotides other than U in the center position which show significant, discernable cleavage.

In the case of the ribozymes of the "hairpin" type, a preferred target sequence is AGUC.

These target sequences are important in the construction and functioning of the polyribozymes, not only because they indicate the positions of cleavage of the substrate but also because they define the position at which the catalytic region must be inserted in the complementary sequence. In fact, each catalytic region of the polyribozyme must be situated at a site in the complementary sequence which corresponds to a XUX site of the transcript. For example, if one XUX site is situated at position 412 of the C_1 gene and another at position 711, a catalytic region is inserted at the position corresponding to 412 in the complementary sequence, and another at 711. In other words, the X and X' hybridising sequences which flank the catalytic domain in each "R" unit, are chosen so as to be complementary to the bases surrounding the XUX site targeted in the substrate.

The motif XUX is a motif which occurs very frequently in the RNA sequences. For example, on average there is a GUC motif every 64 bases in a sequence having a random and equal distribution of bases. This signifies that the substrate usually contains a plurality of XUX cleavage sites. As indicated above, the catalytic regions of the polyribozymes are situated at positions of the complementary sequence which correspond to the XUX sites. However, it is not necessary to include a catalytic region for each XUX target sequence of the substrate in order to obtain an efficient cleavage according to the invention. The total number of catalytic regions included in the polyribozyme is normally equal to or smaller than the total number of XUX sites present in the transcript target. The polyribozyme variant of the invention may thus contain a very variable number of catalytic regions. For example, in the case of C_1 of TLCV, the polyribozyme may contain from about 2 to about 6 catalytic regions, when the target sequence is GUC. In the case in which it is decided to include a smaller number of catalytic regions in the complementary sequence than the number of XUX sites in the substrate, the choice of the sites selected may be made by respecting the following criteria :

a) the distance between the 2 XUX sites targeted and, consequently, between 2 catalytic regions in the polyribozyme must be long enough to enable the hybridizing arms of the polyribozyme situated between the corresponding catalytic regions to hybridize with the substrate in a stable manner and to prevent the catalytic regions hybridizing with themselves. A distance of at least 8 bases, and preferably at least

14 bases, for example about 20 bases is particularly advantageous. Of course, this criterion must only be taken into consideration when the substrate contains XUX sites very close together. Otherwise, if the XUX sites of the substrate are separated from each other by more than 8 to 20 bases, this selection criterion is not important.

b) the XUX sites targeted are preferably situated in a part of the substrate which does not have significant secondary structure. This facilitates the access of the polyribozyme to the substrate and increases its efficacy. Three-dimensional folding analyses may be carried out.

c) the XUX sites targeted may form part of the regions of homology conserved between different strains or variants or mutants of one and the same virus, or between different related viruses. This aspect of the invention is considered in detail later.

d) another selection criterion of the XUX sites targeted is the absence of homology with endogenous genes of the plant to be transformed. In fact, although they are rare, some viruses possess sequences which find a homology in the genome of plants. It is thus important to avoid XUX sites situated within such a sequence.

In the ribozymes of the invention, the hybridising X' and X motifs within any one "R" unit are often complementary to adjacent, contiguous partions of the substrate. In this case, the ribozyme can be thought of as "symetric", and the catalytic domain acts at a unique site determined by the complementarity of its two flanking arms. A preferred

example of this symetric type of ribozyme is the "purely complementary" polyribozyme in which the 3' hybridising arm of a given "R" unit and the 5' hybridising arm of the adjacent "R" unit are complementary to sequences which are contiguous in the (-)sense RNA transcript or in the DNA genome.

In other words, in this embodiment, the aggregate of the hybridising arms gives a sequence of uninterrupted complementarity to the target sequence. This type of polyribozyme may be considered as a complementary sequence having, inserted at distinct sites in that sequence, a plurality of ribozyme catalytic regions. The complementary sequence may hybridise with the entire length of the target transcript. On the other hand, it may hybridise with only a fragment of the targeted transcript. The fragment in question must be long enough to allow the inclusion of at least 2 catalytic regions in the corresponding sequence of the polyribozyme. In general, for this variant, the length of the complementary sequence, not counting the catalytic regions (the sum of the hybridising arms), may vary from about 40 to 2000 bases. Lengths of 400 to 1000 is particularly preferred.

According to a variant of the invention, rather than being "symetrical" as described above, the ribozyme may be, or may possess at least one "R" unit which is "asymetrical". This signifies that the X and X' hybridising arms within a given "R" unit are complementary to two sequences which are non-contiguous in the substrate. This in fact means that the catalytic domain of the "asymetric" "R" unit can act at two distinct XUX sites in the substrate, one being the XUX site flanked by the sequence complementary to its 5' arm, and the other being the

XUX site flanked by the sequence complementary to its 3' arm. This type of ribozyme may be specifically constructed, or may be the result of a spontaneous re-arrangement during synthesis. The locations of the sequence to which, on the one hand, the 3' flanking arm hybridises, and the sequence to which, on the other hand, the 5' flanking arm hybridises, may be separated by hundreds of bases or by as little as 10 or 20 bases. This type of structure is advantageous since it allows a significant "economy" in catalytic regions : for example five "asymetric" "R" units can achieve the same result as 10 symetric units.

According to another highly preferred embodiment of the invention, in the polyribozyme having the formula :

$$R_1\text{-}R_2\text{-}R_3\text{-----}R_n,$$

the 3' hybridising arm (X') of a given "R" unit and the 5' hybridising arm of the adjacent "R" unit are complementary to non-contiguous portions of the genome or of the (-)sense RNA transcript. This type of polyribozyme will be referred to hereinafter as a "short arm polyribozyme".

The structure of these polyribozymes is basically a sequence complementary to the target sequence in which ribozyme catalytic regions are embedded and which has undergone deletions in the complementary sequence. The distance between two catalytic regions in the polyribozyme is shorter than the distance between the two corresponding XUX sites in the target sequence. The distance between ribozymes can be reduced significantly with respect to the distance between the corresponding target sites in the substrate, for example, if two adjacent targeted XUX

sites in the substrate are separated by a distance of 1000 nucleotides, the distance between the catalytic regions of the "short arm ribozyme" may be as little as 30 or 40 nucleotides. Reductions of over 90 % of the length of the hybridising sequence may therefore be made. The minimum length between two adjacent catalytic regions is normally about 8 nucleotides and preferably at least 20. The present inventors have shown that such shortened hybridising sequences do not, surprisingly, prevent the polyribozyme from efficiently cleaving the substrate and can, in fact, in many cases improve the efficiency of the inactivation.

For example, the reduction in the length of the complementary sequence avoids problems associated with the formation of secondary structure leading to the inactivation of the ribozyme. Thus, for a given length of ribozyme molecule, more catalytic regions may be inserted targeting more cleavage sites in the substrate.

Furthermore, the use of the short arm polyribozyme means that efficient polyribozymes may be constructed even if only parts of the sequence of the substrate are known. It is sufficient to know the 10 or 15 bases surrounding an XUX target site.

Another important advantage is the possibility to use, as the shortened hybridising arms, exclusively those sequences which are conserved in different viruses, for example viruses of the same type. In this way, different viruses may be inactivated by the same polyribozyme even if the sequences between the conserved regions are totally dissimilar.

Finally the smaller molecule allows an easier and more economical synthesis. The functional nature of the "short arm" ribozymes of the invention prove that,

in a polyribozyme, the different catalytic regions can act independently of each other.

Any one polyribozyme of the invention may include a mixture of "long arm" and "short arm" ribozymes, and also of "symetric" and asymetric" ribozymes. Alternatively, it may include only long arm ribozymes, or only short arm, or only asymetric, etc... The structure of the ribozyme can therefore be optimised, and tested.

According to yet a further embodiment of the invention, the hybridising arms of the polyribozyme may be derived from at least two different types of virus. Such polyribozymes are referred to hereinafter as "chimeric polyribozymes". As an example of this type of construction, mixtures of TLCV and TYLCV derived sequences can be cited, for example : the Sardinian, Israeli or Tunisian strain. This type of polyribozyme ensures the inactivation of both types of virus, if present together, or allows the use of a single construction in different geographical areas.

According to a further variant of the invention, the polyribozymes may include, between two consecutive "R" units, a non-complementary sequence (A) which has a length of at least 4 bases, and which is non-complementary to the (-)sense RNA transcript or DNA genome.

The non-complementary sequence of the polyribozyme may have a precise function, for example, it may be constituted by a coding sequence which can be used to select transformants or also a sequence containing a ribozyme which acts on a substrate other than the primary target of the DNA virus or which is cis acting on a part of the polyribozyme. It typically contains a polylinker for cloning, or an adaptor to facilitate construction of chimeric polyribozyme. The

non-complementary sequence usually has a length comprised between 4 and 500 bases, for example 20 to 100 bases. When there is a plurality of non-complementary sequences, they may together constitute as much as about 90% of the length of the polyribozyme, for example 50%. These non-complementary sequences normally replace portions of the complementary sequences X and X', but leave at least 4 complementary bases immediately next to the catalytic domain.

According to a most preferred embodiment, the ribozymes of the invention are constructed so that the hybridising arms are complementary to a region or to regions which is (are) highly conserved between variants or mutants of the same strain, between strains of the same virus type or even between viruses of different types.

Comparisons of deduced amino-acid sequences encoded by a different open-reading frames of geminiviruses, that is V1, V2, C1, C2, C3 and C4, has been made (see for example references cited in table 1). For the C1 replicase polypeptide, the highest homology occurs between TLCV and TYLCV-I with a homology of 83 %. Between TLCV and TYLCV-S there is 79 % amino-acid identity over the C1 open-reading frame.

If this comparison is made for the Israeli, Sardinian and Tunisian isolates of TYLCV against TLCV, similar homologies are found (see table 1).

TABLE 1 : PAIRWISE COMPARISONS OF DEDUCED AMINO-ACID SEQUENCES ENCODED BY C1 ORF OF GEMINIVIRUSES :

Virus	Amino-acid identity (%)
1 : TYLCV-S/TYLCV-I	77
2 : TYLCV-S/TYLCV-T	75
3 : TYLCV-S/ACMVA	74
4 : TYLCV-S/ICMVA	76
5 : TYLCV-S/ABMVA	67
6 : TYLCV-S/TGMVA	67
7 : TYLCV-S/BGMVA	63
8 : TYLCV-S/SqLCV	53
9 : TYLCV-I/TYLCV-T	78
10 : TYLCV-I/ACMVA	73
11 : TYLCV-I/ICMVA	75
12 : TYLCV-T/ACMVA	72
13 : TYLCV-T/ICMVA	77
14 : ACMVA/ICMVA	74
15 : TLCV/TYLCV-I	83
16 : **TLCV/TYLCV-S**	**79**
17 : TLCV/BGMVA	64
18 : TLCV/PYMVA	68
19 : TLCV/ABMVA	68
20 : TLCV/TGMVA	69
21 : TLCV/ACMVA	78
22 : TLCV/BCTV	61
23 : TLCV/TobYDV	25
24 : TLCV/MSV	25
25 : TLCV/WDV	24

Legend for Table 1 :

For 1 to 14, amino-acid identity is given in percent derived with the program BestFit of the UWGCG-Sequence analysis package. The word size (K-tuple) was 2, the gap weight was 2.0 and gaplength weight was 0.1. The program FastA yielded the same relationship.

For 15 to 25, amino-acid identity was calculated using PALIGN program of the PC/GENE sequence analysis package using the structure-genetic comparison matrix (open gap cost value, 10 ; unit gap cost value, 10).

ABMVA : Abutilon Mosaïc Virus DNA A ; ACMVA : African Cassava Mosaïc Virus DNA A ; BGMVA : Bean Golden Mosaïc Virus DNA A ; ICMV : Indian Cassava

Mosaïc Virus DNA A ; SqlCV : Squash Leaf Curl Virus DNA A ; PYMVA : Potato Yellow Mosaïc Virus DNA A ; TGMVA : Tomato Golden Mosaïc Virus DNA A ; TYLCV-I : Tomato Yellow Leaf Curl Virus Israeli isolate ; TYLCV-S : Tomato Yellow Leaf Curl Virus Thai isolate ; TLCV : Tomato Leaf Curl Virus ; BCTV : Beet Curly Top Virus ; TobYDV : Tobacco Yellow Dwarf Virus ; MSV : Maize Streak Virus ; WDV : Wheat Dwarf Virus.

The results summarized in the table have been given by Dry et al (J. Gen. Virol. (1993) 74:147) and Kheyr-pour et al. (Nucl. Acid. Res. (1991) 19:6763).

The degree of sequence diversity between the 3 isolates of TYLCV and TLCV is in pronounced constrast to the usual very high sequence similarity between different geminivirus isolates or strains, for example : the DNA sequences of the Kenyan and Nigerian isolates of ACMV show 96 % identity and Kenyan, Nigerian and South African isolates of MSV vary maximally by about 2 %. In spite of the pronounced sequence diversity between the 3 isolates of TYLCV and TLCV, the present inventors carried out a comparison of the C1 RNA transcript and identified three very highly conserved regions, two of which are shown in figure 9. These regions are the following (the following numerotation is the TLCV numerotation of the complementary sense transcript) :

1. 365 to 435 approximately
2. 634 to 737 "
3. 895 to 972 "

The nucleotide sequences of the C1 gene in three TLCV isolates (from Australia, Israel and Sardinia, respectively) are shown in Figure 1. The complete nucleotide sequence of the C1 gene from TLCV is disclosed in Mullineaux et al., Virology, 193 : 414-423, 1993 and Dry et al, J. Gen. Virol., 74 : 147-151, 1993. The Israeli isolate is disclosed by Navot et al in Virology, 185 : 151-161, 1991 and the

Sardinian isolate is disclosed by Khyer-Pour et al, Nucl. Acid. Res. 19 : 6763-6769, 1991.

Polyribozymes constructed with target sites in these conserved regions may therefore be used to ensure that all related strains or variants are inactivated. As pointed out above, if the polyribozyme is the deleted short arm version, whose hybridising arms correspond exclusively to the conserved regions, wide sequence diversity between the conserved regions does not affect the efficient hybridisation of the polyribozyme to the substrate.

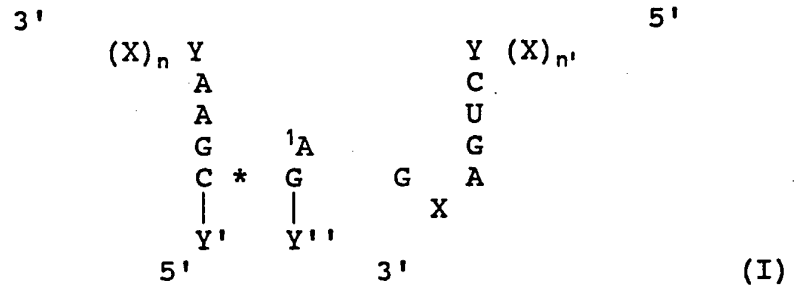
According to this aspect of the invention, a "conserved region" is considered as having a 100 % homology over a distance of about 20 bases. Generally speaking, the homology may be around 90 % over 30 bases. Ribozymes R1, R4, R5 and R2 are designed so that the target sites lie within these conserved regions, as shown in the examples below.

According to this embodiment, therefore, the present invention further relates to a ribozyme comprising a hybridising region and a catalytic region wherein the hybridising region is capable of hybridising to at least one part of a target mRNA sequence encoding the C1 gene of at least two variants of particular geminivirus and in particular at least two variants of TLCV, and wherein said catalytic region is capable of cleaving said target mRNA sequence from each of said variants thereby susbtantially reducing replication of said geminivirus (e.g. TLCV, TYLC and their mutants, derivatives and variants).

The polyribozymes of the invention are usually constituted of RNA. Nonetheless, it is possible to replace some parts of the polyribozyme by DNA, for example the hybridizing arms or parts of these arms,

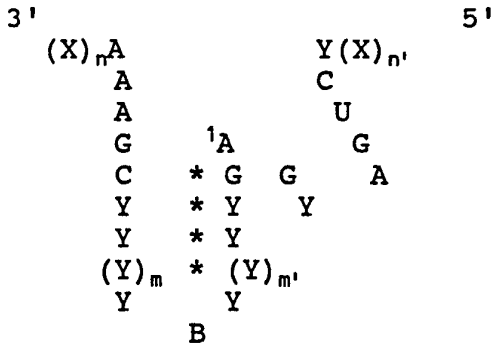
or also a part of the catalytic region, in particular the "loop", provided that the catalytic activity is maintained (see, for example, the substitution of the RNA by DNA described in the international patent application WO-9119789).

As far as the catalytic region is concerned, the ribozymes of the present invention can be represented as a molecule of formula (I) :



wherein, $(X)_n$ and $(X)_n'$ represent ribonucleotide sequences substantially complementary to a mRNA sequence of a DNA virus; the sum of n and n' may be the same or different; an (*) represents a base pair between complementary ribonucleotides ; Y represents any ribonucleotide and each Y may be the same or different ; Y' and Y" represent ribonucleotides of complementary sequence along at least part of their length to allow base pairing between the oligoribonucleotides, or Y' and Y" together form a single RNA sequence wherein at least part of said sequence comprises a stem formed by base pairing between complementary nucleotides; and optionally, an additional nucleotide selected from any one of A, G, C or U is inserted after ^1A.

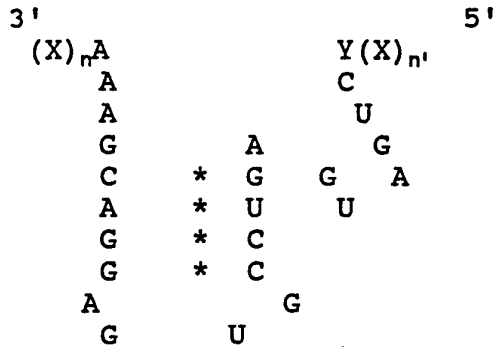
In another embodiment, the ribozyme is as set forth in formula (II) :



(II)

wherein $(X)_n$ and $(X)_n'$ represent ribonucleotide sequences substantially complementary to a mRNA of a DNA virus and in particular a geminivirus such as TLCV ; Y represents any ribonucleotide and each Y residue may be the same or different ; an (*) represents a base pair between complementary ribonucleotides; n and n' are as previously defined; B represents a bond, a base pair, a ribonucleotide, or an oligoribonucleotide containing at least 2 ribonucleotides ; m and m' are 1 or more and may be the same or different ; and optionally, an additional nucleotide selected from any one of A, G, C or U is inserted after ^1A.

In a preferred embodiment, the ribozyme is as set forth in formula (III) :



(III)

wherein $(X)_n$ and $(X)_n'$ represent a ribonucleotide sequence substantially complementary to a mRNA of a DNA virus and in particular a geminivirus such a

TLCV ; Y represents any ribonucleotide and each Y residue may be the same or different an (*) represents a base pair between complementary ribonucleotides ; n and n' are as previously defined.

Preferably, the DNA virus is a single stranded DNA virus. Preferably, the single stranded DNA virus is a geminivirus. Most preferably, the geminivirus is TLCV and $(X)_n$ and $(X)_{n'}$ represent a sequence capable of hybridising to a region of C1 gene mRNA.

In a most preferred embodiment, $(X)_n$ and $(X)_{n'}$ hybridise to the C1 gene mRNA transcript of TLCV or TYLCV as set forth in Figure 1.

Particulary preferred ribozymes of the present invention are specifie to the C1 gene of TLCV and its variants and comprise the nucleotide sequences set forth below :

3'- GUCCAGUCGUGUAAAAAGCAGGAGUGCCUGAGUAGUCGUAGGCUU
GUAAGUC-5' ;

3'- GAGGAAAAAAUAGAAAAGCAGGAGUGCCUGAGUAGUCAAGAAAAC
UAGCUCA-5' ;

3'- GAGUUGUAAACUACAAAGCAGGAGUGCCUGAGUAGUCUAACUUCU
AGUACUA-5'.

and

5'- GATTTAGCTCCCTCTGATGAGTCCGTGAGGACGAAAATGTTCGGATG
GAAATGTGCTCTGATGAGTCCGTGAGGACGAAACCTGGTTGGGGAT-3'

Although the catalytic regions illustrated above and in Figure 7 have a conserved structure and sequence, it has been observed that some nucleotides may be deleted, inserted, substituted or modified without prejudice to the activity of the ribozyme. The invention comprises the use of these modified catalytic regions in the polyribozyme provided that their catalytic activity is conserved. This activity can be verified by using the tests described below.

For example, one or more nucleotides of the catalytic region II illustrated in formula I, II and III may be replaced by nucleotides containing bases such as adenine, guanine, cytosine, methylcytosine, uracil, thymine, xanthine, hypoxanthine, inosine or other methylated bases. The "conserved" bases C-G which together form the first base pair of the catalytic loop, can be replaced by U-A (Koizumi et al., FEBS Letts. 228, 2, 228-230, 1988).

The nucleotides of the catalytic region illustrated in Figure 7 can also be modified chemically. The nucleotides are composed of a base, a sugar and a monophosphate group. Each of these groups can thus be modified. Such modifications are described in "Principles of Nucleic Acid Structure" (Ed. Wolfram Sanger, Springer Verlag, New York, 1984). For example, the bases may bear substituents such as halogeno, hydroxy, amino, alkyl, azido, nitro, phenyl groups, etc.. The sugar moiety of the nucleotide may also be subjected to modifications such as the replacement of the secondary hydroxyl groups by halogeno, amino or azido groups or even to 2' methylation.

The phosphate group of the nucleotides may be modified by the replacement of an oxygen by N, S or C, giving rise to a phosphoramidate, phosphorothioate and phosphonate, respectively. These latter may exhibit useful pharmacokinetic properties.

The bases and/or the nucleotides of the catalytic region may also bear substituents such as amino acids, for example, tyrosine or histidine.

According to a variant of the invention, the ribozyme may comprise as catalytic region one or more structures such as those illustrated in Figure 17. This structure, called "minizyme", is described in the international patent application WO-A-9119789. It

represents a catalytic region of the "hammerhead" type, the "loop" of which has been replaced by a "P" group. P may be a covalent link between G and ^{1}A, one or more nucleotides (RNA or DNA, or a mixture, or even derivatives described above) or any atom or group of atoms other than a nucleotide which does not affect the catalytic activity. When P represents a plurality of nucleotides, it may contain internal base pairings. The sequence and the number of nucleotides constituting the group "P" is not critical and may vary from 1 to 20 nucleotides for example, and preferably from 1 to 6. It is preferable to select a sequence lacking internal base pairings of the Watson-Crick type.

The catalytic activity of the ribozymes of the invention may be verified in vitro by placing the ribozyme, or a sequence which after transcription will give rise to the ribozyme, in contact with the substrate, followed by demonstration of the cleavage. The experimental conditions for the in vitro cleavage reaction are the following : a temperature comprised between 4 and 60°C, and preferably between 20 and 55°C, a pH comprised between about 7.0 and 9.0, in the presence of divalent metals, such as Mg^{2+}, at a concentration of 1 to 100 mM (preferably 1 to 20 mM). The polyribozyme is usually present in an equimolar ratio with the substrate, or in excess. The in vitro cleavage reactions are advantageously carried out according to the procedure described by Lamb and Hay (J. Gen. Virol., 1990, 71, 2257-2264). This article also describes suitable conditions for in vitro transcription for the production of ribozymes from oligodeoxyribonucleotides inserted into plasmids.

The in vivo cleavage conditions are those existing naturally in the cell.

In accordance with the embodiments and aspects of the invention as set forth above, the ribozyme is said to "hybridise" to a target mRNA sequence or to have "complementarity" hybridising arms. Such hybridisation and complementarity is definied herein as an extent of duplex formation sufficient to form a stable enough structure so that cleavage with the target triplet can occur. Depending on the target sequence, the ribozyme and the secondary structure of the mRNA, preferably less than about 5-10, more preferably less than about 3 to 5 and even more preferably less than 1 to 3 bases would be mismatched between the target mRNA sequence and one or both hybridising arms of the ribozyme. Over the whole length of the hybridising arm, not more than 10 % of mismatches should occur. Accordingly, the present invention extends to ribozymes described herein and in particular Rz1, Rz2, Rz3, Rz4 and Rz5, to the polyribozymes containing them, and to any or all functional mutants, derivatives, parts, homologues or analogues thereof. Examples of such mutants include single or multiple nucleotide substitutions, deletions and/or additions to the hybridising regions and/or catalytic regions of the ribozymes provided such modified ribozymes are still functional in cleaving the target mRNA sequence.

Put in alternative terms, the present invention extends to a ribozyme having a hybridising domain and a catalytic domain, wherein the hybridising domain comprises a sequence of nucleotides substantially complementary to a sequence of nucleotides in a mRNA molecule encoding the C1 gene of TLCV or TYLCV or which hybridises to said TLCV or TYLCV mRNA sequence in vitro under high stringency conditions and wherein said catalytic region is capable of cleaving said target mRNA sequence. This aspect of the present

invention is conveniently determined by the extent of cleavage of the target mRNA sequence in vitro.

For the purposes of the present invention, the stringency conditions set forth in Sambrook et al, Molecular Cloning : A laboratory Manual, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, USA, pages 387-389 at paragraph 11 are considered high stringent conditions. Alternatively, medium stringency may be used involving 0.25-0.5 % w/v SDS at 45° C for 2-3 hours. The alternative conditions are applicable depending on concentration, purity and source of nucleic acid molecules.

The present invention extends to genetic constructs comprising the ribozymes herein described. These genetic constructs generally comprise a vector sequence as a vehicle for transferring the ribozyme to a host cell and a promoter sequence to direct transcription of the ribozyme and a terminator. A preferred promoter includes but is not limited to a 35S Cauliflower Mosaïc Virus (CaMV) or to a double 35S CaMV promoter. The CaMV promoter is described by Odell et al, Nature 313 : 810-812, 1985. The ribozymes are generally in the complementary form such that upon transcription, the ribozyme is produced. The genetic constructs of the present invention are used to generate transgenic plants capable of constitutively, developmentally or inducibly (e.g. in response to certain stimuli) expressing the ribozymes herein described. Most preferred transgenic cells are rendered substantially resistant to TLCV infection.

The ribozymes and genetic constructs of the present invention can be conveniently tested in vivo using virus-sensitive plants. A particularly useful indicator plant is the tobacco plant. The analysis may be qualitative such as by screening for severity of

symptoms or quantitative where samples are analysed, for example, by measuring the level ribozyme expression or level of target mRNA. A combination of qualitative or quantitative analyses may also be employed.

All of the known means for introducing foreign DNA into plants may be used, for example Agrobacterium, electroporation, protoplast fusion, bombardment with a particle gun, or penetration of DNA into cells such as pollen, the microspore, the seed and the immature embryo, viral vectors such as the Geminiviruses or the satellite viruses. Agrobacterium tumefaciens and rhizogenes constitute the preferred means. In this case, the sequence coding for the polyribozyme is introduced into a suitable vector together with all of the regulatory sequences necessary such as promoters, terminators, etc....as well as any sequence necessary for selecting the transformants.

In one approach, tobacco cells are transformed using standard procedures employing Agrobacterium with the genetic construct to be tested. Generally, Agrobacterium tumefaciens mediated transformation of tobacco is carried out essentially as described by Horsch et al., Science 227 : 1229-1231, 1985, although conveniently strain LBA 4404 is used and the plantlets are selected on Kanamycin at a concentration of 250 µl/ml. The transformed cells are regenerated into small plants and samples removed to screen for expression. The plants are allowed to self-fertilise and to seed and the transgenic seeds are then used to generate new plants. The transgenic plants along with suitable controls are then exposed to the virus and the degree of symptom development analysed. Alternatively or in addition to, a quantitative

assessment is made by biochemical analysis. This approach is particularly useful for screening for ribozymes against geminiviruses and in particular TLCV. Once suitable ribozyme genetic constructs are identified using this procedure, these particular constructs can then be used to generate, for example, in the case of TLCV and TYLCV, transgenic tomato plants. Similar transformation and screening procedures are employed.

The resistant nature of the transgenic plants can be tested by carrying out a self-fertilisation, or a cross with a non transformed genotype, on a primary descendant to obtain T_1. Subsequently, T_1 plants are inoculated with the DNA virus by Agroinoculation. Degree of resistance (i.e. "complete resistance" meaning total absence of symptoms, "tolerance" meaning that the plant can be infected and shows symptoms but recovers, "sensitive" meaning that the plant exhibits symptoms and replicates the virus) is then noted.

The present invention is particularly directed to transgenic tomato plants which exhibit at least some resistance to TLCV and TYLCV infection. By resistance to infection is meant a qualitative reduction in symptoms (generally due to inhibition of virus replication) by more than approximately 30 %, preferably more than approximately 50 % and even more than approximately 70 %. Due to the variability of biological systems, 100 % inhibition of TLCV replication is difficult to achieve although the effectiveness of the ribozyme can be enhanced by various molecular biological techniques.

According to this aspect of the present invention, there is provided a method of generating transgenic tomato plants exhibiting reduced symptoms following exposure to TLCV compared to non-transgenic

plants, said method comprising transforming tomato plant cells with a genetic construct encoding a ribozyme as hereinbefore described capable of cleaving C1 mRNA from TLCV, regenerating plants from said transformed cells, self-fertilising said regenerated plants and obtaining seeds there from and then growing transgenic plants from said seeds.

The present invention extends to transgenic plants exhibiting resistance to a plant DNA virus, more particularly a geminivirus and even more particularly TLCV. In one preferred embodiment, the transgenic plants are tomato plants. The subject invention also extends to reproductive material and seeds from such transgenic plants (e.g. tomato plants). The ribozymes may be expressed constitutively, developmentally or inducibly.

The present invention is further described by reference to the following non-limiting figures and examples.

In the figures :

- Figure 1 is a representation of the nucleotide sequences of the complementary-sense DNA strands from three different isolates of TLCV : top row, TLCV (Australia) ; middle row, TLCV (Israel) ; bottom row TLCV (Sardinia). The sequence is represented in a 5'->3' direction.

- Figure 2 is a schematic representation of pRIBO5BIN comprising a CaMV35S-1568bp TLCV fragment (containing Rz1, Rz2 and Rz3)-CaMVpolyA expression cassette cloned into BamHI cut Bin19. The insertion sites of the 3 ribozymes Rz1, Rz2 and Rz3 are indicated at positions 1111, 2056 and 2355. This numbering is the virion-sense numbering and corresponds to positions 1654, 711 and 412,

respectively, using the (-)sense numbering as illustrated in figures 8 and 10.

- Figure 3 is a schematic representation of pRIBO5BINDel comprising a CaMV35S-1346bp TMCV fragment (containing Rz1, Rz2 and Rz3)-CaMVpolyA expression cassette cloned into BamHI cut Bin19.

- Figure 4 is a schematic representation of pRIBO7BIN comprising a CaMV35S-396bp TLCV fragment (containing Rz1 and Rz2)-CaMVpolyA expression cassette cloned into BamHI cut Bin19.

- Figure 5 is a schematic representation of pRIBO2BIN comprising a CaV35S-30bp TLCV fragment (containing Rz1)-CaMVpolyA expression cassette cloned into BamHI cut Bin19.

- Figure 6 is a schematic representation of the location of primers used for RT-PCR analysis of ribozyme construct expression in transgenic tobacco.

- Figure 7 is a schematic representation of a ribozyme model (from Haselhoff and Gerlach, Nature, 1988). Substrate RNA can have any sequence (**X**) flanking GUC at the cleavage site, as long as base pairing forms with the ribozyme as shown. The arrow indicates the cleavage site. Conserved bases in ribozymes are boxed. In naturally occuring hammerheads, the substrate and ribozyme portions are connected by a loop, resulting in an intramolecular cleavage.

- Figure 8 shows the nucleotide sequence of the complementary-sense strand of TYLCV-S. Lower case letters indicate the ribozyme cleavage site. The sequence of TYLCV-S was published by Kher-Pour et al. (Nucl. Acid Res. (1991) 19 : 6763)

- Figure 9 shows the regions conserved in the Replicase gene, between TLCV and TYLCV-S : Figure 9(a) shows the regions highly conserved around ribozymes

R1, R4 and R5 ; Figure 9(b) shows the regions highly conserved around ribozyme R2. ":" means a mismatch, a gap means no nucleotide. Lower case letters indicate the ribozyme cleavage site.

- Figure 10 illustrates the nucleotide sequence of the complementary strand of TLCV. Dry et al. (J. Gen. Virol. (1993) 74 : 147) Lower case letters indicate the ribozyme cleavage site.

- Figure 11 shows the target sequences for polyribozymes D, F and E. Lower case letters indicate the ribozyme cleavage site. Upper case letters correspond to the complementary sequences of ribozyme arms.

- Figure 12 is a schematic representation of ribozyme constructs. A hatched line represents the TYLCV-S complementary-sense strand, and a solid line represents TLCV complementary sense strand. R = ribozyme.

- Figure 13 is a schematic physical map of binary vector pGAZE.

- Figure 14 is a schematic physical map of ribozyme plasmids, pGAZE-RzA, pGAZE-polyRzC, pGAZE-polyRzD, pGAZE-polyRzE, pGAZE-polyRzF.

- Figure 15(a) is a schematic representation of pRIBO5BS : 1690bp TLCV C1/C2/C3 complementary fragment (containing 3 ribozymes) blunted into EcoRV cut Bluescript SK+ ; Figure 15(b) is a schematic representation of pRIBO5BIN : 2660bp Bgl II 35S-C1/C2/C3 complementary (containing 3 ribozymes) , CaMVpolyA cassette cloned into BamHI cut BIN 19; open triangles show the position of 22bp ribozyme insertions, E : EcoRI ; H : HindIII ; K : KpnI ; P : PstI ; Sm : SmaI ; B : BamI ; Sl : SalI ; St : SstI ; X : XhoI ; Xb : XbaI ; C : ClaI.

- Figure 16 is a schematic representation of the ribozyme constructs, showing their inter-relationship and the deletions carried out to obtain the "Short-arm" ribozymes, polyE, polyF and polyD.

- Figure 17 illustrates the "Minizyme" structure (WO-A-9119789) wherein the loop of the catalytic region is replaced by an element "P". P may be at least 1 nucleotide (ribonucleotides, deoxyribonucleotides, derivatives or a mixture), provided that the ribonucleotides of the "P" group are not base paired by "Watson -Crick" base pairings when the sequences $(X')_n$ and $(X)_{n'}$ and P are constituted exclusively of ribonucleotides. "P" may also be a bond or any atom or group of atoms which do not affect the catalytic activity of the ribozyme. X has the same meaning as in formula I, II, III.

EXAMPLES

EXAMPLE I - SELECTION OF TARGET mRNA OF TLCV :

To enable selection of a suitable target RNA, the major mRNA transcripts were identified and mapped on the circular genome of an Australian isolate of TLCV [TLCV (Australia), Mullineaux et al., Supra ; Dry et al, Supra]. One of the viral mRNAs derived from the complementary sense strand of the viral DNA occurred at a lower abundance than the other TLCV transcripts. This low abundance mRNA transcript was identified as encoding the C1 gene, the product of which, is essential for replication of the virus. [Davies et al, Trends in Genetics, 5 : 77-81, 1989). The mRNA transcript also encodes three other gene products, designated C2, C3 and C4, whose functions are not

conclusively known. Accordingly, the low abundance mRNA encoding the C1 gene was selected as the target mRNA. The low abundance of this transcript means a higher ratio of ribozyme target sequence, thereby potentially permitting a more substantial decrease in C1 gene product.

EXAMPLE II - SELECTION OF RIBOZYMES BASED ON CONSERVED REGIONS OF C1 GENE BETWEEN TLCV ISOLATES :

The nucleotide sequences of the complementary sense strands of three TLCV isolates [TLVC (Australia), TLCV (Israel) and TLVC (Sardinia)] were compared in order to identify conserved regions for targeting by ribozymes. The sequence comparison is shown in figure 1. Three conserved regions were identified for design of ribozymes. Two of these conserved regions are shown in figure 1. In addition to sequence comparisons, a computer program can be used to predict secondary structure of the target site to enable greater efficacy of the ribozyme.

EXAMPLE III - SYNTHESIS OF RIBOZYMES TARGETING CONSERVED SEQUENCES OF C1 GENE OF TLCV :

Ribozyme 1 (Rz1) :
3'- GUCCAGUCGUGUAAAAAGCAGGAGUGCCUGAGUAGUCGUAGGCUU
GUAAGUC - 5'

Ribozyme 2 (Rz2) :
3'- GAGGAAAAAAUAGAAAAGCAGGAGUGCCUGAGUAGUCAAGAAAACU
AGCUCA - 5'

Ribozyme 3 (Rz3) :
3'- GAGUUGUAAACUACAAAGCAGGAGUGCCUGAGUAGUCUAACUUCUA
GUACUA - 5'

EXAMPLE IV - GENETIC CONSTRUCTS CARRYING Rz1, Rz2 and/or Rz3 : pRIBO5BIN :

A 1568 fragment of TLCV from nucleotide 1075-2643 covering the C1, C2, C3 & C4 reading frames (ORPS) was cloned into pALTER-1 (Promega). Ribozyme sequences 1, 2 & 3 (Rz1, Rz2 and Rz3, respectively) were chemically synthesised as described in Example III and introduced into the TLCV sequence using the Altered sites in vitro mutagenesis system (Promega). The integrity of introduced ribozyme sequences was confirmed by sequencing using the dideoxy chain termination method. Rz1, Rz2 and Rz3 were shown to catalyse in vitro cleavage of a complementary-sense viral RNA transcript generated with 57 RNA polymerase at 50° C and 37° C.

The modified 1634 bp complementary-sense TLCV fragment containing Rz1, Rz2 and Rz3 was cloned in the antisense orientation (relative to C1 ORF transcription) between a double 35S CaMV promoter and a CaMV polyA termination sequence and the whole expression cassette introduced into the BamHI site of the multiple cloning site of the binary vector Bin19. The resulting construct is pRIBO5BIN and is shown in figure 2.

EXAMPLE V - GENETIC CONSTRUCTS CARRYING Rz1, Rz2 and/or Rz3 : pRIBO5BINDel :

This construct was derived from pRIBO5BIN (Example IV) by deletion of a 216 bp region from the BstBI site (nucleotide 2424) to the 3'-end of the TLCV fragment (nucleotide 2643). The resulting construction is pRIBO5BINDel and is shown in figure 3.

EXAMPLE VI - GENETIC CONSTRUCTS CARRYING Rz1, Rz2 and/or Rz3 : pRIBO7BIN :

This construct was derived from pRIBO5BINDel (Example V) by removal of a 950 bp region from the 5'-end of the TLCV fragment (nucleotide 1075) to the XhoI site (nucleotide 2025). The resulting construct is pRIBO7BIN and is shown in figure 4.

EXAMPLE VII - GENETIC CONSTRUCTS CARRYING Rz1, Rz2 and/or Rz3 : pRIBO2BIN :

A 52 bp fragment containing Rz1 was amplified by polymerase chain reaction (PCR) from pRIBO5BIN (Example IV) and cloned in the antisense orientation (relative to C1 ORF transcription) between a double 35S CaMV promoter and a CaMV polyA termination sequence and the whole expression cassette introduced into the BamHI site of the multiple cloning site of the binary vector Bin19. The resulting construct is pRIBO2BIN and is shown in figure 5.

EXAMPLE VIII - EXPRESSION OF RIBOZYME GENETIC CONSTRUCTS IN TRANSGENIC TOBACCO CELL LINES :

The ribozyme constructs described in Examples 5 to 8 were tested for expression in transgenic tobacco plants using PCR analysis.

Leaf samples (150 mg) were collected from transgenic tobacco lines prior to potting out in the glasshouse and frozen in liquid N_2. Samples were ground to a fine powder and vortexed for 30s with 0.5 ml extraction buffer (containing 0.1 M Tris-HCl (pH 9), 0.1 M NaCl, 10 mM EDTA, 1 % w/v SDS and 1 % v/v beta-mercaptoethanol) and 0.5 ml phenol. The

extract was centrifuged at 12 000 g for 10 mn and the supernatant fraction collected. The supernatant fraction was extracted a further time with phenol and then with phenol/chloroform (1:1). The total nucleic acid fraction was precipitated at -20° C with sodium acetate/ethanol and centrifuged at 12 000 g for 10 mn. The pellet was washed with 80 % v/v ethanol, dried and resuspended in 50 µl sterile water.

First strand cDNA reactions were carried as follows. Nucleic acid samples were annealed at 65° C for 5 mn with 100 mg of CaMV Term primer (5'-TTTACAAATACAAATACATACTAAGGGT in a total volume of 5 µl. This primer is complementary to a region within the CaMV termination sequence present in all ribozyme construct (see figure 6) transcripts produced from these transcriptions.

To the annealed template was added 5 µl of reverse trascriptase mix containing 50 mg Tris-HCl (pH 8.3), 50 mM KCl, 10 mM $MgGl_2$, 0.5 mM spermidine, 10 mM DTT, 1 unit ribonuclease inhibitor and 2.25 units AMV reverse transcriptase. The mixture was incubated at 42° C for 60 mn followed by 94° C for 2 mn. Where indicated, control reactions were performed in which the AMV reverse transcriptase was omitted from the reaction mM Tris-HCl (pH 9), 1.5 mM $MgCl_2$, 1.5 mM $MgCl_2$, 0.1 % v/v Triton X-100, 200 µM dNTPs, 250 µM CaMV Term primer (see figure 6), 0.5 units of Taq DNA polymerase and 2 µl of first strand cDNA reaction mix. Two alternative 5'-end primers were used, the positions of which are shown in the attached figure :

C3 primer : 5'-ACTGATCTAATTACATTGTTAATGC

R1B primer : 5'-GCAAGCTTCCTGAATGTTCGGAT

PCR cycling conditions were 94° C for 1 mn, 50-55° C for 1 mn, 72° C for 3 mn for 15-30 cycles. PCR products were analysed on 1.2-1.8 % w/v agarose gels.

Reverse transcriptase (RT)-PCR analysis of RIBO5BIN and RIB05BINDel lines using the C3/CaMV Term primer combination produces DNA products approximately the predicted sizes i.e. 1690bp and 1450bp respectively.

RT-PCR analysis of RIBO5BIN, RIBO5BINDel and RIBO2BIN lines using the R1B/CaMV Term primer combination produces DNA products approximating the predicted sizes i.e. 504bp, 264bp and 235 bp, respectively.

No PCR products were observed in control reactions : (a) no reverse transcriptase (b) nucleic acid samples from plants transformed with vector sequences alone i.e. not containing ribozyme sequences. This demonstrates that the PCR products observed with these primers were derived specifically from RNA transcripts of the introduced ribozyme constructs.

Predicted PCR product sizes using the C3/CaMV Term primer combination are as follows :

1.	RIBO5BIN	1690 bp
2.	RIBO5BINDel	1450 bp
3.	RIBO7BIN	-
4.	RIBO2BIN	-

Predicted PCR product sizes using the RIB/CaMV Term primer combination are as follows :

1. RIBO5BIN	504 bp
2. RIBO5BINDel	264 bp
3. RIBO7BIN	264 bp
4. RIBO2BIN	235 bp

EXAMPLE IX - CONSTRUCTION OF RIBOZYME GENES :

Pairwise comparisons of deduced amino-acid sequences encoded by C1 ORF of geminiviruses were investigated (table 1).
Alignment of nucleotide sequences of TYLCV genomes isolates from Sardinia (Kheyr-Pour et al., NAR (1991) 19 : 6763), Thai (Rochester et al., Virology (1990) 178 : 520), Israeli (Navot et al., Virology (1991) 185 : 151), Australia (Dry et al., J. Gen. Vir. (1993) 74 : 147) shows that there are three conserved regions in an internal fragment of the C1 transcript encoding the polypeptide essential to replication of the virus. This BstBI / Eco 0109I internal fragment (nt 346 to nt 982) of TYLCV-S genome was cloned into a Dra II/ Sma I digested pBluescript II SK+ phagemid (STRATAGENE). This clone was used as template for the introduction of 2 ribozymes in the most highly conserved region. Two and three dimensional folding analyses were also made to retain the more theoretical accessible target sites for cleavage with ribozyme.

The catalytic domain of hammerhead-type RNA (figure 1), in this case satellite RNA of tobacco ringspot virus (STobRV), was used to design ribozymes that form, with a completely unrelated substrate RNA, a hammerhead structure in trans, so that the target RNA is specifically cleaved at the predicted site (Haseloff and Gerlach (1989), Gene, 82 : 43-52). According to the general rules described by these authors, the only requirements for a substrate RNA to

be cleaved by hammerhead-type ribozymes is the presence of the trinucleotide sequence for which self-cleavage has been described (Perriman et al., Gene (1992) 113 : 157). The two positions for the hammerhead-type ribozymes retained on the most highly conserved region of the C1 transcript of TYLCV-S are shown in figures 2 and 3 :

* position R4, nucleotide 407 (target site GUC) ;
* position R5, nucleotide 430 (target site UUC) ;

To introduce the 22 bases of the hammerhead sequence of the satellite RNA of TobRV at these positions, the strategy of rapid insertional mutagenesis of DNA by polymerase chain reaction (PCR) described by Kammann et al. (Nucl. Acid. Res. (1989) 17 : 5404) was used.

This method requires the following synthetic oligonucleotides :

Oligo R4-R5 carrying the 2 hammerhead-type ribozymes :

5' GATTTAGCTCCCTCTGATGAGTCCGTGAGGACGAA**AA**TGTTCGGATG
GAAATGTGCTCTGATGAGTCCGTGAGGACGAA**AC**CTGGTTGGGGAT 3'

The underlined sequences correspond to the hammerhead structure of the satellite of ToRSV. Bold letters correspond to the target site.

This oligo R4-R5 was synthesized on an Applied Biosystems Model 380B DNA synthesizer.

Oligo T7 sequencing primer (Pharmacia) :

5' TTAATACGACTCACTAT 3'

Oligo T3 sequencing primer (Pharmacia) :

5' ATTAACCCTCACTAAAG 3'

In the first PCR (PCR I) step, the sequence between oligo R4-R5 and oligo T3 sequencing primer was amplified. The amplified fragment was purified on a molecular sieving column (PrimeErase Quick ™ kit, STRATAGENE). In the second PCR (PCR II) step, this amplified fragment was used itself as a primer in combination with oligo T7 sequencing primer. The PCR conditions were as follows :

PCR was carried out in a total reaction volume of 100 µl containing 50 mM KCl, 10 mM Tris-HCl pH 8.3,1.5 mM MgCl2, 300 µM dNTP each, 2 U Taq polymerase (Perkin Elmer Cetus), 40 pg template DNA, 100 pM primer each. PCR II was the same as PCR I, except that 5 µl (300 ng of amplified fragment estimated from agarose gel) assay volume of PCR I was directly used for priming. Denaturation : 2 min 94°C ; annealing 2 min 40°C ; synthesis ; 3 min 72°C with 30 cycles for each step.

After purification using agarose electrophoresis, the PCR product was digested with BstBI, filled in using Klenow polymerase and finally digested with Eco0109I. The resulting 679 nt fragment was cloned into a Sma I /Dra II digested Bluescript SK+ vector. The cloning vector containing the cloned sequences was transformed into, and propagated in, the bacterial host XL1 Blue (STRATAGENE), using techniques known in the art (Maniatis, Molecular cloning, A laboratory guide, 2nd edition, 1989, Cold Spring Harbor Laboratory publishers).

The presence of the 2 hammerhead sequences of the satellite RNA of TobRV was verified by sequence analysis using T7 sequencing kit (Pharmacia). The result shows a perfect insertion of catalytic R4 and R5 sites.

EXAMPLE X - CONSTRUCTION OF RIBOZYME PLASMIDS :

Plasmid pRIBO5BS shown in figure 15 was the base point for the construction of the other plasmids. This plasmid is a pBluescript SK+ vector carrying a 1690 bp 5' end - Ase I fragment which corresponds to the C1/C4/C2/C3 complementary fragment of TLCV (figure 4) bearing 3 hammerhead-type ribozymes at positions :

- R1 (nucleotide 412, target site CUU),
- R2 (nucleotide 711, target site CUU) and,
- R3 (nucleotide 1654, target site GUA).

Plasmid pPolyribozyme E is derived from plasmid pRIBO5BS by deleting the 877bp "filled-in using Klenow Polymerase Xho I - Bcl I" fragment corresponding to the region between 2 hammerhead-type sequences at positions R2 and R3, and the 264 bp "5' end - Tth 111I" fragment. The Tth 111I site was filled-in using klenow polymerase. The "Tth III I - HindIII (site of pBluescript SK+ vector") fragment was isolated after separation by electrophoresis on agarose gel. This fragment was cloned into EcoRI filled-in using Klenow Polymerase and Hind III sites of pBluescript SK+ vector, deleted previously from Sac I-Xba I digested using Mung Bean Nuclease fragment.

The polyribozyme E contains 434 bases and includes 3 ribozymes : R1 (position 28), R2 (position 327) and R3 (position 399) as shown in figures 5 and 6 (polyR2E-R2). The size of the arms flanking the catalytic domains of the ribozymes is :

* 27 nucleotides from 5' end to ribozymeR1 ;
* 298 nucleotides between R1 and R2 ;
* 71 nucleotides between R2 and R3 ;
* 35 nucleotides from ribozyme R3 to 3'end.

The polyribozyme F plasmid is derived from pPolyribozyme E plasmid. The 56bp "SmaI (site in

pBluescript SK + vector near 5' end) - Sac I" fragment of the replicase gene of TLCV containing the ribozyme R1 was replaced with the 98bp "BstBI filled-in using klenow polymerase - Sac I" fragment of replicase gene of TYLCV - Sardinian isolate bearing the two ribozymes R4 and R5 as described previously. The exchanged fragments possess a region which is very highly conserved between different strains of TYLCV and TLCV. The nucleotide sequence identity corresponds to 97 % between the 365 nt to 435 nt region of TLCV and the 370 nt to 440 nt region of TYLCV-Sardinian isolate (Figure 3).

The chimeric polyribozyme F contains replicase gene fragments which have two different origins : TYLCV-Sardinian isolate and TLCV-australian isolate. The assembly of these fragments was made at the Sac I site. It is also possible to assemble the two fragments using an artificial polylinker. The inclusion of this non-complementary sequence has no deleterious effect on the catalytic activity of the polyribozyme.

The polyribozyme F contains 479 bases and includes 4 ribozymes : R4 (position 59), R5 (position 83), R2 (position 372) and R3 (position 444), as shown in figures 5 and 6.

The size of the arms flanking the catalytic domains of the ribozymes is :

* 59 nucleotides from 5' end to ribozyme R4 ;
* 22 nucleotides between ribozymes R4 and R5 ;
* 288 nucleotides between ribozymes R5 and R2 ;
* 71 nucleotides between ribozymes R2 and R3 ;
* 35 nucleotides from ribozyme R3 to 3' end.

The chimeric pPolyribozyme D is derived from pPolyribozyme F. The 206bp "SacI-SwaI" fragment originating from TLCV was deleted. The Sac I site was

destroyed by action of T4-DNA polymerase. The polyribozyme D contains 270 bases and includes 4 ribozymes, R4 (position 59), R5 (position 83), R2 (position 163) and R3 (position 235) as shown in figures 5 and 6.

The size of the arms flanking the catalytic domains of the ribozymes is :

* 59 nucleotides from 5' end to ribozyme R4 ;
* 22 nucleotides between ribozymes R4 and R5 ;
* 79 nucleotides between ribozymes R5 and R2 ;
* 71 nucleotides between ribozymes R2 and R3 ;
* 35 nucleotides from ribozyme R3 to 3' end.

The sequences of polyribozyme E, F and D were verified using the T7 sequencing kit supplied by Pharmacia.

The functioning of polyribozymes E, F and D was tested in vitro in accordance with the protocol described in "Protocol and Application Book" from Promega.

No expression signals are contained in the polyribozymes. For this reason, they were placed under the control of double 35S promoter and poly(A) of 35S RNA of CaMV.

To realize these constructions, the expression cassette composed of a double 35S promoter, monoribozyme R1 and poly (A) of 35S of CaMV was isolated from pRIBO2BIN plasmid described above.

The pRIBO2BIN plasmid was digested with Sac I. The Sac I sites were made blunt-ended using T4-DNA polymerase.

The linearized pRIBO2BIN plasmid was then digested with Sal I, the "Sac I filled-in Sal I" fragment which contains the expression cassette was purified on agarose gel and cloned into pBluescript

SK+ vector in "Xba I filled-in using Klenow Polymerase and Sal I" sites.

The Hind III - Bam HI fragment corresponding to monoribozyme R1 was then replaced with HindIII - BamHI fragments containing the polyribozymes E, F and D isolated from pPolyribozymes E, F and D respectively. The Hind III - BamHI fragment is in inverse orientation in the expression vector.

The expression cassette with polyribozymes E, F or D were cloned into the binary vector pGAZE (figure 7) derived from pGA492 (An, Plant Physiol. (1986) 81 : 86). The pGA 492 vector was double digested with Sac I and Sca I, made blunt-ended using T4-DNA polymerase and ligated after agarose gel purification.

The eliminated fragment corresponds to nearly the entire chloramphenicol acetyl transferase gene. The Hind III site was also converted into EcoRI site with HindIII - EcoRI adaptors supplied by Stratagene. As for pGA492, pGAZE contains the cis-acting elements required for plant transformation, including T-DNA border, a selectable marker expressible in plants (kanamycin resistance), cloning sites, and a wide host range replicon. The kanamycin resistant marker is constructed from nopaline synthase control regions and neomycin phosphotransferase coding region of Tn5.

The pGAZE vector was digested with EcoRI and filled-in using Klenow-polymerase. The "SacI filled-in - Sal I" fragments containing the expression cassettes with polyribozymes E, F or D were cloned into the. EcoRI filled-in site of pGAZE.

The obtained recombinant binary vectors, pGAZE-polyribozyme E, pGAZE-polyribozyme F and pGAZE-polyribozyme D (figure 7) were introduced into the desarmed strain LBA4404 of Agrobacterium tumefaciens (Hoekema et al., Nature (1983) 303 : 179) by direct

transformation (protocol derived from Holsters et al., Mol. Gen. Genet. (1978) 163 : 181)

For ribozyme A, which corresponds to monoribozyme R1 including the catalytic domainand flanking arms 15 bases long, and polyribozyme C described above (example V) expression cassettes were introduced in pGAZE and/or pGA492 as mentioned above.

Thus, pGAZE-ribozyme A, pGA492-ribozyme A and pGAZE-polyribozyme C (Figure 8) were obtained. The genes of interest of all the constructions described above were transferred into tomato using Agrobacterium based co-cultivation system. Tomato transformation and regeneration was carried out as described by Fillatti et al (Biotechnology (1987) 5 : 726).

EXAMPLE XI - MOLECULAR ANALYSIS OF TRANSGENIC PLANTS

*** RNA analysis :**

Total RNA is isolated from young leaves of transgenic and non-transgenic plants grown in the greenhouse according to Chandler et al. (Plant Physiol. (1983) 74 : 47). RNA is separated on agarose-formaldehyde gels and transferred to a nitrocellulose membrane. The blot is hybridized with labelled fragment or entire gene of interest. To check whether equal amounts of RNA were applied to each line, the nitrocellulose membrane is stained with methylene blue.

*** DNA analysis :**

For Southern blot analysis, total DNA is isolated from young leaves of transgenic and non-transgenic plants grown in the greenhouse according to Dellaporta et al. (Plant Mol. Biol. Rep. (1983) 1 : 19), digested with restriction enzymes according to the

manufacturer's instructions, separated by electrophoresis on agarose gel and transferred to nitrocellulose by capillary action. The blot is probed with labelled fragment or entire gene of interest.

EXAMPLE XII - EVALUATION OF RESISTANCE TO TYLCV FOR TRANSFORMED TOMATO PLANTS WITH THE DIFFERENT CONSTRUCTIONS BY AGROINOCULATIONS

Since TYLCV or its cloned DNA are not transmitted mechanically, the agroinoculation technique (Grimsley et al., Plant DNA Infections Agents, Hohn and Schell (eds) Springer, Wien, New York (1987) pp 87-107) is used to assay to infectivity of the cloned DNA.

The TYLCV-Sardinian strain genome was inserted into Sst I site of the binary plant transformation vector pBin 19 (Stanley et al., EMBO J. (1986)5 : 1761) and a BamHI-fragment of about 600 bp extending from polylinker of pBin 19 through the intergenic region of TYLCV to the BamHI site at map position 152 of TYLCV was deleted. Subsequently a complete genome unit of TYLCV was inserted into the remaining Sst I site yielding a 1,8 mer of the TYLCV-genome in pBin 19. This plasmid was introduced directly into Agrobacterium tumefaciens strain LBA4404. Agrobacterium tumefaciens cultures were grown at 28°C for about 48 hours. The cells were pelleted and washed twice with water and resuspended in 1/10 th of the initial volume of sterile water.

Young transformed tomato plants, at the 3 to 4 leaf stage, are inoculated into the petioles of the three youngest leaves with the concentrated Agrobacteria using a 18-gauge needle.

The plants are placed in a closed growth chamber with 16 h - day light at 24°C under 70 % relative humidity.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

CLAIMS

1. A ribozyme comprising a hybridising region and a catalytic region wherein the hybridising region is capable of hybridising to at least part of a target mRNA sequence transcribed from a DNA molecule associated with a DNA virus capable of infecting plant cells, wherein said catalytic region is capable of cleaving said target mRNA sequence thereby substantially reducing replication, infection and/or assembly of said DNA virus.

2. A ribozyme according to claim 1 wherein the plant DNA virus is a single stranded DNA virus.

3. A ribozyme according to claim 2 wherein the single stranded DNA virus is a geminivirus.

4. A ribozyme according to claim 3 wherein the geminivirus is selected from the group consisting of Maize Streak Virus (MSV), Bean Gold Mosaïc Virus (BGMV), African Cassava Mosaïc Virus (ACMV), Chloris Striate Mosaïc Virus (CSMV), Tomato Yellow Leaf Curl Virus (TYLCV), Tomato Golden Mosaïc Virus (TGMV), Tomato Leaf Curl Virus (TLCV) and Beet Curly Top Virus (BCTV).

5. A ribozyme according to claim 4 wherein the geminivirus is Tomato Leaf Curl Virus (TLCV), Tomato Yellow Leaf Curl Virus (TYLCV) or a mutant, derivative or variant thereof.

6. A ribozyme comprising a hybridising region and a catalytic region wherein the hybridising region is capable of hybridising to at least part of a target mRNA sequence transcribed from a virion-sense DNA molecule of a geminivirus or a DNA molecule complementary to said virion-sense DNA molecule, wherein said catalytic region is capable of cleaving

said target mRNA sequence thereby substantially reducing replication of said geminivirus.

7. A ribozyme according to claim 6 wherein the mRNA transcript is in low abundance compared to other mRNA transcripts directed by the virus.

8. A ribozyme according to claim 7 wherein the mRNA transcript is from the C1, L1, AL1, AC1, or other gene involved in the replication of a geminivirus gene.

9. A ribozyme according to claim 8 wherein the C1 gene is specified by the complementary-sense DNA strand of said geminivirus.

10. A ribozyme according to claim 9 wherein the geminivirus is selected from the group consisting of Maize Streak Virus (MSV), Bean Gold Mosaïc Virus (BGMV), African Cassava Mosaïc Virus (ACMV), Chloris Striate Mosaïc Virus (CSMV), Tomato Yellow Leaf Curl Virus (TYLCV), Tomato Golden Mosaïc Virus (TGMV), Tomato Leaf Curl Virus (TLCV) and Beet Curly Top Virus (BCTV) preferably TLCV, TYLCV or a mutant, derivative or variant thereof.

11. A ribozyme according to claim 10 wherein the hybridising region of said ribozyme is capable of hybridising to a sequence of ribonucleotides flanking a sequence X^0UY, wherein X is any ribonucleotide, U is uracil and Y is adenine, cytosine or uracil, said hybridising region comprising a sequence of nucleotides having substantial identity with a part or region of the sequence illustrated in figure 1.

12. A ribozyme according to claim 11 selected from the group consisting of :

3'- GUCCAGUCGUGUAAAAAGCAGGAGUGCCUGAGUAGUCGUAGGCUU GUAAGUC-5' ;

3'- GAGGAAAAAAUAGAAAAGCAGGAGUGCCUGAGUAGUCAAGAAAAC UAGCUCA-5' ;

3'- GAGUUGUAAACUACAAAGCAGGAGUGCCUGAGUAGUCUAACUUCU
AGUACUA-5'.

and

5'- GATTTAGCTCCCTCTGATGAGTCCGTGAGGACGAAAATGTTCGGATG
GAAATGTGCTCTGATGAGTCCGTGAGGACGAAACCTGGTTGGGGAT-3'

13. Nucleic acid compound having endoribonuclease activity and being capable of inhibiting the replication, infection or assembly of a DNA virus, said compound comprising at least one unit, "R", having the general formula :

5' [X-Y-X'] 3'

in which :

- X and X' each represent, independently, a nucleotide sequence having at least 4 bases and being complementary to at least a part of either the viral genome, or the (-)sense RNA transcript of a DNA virus ;

- Y represents the catalytic region of a ribozyme.

14. Nucleic acid compound according to claim 13 comprising one "R" unit only and having the general formula :

[X-Y-X']

X, X' and Y being as previously defined.

15. Nucleic acid compound according to claim 13, comprising a plurality of "R" units and having the general formula :

R_1-R_2--------R_n

R being as previously defined, and n > 1.

16. Nucleic acid compound according to claim 14 or 15 wherein the X and X' sequences within any one "R" unit are complementary to adjacent, contiguous portions of the DNA virus genome or (-)sense RNA transcript.

17. Nucleic acid compound according to claims 15 and 16 wherein the 3' X' sequence of a given "R" unit and the 5' X sequence of the adjacent "R" unit are complementary to sequences which are contiguous in the DNA genomes or (-)sense RNA transcript.

18. Nucleic acid compound according to claim 14 or 15 characterised in that the X and X' sequences within any one "R" unit are complementary to non-contiguous portions of the genome or of the (-)sense RNA transcript.

19. Nucleic acid compound according to claim 15 wherein the 3' X' sequence of a given "R" unit and the 5' X sequence of the adjacent "R" unit are complementary to non-contiguous portions of the genome or of the (-)sense RNA transcript.

20. Nucleic acid compound according to any one of claims 13 to 19 wherein a sequence "A" having at least 4 bases and being non-complementary to the viral genome or the (-)sense RNA transcript, is included between 2 consecutive "R" units.

21. Nucleic acid compound according to claim 20 wherein the non-complementary sequence "A" is a polylinker or a spacer.

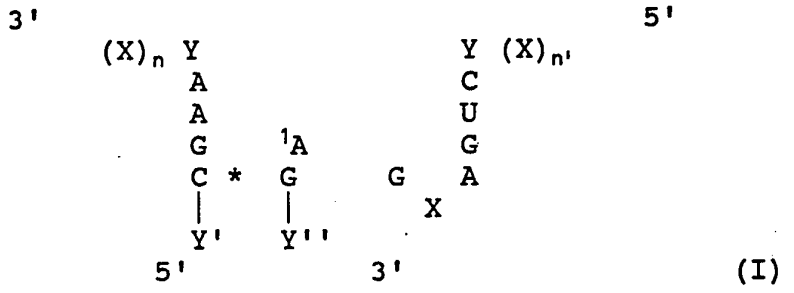
22. Nucleic acid compound according to any of claims 15 to 21 comprising X and X' sequences complementary to the genome or to the (-) sense transcripts of at least 2 different types or isolates of DNA virus.

23. Nucleic acid compound according to any of claims 15 to 21 wherein at least one of the "R" units

contains X and X' sequences which are complementary to regions highly conserved between DNA viruses, for example between DNA viruses of the same type.

24. Nucleic acid compound according to any of the preceding claims wherein the ribozyme catalytic region "Y" is a hammerhead catalytic region.

25. A ribozyme of Formula (I) :

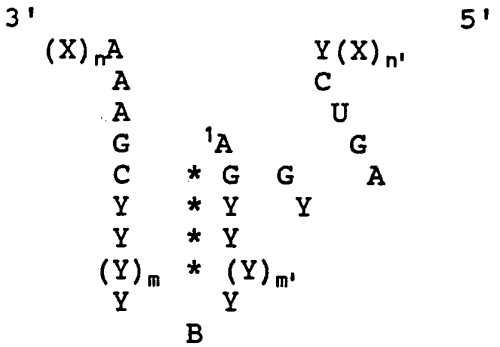


(I)

wherein, $(X)_n$ and $(X)_{n'}$ represent ribonucleotide sequences substantially complementary to a mRNA sequence of a DNA virus ; the sum of n and n' may be the same or different ; an (*) represents a base pair between complementary ribonucleotides ; Y represents any ribonucleotide and each Y may be the same or different ; Y' and Y'' represent ribonucleotides of complementary sequence along at least part of their length to allow base pairing between the oligoribonucleotides, or Y' and Y'' together form a single RNA sequence wherein at least part of said sequence comprises a stem formed by base pairing between complementary nucleotides ; and optionally, an additional nucleotide selected from any one of A, G, C or U is inserted after ^1A.

26. A ribozyme according to claim 25 of formula (II) :

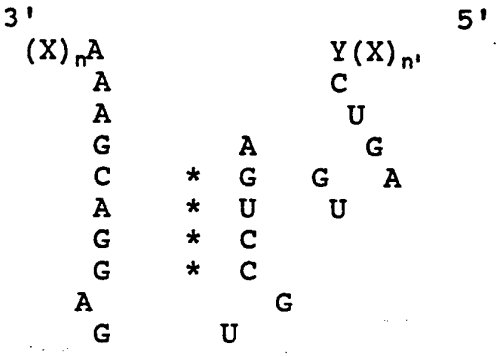
55



(II)

wherein, $(X)_n$ and $(X)_{n'}$ represent ribonucleotide sequences substantially complementary to a mRNA of a DNA virus and in particular a geminivirus such as TLCV or TYLCV ; Y represents any ribonucleotide and each Y residue may be the same or different ; an (*) represents a base pair between complementary ribonucleotides ; n and n' are as previously defined ; B represents a bond, a base pair, a ribonucleotide or an oligoribonucleotide containing at least 2 ribonucleotides ; m and m' are 1 or more and may be the same or different ; and optionally, an additional nucleotide selected from any one of A, G, C or U is inserted after ^1A.

27. A ribozyme according to claim 26 of Formula (III) :



(III)

wherein, $(X)_n$ and $(X)_{n'}$ represent ribonucleotide sequences substantially complementary to a mRNA of a DNA virus and in particular a geminivirus such as

TLCV ; Y represents any ribonucleotide and each Y residue may be the same or different ; an (*)represents a base pair between complementary ribonucleotides ; n and n' are as previously defined.

28. A ribozyme according to claim 27 selected from the group consisting of :

3'- GUCCAGUCGUGUAAAAAGCAGGAGUGCCUGAGUAGUCGUAGGCUU GUAAGUC-5' ;

3'- GAGGAAAAAAUAGAAAAGCAGGAGUGCCUGAGUAGUCAAGAAAAC UAGCUCA-5' ;

3'- GAGUUGUAAACUACAAAGCAGGAGUGCCUGAGUAGUCUAACUUCU AGUACUA-5'.

and

5'- GATTTAGCTCCCTCTGATGAGTCCGTGAGGACGAAAATGTTCGGATG GAAATGTGCTCTGATGAGTCCGTGAGGACGAAACCTGGTTGGGGAT-3'

29. A genetic construct comprising a complementary sequence of the ribozyme according to claim 1, 6, 13, 25 or 28 and a vector sequence.

30. A genetic construct according to claim 29 further comprising a promoter operably linked to said complementary ribozyme sequence.

31. A genetic sequence selected from the group consisting of pRIBO5BIN, pRIBO5BINDel, pRIBO7BIN, pRIBO2BIN, pGAZE-RzA, pGAZE-polyRzC, pGAZE-polyRzD, pGAZE-polyRzE, pGAZE-polyRzF, pGA492-RzA and pGA492polyRzC.

32. A transgenic plant comprising cells capable of reducing replication, infection and/or assembly of a plant DNA virus, said plant cells expressing a ribozyme according to any one of claims 1, 6, 13, 25 or 28.

33. A transgenic plant according to claim 32 wherein said plant DNA virus is a single stranded DNA virus.

34. A transgenic plant according to claim 33 wherein said single stranded DNA virus is a geminivirus.

35. A transgenic plant according to claim 34 wherein said geminivirus is TLCV or TYLCV.

36. A transgenic plant according to claim 35 wherein the ribozyme expressed is selected from the group consisting of RIBO5, RIBO5Del, RIBO7, RIBO2, RzA, polyRzC, polyRzD, polyRzE, polyRzF.

37. A transgenic plant according to any one of claims 3 to 36 wherein said plant is a tomato plant.

38. Process for conferring resistance to a DNA virus on a plant, by the introduction into the plant of a ribozyme according to any one of claims 1, 6, 13, 25 or 28, for example by stable genetic transformation of a part of the plant by a DNA sequence encoding the ribozyme, followed by regeneration of a transgenic plant.

FIGURE 1

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TTAAAGTTTGCAGAGAACTCCATGAGAATGGGGAGCCTCATCTCCACGTG
TCAAAGTTTGCAAAGAACTCCACGAGAATGGGGAACCACATCTCCATGTG
TCAAAATTTGCAGAGAACTACATGAAAATGGGGAACCTCATCTCCATATT

CTTATCCAGTTCGAAGGCAAGTTCCAGTGCAAGAATCAACGATTCTTCGA
CTTATCCAATTCGAAGGCAAATACCAATGTAAGAACCAACGGTTCTTCGA
CTCATCCAATTCGAAGGAAAATACAATTGTACCAATCAACGATTCTTCGA

CCTGGTCTCCCCCACCAGGTCAGCACATTTCCATCCGAACATTCAGGGAG
CTTGGTATCCCCAAACAGGTCAGCACATTTCCATCCGAACATTCAGGCAG
CCTGGTATCCCCAACCAGGTCAGCACATTTCCATCCGAACATTCAGGGAG

CTAA---GAGCTCGTCAGACGTCAAGTCCTATCTGGAGAAGGACGGAGAC
CTAA---GAGCTCAACAGATGTCAAGACCTACGTGGAGAAAGACGGAAAC
CTAAATCGAGCTC--C-GACGTCAAGTCCTATATCGACAAGGACGGAGAT

ACCCTCGAATGGGGAGAGTTTCAGATCGATGGACGATCTGCAAGAGGGGG
TTCATTGATTTTGGAGTTTCCCAAATCGATGGCAGATCAGCTAGAGGAGG
GTTCTTGAATGGGGTACTTTCCAGATCGACGGACGATCTGCTAGGGGAGG

ACAACAATCAGCCAATGACGCTTACGCCCAGGCGCTTAACACTGGAAGTA
TCAGCAATCTGCCAACGACGCATATGCCGAAGCACTCAATTCAGGCAGTA
ACAACAGACAGCCAACGACGCTTACGCAAAGGCAATTAACGCAGGAAGTA

AGTCAGAGGCTCTTAACGTCCTTAGGGAATTAGCCCCTAAGGATTATGTT
AATCCGAGGCCCTCAATATATTAAAAGAGAAGGCCCCAAAGGACTATATT
AGTCGCAGGCTCTTGATGTAATTAAAGAATTAGCGCCTAGAGATTACGTT

TTACAATTTCATAATTTAAATAGTAATTTAGATAGGATTTT---TACACC
TTACAATTTCATAATTTAAGTTCAAATTTAGATAGGATTTT---TAGTCC
CTACATTTTCATAATATAAATAGTAATTTAGATAAGGTTTTCCAGGTGCC

TCCGTTGGAGGTTTATGTTTCTCCTTTTTTATCTTCTTCTTTTGATCGAG
TCCTTTAGAAGTTTATGTTTCTCCATTTCTTTCTTCTTCTTTTAATCAAG
TCCG---GCACCTTATGTTTCTCCTTTTTTATCTTCTTCTTTCGATCAAG

TTCCAGAAGAACTCGAGGAGTGGGTCGCCGAGAATGTGAAGGATGCCGCT
TTCCAGATGAACTTGAAGAGTGGGTCGCCGAGAACGTCGTGTATTCCGCT
TTCCTGATGAACTTGAACACTGGGTTTCCGAGAACGTCATGGATGCCGCT

GCGCGGCCTTTGAGACCCATAAGTATAGTAATAGAGGGTGAGTCTAGAAC
GCGCGGCCATGGAGACCCATAAGTATTGTCATTGAGGGTGATAGCAGAAC
GCGCGGCCTTGGAGACCGGTGAGTATAGTGATTGAGGGTGACAGCCGGAC
```

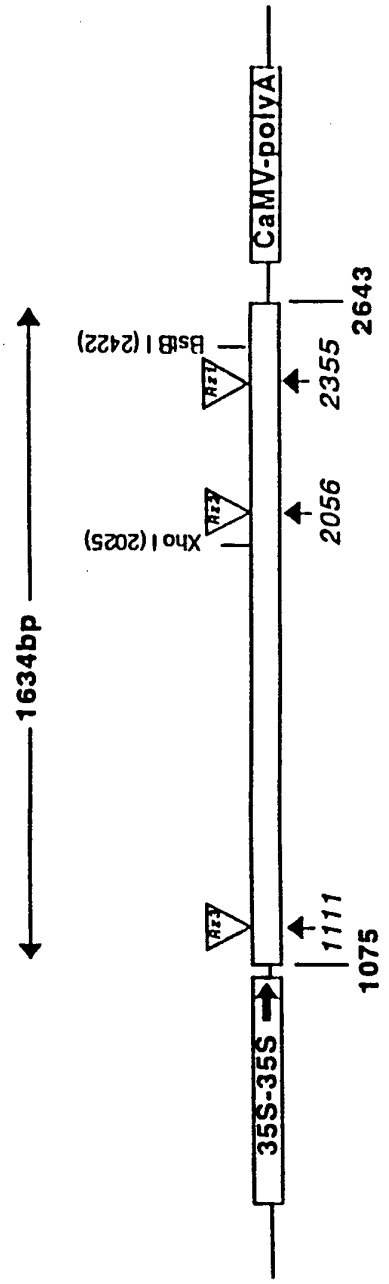

CaMV-polyA
2643
BsiB I (2422)
2355
2056
Xho I (2025)
1634bp
1111
1075
35S-35S

FIGURE 2

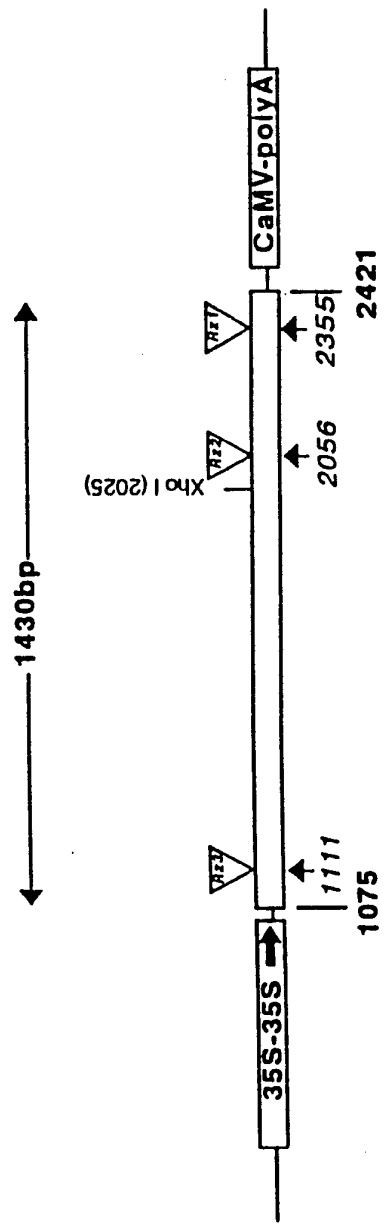


FIGURE 3

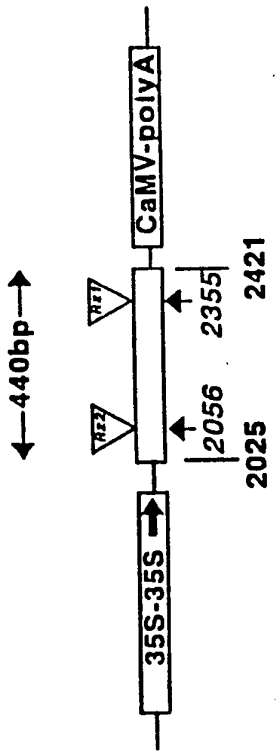


FIGURE 4

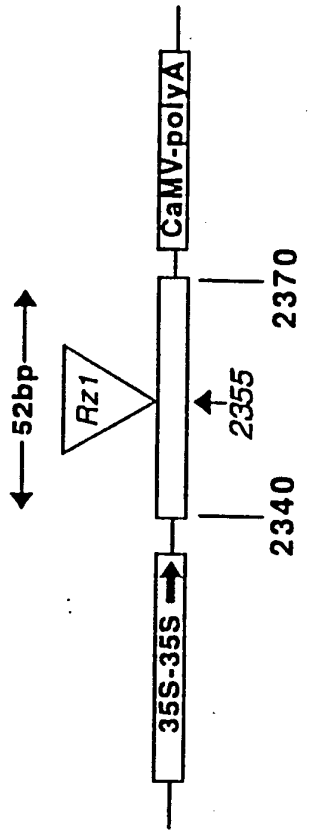


FIGURE 5

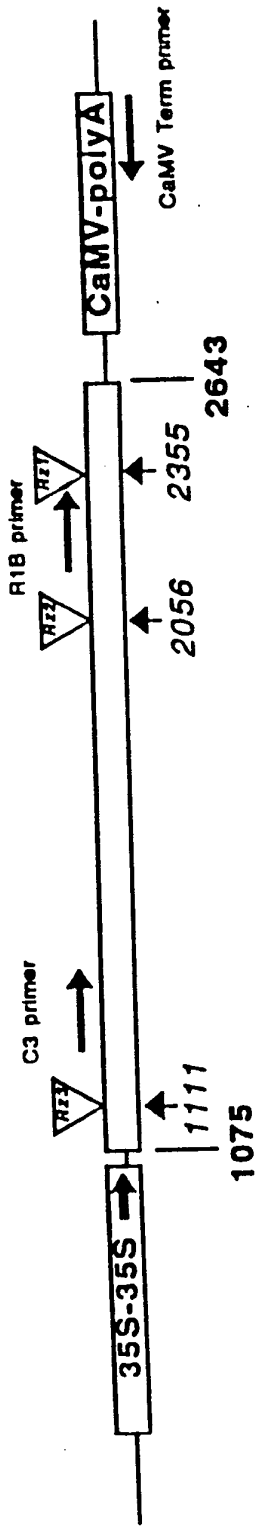

CaMV-polyA
CaMV Term primer
2643
2355
R1B primer
2056
C3 primer
1111
1075
35S-35S

FIGURE 6

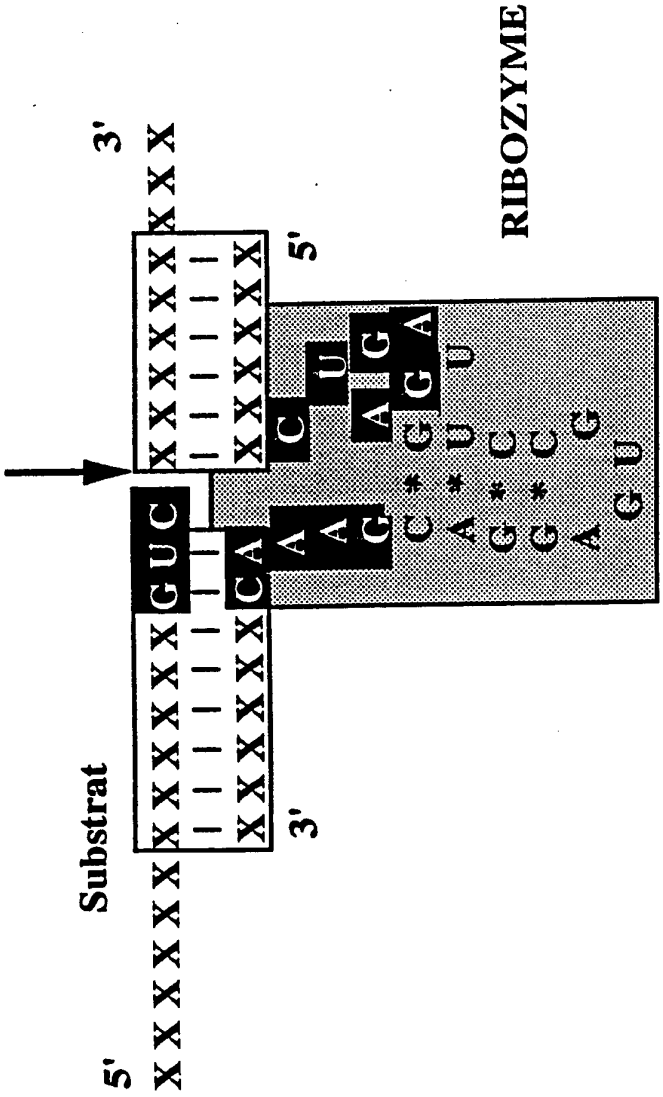


FIGURE 7

FIGURE 8

```
                |   10        |   20        |   30        |   40        |   50        |   60
   1 TACGGATGGC CGCTTTACCA AAAAAAAATT AAAAAAATAA TTTATTACTT TGAATAATTA 60
  61 CATCTATGCC ATTTAGGGGG CATCATATAT ATTGCCCCCC AATTCCCCCA ATTGCTGGAT 120
 121 ACTTTGAGTG TCCCCCGATT CAGAACGACA GCAAAAATGC CAAGATCAGG TCGTTTTAGT 180
 181 ATCAAGGCTA AAAATTATTT CCTTACATAT CCCAAATGTG ATTTAACAAA AGAAAATGCA 240
 241 CTTTCCCAAA TAACAAACCT ACAAACACCC ACAACACAAT TATTCATCAA AATTTGCAGA 300
 301 GAACTACATG AAAATGGGGA ACCTCATCTC CATATTCTCA TCCAATTCGA AGGAAAATAC 360
 361 AATTGTACCA ATCAACGATT CTTCGACCTG GTATCCCCAA CCAGgtcAGC ACATTTCCAT 420
 421 CCGAACAttc AGGGAGCTAA ATCGAGCTCC GACGTCAAGT CCTATATCGA CAAGGACGGA 480
 481 GATGTTCTTG AATGGGGTAC TTTCCAGATC GACGGACGAT CTGCTAGGGG AGGACAACAG 540
 541 ACAGCCAACG ACGCTTACGC AAAGGCAATT AACGCAGGAA GTAAGTCGCA GGCTCTTGAT 600
 601 GTAATTAAAG AATTAGCGCC TAGAGATTAC GTTCTACATT TTCATAATAT AAATAGTAAT 660
 661 TTAGATAAGG TTTTCCAGGT GCCTCCGGCA CCTTATGTTT CTCCTTTTTT ATCTTCTTCT 720
 721 TTCGATCAAG TTCCTGATGA ACTTGAACAC TGGGTTTCCG AGAACGTCAT GGATGCCGCT 780
 781 GCGCGGCCTT GGAGACCGGT GAGTATAGTG ATTGAGGGTG ACAGCCGGAC AGGAAAGACA 840
 841 ACGTGGGCCC GTTCATTAGG CCCACATAAT TATTTGTGCG GCCATCTTGA CCTCAGTCAA 900
 901 AAAGTATACA GCAATAATGC TTGGTATAAC GTCATTGATG ACGTCGACCC GCATTATTTA 960
 961 AAACACTTTA AAGAATTTAT GGGGGCCCAA AGAGATTGGC AAAGCAACAC AAAGTATGGC 1020
1021 AAGCCCATTC AAATTAAAGG AGGCATTCCC ACTATCTTCC TATGcAATCC AGGCCCACAA 1080
1081 TCATCAtTTA AAGAATATCT CGACGAAGAA AAAAATCAAG CATTAAAAAA CTGGGCTACT 1140
1141 AAGAATGCAA TCTTCGTCAC CATCCACCAG CCATTGTTCG CAGATACCAA TCAAAATACA 1200
1201 ACATCACATC GCCAAGAAGA GGCAAGTGAG GCGTAGAAGG GTAGATCTGG ACTGTGGCTG 1260
1261 CTCTTATTAC ATACACTTAG ACTGCaTAAA TCATGGATTT ACGCACAGGG GAGTACATCA 1320
1321 CTGCGCATCA AGCAACGAGT GGCGTTTATA CCTTCGGGAT AACAAATCCC CTATATTTCA 1380
1381 CGATAACCAG ACACAATCAG AACCCATTCA ACAACAAATA CAACACACTA ACATTCCAAA 1440
1441 TCAGATTCAA CCACAACTTG AGGAAGGAAC TGGGGATTCA CAAATGTTTT CTCAACTTCC 1500
1501 ACATCTGGAC GACCTTACAG TCTCCGACTG GTCATTTTTT AAGAGTCTTT AAATATCAAG 1560
1561 TCTGTAAATA TTTGAATAAT TTGGGTGTAA TTTCTTTAAA TAATGTTGTT AGAGCAGTTG 1620
1621 ATTATGTATT GTTTCATGTA TTTGAAAGAA CAATTGATGT AACTGAAAAT CATGAAATAA 1680
1681 AATTTAATTT TTATT                                                  1695
                |   10        |   20        |   30        |   40        |   50        |   60
```

FIGURE 9 (a)

TLCV nt365 AATCAACGATTCTTCGACCTGGT-TCCCC-
TYLCV-S nt370 AATCAACGATTCTTCGACCTGGT-TCCCC-

TLCV ACCAGGTCAGCACAT**ttc**CATCCGAACATTCAGGG
TYLCV-S ACCAG**gtc**AGCACATTTCCATCCGAAC**Attc**AGGG

TLCV AGCTAA nt435
TYLCV-S AGCTAA nt340

FIGURE 9 (b)

```
TLCV       nt634 ATTT CATA---TAAATAGTAATTTAGATA----TTT
TYLCV-S    nt638 ATTT-CATA---TAAATAGTAATTTAGATA ---TTT

TLCV       T---    CCTCC----------TTATGTTTCTCCTTTTTTAT
TYLCV-S    T-------CCTCC------    TTATGTTTCTCCTTTTTTAT

TLCV       CttcTTCTTT-GATC-AGTTCC-    GA-    GAAC nt737
TYLCV-S    CTTCTTCTTT-GATC-AGTTCC----GA----GAAG nt748
```

FIGURE 10

```
                    |   10        |   20        |   30        |   40        |   50        |   60
      1 GACGGATGGC CGCTTGAGGG ATTTGAATTT TGAATTATTT ACTTTTGAGA ATTCTCCGAA 60
     61 TTACCAAAAT GCCATACGGT GTCCAACTAT AAATAAGACC CCAGACACCG ATTCATTTAC 120
    121 CAAGTTGAGA GACACCAATT GCTAAAACGC AATGACTAGA CCAAAGTCAT TCCGTATAAA 180
    181 TGCTAAGAAT TATTTCCTCA CTTATCCCAA ATGCTCTCTC ACTAAAGAAG AAGCCCTTTC 240
    241 CCAATTAAAT AACCTAGAAA CCCCAACATC CAAAAAATAT ATTAAAGTTT GCAGAGAACT 300
    301 CCATGAGAAT GGGGAGCCTC ATCTCCACGT GCTTATCCAG TTCGAAGGCA AGTTCCAGTG 360
    361 CAAGAATCAA CGATTCTTCG ACCTGGTCTC CCCCACCAGG TCAGCACATt tcCATCCGAA 420
    421 CATTCAGGGA GCTAAGAGCT CGTCAGACGT CAAGTCCTAT CTGGAGAAGG ACGGAGACAC 480
    481 CCTCGAATGG GGAGAGTTTC AGATCGATGG ACGATCTGCA AGAGGGGGAC AACAATCAGC 540
    541 CAATGACGCT TACGCCCAGG CGCTTAACAC TGGAAGTAAG TCAGAGGCTC TTAACGTCCT 600
    601 TAGGGAATTA GCCCCTAAGG ATTATGTTTT ACAATTTCAT AATTTAAATA GTAATTTAGA 660
    661 TAGGATTTTT ACACCTCCGT TGGAGGTTTA TGTTTCTCCT TTTTATCTt cTTCTTTTGA 720
    721 TCGAGTTCCA GAAGAACTCG AGGAGTGGGT CGCCGAGAAT GTGAAGGATG CCGCTGCGCG 780
    781 GCCTTTGAGA CCCATAAGTA TAGTAATAGA GGGTGAGTCT AGAACAGGGA AGACTGTTTG 840
    841 GGCGCGTTCA TTGGGTCCAC ATAATTATTT ATGTGGGCAC CTTGATTTAA GCCCTAAAGT 900
    901 GTATTCAAAT GATGCGTGGT ATAACGTCAT TGATGACGTT GATCCGCATT ATCTGAAACA 960
    961 TTTCAAAGAA TTCATGGGGG CCCAGAGAGA TTGGCAAAGC AACACCAAGT ACGGCAAGCC 1020
   1021 AGTTCAAATT AAAGGTGGAA TACCCACTAT CTTCCTCTGC AATCCAGGAC CCAACTCCAG 1080
   1081 CTATAAAGAG TACCTCGATG AGGAGAAAAA CAGCGCATTA AAAGCCTGGG CTTTAAAAAA 1140
   1141 TGCAGAATTC ATCACCCTCA ACGAGCCACT ATACTCAGGT ACCTATCAAG GTCCAACACA 1200
   1201 AAATAGCGAA GAAGAGGTCC ATCCGGAGGA AGAGAATTGA TTTGCCGTGC GGGTGTTCTT 1260
   1261 ACTACCTGCA TATCAACTGC CATAACCATG GATTCACGCA CAGGGAACCC CATCACTGCT 1320
   1321 CGTCAAGCGA TGAATGGCGA GTATATCTGG AGGGTTCCAA ATCCCCTCTA TTTCAAGATC 1380
   1381 ATAAAGCACC ACAAGCGCCC GTTCAACTAC AACCACGACA TCATTCAAGT CCGGATACAG 1440
   1441 TTCAATCACA ACCTGAGGAG AGCATTGGCG ATACACAAAT GCTTCCTGGA CTTCACAGTT 1500
   1501 TTCACTCGCT TACAACCAGC GACTTGGCGT TTCTTGAGGG TATTTAAGAC CCAAGTCATG 1560
   1561 AAGTATTTAG ATAGTTTAGG AGTTATTAGC ATTAACAATG TAATTAGATC AGTTGATCAT 1620
   1621 GTATTGTATA ATGTACTAGA CTCAACATTT GatgTAATTG AAGATCATGA TATAAAATTT 1680
   1681 AATTTTTATT AATT                                                  1694
                    |   10        |   20        |   30        |   40        |   50        |   60
```

FIGURE 11 (a)

POLYRIBOZYME D

```
                  |    10       |    20       |    30       |    40       |    50       |    60
  1 CGAAGGAAAA TACAATTGTA CCAATCAACG ATTCTTCGAC CTGGTATCCC CAACCAGgtc 60
 61 AGCACATTTC CATCCGAACA ttcAGGGAGC TAAATCGAAA TAGTAATTTA GATAGGATTT 120
121 TTACACCTCC GTTGGAGGTT TATGTTTCTC CTTTTTTATC ttcTTCTTTT GATCGAGTTC 180
181 CAGAAGAACT CGAGATCATG TATTGTATAA TGTACTAGAC TCAACATTTG ATgtaATTGA 240
241 AGATCATGAT ATAAAATTTA ATTTTTATTA                                   270
                  |    10       |    20       |    30       |    40       |    50       |    60
```

FIGURE 11 (b)

POLYRIBOZYME E

```
          |   10        |   20        |   30        |   40        |   50        |   60
    1 GGTCTCCCCC ACCAGGTCAG CACATttcCA TCCGAACATT CAGGGAGCTA AGAGCTCGTC 60
   61 AGACGTCAAG TCCTATCTGG AGAAGGACGG AGACACCCTC GAATGGGGAG AGTTTCAGAT 120
  121 CGATGGACGA TCTGCAAGAG GGGGACAACA ATCAGCCAAT GACGCTTACG CCCAGGCGCT 180
  181 TAACACTGGA AGTAAGTCAG AGGCTCTTAA CGTCCTTAGG GAATTAGCCC CTAAGGATTA 240
  241 TGTTTTACAA TTTCATAATT TAAATAGTAA TTTAGATAGG ATTTTTACAC CTCCGTTGGA 300
  301 GGTTTATGTT TCTCCTTTTT TATCttcTTC TTTTGATCGA GTTCCAGAAG AACTCGAGAT 360
  361 CATGTATTGT ATAATGTACT AGACTCAACA TTTGATgtaA TTGAAGATCA TGATATAAAA 420
  421 TTTAATTTTT ATTA                                                  434
          |   10        |   20        |   30        |   40        |   50        |   60
```

FIGURE 11 (c)

POLYRIBOZYME F

```
      |   10      |   20      |   30      |   40      |   50      |   60
  1 CGAAGGAAAA TACAATTGTA CCAATCAACG  ATTCTTCGAC CTGGTATCCC CAACCAGgtc 60
 61 AGCACATTTC CATCCGAACA ttcAGGGAGC  TAAATCGAGC TCGTCAGACG TCAAGTCCTA 120
121 TCTGGAGAAG GACGGAGACA CCCTCGAATG  GGGAGAGTTT CAGATCGATG GACGATCTGC 180
181 AAGAGGGGGA CAACAATCAG CCAATGACGC  TTACGCCCAG GCGCTTAACA CTGGAAGTAA 240
241 GTCAGAGGCT CTTAACGTCC TTAGGGAATT  AGCCCCTAAG GATTATGTTT TACAATTTCA 300
301 TAATTTAAAT AGTAATTTAG ATAGGATTTT  TACACCTCCG TTGGAGGTTT ATGTTTCTCC 360
361 TTTTTTATCt tcTTCTTTTG ATCGAGTTCC  AGAAGAACTC GAGATCATGT ATTGTATAAT 420
421 GTACTAGACT CAACATTTGA TgtaATTGAA  GATCATGATA TAAAATTTAA TTTTTATTA  479
      |   10      |   20      |   30      |   40      |   50      |   60
```

FIGURE 12 (a)

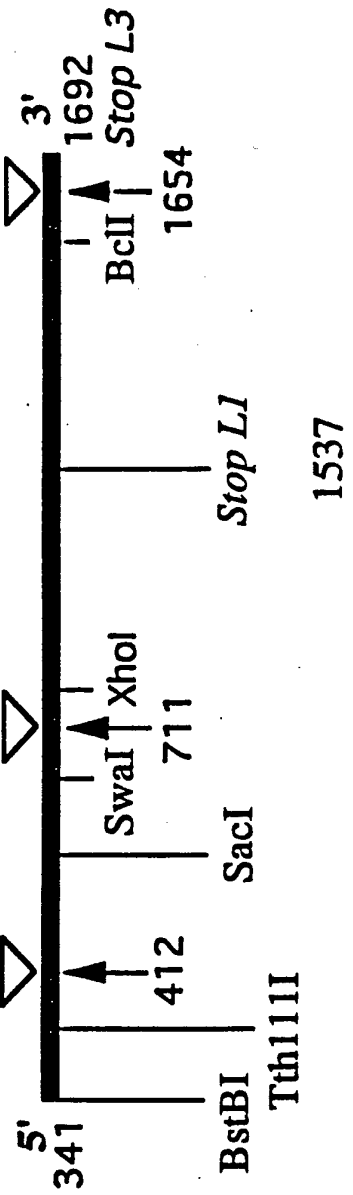
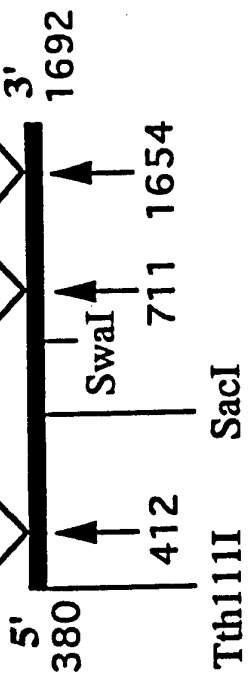
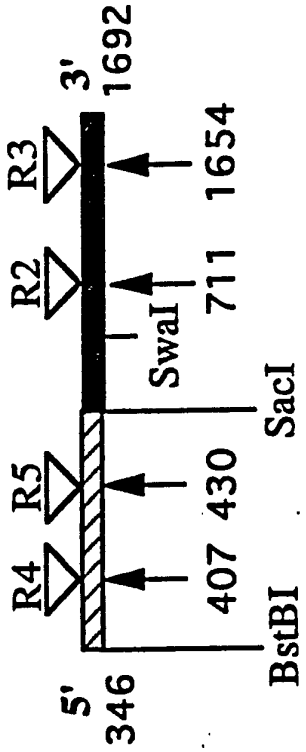


FIGURE 12 (b)



Polyribozyme E
(497 bp)

FIGURE 12 (c)



Polyribozyme F
(563 bp)

FIGURE 12 (d)

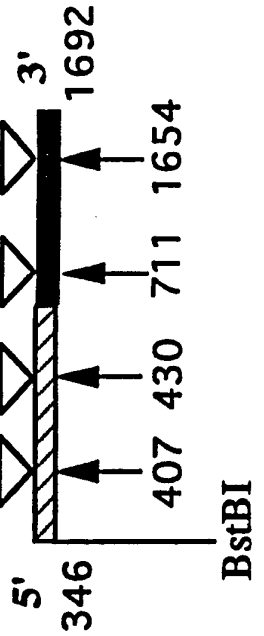

R3
R2
R5
R4
3'
1692
1654
711
430
407
5'
346
BstBI
Polyribozyme D
(354 bp)

FIGURE 12 (e)

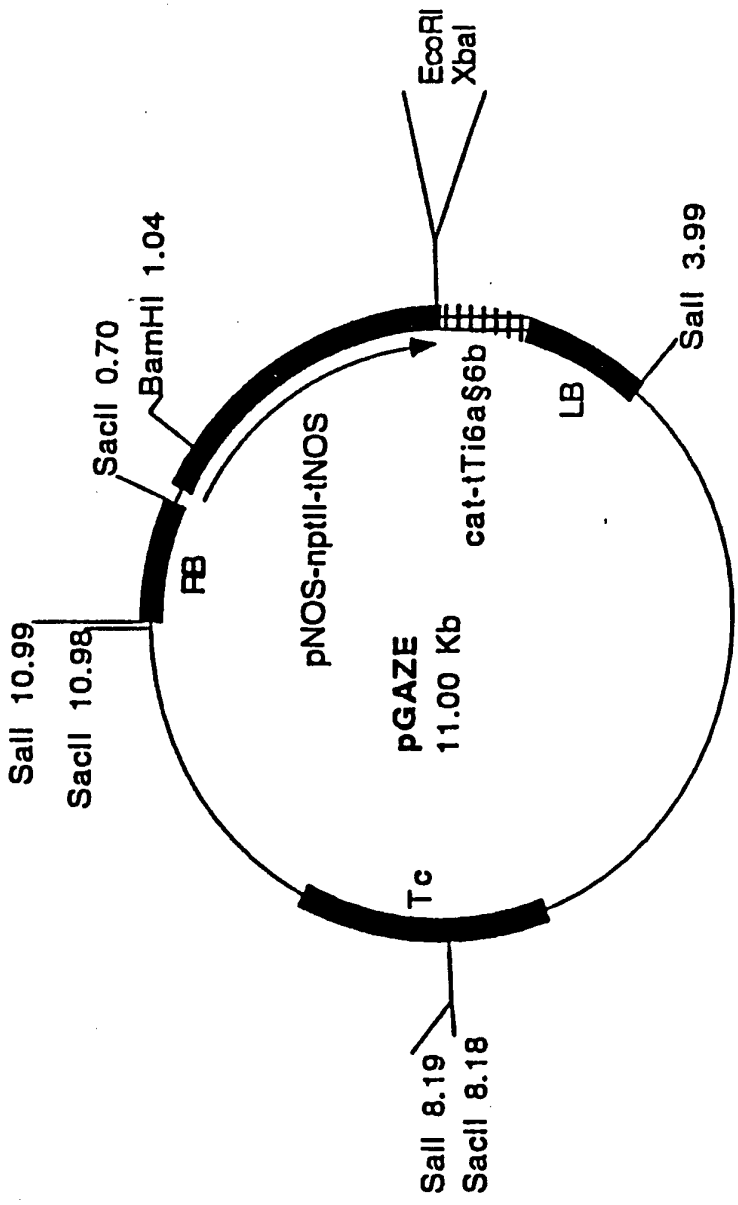

EcoRI
XbaI
BamHI 1.04
SacII 0.70
SalI 3.99
LB
cat-tTi6a§6b
pNOS-nptII-tNOS
RB
pGAZE
11.00 Kb
SalI 10.99
SacII 10.98
Tc
SalI 8.19
SacII 8.18

FIGURE 13

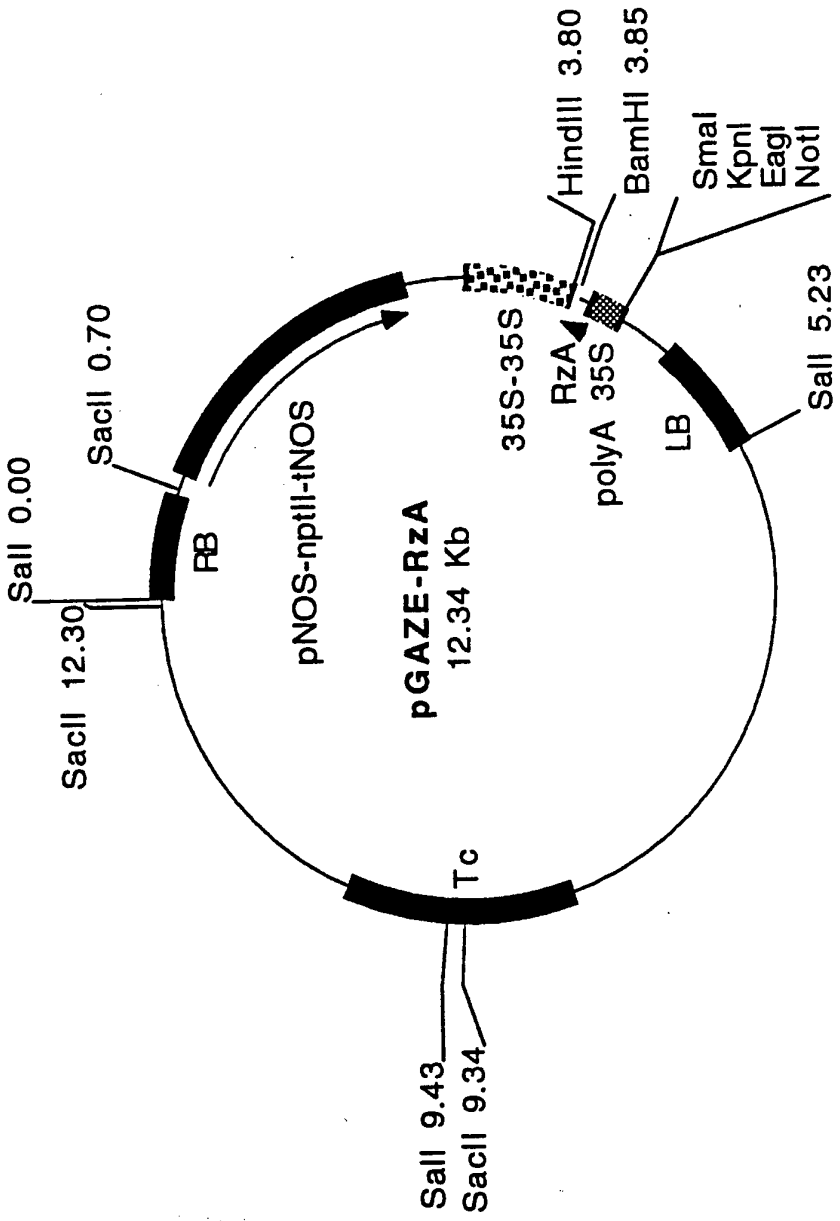

HindIII 3.80
BamHI 3.85
SmaI
KpnI
EagI
NotI
SalI 5.23
SacII 0.70
SalI 0.00
SacII 12.30
RB
pNOS-nptII-tNOS
35S-35S
RzA
polyA 35S
LB
pGAZE-RzA
12.34 Kb
Tc
SalI 9.43
SacII 9.34

FIGURE 14 (a)

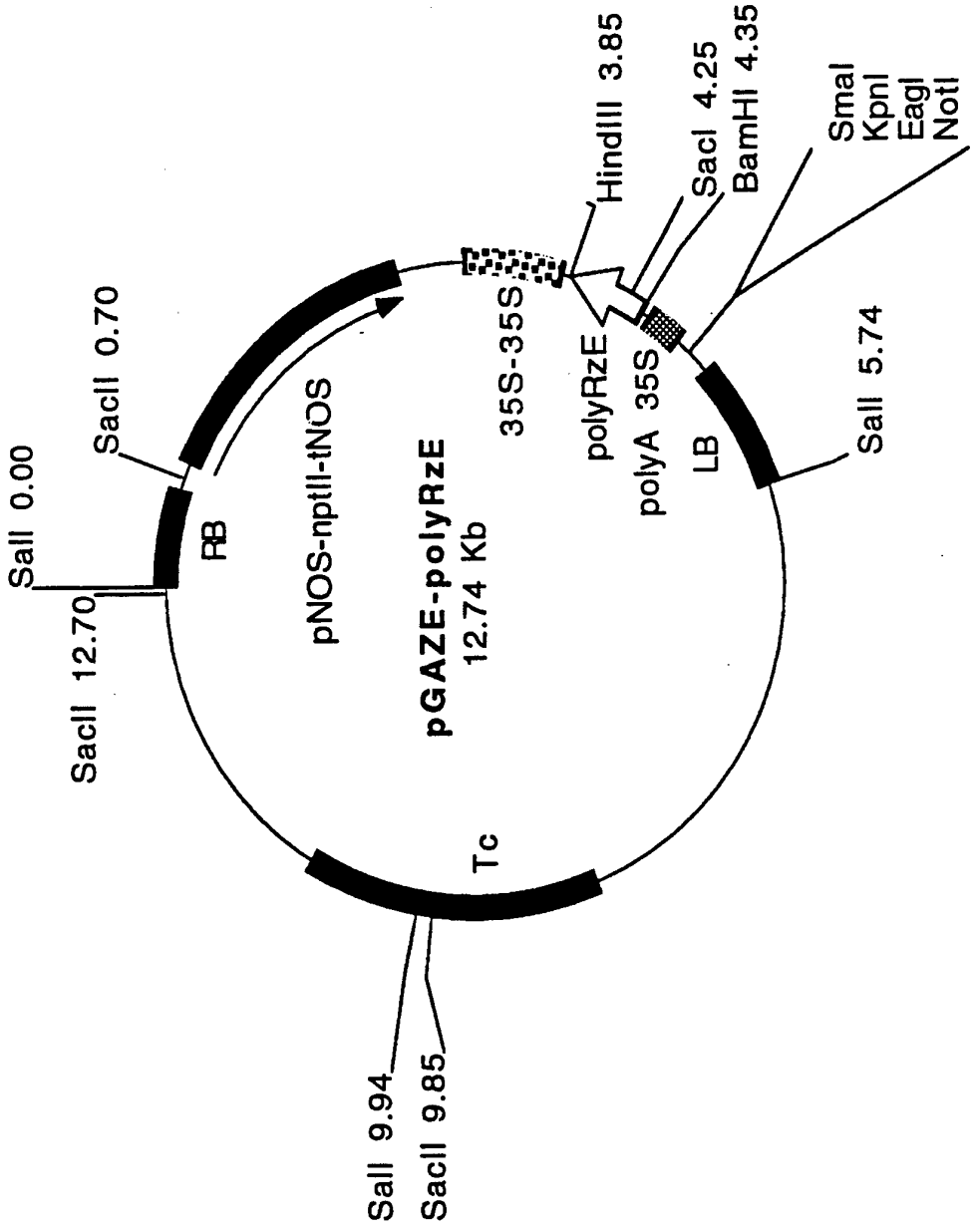

SacII 0.70
SalI 0.00
SacII 12.70
RB
pNOS-nptII-tNOS
pGAZE-polyRzE
12.74 Kb
35S-35S
polyRzE
polyA 35S
LB
Tc
SalI 9.94
SacII 9.85
HindIII 3.85
SacI 4.25
BamHI 4.35
SmaI
KpnI
EagI
NotI
SalI 5.74

FIGURE 14(b)

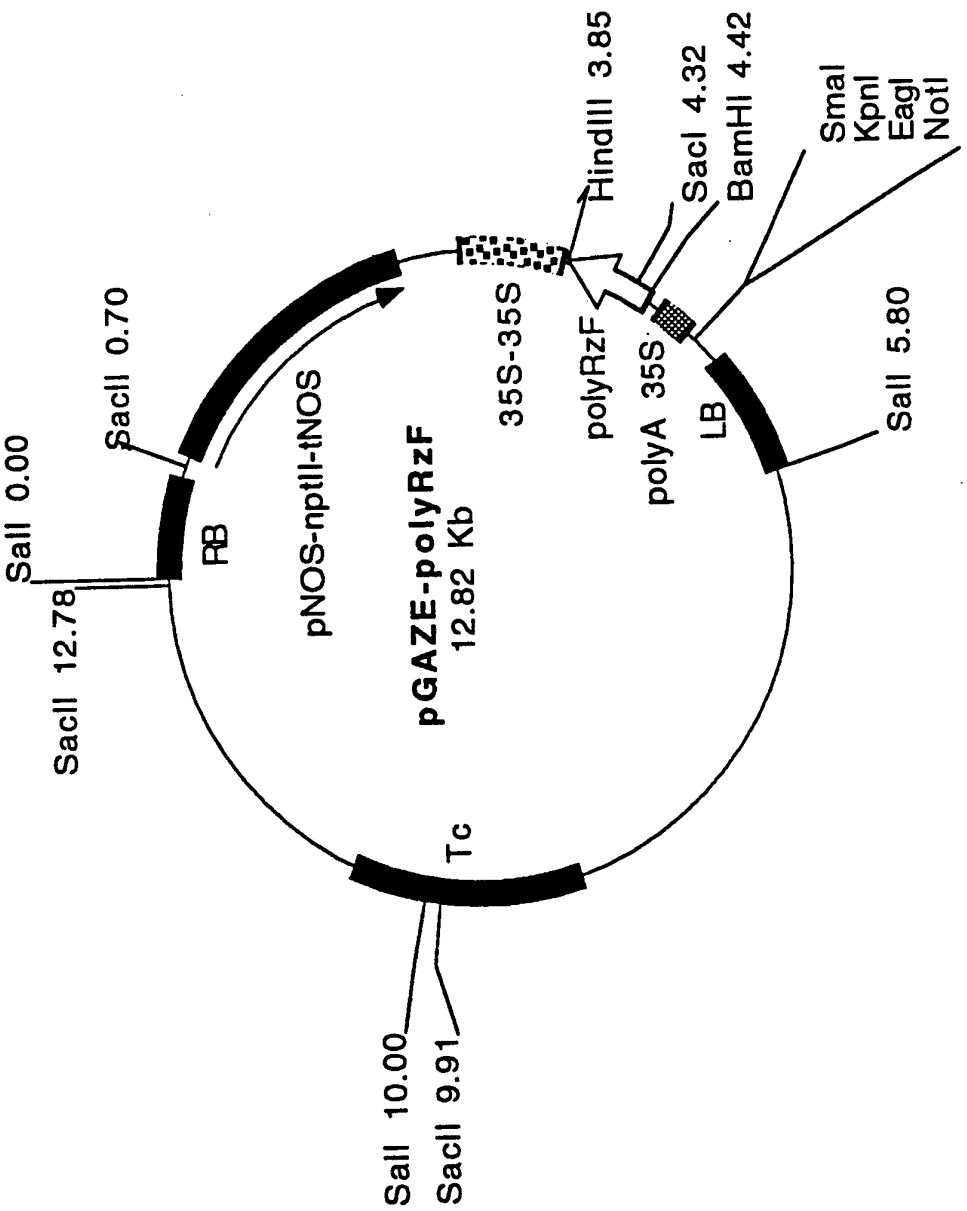

pGAZE-polyRzF
12.82 Kb
SalI 0.00
SacII 0.70
SacII 12.78
RB
pNOS-nptII-tNOS
35S-35S
HindIII 3.85
polyRzF
SacI 4.32
polyA 35S
BamHI 4.42
LB
SmaI
KpnI
EagI
NotI
SalI 5.80
Tc
SalI 10.00
SacII 9.91

FIGURE 14 (c)

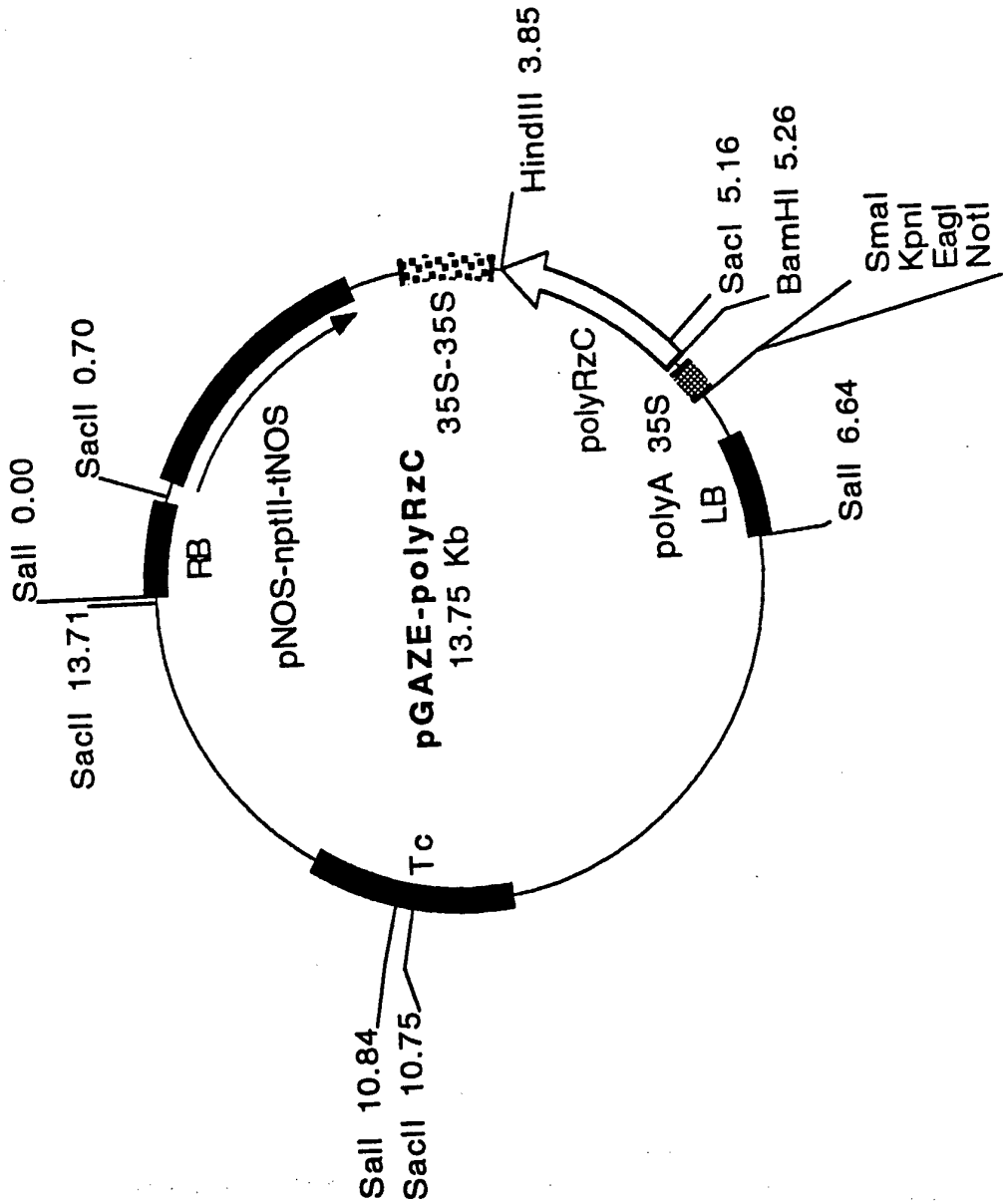

HindIII 3.85
SacI 5.16
BamHI 5.26
SmaI
KpnI
EagI
NotI
SalI 6.64
SacII 0.70
SalI 0.00
SacII 13.71
SalI 10.84
SacII 10.75
RB
pNOS-nptII-tNOS
35S-35S
pGAZE-polyRzC
13.75 Kb
polyRzC
polyA 35S
LB
Tc

FIGURE 14 (d)

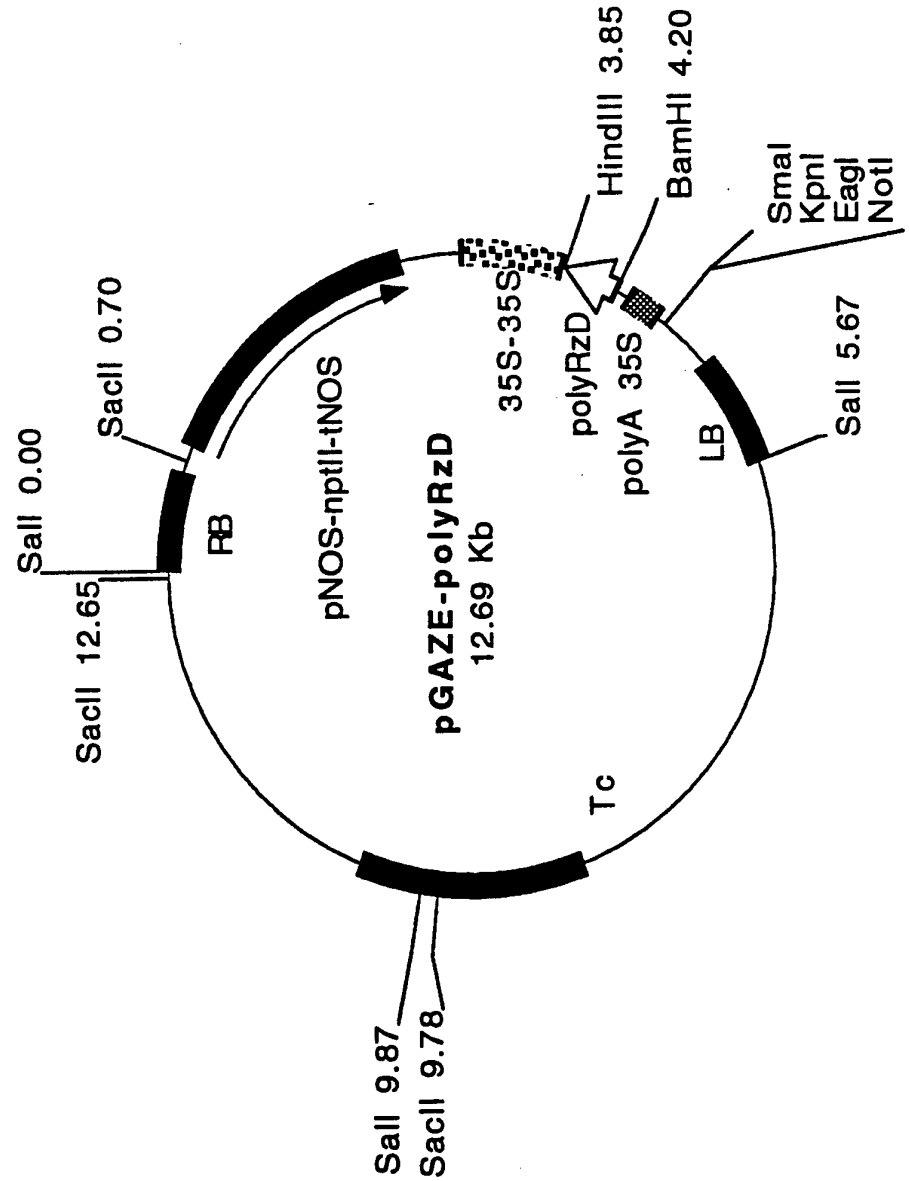

HindIII 3.85
BamHI 4.20
SmaI
KpnI
EagI
NotI
SacII 0.70
SalI 0.00
SacII 12.65
SalI 9.87
SacII 9.78
SalI 5.67
RB
pNOS-nptII-tNOS
35S-35S
polyRzD
polyA 35S
LB
pGAZE-polyRzD
12.69 Kb
Tc

FIGURE 14 (e)

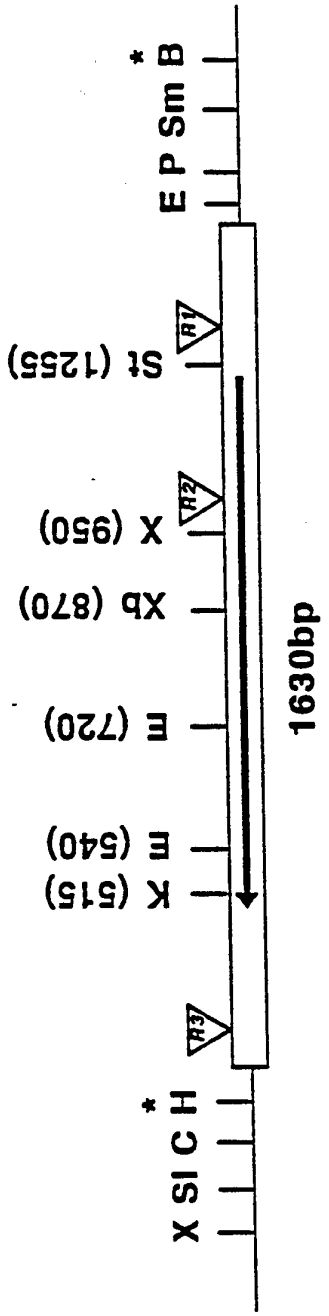

pRIBO5BS -
X Sl C H *
R3
K (515)
B (540)
E (720)
Xb (870)
X (950)
R2
St (1255)
R1
E P Sm B *
1630bp

FIGURE 15 (a)

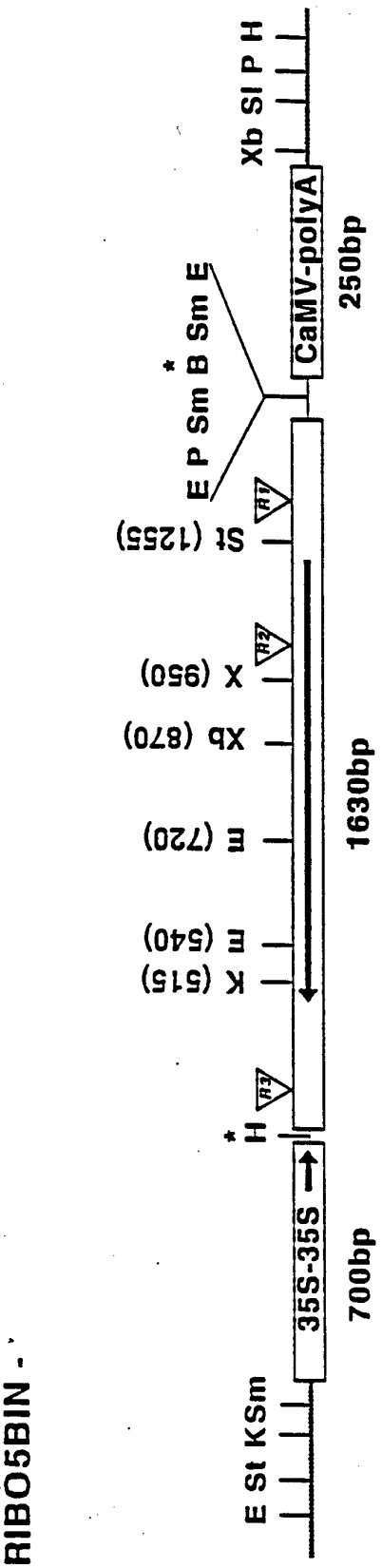

pRIBO5BIN -
E St KSm
35S-35S
700bp
H
K (515)
E (540)
E (720)
Xb (870)
X (950)
St (1255)
E P Sm B Sm E
CaMV-polyA
250bp
Xb Sl P H
1630bp

FIGURE 15 (b)

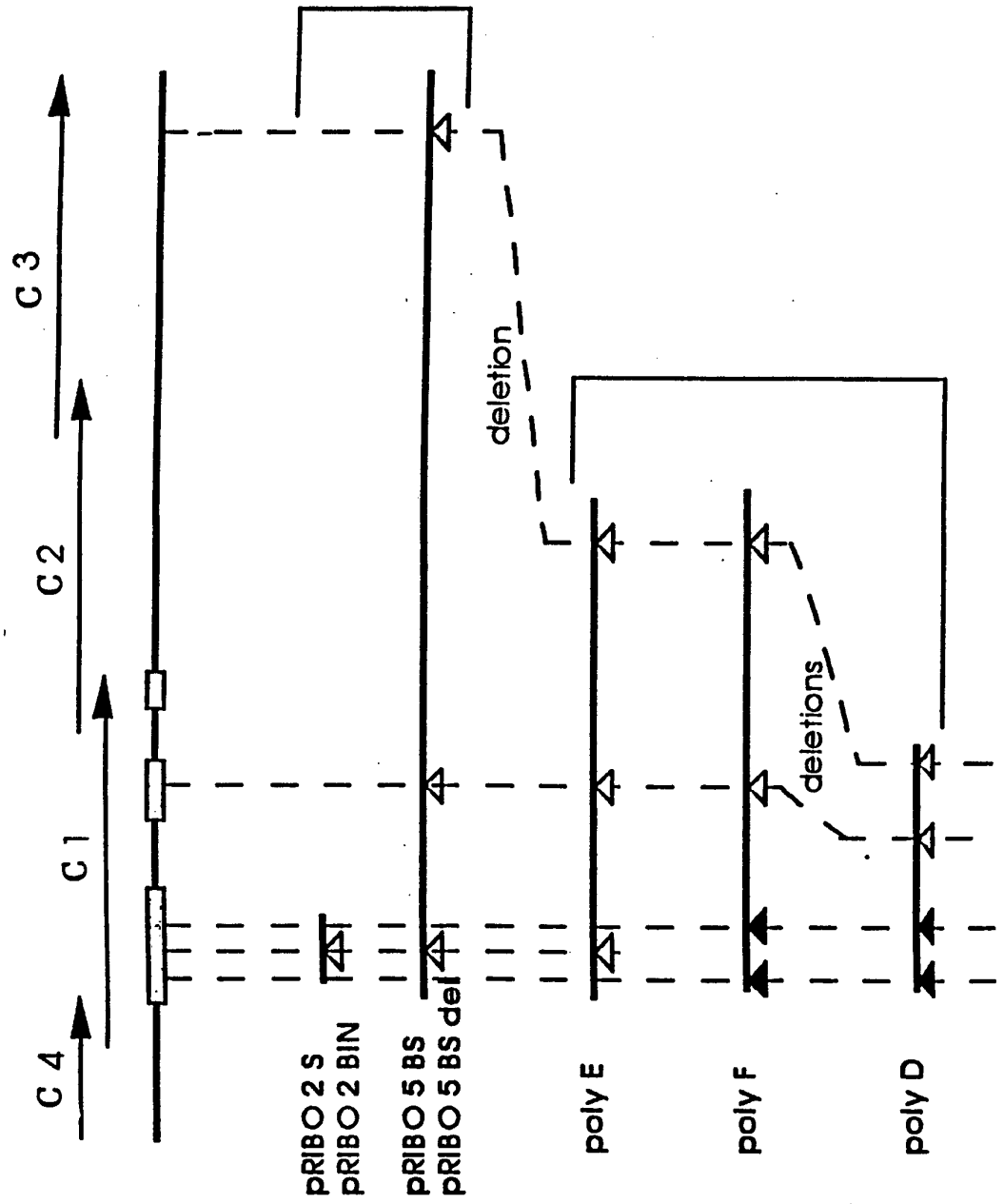

C 3
C 2
C 1
C 4
deletion
deletions
pRIBO 2 S
pRIBO 2 BIN
pRIBO 5 BS
pRIBO 5 BS del
poly E
poly F
poly D

FIGURE 16

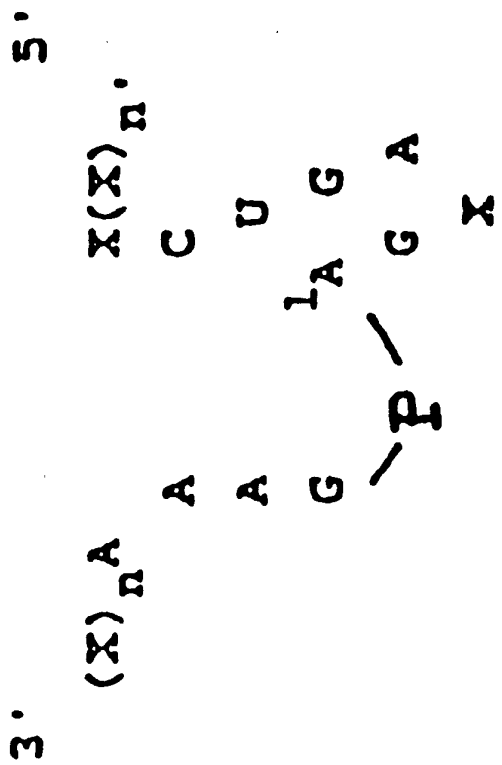


FIGURE 17

INTERNATIONAL SEARCH REPORT

Interr al Application No
PCT/EP 93/01946

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/11 C12N9/00 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,92 06693 (FOX CHASE CANCER CENTER) 30 April 1992 see page 5, line 19 - line 31; figure 5 ---	13,14
X	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS vol. 189, no. 2 , 15 December 1992 , DULUTH, MINNESOTA US pages 743 - 748 VON WEIZSÄCKER, F., ET AL. 'Cleavage of hepatitis B virus RNA by three ribozymes transcribed from a single DNA template' see the whole document --- -/--	13,14

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 March 1994	04. 05. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Maddox, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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