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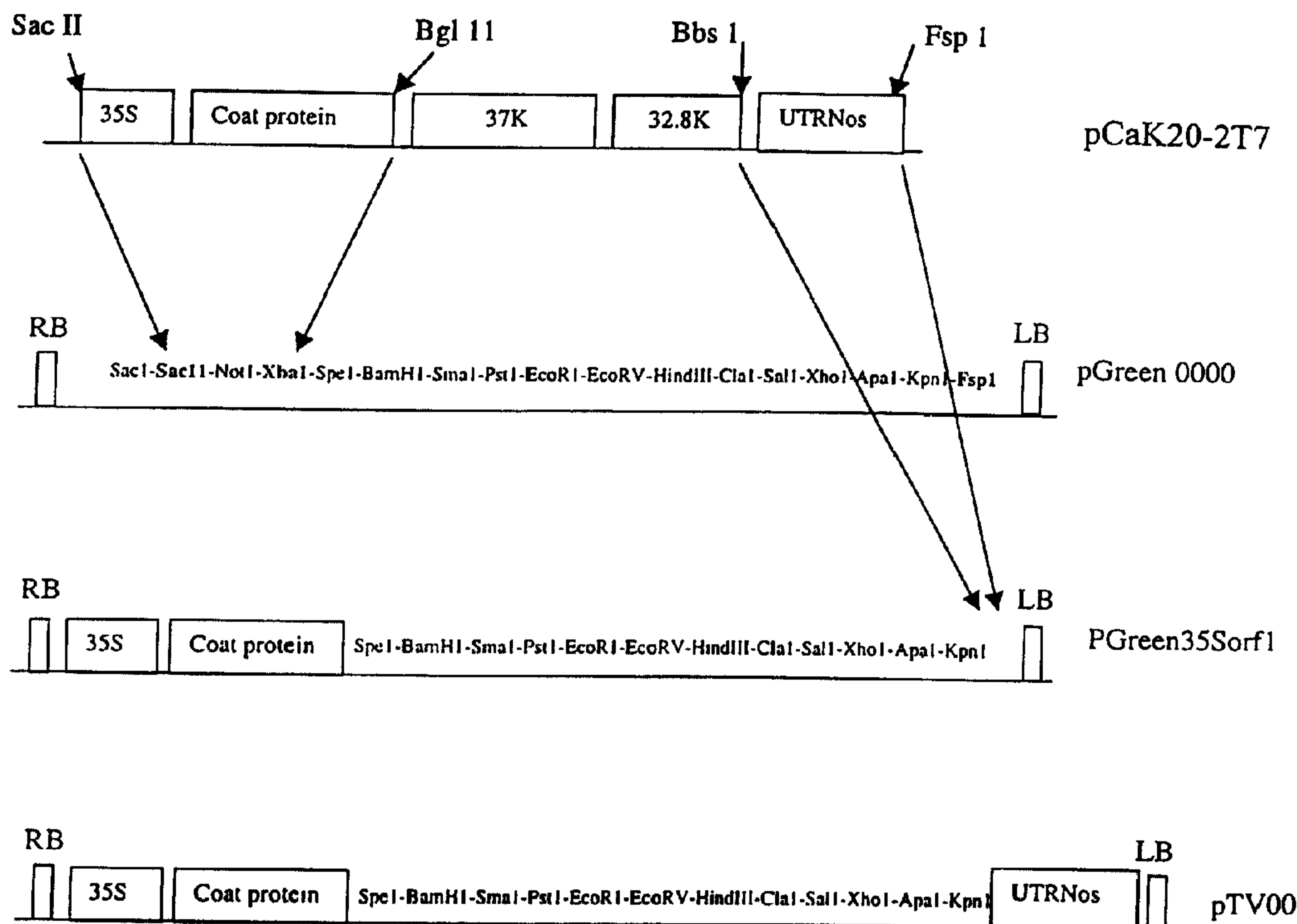
pTV00 Construction;



(57) Abrégé/Abstract:
Disclosed are nucleic acid vectors which comprise: (a) a transfer nucleotide sequence comprising (i) a plant active promoter, operably linked to (ii) a recombinant tobacco rattle virus (TRV) cDNA (preferably derived from TRV RNA2) which includes at least cis acting elements permitting replication of said cDNA; a subgenomic promoter operably linked to a sequence encoding a

(57) **Abrégé(suite)/Abstract(continued):**

TRV coat protein; and a heterologous nucleotide sequence which is foreign to said virus;(b) border sequences which permit the transfer of the transfer nucleotide sequence into a plant genome. Such vectors may be used as expression vectors or for achieving viral induced gene silencing (VIGS) of a target gene, wherein the heterologous nucleotide sequence is a targeting sequence which corresponding to that gene. Example vectors include pTV00 and vectors which are derived from pTV00 and have the characteristics thereof. Also disclosed are associated processes, methods, viruses or viral particle, kits, host cells and plant tissues.

ABSTRACT

Disclosed are nucleic acid vectors which comprise: (a) a transfer nucleotide sequence comprising (i) a plant active promoter, operably linked to (ii) a recombinant tobacco rattle virus (TRV) cDNA (preferably derived from TRV RNA2) which includes at least cis acting elements permitting replication of said cDNA; a subgenomic promoter operably linked to a sequence encoding a TRV coat protein; and a heterologous nucleotide sequence which is foreign to said virus;(b) border sequences which permit the transfer of the transfer nucleotide sequence into a plant genome. Such vectors may be used as expression vectors or for achieving viral induced gene silencing (VIGS) of a target gene, wherein the heterologous nucleotide sequence is a targeting sequence which corresponding to that gene. Example vectors include pTV00 and vectors which are derived from pTV00 and have the characteristics thereof. Also disclosed are associated processes, methods, viruses or viral particle, kits, host cells and plant tissues.

VIRAL VECTORS

TECHNICAL FIELD

5 The present invention relates generally to recombinant plant viral nucleic acids, and methods of use thereof.

PRIOR ART

10 Recombinant plant viral nucleic acids are of interest generally for their utility as expression vectors in plants.

Additionally, such nucleic acids can be used to initiate virus induced gene silencing (VIGS). This phenomenon is based on the 15 observation that virus infection in plants can initiate sequence-specific nucleic-acid based defence mechanisms that resemble either transcriptional, or post-transcriptional gene silencing (PTGS) (Covey, Al-Kaff 1997; Ratcliff, Harrison et al. 1997; Al-Kaff, Covey et al. 1998). PTGS is also manifest as an inhibition of nuclear gene 20 expression when a virus is modified to carry sequence from a nuclear expressed gene (Kumagai, Donson et al. 1995; Kjemtrup, Sampson et al. 1998; Ruiz, Voinnet et al. 1998). PTGS can also cause recovery from viral infection when a plant expressing a transgene derived from viral cDNA is infected by a homologous virus (Lindbo, Silva-Rosales 25 et al. 1993; Guo and Garcia 1997). Both the inhibition of nuclear gene expression, and recovery from viral infection are caused by sequence-specific RNA degradation.

Because modified viruses inhibit the expression of homologous plant 30 genes, VIGS can be used to induce an apparent null-phenotype or a loss of function and therefore identify the function of any gene. Viruses that have been modified in this manner include tobacco mosaic virus (TMV) (Kumagai, Donson et al. 1995) potato virus X (PVX) (Ruiz, Voinnet et al. 1998), and tomato golden mosaic virus (Kjemtrup, 35 Sampson et al. 1998).

DISCLOSURE OF THE INVENTION

The present invention is concerned with novel recombinant plant viral 40 nucleic acids.

In preferred forms the present invention is concerned with providing VIGS-based methods and materials which may be more suitable as a tool for functional genomics than those which have been used in the past. 45 For instance TMV, PVX and TGMV infections cause significant symptoms, such as a chlorosis, leaf-distortion and necrosis. Phenotypes caused by VIGS of a plant gene can therefore be hard to differentiate from these viral symptoms. Secondly, like most viruses, TMV, PVX and TGMV form mosaic, vein-based infections, and therefore do not cause 50 confluent VIGS across the whole leaf. Leaves may therefore contain a mixture of cells with and without VIGS, complicating interpretation of any phenotype. Thirdly, TMV, PVX and TGMV do not infect meristems (Matthews 1991) and can not therefore inhibit expression of genes that determine the identity and development of plant tissue. 55 Finally, although the first plant genome to be fully sequenced will

be that of *Arabidopsis thaliana*, TMV, PVX and TGMV vectors do not infect this plant. Therefore the potential of VIGS to identify gene function in Arabidopsis is limited with available technology. A VIGS vector which overcame one or more of these drawbacks would therefore represent a contribution to the art.

The present inventors have developed novel recombinant cDNA viral constructs based on tobacco rattle virus (TRV) which in preferred forms are particularly adapted for use with VIGS. Such vectors may induce few or no symptoms, cause confluent VIGS across the leaf, operate in Nicotiana species and in Arabidopsis, and inhibit gene expression in meristems.

A viral expression vector based on TRV has previously been described in which non-viral proteins were expressed from a sub-genomic promoter (Ratcliff, MacFarlane et al. 1999). The viral RNA was synthesised *in vitro* and then inoculated into the plant. The TRV vectors of the present invention include *inter alia* modifications to facilitate both the insertion of plant gene sequences and the subsequent infection of plants. Other TRV based vectors are disclosed by Hamilton & Baulcombe (1989) J. Gen. Virol 70: 963-968 and Mueller et al (1997) J. Gen. Virol 78: 2085-2088.

Thus in a first aspect of the present invention there is disclosed a nucleic acid vector which comprises:
(a) a transfer nucleotide sequence comprising (i) a plant active promoter, operably linked to (ii) a recombinant tobacco rattle virus (TRV) cDNA which includes at least cis acting elements permitting replication of said cDNA; a subgenomic promoter operably linked to a sequence encoding a TRV coat protein; and a heterologous nucleotide sequence which is foreign to said virus;
(b) border sequences which permit the transfer of the transfer nucleotide sequence into a plant cell nucleus.

The transfer nucleotide sequence is situated between the border sequences and is capable of being inserted into a plant genome under appropriate conditions. Generally this may be achieved by use of so called "agro-infiltration" which uses Agrobacterium-mediated transient transformation. Briefly, this technique is based on the property of *Agrobacterium tumafaciens* to transfer a portion of its DNA ("T-DNA") into a host cell where it may become integrated into nuclear DNA. The T-DNA is defined by left and right border sequences which are around 25 nucleotides in length. In the present invention the border sequences are included around the transfer nucleotide sequence (the T-DNA) with the whole vector being introduced into the plant by agro-infiltration, optionally in the form of a binary-transformation vector.

By "plant active promoter" is meant a sequence of nucleotides from which transcription may be initiated of DNA operably linked downstream (i.e. in the 3' direction on the sense strand of double-stranded DNA). "Operably linked" means joined as part of the same nucleic acid molecule, suitably positioned and oriented for transcription to be initiated from the promoter. Nucleic acid operably linked to a promoter is "under transcriptional initiation

regulation" of the promoter.

The cDNA includes cis acting elements permitting replication of said cDNA. However the vector need not include all of the sequence required to replicate and move within the plant. The vectors of the present invention will generally require supplementary proteins and/or nucleic acids from TRV in order to achieve this. Thus the cDNA may correspond to part of TRV RNA 2, and will thus require proteins encoded by TRV RNA1 for replication.

The TRV coat protein (as with other defined or recited sequences herein) need not be 'wild-type', but may optionally be a variant (e.g. mutant, or other variant, or a substantially homologous derivative) provided that its function (to encapsulate and permit movement of the TRV genome) is not negated. By "Substantially homologous" is meant that the sequence in question shares at least about 70%, or 80% identity, most preferably at least about 90%, 95%, 96%, 97%, 98% or 99% identity with the reference sequence. Identity may be at the nucleotide sequence and/or encoded amino acid sequence level. Homology may be over the full-length of the relevant sequence shown herein (e.g. in the sequence Annex) or may be over a part of it. Identity may be determined by the TBLASTN program, of Altschul *et al.* (1990) *J. Mol. Biol.* 215: 403-10, or BestFit, which is part of the Wisconsin Package, Version 8, September 1994, (Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA, Wisconsin 53711). Preferably sequence comparisons are made using FASTA and FASTP (see Pearson & Lipman, 1988. Methods in Enzymology 183: 63-98). Parameters are preferably set, using the default matrix, as follows: Gapopen (penalty for the first residue in a gap): -12 for proteins / -16 for DNA; Gapext (penalty for additional residues in a gap): -2 for proteins /-4 for DNA; KTUP word length: 2 for proteins / 6 for DNA.

The heterologous nucleotide sequence is foreign (non-native) to TRV, which is to say that it does not occur naturally in the TRV viral genome at the position in which it is present in the VIGS vector. The sequence will generally be either a cloning site (to permit the insertion of a desired sequence) or a desired sequence itself.

Some preferred embodiments of the invention will now be discussed.

Vector

This is preferably based on plant binary transformation vector pGreen (see Materials and Methods below). The vector may be an expression vector (for transcription of a desired sequence, which may then be translated). Alternatively (and preferably) the vector is a "VIGS vector", by which is meant one which is adapted to cause or permit virus induced gene silencing of a desired target nucleotide sequence corresponding to a sequence included in the vector.

Nucleic acid vectors according to the present invention may be provided isolated and/or purified, in substantially pure or homogeneous form, or free or substantially free of other nucleic acid. The term "isolated" encompasses all these possibilities.

Generally speaking, in the light of the present disclosure, those skilled in the art will be able to construct vectors according to the present invention. Such vectors may include, in addition to the promoter, a suitable terminator or other regulatory sequence such as to define an expression cassette consisting of the recombinant TRV cDNA and the heterologous nucleotide sequence. For further details see, for example, *Molecular Cloning: a Laboratory Manual:* 2nd edition, Sambrook *et al*, 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Protocols in Molecular Biology*, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992. Specific procedures and vectors previously used with wide success upon plants are described by Bevan, Nucl. Acids Res. (1984) 12, 8711-8721), and Guerineau and Mullineaux, (1993) Plant transformation and expression vectors. In: Plant Molecular Biology Labfax (Croy RRD ed) Oxford, BIOS Scientific Publishers, pp 121-148.

Plant promoter

Suitable promoters will be well known to those skilled in the art and include the Cauliflower Mosaic Virus 35S (CaMV 35S) gene promoter that is expressed at a high level in virtually all plant tissues. The promoter may in principle be an inducible promoter such as the maize glutathione-S-transferase isoform II (GST-II-27) gene promoter which is activated in response to application of exogenous safener (WO93/01294, ICI Ltd). The GST-II-27 gene promoter has been shown to be induced by certain chemical compounds which can be applied to growing plants. Another suitable promoter may be the DEX promoter (Plant Journal (1997) 11: 605-612).

Recombinant TRV cDNA

This is preferably based on a modified, reduced, cDNA clone of TRV RNA2. In the Examples herein the strain used is ppk20. However any appropriate strain, which can give rise to replicating, infectious viral transcripts, could be used (see e.g. Macfarlane, 1999 for further examples).

Within the cDNA it is preferable that non-essential ORFs or other sequences are deleted, provided that the cDNA can still be used to generate replicating, infectious transcripts. Preferably, where the cDNA is based on TRV RNA2 of ppk20, two open reading frames (37K and 32.8K) are deleted to leave only the 5′ and 3′ untranslated regions and the viral gene encoding the coat-protein. The deleted ORFs are replaced by a heterologous nucleotide sequence between the coat protein and the untranslated region (UTR). The sequence is shown in the Sequence appendix (No. 1). Naturally substantially homologous variants of the sequence are also included within the scope of the invention. In particular, vectors derived from pTV00 and having the characteristics (described herein) of that vector, are also embraced.

Vectors based on TRV RNA2 require proteins encoded by TRV RNA1 for

replication, which can be achieved as described below.

Heterologous nucleotide sequence.

5 This can in principle be a single or multiple cloning site (i.e. a sequence encoding two or more restriction endonuclease target sites) to facilitate the incorporation of a desired nucleotide sequence.

For expression vectors according to the present invention, the
10 sequence will generally include or be operably linked to a subgenomic promoter which is recognised by a TRV-effective replicase (e.g. the PEBV CP subgenomic promoter) and an ORF sequence which it is desired to express and which is therefore transcribed as a subgenomic RNA.

15 For VIGS vectors the sequence will be a "targeting sequence" which corresponds to a sequence in a target gene, either in the sense or anti-sense orientation, or a sequence which has sufficient homology to a target sequence for down-regulation of expression of the target gene to occur. Such a targetting sequence may be included in the
20 vector anywhere in the viral cDNA irrespective of the location of any subgenomic promoter (provided it does not interfere with the cis-acting replication elements or the coat protein). Generally speaking it will be preferable for VIGS vectors according to the present invention not to include a subgenomic promoter within or operably
25 linked to the heterologous gene sequence. Such preferred vectors have the advantage that they are more stable (reduced likelihood of self-recombination) that those of the prior art such as those described by Ratcliff, MacFarlane et al. (1999) *supra* which had more than one subgenomic promoter.
30

In general the targeting sequence may be derived from a plant nuclear gene or transgene, or a gene on an extrachromosomal element such as a plastid.

35 VIGS is particularly preferred for investigating gene function in that it can be used to impose an intermediate or a null phenotype for a particular gene, which can provide information about the function of that gene *in vivo*. In such cases the targeting sequence may not be known, but the methods of the present invention may be used to
40 identify it with a particular phenotype.

The complete sequence corresponding to the coding sequence (in reverse orientation for anti-sense) need not be used. For example fragments of sufficient length may be used. It is a routine matter
45 for the person skilled in the art to screen fragments of various sizes and from various parts of the coding sequence to optimise the relationship between target and targeting sequence. It may be preferable that there is complete sequence identity between the targeting sequence in the vector and the target sequence in the
50 plant, although total complementarity or similarity of sequence is not essential. One or more nucleotides may differ in the targeting sequence from the target gene. Thus, a targeting sequence employed in a construct in accordance with the present invention may be a wild-type sequence (e.g. gene) selected from those available, or a
55 substantially homologous mutant, derivative, variant or allele, by

way of insertion, addition, deletion or substitution of one or more nucleotides, of such a sequence. Such a sequence need not include an open reading frame or specify an RNA that would be translatable.

5 A further possibility is to target a conserved sequence of a gene, e.g. a sequence that is characteristic of one or more genes in one or more pathogens against which resistance is desired, such as a regulatory sequence.

10 Other aspects of the invention will now be discussed.

One aspect of the present invention is a process for producing a vector as described above, the process being substantially as set out in the Examples hereinafter. A further aspect is a process for
15 producing a vector as described above, which process comprises the step of cloning a heterologous nucleotide sequence which is a targeting sequence into the vector.

Thus one aspect of the present invention includes a method of
20 silencing a target gene in a plant tissue using VIGS which method comprises the steps of introducing a vector as described above into the plant, wherein said vector includes a heterologous nucleotide sequence which is a targeting sequence.

25 "Plant tissue" is any tissue of a plant in planta or in culture, including the whole plant an organ thereof, a cutting, or any group of plant cells organised into a structural and functional unit.

"Silencing" is a term generally used to refer to suppression of
30 expression of a gene. The degree of reduction may be so as to totally abolish production of the encoded gene product, but more usually the abolition of expression is partial, with some degree of expression remaining. The term should not therefore be taken to require complete "silencing" of expression. It is used herein where
35 convenient because those skilled in the art well understand this.

The method may be preferably used to cause confluent VIGS of the target gene across a whole leaf and/or to silence a target gene in meristematic tissue.

40

As discussed above, for introduction into the plant, the vector may be in the form of an *Agrobacterium* binary vector. The vector is introduced into the plant cell by *Agrobacterium*-mediated T-DNA transfer, the transfer sequence may be integrated transiently into
45 the plant (cell) genome, and is then transcribed to RNA from the plant promoter. In the published vector of Ratcliff, MacFarlane et al. (1999), the viral cDNA and any cDNA inserted after the sub-genomic promoter was transcribed to infectious RNA *in vitro* by T7 RNA polymerase and subsequently introduced into the plant.

50

TRV RNA 2 and all derived constructs require proteins encoded by TRV RNA1 for replication within and movement though out the plant. TRV RNA1 infections can be initiated either by rub-inoculating the plant with purified RNA 1 (Matthews 1991), or by transient *Agrobacterium*
55 mediated expression in the plant of the plasmid pBINTRA6, which

contains a CaMV 35S driven infectious clone of TRV PPK20 RNA 1 (see Materials and Methods).

The present invention may particularly be applied in plants which are natural hosts (compatible with) TRV. By "compatible" is meant capable of operating with the other components of a system, in this case TRV must be capable of replicating in the plant in question. These include *Arabidopsis thaliana*. Others include (but are not limited to) *Allium cepa; Amaranthus caudatus; Amaranthus retroflexus; Antirrhinum majus; snap-dragon; Arachis hypogaea; Avena sativa; Bellis perennis; Beta vulgaris; Brassica campestris; Brassica campestris ssp. napus; Brassica campestris ssp. pekinensis; Brassica juncea; Calendula officinalis; Capsella bursa-pastoris; Capsicum annuum; Catharanthus roseus; Cheiranthus cheiri; Chenopodium album; Chenopodium amaranticolor; Chenopodium foetidum; Chenopodium quinoa; Coriandrum sativum; Cucumis melo; Cucumis sativus; Glycine max; Gomphrena globosa; Gypsophila elegans; Helianthus annuus; Hyacinthus; Hyoscyamus niger; Lactuca sativa; Lathyrus odoratus; Linum usitatissimum; Lobelia erinus; Lupinus mutabilis; Lycopersicon esculentum; Lycopersicon pimpinellifolium; Melilotus albus; Momordica balsamina; Myosotis sylvatica; Narcissus pseudonarcissus; Nicandra physalodes; Nicotiana benthamiana; Nicotiana clevelandii; Nicotiana glutinosa; Nicotiana rustica; Nicotiana sylvestris; Nicotiana tabacum; Nicotiana edwardsonii; Ocimum basilicum; Petunia hybrida; Phaseolus vulgaris; Phytolacca americana; Pisum sativum; Raphanus sativus; Ricinus communis; Salvia splendens; Senecio vulgaris; Solanum melongena; Solanum nigrum; Solanum tuberosum; Spinacia oleracea; Stellaria media; Trifolium pratense; Trifolium repens; Tropaeolum majus; Tulipa; Vicia faba; Vicia villosa; Viola arvensis.*

Target genes include those which confer 'unwanted' traits in the plant and which it may therefore be desired to silence using VIGS. Examples include ripening specific genes in tomato to improve processing and handling characteristics of the harvested fruit; genes involved in pollen formation so that breeders can reproducibly generate male sterile plants for the production of F1 hybrids; genes involved in lignin biosynthesis to improve the quality of paper pulp made from vegetative tissue of the plant; gene silencing of genes involved in flower pigment production to produce novel flower colours; gene silencing of genes involved in regulatory pathways controlling development or environmental responses to produce plants with novel growth habit or (for example) disease resistance; elimination of toxic secondary metabolites by gene silencing of genes required for toxin production.

A further aspect provides a process which includes introducing the vector into a plant, optionally including the further step of introducing a source of proteins encoded by TRV RNA1 into the plant.

A further aspect of the present invention provides a method which includes causing or allowing transcription from a construct as disclosed within the genome of a plant cell to produce a cytoplasmically-replicating RNA.

A further aspect of the present invention provides a method of reducing or suppressing or lowering the level of a target gene in a plant cell, the method including causing or allowing transcription from a vector as disclosed above.

In preferred forms the present invention is concerned with providing VIGS-based methods are useful in functional genomics. Thus in one aspect of the present invention, the target gene may be of unknown phenotype, in which case the VIGS system may be employed to analyse the phenotype by generating a widespread null (or nearly null) phenotype. The target gene may be essential, which is to say that the null phenotype is lethal to the cell or tissue in question.

This aspect of the invention may comprise a method of characterising a target gene comprising the steps of:
(a) silencing the target gene in a part or at a certain development stage of the plant using the TRV VIGS system described above,
(b) observing the phenotype of the part of the plant in which or when the target gene has been silenced.

Generally the observation will be contrasted with a plant wherein the target gene is being expressed in order to characterise (i.e. establish one or more phenotypic characteristics of) the gene.

The advantage of the TRV system over certain prior art constructs is discussed above. There are also several advantages of the current method over alternative methods in which the targeted gene is inactivated by insertional or other mutagenic procedures. The advantage over mutagenic procedures applies when there is more than one homologous gene carrying out the role of the target gene. Mutagenic procedures will not normally reveal a phenotype in that situation. A second situation where the current invention has advantage over both mutagenic and unregulated gene silencing procedures applies when the target gene has a lethal phenotype. The controllable attribute of the gene silencing will allow the phenotype of such genes to be investigated and exploited more efficiently than using the alternative methods available prior to the disclosure of the current invention.

Nor, for the identification of endogenous genes, would it be necessary to try and generate a transgenic plant in which gene silencing is already activated to observe the effect.

In a further aspect there is disclosed a method of altering the phenotype of a plant comprising use of the silencing method discussed above. Traits for which it may be desirable to change the phenotype include the following: colour; disease or pest resistance; ripening potential; male sterility.

In a further aspect of the present invention there is disclosed a virus or viral particle including, preferably encapsulating, a vector (or transcript from the expression cassette in the vector) according to the present invention.

In a further aspect of the present invention there is disclosed a kit comprising a vector as described above, plus a source of TRV RNA1 polypeptide or vector encoding the same (e.g. pBINTRA6).

5 In a further aspect of the present invention there is disclosed a host cell including a vector according to the present invention. These may be plant cells, or may be microbial (particularly bacterial and especially *Agrobacterium*) cells.

10 In a further aspect there is disclosed a plant, or plant tissue, including, or transiently transformed by, a vector of the present invention.

The invention will now be further described with reference to the 15 following non-limiting Figures and Examples. Other embodiments of the invention will occur to those skilled in the art in the light of these.

FIGURES

20

Figure 1; Schematic illustration of the cloning steps to produce pTV00. 35S is the CaMV 35S promoter; 37K and 32.8K are the open reading frames for the 37K and 32.8K proteins; UTR is the untranslated region; Nos is the nopaline synthase transcriptional 25 terminator; RB is the right border; LB is the left border.

Figure 2.
A; Schematic drawing of TRV RNA1; 5'UTR and 3'UTR are the 5' and 3' untranslated regions respectively; Rep 134 K is the 134KDa replicase 30 protein; Rep 194 K is the 194 KDa read-through replicase protein; MP is the movement protein; 16K is the 16 KDa protein. B; The relative positions of the PCR1 and PCR2 cDNA fragments.

Figure 3.
35 Schematic illustration of the cloning strategy for pBSTRF16.

Figure 4.
Schematic representation of the cloning strategy for the introduction of intron 3 from *A. thaliana* NIA1 gene to TRV RNA 1, to obtain 40 pBSTRA3. INT is NIA1 gene intron 3.

Figure 5.
Schematic representation of pBINTRA6. LB and RB respectively are the left border and right border of pBINTRA6 T-DNA.

45

SEQUENCE APPENDICES

1. The full sequence of pTV00.

50 2. PEBV promoter sequence.

3. *N. benthamiana* pds cDNA fragment.

4. *A. thaliana* pds cDNA fragment.

55

5. *N. benthamiana* rubisco cDNA fragment.

6. GFP (PCR amplified).

7. *N. benthamiana* NFL cDNA fragment.

8. The partial sequence of pBINTRA6.

EXAMPLES

General materials and methods

All DNA modifications and digestions were performed using enzymes according to the manufacturers' instructs and following protocols described by Sambrook *et al.* (Sambrook, Fritsch et al. 1989).

The TRV vector pTV00 was derived from pCaK20-2T7, a previously described clone of TRV RNA2 (Hernandez, Mathis et al. 1995). pCaK20-2T7 contains a full-length cDNA of TRV strain PPK20 RNA2 cloned between the cauliflower mosaic virus (CaMV) 35S promoter and the Nos terminator.

For the construction of pTV00 two fragments of pCaK20-2T7 were cloned into the binary plant transformation vector pGreen0000 (described below). The first fragment, of 1536bp, included the CaMV 35S promoter, and cDNA sequence encoding the TRV RNA2 5' untranslated region and coat protein. This fragment was released by digesting pCaK20-2T7 with Bgl II, treating with T4 DNA polymerase , and subsequently digesting with Sac II. The resulting fragment was ligated into the binary plasmid pGreen 0000 that had been cut with Xba I, blunted with T4 DNA polymerase and digested with Sac II, to form the plasmid pGreen35Sorf1.

The second fragment of pCaK20-2T7, containing the 3' untranslated region (UTR) of TRV RNA2 and the Nos terminator, was released by digestion with BbsI and FspI and then blunted with T4 DNA polymerase. This 835bp fragment was ligated into pGreen35Sorf1 at an Fsp I site, to form pTV00. The full sequence of pTV00 is given in Appendix 1. A schematic illustration of the cloning procedure is shown in Figure 1.

These cloning steps had three purposes. Firstly, the TRV RNA 2 cDNA clone was introduced to the T-DNA of a plant binary-transformation vector. This allows Agrobacterium mediated-infection of TRV RNA 2 without the need for *in vitro* transcription as previously described for another TRV RNA2 clone (Ratcliff, MacFarlane et al. 1999). The second effect of these cloning steps was to remove the cDNA sequences that encode the 37K and 32.8K proteins. These proteins are dispensable for TRV infection, and are required for transmission of TRV by nematode vectors (MacFarlane 1999). The third effect was to introduce 12 unique restriction enzyme sites into the genome of the TRV RNA 2. This multiple cloning site (MCS) is more convenient for the insertion of novel sequences to TRV RNA 2 than the single restriction enzyme site present in the previously described TRV RNA 2 vector (Ratcliff, MacFarlane et al. 1999).

A 409bp cDNA fragment of pds was PCR amplified from *N. benthamiana* cDNA using Taq DNA polymerase and the primers 5'ggcactcaactttataaacc and 5'cttcagttttctgtcaaacc. This pds cDNA fragment (Appendix 3) was cloned into the Sma 1 sites of pTV00 and into pGR107, to form pTV.pds
5 and pGR107.pds respectively.

For construction of pTV.apds a 1.7kb fragment of the pds gene was PCR amplified from *A. thaliana* cDNA using Taq DNA polymerase and the primers 5'cccctcgagagatgtcaaatc and 5'cccctcgaggcactttcatctgg. This
10 *A. thaliana* pds cDNA fragment (Appendix 4) was cloned into pTV00 at the Sma 1 site.

A 500bp cDNA fragment of the rubisco small sub-unit was PCR amplified from *N. benthamiana* cDNA using Taq DNA polymerase and the primers
15 5'cagtctagatggcttcctcagttctttcc and 5'cagggatcccacttgacgcacgttgtc. This rubisco cDNA fragment (Appendix 5) was cloned into the Sma 1 sites of pTV00 and pGR107 to form pTV.rubisco and pGR107.rubisco respectively.

20 A 321bp fragment corresponding to the 3' end of GFP (designated P) was PCR amplified using Taq DNA polymerase and the primers 5'aacatcctcggcccacaagtt and 5'gagctcttagagttcgtcatg. This fragment (Appendix 6) was cloned into the Sma 1 sites of pGR107 and pTV00 to form pGR107.P and pTV.P respectively.
25

A 421bp cDNA fragment of the NFL gene was PCR amplified from *N. benthamiana* using Taq DNA polymerase and the primers 5'tggacccagaggctttctc and 5'cttcttgtgagagagcgtca. This NFL cDNA fragment (Appendix 7) was cloned into pGR107 and pTV00 at the Sma 1
30 sites to form pGR107.NFL and pTV.NFL respectively.

pBINTRA6, a full length infectious clone of TRV (strain PPK20) RNA1, was constructed as follows.

35 Total RNA was prepared from TRV (strain ppk20) infected *N. benthamiana* plants as previously described (Devic, Jaegle et al. 1989). Full length cDNA corresponding to TRV RNA1 was prepared from this RNA using Superscript Reverse Transcriptase (Gibco) and the primer TRV2 5'gggggatccgggcgtaataacgcttacg3' which anneals to the 3'
40 end of TRV RNA1. A schematic drawing of TRV RNA1 is shown in Figure 2. All primers in this work were derived from the sequence of a closely related TRV strain SYM (Hamilton, Boccara et al. 1987) The full-length cDNA was used as a template for PCR amplification of two overlapping fragments, PCR1 and PCR2, which together cover all of TRV
45 RNA1.

PCR1, a 3.2 kb fragment, was amplified using Expand HiFi polymerase (Roche). The primers were: TRV1 'gggggatccataaaacatttcaatcctttg3'(which anneals to positions 1-21 of
50 TRV) and TRV4U 5'ttagcaccagctatctgagcgc3' (positions 3168-3189). PCR2, a 4.1 kb product, was also amplified using Expand HiFi polymerase (Roche) and the primers TRV4D 5'gttccaaccagacaaacgtatgg3' (positions 2698-2720) and TRV2 (see above).

55 PCR1 and PCR2 share a 491nt overlap in the replicase open reading

frame (ORF). The primers TRV1 and TRV2 contain BamHI sites to allow cloning of the full-length product (Figure 2). PCR2 was blunt-ended using T4 DNA polymerase, digested with BamHI, and cloned into the plasmid pBAC/SacB1 (Bendahmane, Kanyuka et al. 1999) which had
5 previously digested with BamHI and EheI to form pBSTR3'C. The PCR1 fragment was blunted-ended with T4 DNA polymerase and ligated into HpaI digested-pBSTR3'C, to form pBSTRF16 (Figure 3). pBSTRF16 therefore contains 302bp that are duplicated within the replicase ORF.
10

The 302bp of duplicated replicase sequence was replaced with a 438bp intron. Intron 3 of *Arabidopsis thaliana* Col-0 nitrate reductase NIA1 gene (Wilkinson and Crawford 1993) was amplified using the primers AraF and AraR. AraF is 5'CGTATCTTTGCAA
15 TAACAGgtaataatcctctctcttgatatt3', where the sequence in upper case corresponds to positions 2826-2845 of TRV RNA1 and the sequence in lower case corresponds to positions 1-24 of the intron. Similarly, AraR is 5'TTAAATTGTCCAAGATCAACct gtttaacacaagtcaacgtc3' where the sequence in upper case corresponds to positions 2846-2864 of TRV RNA
20 1 and the sequence in lower case corresponds to positions 416-438 of the intron. The PCR amplified intron 3 fragment was therefore flanked by the AGGT intron splice-sites, and 19bp of TRV (exon) sequence (Figure 4). Two TRV-exons (exon 1 and exon 2) that flank the intron insertion site were then PCR amplified. For exon 1 the primers were
25 TRV2D 5'tcgcacaaaaccaaggtgatag3' (positions 1772-1793) and Ara5'R 5'ggattatt acCTGTTATTGCAAAGATACGTCTG3' where the sequence in lower case corresponds to positions 1-10 of the intron and sequence in upper case corresponds to positions 2822-2845 of TRV RNA1. Exon 1 was amplified as a 1.07kb fragment from pBSTR16. For exon 2 the primers
30 were Ara3'F 5'tgttaaacagGTTGATC TTGGACAATTTAAGTGC3', where the sequence in upper case corresponds to positions 2846-2868 of TRV RNA1 and the sequence in lower case corresponds to positions 428-438 of the intron, and TRV4U (see above). Exon 2 was amplified as a 0.35kb fragment from PCR 1 (see above). Exon 1, intron3 and exon 2 were all
35 amplifed using Pfu polymerase (Promega). To introduce intron 3 to the TRV RNA 1 genome, chimeric PCR was performed with Pfu polymerase and the primers TRV2D and TRV4U using a mixture of exon 1, intron 3 and exon 2 as template to give a 1.8kb fragment.

40 This 1.8kb intron-containing-fragment was digested with ApaI and SalI and cloned in pBSTRF16 using ApaI-partial digestion and SalI, thus replacing the region that included duplicated sequence, and forming pBSTRA3 (Figure 4).

45 To transfer the cloned RNA1 to a binary transformation vector, the 7.2kb fragment corresponding to TRV RNA 1 was released from pBSTRA3 with BamHI and cloned into the BamHI site between the CaMV 35s promoter and the CaMV terminator on the plasmid pBIN61 to form pBINTRA6 (Figure 5). pBIN61 is a modified version of the pBIN19
50 (Frisch, Harris-Haller et al. 1995) binary vector that carries a transcription cassette comprising the CaMV 35S promoter and terminator. To construct the pBIN61 binary vector, the transcription cassette containing the CaMV 35S promoter and terminator was released by digestion with *Kpn*I and *Xho*I from the plasmid pJIT61 (kindly
55 provided by P. Mullineaux, JIC, Norwich, UK). The transcription

cassette was then ligated to the pBIN19 plasmid vector digested with *Kpn*I and *Sal*I to create pBIN61.

Agrobacterium strain GV3101 containing pBINTRA6 was infiltrated into
5 *N. benthamiana* leaves causing a TRV RNA 1 infection. The partial sequence of pBINTRA6 is given in Appendix 8.

The kanamycin resistance gene (NptI) from pACYC177 (Chang and Cohen 1978) was cloned as a NheI-NcoI fragment into the SpeI-BspHI sites of
10 pBluescript SKII+, creating intermediate I. The NcoI site was introduced and restriction sites that would have been duplicated in the pGreen polylinker were removed by site-directed mutagenesis (mutagenic oligos: XhoI (5' cgtcttgctcaaggccgcgat 3'), ClaI (5' cgacaatcta ccgattgtatg 3'), SmaI (5' ctgcgatcccagggaaaacag 3'),
15 HindIII (5'aaatgcataaagtttt gccat 3') and NcoI (5' tggttgtaaccatggcagagca 3'). Two complementary oligos (5'gaattcagatcta3' and 5'acatgtagatctg3' respecively) were annealed and inserted between the EcoRI and AflIII sites, to introduce a unique BglII site, this was intermediate II. The pSa-ori sequence
20 from pJIT134Sa-Bam was inserted as a BamHI-SmaI fragment into the BamHI-SmaI sites (remaining from the original pBluescript plasmid) of intermediate II. These sites, along with intervening PstI and NotI sites were removed by successive rounds of treatment with T4 DNA polymerase I (T4 polI) and re-ligation. The StuI site in the NptI
25 promoter and ClaI sites, introduced when the pSa-ori was inserted, were removed by transformation into E.coli strain SCS110 (dam;dcm), digestion with T4 polI and re-ligation. This produced the pGreen backbone that was ready to receive the T-DNA cassette.

30 The complementary olignucleotides (5'-catgaaggccttgacaggatatattggcgggtaaactaa gtcgctgtatgtgtttgtttgagatct-3' and 5'-catgagatctcaaacaaacacatacagcgacttagtttacccg ccaatatatcctgtcaaggcctt -3') were annealed to produce a DNA fragment consisting of a StuI site, the RB sequence, the RB "overdrive"
35 sequence and a BglII site. This RB DNA fragment was inserted into the AflIII site of pBluescript SKII- and its orientation determined by sequencing. A recombinant plasmid (intermediate A) was selected which had the orientation of the RB fragment such that the StuI site was nearest to the SK multiple cloning site.

40

Two further oligonucleotides (5'-tccacacattatacgagccgatgattaattgtcaacagatcttggcag gatatattgtggtgtaaacgttaac-3' and 5'-ggtaacgtttacaccacaatatatcctgccaagatctgttgacaatta
45 atcatcggctcgtataatgtgtgga-3') were then annealed to produce an LB DNA fragment consisting of HpaI and BglII site and LB sequence. This fragment was inserted between the two SspI sites of intermediate A, simultaneously deleting the pBluescript SKII- f1 ori. This 815bp BglII fragment was cloned into the pGreen backbone to produce
50 pGreen0000.

All work involving virus infected material was carried out in containment glasshouses under MAFF license PHF 1420c/1773(12/1996). *N. benthamiana* and *A. thaliana* plants were germinated on a 1:1
55 mixture of JIC compost and peat, then grown individually in pots at

25°c during the day and 20°c during the night. Supplementary winter lighting from halogen quartz iodide lamps provided a 16 hour day length.

5 Virus infections on *N. benthamiana* were achieved by Agrobacterium-mediated transient gene expression of infectious constructs from the T-DNA of a binary plasmid (e.g. pGR107, pTV00 or pBINTRA6). Agrobacterium was grown to an OD of 600 in L broth. The culture was then centrifuged and re-suspended in 10mM MgCl2, 10mM MES and 150mM 10 acetosyringone, and kept at room temperature for 2 hours. The culture was then infiltrated to the underside of a leaf using a 2ml syringe without a needle.

15 For virus infections on *A.thaliana*, Agrobacterium cultures carrying pBINTRA6 and pTV.apds were first infiltrated to *N. benthamiana*, as described above. A week later, systemically infected leaves were ground in 50mM phosphate buffer pH7 using a pestle and mortar. The solution was then centrifuged for 1 minute at 3000 rpm and the 20 supernatant was rubbed onto the leaves of carborundum-dusted plants.

For RNA infection, total RNA was purified from infected plants as previously described (Devic, Jaegle et al. 1989). 5m of this RNA was 25 rubbed onto the leaves of carborundum dusted plants.

Total RNA from *N. benthamiana* was prepared using Tri-reagent (Sigma). Genomic DNA was removed from this RNA by incubation with DNase 30 (Sigma) at 37°c for 2 hours followed by phenol extraction. This RNA was used as a template for first-strand cDNA synthesis using random hexanucleotide primers and Super-Script Reverse Transcriptase (Roche) according to the manufacture's instructions. This cDNA was diluted 2000 fold in sterile distilled water, and used as a template for 35 quantitative PCR in an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems) with 2X Taqman Universal PCR Mastermix (PE Applied Biosystems). For rubisco amplification, the primer sequences were cgtcaagtgcagtgcatcagt and gacaatagggtaagttgtcctaatatgaaa and the probe sequence was cattgcctccaagcctgacgga. Each sample was 40 quantitatively standardised by amplification of 25S ribosomal cDNA using the primers accacagggataactggcttgt and ccgacatcgaaggatcaaaaa with the probe cagccaagcgtcatagcgacgttg. The probe DNA was modified to contain 5' FAM fluorescent reporter and 3' Tamra quencher (MGW). To confirm that PCR amplification corresponded to mRNA levels and not 45 from genomic or contaminating DNA, additional reactions were performed without template cDNA and with RNA that had not been treated with reverse-transcriptase.

Example 1 - comparison of silencing of pds gene by TRV and PVX VIGS 50 vectors

To assess whether constructs derived from TRV have advantages over previously described virus vectors, we compared symptoms and VIGS caused by TRV and another virus. PVX was used for this comparison, 55 because it inhibits expression of a wide range of plant genes (Ruiz,

Voinnet et al. 1998; Jones, Hamilton et al. 1999). Furthermore, as with TRV, an infectious PVX cDNA construct is available under control of the CaMV 35s promoter on the binary transformation plasmid "pGreen" (pGR107, (Jones, Hamilton et al. 1999)). As both TRV and PVX derivatives are therefore introduced to plants in the same way, any differences can be attributed to the virus itself, rather than the means of inoculation.

We next compared the ability of TRV and PVX derived constructs to inhibit plant gene expression in *N. benthamiana*. Identical plant cDNA sequences were inserted into pTV00 and pGR107 to compare the efficiency of TRV and PVX at silencing the same plant gene. cDNA sequences from two plant genes were used for this comparison.

The first cDNA we used was part of the phytoene desaturase (pds) gene. Pds is essential for production of carotenoids that protect plants from photobleaching (Demmig-Adams and Adams 1992). Plant tissue in which pds is inhibited therefore turns white due to photobleaching. This whitening phenotype provides a useful visual marker for assessing the extent and severity of VIGS (Kumagai, Donson et al. 1995; Ruiz, Voinnet et al. 1998). A further rationale for choosing pds as a target of VIGS is that the copy number and expression level of the pds gene is low (Ruiz, Voinnet et al. 1998). VIGS of pds therefore indicates the potential of a virus to inhibit the expression of a low abundance mRNA. For these experiments a 409bp cDNA fragment of pds (Appendix 3) was PCR amplified from *N. benthamiana* cDNA and cloned in the sense orientation into pTV00 and pGR107, to form pTV.pds and pGR107.pds respectively.

Leaves of 3 week old *N. benthamiana* plants were infiltrated with Agrobacterium containing either pGR107.pds, to cause PVX.pds infections, or with Agrobacterium containing pBINTRA6 and pTV.pds, to cause TRV.pds infections. As previously reported (Ruiz, Voinnet et al. 1998), after 3 weeks plants infected with PVXpds developed symptoms of photobleaching that are typical of pds inhibition. Stems, axillary shoots, and sepals were affected. In leaves the photobleaching was initiated and predominantly maintained around the veins. However, a large proportion of the leaf surface remained green. Plants infected with TRV.pds also developed photobleaching symptoms. Again, stem, axillary shoots, sepals and leaves were affected. However, unlike leaves infected with PVX.pds, leaves infected with TRV.pds became predominantly white, indicating widespread silencing of pds gene by the TRV based construct.

These data are typical of 10 repeats, and show that when TRV and PVX derivatives carry the same fragment of the pds gene, the TRV construct inhibits pds activity in a higher proportion of leaf tissue than the PVX vector.

Example 2 - comparison of silencing of rubisco by TRV and PVX VIGS vectors

A second comparison of VIGS caused by TRV and PVX was made using the small sub-unit of ribulose -1,5-bisphosphate carobxylase oxygenase (rubisco). Antisense or PVX.rubisco induced inhibition of rubisco

causes chlorotic and stunting symptoms (Rodermel, Abbott et al. 1988; Jones, Hamilton et al. 1999). A further rationale for choosing rubisco as a target of VIGS is that the copy number and expression level of the rubisco genes is very high. In contrast to pds, VIGS of rubisco would therefore indicate the potential of a virus to inhibit the expression of a high abundance endogenous mRNA. A 500bp cDNA fragment of the rubisco small sub-unit (Appendix 4) was PCR amplified from *N. benthamiana* cDNA and cloned in the sense orientation into pTV00 and pGR107 to form pTV.rubisco and pGR107.rubisco respectively.

Leaves of 3 week old *N. benthamiana* plants were infiltrated either with Agrobacterium containing pGR107.rubisco, to cause a PVX.rubisco infection, or with Agrobacterium containing pBINTRA6 and pTV.rubisco, to cause a TRV.rubisco infection.

Three weeks after infiltration, the upper leaves of plants that were infected with PVX.rubisco developed very pale green or yellow patches. However, as with PVX induced inhibition of pds, leaves were not uniformly affected. Similar areas of pale green developed on stems, axillary shoots and sepals. These pale green areas were distinct and different from the mosaic normally associated with PVX infection. Plants that were infected with TRV.rubisco also developed pale green or yellow tissue in systemically infected leaves, stems, axillary shoots and sepals. However, in contrast to PVX.rubisco infected plants, in TRV.rubisco infected leaves there was no mosaic, and the pale green or yellow symptoms appeared uniform across the leaf. This experiment was repeated more than 10 times, with the same result.

To confirm that the symptoms associated with PVX.rubisco and TRV.rubisco infection were caused by inhibition of rubisco, and to quantify any reduction in rubisco mRNA caused by each construct, rubisco mRNA levels were measured by Taqman PCR (PE Applied Biosystems). In these experiments, total RNA from infected plants was used as a template for first-strand cDNA synthesis. Quantitative PCR was then used to assess the ratio of rubisco cDNA to that of ribosomal cDNA in PVX.rubisco or TRV.rubisco infected plants, and in uninfected plants. The rubisco sequence carried by the PVX and TRV constructs was not PCR amplified in these experiments as the primers used were outside the region carried by in the virus vectors (see Materials and Methods). Nine separate plants were used for each treatment, and each plant was analysed three times.

In leaves infected with PVX.rubisco there was a 37 fold reduction in rubisco mRNA compared to the levels in mock inoculated plants (Student's T-Test; $P=1.96\ 10^{-7}$). However, in leaves infected with TRV.rubisco there was an even greater reduction in rubisco levels of 200 fold compared to mock inoculated plants ($P=2.1\ 10^{-7}$). These data show that the TRV based construct is 5.4 (P=0.02) fold more efficient at suppressing rubisco accumulation than the PVX based construct carrying an identical rubisco sequence.

<u>Example 3 - comparison of silencing in *A. thaliana* by TRV and PVX VIGS vectors</u>

One disadvantage of currently available virus vectors is that they have a limited host range. For example, neither PVX nor TMV vectors infect the model plant *A. thaliana*. In an attempt to overcome this limited host range, some authors have made transgenic *A. thaliana* plants that express a full-length infectious PVX cDNA carrying endogenous plant gene sequence. This strategy was pursued because PVX replicates in *A. thaliana* protoplasts, but does not infect whole plants. However, even when PVX is expressed from a transgene and replicating in every cell, there was little or no inhibition of homologous Arabidopsis gene expression (unpublished data).

We tested whether constructs based on TRV would inhibit gene expression in *A. thaliana*. A fragment of the pds gene was PCR amplified from *A. thaliana* cDNA (Appendix 5) and cloned the sense orientation into pTV00 to form pTV.apds. No TRV infections could be established by infiltrating *A. thaliana* with Agrobacterium carrying pBINTRA6 and pTV.pds or pTV00. Therefore, TRV.apds infections were established by infiltrating Agrobacterium that contained pBINTRA6 and pTV.apds to *N. benthamiana*. Infectious sap from these *N. benthamiana* plants was subsequently used to inoculate *A. thaliana* ecotype Col-0 (see Materials and Methods). As with *N. benthamiana*, *A. thaliana* plants infected with TRV developed no symptoms. However, *A. thaliana* plants infected with TRV.apds developed confluent photobleaching in systemic leaves that is typical of pds inhibition. These data suggest that unlike PVX, TRV can inhibit gene expression in a range of plant species including *A. thaliana*.

Example 4 - comparison of silencing of GFP transgene in meristematic tissue by TRV and PVX VIGS vectors

A sub-set of genes that control identity and development of newly forming plant tissues are expressed in meristematic regions. Understanding the function of these genes is of particular interest as they determine characteristics such as fruit and flower production. However, PVX, TMV and TGMV do not infect meristems (Matthews 1991), and therefore cannot be used to inhibit meristem-expressed genes.

We assessed whether our TRV based vector would overcome this limitation by comparing the ability of the PVX and TRV derived constructs to inhibit the expression of two genes in meristems. The first of these genes encodes the green fluorescent protein, GFP (Chalfie, Tu et al. 1994). A 321bp fragment corresponding to the 3' end of GFP (designated P; Appendix 6) was PCR amplified and cloned in the sense orientation into pGR107 and pTV00 to form pGR107.P and pTV.P respectively. For these experiments *N. benthamiana* plants were used that express GFP from a CaMV 35S driven transgene (line 16c, (Ruiz, Voinnet et al. 1998)). In UV light these plants fluoresce green due to expression of the GFP transgene. In contrast, non-transgenic plants, and 16c plants in which GFP expression is inhibited, are red under UV illumination, due to fluorescence from chlorophyll.

Three week old 16c *N. benthamiana* plants were infiltrated with Agrobacterium containing either pGR107.P, to cause a PVX.P infection,

or pBINTRA6 and pTV.P to cause a TRV.P infection, or with water. As previously reported, 3 weeks after infiltration the leaves, stems and axillary shoots of plants infected with PVX.P had lost all green fluorescence, and appeared red under UV light (Figure 5A) (Ruiz, Voinnet et al. 1998). Similarly, 16c plants that were infected with TRV.P also lost GFP expression in leaves, stems and axillary shoots. We then assessed the level of GFP expression in vegitative meristematic regions using confocal laser-scanning microscopy. As previously reported (Ruiz, Voinnet et al. 1998) PVX.P did not inhibit GFP expression in this region, as the meristem and surrounding leaf primordia in these plants remained green-fluorescent. In contrast, TRV.P inhibited GFP expression in the meristematic dome and surrounding leaf primordia.

Example 5 - comparison of silencing of LFY endogenous gene in meristematic tissue by TRV and PVX VIGS vectors

We next compared the ability of PVX and TRV derived constructs to inhibit the expression of an endogenous gene in meristems. The gene we chose was NFL, the Nicotiana floricola homologue of the *A. thaliana* gene leafy (LFY). LFY is important for determination of floral organs. *A. thaliana lfy* mutants have two characteristics; additional inflorescence shoots are formed in the place of early flowers, and flowers that develop later are abnormal and contain shoot-inflorescence tissue (Weigel, Alvarez et al. 1992). We therefore predicted that inhibiting NFL expression would have similar effects on flower formation in *N. benthamiana*. A 421bp cDNA fragment of the NFL gene was PCR amplified from *N. benthamiana* cDNA (Appendix 7) and cloned into in the sense orientation into pGR107 and pTV00 to form pGR107.NFL and pTV.NFL respectively.

Three week-old *N. benthamiana* plants were infiltrated with water (mock), or with Agrobacterium containing pGR107 or pGR107.NFL to cause PVX or PVX.NFL infections, or pBINTRA6 and pTV00 or pTV.NFL to cause a TRV or TRV.NFL infections respectively. Six plants were used for each treatment. The arrangement and formation of leaves, flowers and branches was recorded after 12 weeks. In *N. benthamiana,* organs are formed at nodes around the main stem. Each node typically consists of a leaf, a primary branch, and a flower. The branches vary in size and complexity, and may give rise to secondary, or occasionally tertiary branches, before producing flowers. Floral organs such as petals, sigma and stamen are formed after a whorl of leaves known as sepals. On each plant, the number of primary, secondary and tertiary branches was recorded, as well as the number of correct and incorrectly formed flowers. Although there was structural variation between plants, there were no consistent changes between mock inoculated plants, and plants that were infected with PVX, PVX.NFL or TRV. All plants produced secondary branches. One TRV and one PVX.NFL infected plant each produced a single tertiary branch. All flowers on these plants were normal compared to mock inoculated plants. In contrast, plants infected with TRV.NFL had a more complex and branched structure. Each of these plants produced secondary and tertiary branches. Five of the six plants also produced quartenary branches. These additional branches were formed in place of flowers. Many of the flowers that were formed on TRV.NFL infected

plants were abnormal. Flower defects ranged from repeated whorls of sepals, to floral structures containing single or multiple inflorescence branches which themselves gave rise to more floral structures. These data are reminiscent of floral abnormalities in Arabidopsis *lfy* mutants, and are entirely consistent with an inhibition of NFL expression in the meristematic regions of *N. benthamiana*.

Taken together, these two experiments with GFP and NFL show that unlike other viral vectors, constructs based on TRV can inhibit gene expression in meristems.

Example 6 - TRV as an expression vector

The TRV derived virus vector pTV00 has been specifically designed to inhibit rather than direct protein synthesis. For certain purposes it may be advantageous to modify pTV00 to allow protein expression from this vector. If the multiple cloning site of pTV00 was immediately preceded by a sub-genomic promoter that was recognised by the replicase protein of TRV RNA 1, then proteins would be translated from a sub-genomic RNA.

For example, total RNA could be prepared from PEBV infected *N. benthamiana* plants as previously described (Devic, Jaegle et al. 1989). cDNA corresponding to the coat protein sub-genomic promoter of PEBV could be prepared and then PCR amplified from this total RNA using Superscript Reverse Transcriptase (Gibco) followed by Pfu polymerase (Promega) with the primers ggatccgcacacaaggtta and gggcgcgccctcgttaac. This PEBV sub-genomic promoter cDNA fragment (Appendix 2) could be cloned into pTV00 that was previously digested with Spe 1 and blunted with T4 DNA polymerase, to form pTV1.0. Open reading frames cloned in sense and in frame in the remainder of the multiple cloning site would then be expressed. For example, GFP cDNA sequence (Chalfie, Tu et al. 1994), could be inserted into the BamHi site, to achieve GFP expression in any host plant such as *N. benthamiana*.

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Inasmuch as they may be required by the person skilled in the art to practice the present invention, all citations are specifically included herein by cross-reference.

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25

SEQUENCE LISTING

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(viii) PATENT AGENT INFORMATION:
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(B) REFERENCE NUMBER: 04900-36

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GGCACTCAAC TTTATAAACC 20

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CTTCAGTTTT CTGTCAAACC 20

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CCCCTCGAGA GATGTCAAAT C 21

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CCCCTCGAGG CACTTTCATC TGG 23

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CAGTCTAGAT GGCTTCCTCA GTTCTTTCC 29

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CAGGGATCCC ACTTGACGCA CGTTGTC 27

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AACATCCTCG GCCCACAAGT T 21

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GAGCTCTTAG AGTTCGTCAT G 21

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TGGACCCAGA GGCTTTCTC 19

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CTTCTTGTGA GAGAGCGTCA 20

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GGGGGGATCC GGGCGTAATA ACGCTTACG 29

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GGGGGGATCC ATAAAACATT TCAATCCTTT G 31

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

TTAGCACCAG CTATCTGAGC GC 22

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GTTCCAACCA GACAAACGTA TGG 23

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CGTATCTTTG CAATAACAGG TAATAATCCT CTCTCTTGAT ATT 43

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

TTAAATTGTC CAAGATCAAC CTGTTTAACA CAAGTCAACG TC 42

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

TCGCACAAAA CCAAGGTGAT AG 22

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

GGATTATTAC CTGTTATTGC AAAGATACGT CTG 33

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

TGTTAAACAG GTTGATCTTG GACAATTTAA GTGC 34

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

CGTCTTGCTC AAGGCCGCGA T 21

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

CGACAATCTA CCGATTGTAT G 21

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

CTGCGATCCC AGGGAAAACA G 21

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

AAATGCATAA AGTTTTGCCA T 21

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

TGGTTGTAAC CATGGCAGAG CA 22

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

GAATTCAGAT CTA 13

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

ACATGTAGAT CTG 13

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

CATGAAGGCC TTGACAGGAT ATATTGGCGG GTAAACTAAG TCGCTGTATG TGTTTGTTTG 60

AGATCT 66

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

CATGAGATCT CAAACAAACA CATACAGCGA CTTAGTTTAC CCGCCAATAT ATCCTGTCAA 60

GGCCTT 66

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 73 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

TCCACACATT ATACGAGCCG ATGATTAATT GTCAACAGAT CTTGGCAGGA TATATTGTGG 60

TGTAAACGTT AAC 73

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 73 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

GGTAACGTTT ACACCACAAT ATATCCTGCC AAGATCTGTT GACAATTAAT CATCGGCTCG 60

TATAATGTGT GGA 73

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

CGTCAAGTGC AGTGCATCAG T 21

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

GACAATAGGG TAAGTTGTCC TAATATGAAA 30

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

CATTGCCTCC AAGCCTGACG GA 22

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

ACCACAGGGA TAACTGGCTT GT 22

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

CCGACATCGA AGGATCAAAA A 21

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

CAGCCAAGCG TCATAGCGAC GTTG 24

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5592 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

TTTTTATCCC CGGAAGCCTG TGGATAGAGG GTAGTTATCC ACGTGAAACC GCTAATGCCC 60

CGCAAAGCCT TGATTCACGG GGCTTTCCGG CCCGCTCCAA AAACTATCCA CGTGAAATCG 120

CTAATCAGGG TACGTGAAAT CGCTAATCGG AGTACGTGAA ATCGCTAATA AGGTCACGTG 180

AAATCGCTAA TCAAAAAGGC ACGTGAGAAC GCTAATAGCC CTTTCAGATC AACAGCTTGC 240

AAACACCCCT CGCTCCGGCA AGTAGTTACA GCAAGTAGTA TGTTCAATTA GCTTTTCAAT 300

TATGAATATA TATATCAATT ATTGGTCGCC CTTGGCTTGT GGACAATGCG CTACGCGCAC 360

CGGCTCCGCC CGTGGACAAC CGCAAGCGGT TGCCCACCGT CGAGCGCCAG CGCCTTTGCC 420

CACAACCCGG CGGCCGGCCG CAACAGATCG TTTTATAAAT TTTTTTTTTT GAAAAAGAAA 480

AAGCCCGAAA GGCGGCAACC TCTCGGGCTT CTGGATTTCC GATCCCCGGA ATTAGATCTC 540

AAACAAACAC ATACAGCGAC TTAGTTTACC CGCCAATATA TCCTGTCAAG GCCTTCATGT 600

TCTTTCCTGC GTTATCCCCT GATTCTGTGG ATAACCGTAT TACCGCCTTT GAGTGAGCTG 660

ATACCGCTCG CCGCAGCCGA ACGACCGAGC GCAGCGAGTC AGTGAGCGAG GAAGCGGAAG 720

AGCGCCCAAT ACGCAAACCG CCTCTCCCCG CGCGTTGGCC GATTCATTAA TGCAGCTGGC 780

ACGACAGGTT TCCCGACTGG AAAGCGGGCA GTGAGCGCAA CGCAATTAAT GTGAGTTAGC 840

TCACTCATTA GGCACCCCAG GCTTTACACT TTATGCTTCC GGCTCGTATG TTGTGTGGAA 900

TTGTGAGCGG ATAACAATTT CACACAGGAA ACAGCTATGA CCATGATTAC GCCAAGCTCG 960

GAATTAACCC TCACTAAAGG GAACAAAAGC TGGAGCTCCA CCGCGGTGGA GCTCCACCGG 1020

GGAAACCTCC TCGGGATTCC ATTGCCCAGC TATCTGTCAC TTTATTGAGA AGATAGTGGA 1080

AAAGGAAGGT GGCTCCTACA AATGCCATCA TTGCGATAAA GGAAAGGCCA TCGTTGAAGA 1140

TGCCTCTGCC GACAGTGGTC CCAAAGATGG ACCCCCACCC ACGAGGAGCA TCGTGGAAAA 1200

AGAAGACGTT CCAACCACGT CTTCAAAGCA AGTGGATTGA TGTGATATCT CCACTGACGT 1260

AAGGGATGAC GCACAATCCC ACTATCCTTC GCAAGACCCT TCCTCTATAT AAGGAAGTTC 1320

ATTTCATTTG GAGAGGCTAG ATAAAACATT GCACCTATGG TGTTGCCCTG GCTGGGGTAT 1380

GTCAGTGATC GCAGTAGAAT GTACTAATTG ACAAGTTGGA GAATACGGTA GAACGTCCTT 1440

ATCCAACACA GCCTTTATCC CTCTCCCTGA CGAGGTTTTT GTCAGTGTAA TATTTCTTTT 1500

TGAACTATCC AGCTTAGTAC CGTACGGGAA AGTGACTGGT GTGCTTATCT TTGAAATGTT 1560

ACTTTGGGTT TCGGTTCTTT AGGTTAGTAA GAAAGCACTT GTCTTCTCAT ACAAAGGAAA 1620

ACCTGACGTA TCGCTTACGA AAGTAGCAAT GAAAGAAAGG TGGTGGTTTT AATCGTACCG 1680

CAAAAAACGA TGGGGTCGTT TTAATTAACT TCTCCTACAA GCGTCTAAAC GGACGTTGGG 1740

GTTTTGCTAG TTTCTTTAGA GAAAACTAGC TAAGTCTTTA ATGTTATCAT TAGAGATGGC 1800

ATAAATATAA TACTTGTGTC TGCTGATAAG ATCATTTTAA TTTGGACGAT TAGACTTGTT 1860

GAACTACAGG TTACTGAATC ACTTGCGCTA ATCAACATGG GAGATATGTA CGATGAATCA 1920

TTTGACAAGT CGGGCGGTCC TGCTGACTTG ATGGACGATT CTTGGGTGGA ATCAGTTTCG 1980

TGGAAAGATT TGTTGAAGAA GTTACACAGC ATAAAATTTG CACTACAGTC TGGTAGAGAT 2040

GAGATCACTG GGTTACTAGC GGCACTGAAT AGACAGTGTC CTTATTCACC ATATGAGCAG 2100

TTTCCAGATA AGAAGGTGTA TTTCCTTTTA GACTCACGGG CTAACAGTGC TCTTGGTGTG 2160

ATTCAGAACG CTTCAGCGTT CAAGAGACGA GCTGATGAGA AGAATGCAGT GGCGGGTGTT 2220

ACAAATATTC CTGCGAATCC AAACACAACG GTTACGACGA ACCAAGGGAG TACTACTACT 2280

ACCAAGGCGA ACACTGGCTC GACTTTGGAA GAAGACTTGT ACACTTATTA CAAATTCGAT 2340

GATGCCTCTA CAGCTTTCCA CAAATCTCTA ACTTCGTTAG AGAACATGGA GTTGAAGAGT 2400

TATTACCGAA GGAACTTTGA GAAAGTATTC GGGATTAAGT TTGGTGGAGC AGCTGCTAGT 2460

TCATCTGCAC CGCCTCCAGC GAGTGGAGGT CCGATACGTC CTAATCCCTA GGGATTTAAG 2520

GACGTGAACT CTGTTGAGAT CCTAGAACTA GTGGATCCCC CGGGCTGCAG GAATTCGATA 2580

TCAAGCTTAT CGATACCGTC GACCTCGAGG GGGGGCCCGG TACCCAATTC GCCCTATAGT 2640

GAGTCGTATT ACAATTCACT GGCCGTCGTT TTACAACGTC GTGACTGGGA AAACCCTGGC 2700

GTTACCCAAC TTAATCGCCT TGCAGCACAT CCCCCTTTCG CCAGCTGGCG TAATAGCGAA 2760

GAGGCCCGCA CCGATCGCCC TTCCCAACAG TTGCGAAGAC ATTAAACTAC GGTTCTTTAA 2820

GTAGATCCGT GCCTGAAGTT TTAGGTTCAA TTTAAACCTA CGAGATTGAC ATTCTCGACT 2880

GATCTTGATT GATCGGTAAG TCTTTTGTAA TTTAATTTTC TTTTTGATTT TATTTTAAAT 2940

TGTTATCTGT TTCTGTGTAT AGACTGTTTG AGATCGGCGT TTGGCCGACT CATTGTCTTA 3000

CCATAGGGGA ACGGACTTTG TTTGTGTTGT TATTTTATTT GTATTTTATT AAAATTCTCA 3060

ACGATCTGAA AAAGCCTCGC GGCTAAGAGA TTGTTGGGGG GTGAGTAAGT ACTTTTAAAG 3120

TGATGATGGT TACAAAGGCA AAAGGGGTAA AACCCCTCGC CTACGTAAGC GTTATTACGC 3180

CCTCGAGTAT CGAATTGCTG CAGGCATGCA AGCGATCCCC GATCGTTCAA ACATTTGGCA 3240

ATAAAGTTTC TTAAGATTGA ATCCTGTTGC CGGTCTTGCG ATGATTATCA TATAATTTCT 3300

GTTGAATTAC GTTAAGCATG TAATAATTAA CATGTAATGC ATGACGTTAT TTATGAGATG 3360

GGTTTTTATG ATTAGAGTCC CGCAATTATA CATTTAATAC GCGATAGAAA ACAAAATATA 3420

GCGCGCAAAC TAGGATAAAT TATCGCGCGC GGTGTCATCT ATGTTACTAG ATCGGGAATT 3480

GCCAAGCTGC TTGGCACTGG CCGTCGTTTT ACAACGTCGT GACTGGGAAA ACCCTGGCGT 3540

TACCCAACTT AATCGCCTTG CAGCACATCC CCCTTTCGCC AGCTGGCGTA ATAGCGAAGA 3600

GGCCCGCACC GATCGCCCTT CCCAACAGTT GCGCAGCCTG AATGGCGAAT GGCGCGAAAT 3660

TGTAAACGTT AATGTTAACG TTACACCACA ATATATCCTG CCAAGATCTC ATGTGAGCAA 3720

AAGGCCAGCA AAAGGCCAGG AACCGTAAAA AGGCCGCGTT GCTGGCGTTT TTCCATAGGC 3780

TCCGCCCCCC TGACGAGCAT CACAAAAATC GACGCTCAAG TCAGAGGTGG CGAAACCCGA 3840

CAGGACTATA AAGATACCAG GCGTTTCCCC CTGGAAGCTC CCTCGTGCGC TCTCCTGTTC 3900

CGACCCTGCC GCTTACCGGA TACCTGTCCG CCTTTCTCCC TTCGGGAAGC GTGGCGCTTT 3960

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CTCATAGCTC ACGCTGTAGG TATCTCAGTT CGGTGTAGGT CGTTCGCTCC AAGCTGGGCT    4020
GTGTGCACGA ACCCCCCGTT CAGCCCGACC GCTGCGCCTT ATCCGGTAAC TATCGTCTTG    4080
AGTCCAACCC GGTAAGACAC GACTTATCGC CACTGGCAGC AGCCACTGGT AACAGGATTA    4140
GCAGAGCGAG GTATGTAGGC GGTGCTACAG AGTTCTTGAA GTGGTGGCCT AACTACGGCT    4200
ACACTAGAAG GACAGTATTT GGTATCTGCG CTCTGCTGAA GCCAGTTACC TTCGGAAAAA    4260
GAGTTGGTAG CTCTTGATCC GGCAAACAAA CCACCGCTGG TAGCGGTGGT TTTTTTGTTT    4320
GCAAGCAGCA GATTACGCGC AGAAAAAAAG GATCTCAAGA AGATCCTTTG ATCTTTTCTA    4380
CGGGGTCTGA CGCTCAGTGG AACGAAAACT CACGTTAAGG GATTTTGGTC ATGGTTACAA    4440
CCAATTAACC AATTCTGATT AGAAAAACTC ATCGAGCATC AAATGAAACT GCAATTTATT    4500
CATATCAGGA TTATCAATAC CATATTTTTG AAAAAGCCGT TTCTGTAATG AAGGAGAAAA    4560
CTCACCGAGG CAGTTCCATA GGATGGCAAG ATCCTGGTAT CGGTCTGCGA TTCCGACTCG    4620
TCCAACATCA ATACAACCTA TTAATTTCCC CTCGTCAAAA ATAAGGTTAT CAAGTGAGAA    4680
ATCACCATGA GTGACGACTG AATCCGGTGA GAATGGCAAA AGTTTATGCA TTTCTTTCCA    4740
GACTTGTTCA ACAGGCCAGC CATTACGCTC GTCATCAAAA TCACTCGCAT CAACCAAACC    4800
GTTATTCATT CGTGATTGCG CCTGAGCGAG ACGAAATACG CGATCGCTGT TAAAAGGACA    4860
ATTACAAACA GGAATCGAAT GCAACCGGCG CAGGAACACT GCCAGCGCAT CAACAATATT    4920
TTCACCTGAA TCAGGATATT CTTCTAATAC CTGGAATGCT GTTTTCCCTG GGATCGCAGT    4980
GGTGAGTAAC CATGCATCAT CAGGAGTACG GATAAAATGC TTGATGGTCG GAAGAGGCAT    5040
AAATTCCGTC AGCCAGTTTA GTCTGACCAT CTCATCTGTA ACATCATTGG CAACGCTACC    5100
TTTGCCATGT TTCAGAAACA ACTCTGGCGC ATCGGGCTTC CCATACAATC CATAGATTGT    5160
CGCACCTGAT TGCCCGACAT TATCGCGAGC CCATTTATAC CCATATAAAT CAGCATCCAT    5220
GTTGGAATTT AATCGCGGCC TGGAGCAAGA CGTTTCCCGT TGAATATGGC TCATAACACC    5280
CCTTGTATTA CTGTTTATGT AAGCAGACAG TTTTATTGTT CATGATGATA TATTTTTATC    5340
TTGTGCAATG TAACATCAGA GATTTTGAGA CACAACGTGG CTTTGTTGAA TAAATCGAAC    5400
TTTTGCTGAG TTGAAGGATC AGATCACGCA TCTTCCCGAC AACGCAGACC GTTCCGTGGC    5460
AAAGCAAAAG TTCAAAATCA CCAACTGGTC CACCTACAAC AAAGCTCTCA TCAACCGTGC    5520
TCCCTCACTT TCTGGCTGGA TGATGGGGCG ATTCAGGCGA TCCCCATCCA ACAGCCCGCC    5580
GTCGAGCGGG CT                                                        5592
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(2) INFORMATION FOR SEQ ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 249 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

GGATCCGCAC ACAAGGTTAA AAACGCTGTA GTAATACATG CGCAAGAACA GGCTGAGCAT 60

CTTGTTCTGG GGTTTCACAC TATCTTTAGA GAAAGTGTTA AGTTAATTAA GTTATCTTAA 120

TTAAGAGCAT AATTATACTG ATTTGTCTCT CGTTGATAGA GTCTATCATT CTGTTCTAAA 180

AATTTGACAA CTCGGTTTGC TGACCTACTG GTTACTGTAT CACTTACCCG AGTTAACGAG 240

GGCGCGCCC 249

(2) INFORMATION FOR SEQ ID NO: 39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 409 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

TCTGTCAAAC CATATATGGA CATTTATCAC AGGAACTCCC ACTAGCTTCT CCAACTTTTG 60

GAAATATGGG ATCTCTTTCC AGTCTTCAGG CAAAAGAAGC TTCAAGATAT CCACTGGAGT 120

GGCAAACACA AAAGCATCTC CTTTAATTGT ACTGCCATTA TTCTGTATAA AACATTTGAC 180

ACTTCCATCC TCATTCAGCT CGATCTTTTT TATTCGTGAG TTTAGTCTGA CTTGGCCACC 240

TTTTGACTCA ATATGTTCCA CAATCGGCAT GCAAAGTCTC TCAGGAGGGT TACCATCTAA 300

AAAGGCCATT TTTGAACCAT GTTTCTCCTG AAGAAATCTG TTCAAAGCAA TCAAGATGCA 360

CTGCATCGAA AGCTCGTCAG GGTTTATAAA GTTGAGTGCC TCTAGACTG 409

(2) INFORMATION FOR SEQ ID NO: 40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1773 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

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CTCGAGAGAT GTCAAATCTG TGAGATTCAT TTACTGAAGA GAGTAAGGTT AAGTTTGGAT    60
TGCAATGAGA TTTATACACA AATAATAATG CTTACAAGCA AATCATCTTT AAGTTTTGTC   120
CTCTTCTCAT GATGATGATA CTGTTGCCTC CGACAACTTT CTTGGTCCAG ACGCAGCCAG   180
TAGCTCGTAA TCCTGAACAA TAGACTGAGA GCAGAATTTG CCAGAGAGGA CAGCGCCTTC   240
CATGGAAGCT AAGTACTTCT GTTTTGTGTA ATCTCCAGCT AAGTAGAATC CTTCAATAGG   300
TGATCTTTGT AGAGGACGAC ATGGTTCACA GTTTGGGATG GTCTTGTACA CAGATCTTGG   360
AGTCTTAACG ACATGGTACT TCAGAATTTT AGCTTTGCTT TGGTCAGCTG AGATTTCATC   420
AGGGAAGAGT TTCTCAAGTT CTTTCATTGT TGCATCTATG ATGTCAGAAT CAGTCCGTGA   480
TATCCATTCC TCTGCTGGTG CAAATACTAG CTCCAGCATT GACCGGTTAG GATCGTAATA   540
TTCCTTACAA GTTAAGGACA TGTCGGCATA CACGCTCAGA AGGTTACTTC TGCTAAAGAG   600
TAGGTGATCA TATGTGTTCT TCAGTTTTCG ATCAAACCAT ATATGAACAT TAATAACTGG   660
TACTCCAACT AATTTATCCA ATTTCTTGAA GTACGGTATT TCTTTCCAGG GATCTGGTAA   720
AAGGAGCTTC AGGATATCGA CTGGAGCGGC AAACACATAA GCGTCTCCTT CGACAGTGCT   780
TCCATTAGTG AGTAAGAAAC TCTTAACCGT GCCATCGTCA TTGAGCTCAA TTTTCTTTAT   840
CCTAGAATTA AGTTGCACTT CCCCACCTAG TGATCGAATA TGATCCACTA CTGGCATACA   900
AAGCCTTTCC GGAGGATTAC CATCCAAGAA TGCCATCTTG GAACCATGTT TTTCCTGAAG   960
AAACCGGTTC AAAGCTATCA AAATGCATTG CATTGACAGT TCATCAGGGT TTATAAAGTT  1020
TAGCGCCTTT GACATGGCAA TAAACACCTC GTCGGTCACG CGCTCAGGTA CTCCCTGCTT  1080
TTCCATCCAT TCTTTGACTG ATAAACCATC TTGGGCCTCA ACATAAGCCT GACCGCCGAC  1140
CATGGCTGGC AAAAGTCCAA TAGCAAACTT TATTTTCTCT GGCCATGTCA GCATCTCGTT  1200
GTTCCGCAAA ATAGCCCAAA TACCATTTAA GGGTGCTGGT AGGACATCTG GGAAGTCAAA  1260
TCTACTAAAT TCTCCAGGTT TACTTGGCAT AGCAAAAATC ATGGAGTGTT CCTTCCACTG  1320
CAACCGATCA TTGATCCCAA GTTCTCCAAA TAAATTCTGC ACATTCGGAT AAGCACCGAA  1380
GAAAATATGT AAACCAGTCT CATACCAGTC CCCATCTTCA TCCTTCCATG CAGCTATCTT  1440
TCCACCAAGA ACATCTCTTG CTTCAAGCAA CAGAGGTTTG TGGCCTGCAT CAGCCAGGTA  1500
CTTTGCAGTT GACAATCCAG CCAATCCAGC ACCAGCAATT ACAACTTTCA AAGGCTTAGC  1560
AGGACGAGGA GCACTACGGA AGGATGCAGA TAAACTAGCA GCTTCCAAGA AATTGACAGT  1620
GTTCTCTAGC TCTGGCCTTG GAATATCCAC ACAAACTACC TGCAAAGGAC CAGCAGTACT  1680
CCTCCTCCTT GTTCTTGTCT TAAGCGCTTG AGAAGTGGGA ACCCTAAAGC TATGTCCCAT  1740
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(2) INFORMATION FOR SEQ ID NO: 41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 499 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

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GCAGGTGTGG CCACCAATTA ACAAGAAGAA GTACGAGACT CTCTCATACC TTCCTGATTT 240

GAGCCAGGAG CAATTGCTTA GTGAAGTTGA GTACCTTTTG AAAAATGGAT GGGTTCCTTG 300

CTTGGAATTC GAGACTGAGC ACGGATTTGT CTACCGTGAA AACAACAAGT CACCAGGATA 360

CTATGATGGC AGATACTGGA CCATGTGGAA GCTACCTATT TCGGATGCAC TGATGCCACC 420

CAAGTGTTGG CTGAGGTGGA AGAGGCGAAG AAGGCATACC CACAGGCCTG GATCCGTATC 480

ATTGGATTCG ACAACGTGG 499

(2) INFORMATION FOR SEQ ID NO: 42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 321 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

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GATGGAAGCG TTCAACTAGC AGACCATTAT CAACAAAATA CTCCAATTGG CGATGGCCCT 180

GTCCTTTTAC CAGACAACCA TTACCTGTCC ACACAATCTG CCCTTTCGAA AGATCCCAAC 240

GAAAAGAGAG ACCACATGGT CCTTCTTGAG TTTGTAACAG CTGCTGGGAT TACACATGGC 300

ATGGATGAAC TATACAAATA A 321

(2) INFORMATION FOR SEQ ID NO: 43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 421 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43:

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CGCCGCAGCC TCTATCGGCG GCCTATTCCA TTAGGACAAG GGAGTTAGGA GGGCTAGAGG 180

AGTTGTTTCA AGCTTACGGT ATACGTTATT ACACTGCTGC TAAAATAGCG GAGCTAGGTT 240

TTACGGTGAA TACTCTATTG GACATGAAAG ATGAGGAACT TGATGATATG ATGAATAGCC 300

TTTCACAGAT TTTCAGATGG GAACTCCTCG TCGGAGAAAG GTACGGTATC AAAGCTGCAA 360

TCAGGGCGGA ACGGCGGAGG CTTGAGGAGG AAGAACTACG GCGGCGCAGC CACCTTCTGT 420

C 421

(2) INFORMATION FOR SEQ ID NO: 44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8976 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44:

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GCTATCATTC AAGATGCCTC TGCCGACAGT GGTCCCAAAG ATGGACCCCC ACCCACGAGG 240

AGCATCGTGG AAAAAGAAGA CGTCCCAACC ACGTCTTCAA AGCAAGTGGA TTGATGTGAC 300

ATCTCCACTG ACGTAAGGGA TGACGCACAA TCCCACTATC CTTCGCAAGA CCCTTCTTCT 360

ATATAAGGAA GTTCATTTCA TTTGGAGAGG ACAGCCCAAG CTTTCTAGAG GATCCATAAA 420

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GAACGTACTT ATCACCTACA GTTTTATTTT GTTTTTCTTT TTGGTTTAAT CTATCCAGCT 540

TAGTACCGAG TGGGGGAAAG TGACTGGTGT GCCTAAAACC TTTTCTTTGA TACTTTGTAA 600

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ACGAGATGTC TGCTGAGCAG AGGAGTCATT TCTTTGACTT GATGCTGACT AAACCTGATT 720

GTGAGATCGG GCAAATGATG CAAAGAGTTG TTGTTGATAA AGTCGATGAC ATGATTAGAG 780

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AGTTGATGGA AATTTATCCT GAATTCAATA TCGTGTTTAA AGACGACAAA AACATGGTTC 900

ATGGGTTTGC GGCTGCTGAG CGAAAACTAC AAGCTTTATT GCTTTTAGAT AGAGTTCCTG 960

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GACAGATATT TCTTACTGCT ATTGGTGATC AAGCTAGAAG TGGTAAGAGA CAGATGTCGG 1140

AGAATGAGCT GTGGATGTAT GACCAATTTC GTGAAAATAT TGCTGCGCCT AACGCGGTTA 1200

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GCGCGCAGTA TGCGATAGCT CTTCACAGCC TGTATGACTT CAAGTTGAAA GACTTGATGG 1320

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GGAAGATCTA TTTCGGTTTT GAGAAAGATC CTTCCTTTTC TTACATTCAT GACTGGGAAG 1500

AGTACAAGAA GTATCTACTG GGGAAGCCAG TGAGTTACCA AGGGAATGTG TTCTACTTCG 1560

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ACCAGCAGCT GACCATAAGC AATGTTAAAT CATACTTGAG TTCAAATAAT TGGGTCTTAT 1860

TCATAAACGG GGCGGCCGTG AAGAACAAGC AAAGTGTAGA TTCTCGAGAT TTACAGTTGT 1920

TGGCTCAAAC TTTGCTAGTG AAGGAACAAG TGGCGAGACC TGTCATGAGG GAGTTGCGTG 1980

AAGCAATTCT GACTGAGACG AAACCTATCA CGTCATTGAC TGATGTGCTG GGTTTAATAT 2040

CAAGAAAACT GTGGAAGCAG TTTGCTAACA AGATCGCAGT CGGCGGATTC GTTGGCATGG 2100

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CGGTGAGAAC TTCTCCACCA TCGGTGGTCG GTTCATCTCG AAGCGGACTG GGTCTGTTGT 2580

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(2) INFORMATION FOR SEQ ID NO: 45:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 45:

GGATCCGCAC ACAAGGTTA 19

(2) INFORMATION FOR SEQ ID NO: 46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46:

GGGCGCGCCC TCGTTAAC 18

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS.

1 A nucleic acid vector which comprises:
(a) a transfer nucleotide sequence comprising (i) a plant active
5 promoter, operably linked to (ii) a recombinant tobacco rattle virus (TRV) cDNA which includes at least cis acting elements permitting replication of said cDNA; a subgenomic promoter operably linked to a sequence encoding a TRV coat protein; and a heterologous nucleotide sequence which is foreign to said virus;
10 (b) border sequences which permit the transfer of the transfer nucleotide sequence into a plant genome.

2 A vector as claimed in claim 1 wherein the plant promoter is the Cauliflower Mosaic Virus 35S gene promoter.
15

3 A vector as claimed in claim 1 wherein the border sequences are derived from *Agrobacterium tumefaciens*.

4 A vector as claimed in claim 1 wherein the cis acting elements
20 require a replicase not encoded by the vector for replication.

5 A vector as claimed in claim 1 wherein the recombinant TRV cDNA is derived from TRV RNA2.

25 6 A vector as claimed in claim 5 wherein the recombinant TRV cDNA encodes the TRV coat protein.

7 A vector as claimed in claim 6 wherein non-essential ORFs are deleted from the recombinant TRV cDNA.
30

8 A vector as claimed in claim 7 wherein the 37K and 32.8K ORFs are deleted from the recombinant TRV cDNA.

9 A vector as claimed in claim 1 wherein the heterologous
35 nucleotide sequence is a multiple cloning site.

10 A vector as claimed in claim 1 wherein the heterologous nucleotide sequence does not include, and is not operably linked to, a subgenomic promoter.
40

11 A vector as claimed in claim 1 wherein the heterologous nucleotide sequence is a targeting sequence which corresponds to a

sequence in a target gene

12 A vector as claimed in claim 11 wherein the target gene is a plant nuclear gene.

5

13 A vector as claimed in claim 11 wherein the targeting sequence which corresponds to a conserved sequence of a target gene.

14 A vector as claimed in claim 1 which is an expression vector.

10

15 A vector as claimed in claim 1 which is a VIGS vector.

16 A vector which is pTV00 as described herein.

15 17 A vector which is derived from pTV00 as described herein and has the characteristics thereof.

18 A process for producing a vector as claimed in claim 11, which process comprises the step of cloning a heterologous nucleotide
20 sequence which is a targeting sequence into a multiple cloning site in the vector.

19 A method of silencing a target gene in a plant tissue using VIGS, which method comprises the step of introducing a vector claimed
25 in claim 11 into the plant tissue.

20 A method as claimed in claim 19 for achieving substantially confluent VIGS of the target gene across a leaf.

30 21 A method as claimed in claim 19 for achieving VIGS of a target in meristematic tissue.

22 A method as claimed in claim 19 wherein the vector is introduced by *Agrobacterium*-mediated T-DNA transfer.

35

23 A method as claimed in claim 19 wherein the recombinant TRV cDNA in the vector is derived from TRV RNA2, and proteins encoded by TRV RNA1 are also introduced into the plant tissue.

40 24 A method as claimed in claim 23 wherein proteins encoded by TRV RNA1 are introduced by rub-inoculating the plant with purified RNA 1.

25 A method as claimed in claim 19 wherein the plant tissue is *Arabidopsis thaliana*.

5

26 A process which includes the step introducing a vector as claimed in claim 4 into plant tissue, and optionally further includes the step of introducing one or more proteins encoded by TRV RNA1 into the plant tissue.

10

27 A method which includes causing or allowing transcription from a vector as claimed in claim 1 in plant tissue such as to produce a cytoplasmically-replicating RNA.

15

28 A method of characterising a target gene, which method comprises the steps of:
(a) silencing the target gene in a part, or at a certain development stage, of a plant using a method as claimed in claim 19,
(b) observing the phenotype of the part of the plant in which or when the target gene has been silenced.

20

29 A method as claimed in claim 28 wherein the target gene is an essential gene.

25

30 A method of altering the phenotype of a plant comprising the step of silencing a target gene in the plant using a method as claimed in claim 19.

31 A virus or viral particle including encapsulating an RNA transcript from a vector as claimed in claim 1.

30

32 A kit comprising a vector as claimed in claim 5 plus a source of TRV RNA1 polypeptide, or a vector encoding said polypeptide.

35

33 A host cell including a vector as claimed in claim 1.

34 Plant tissue including or transiently transformed by a vector as claimed in claim 1.

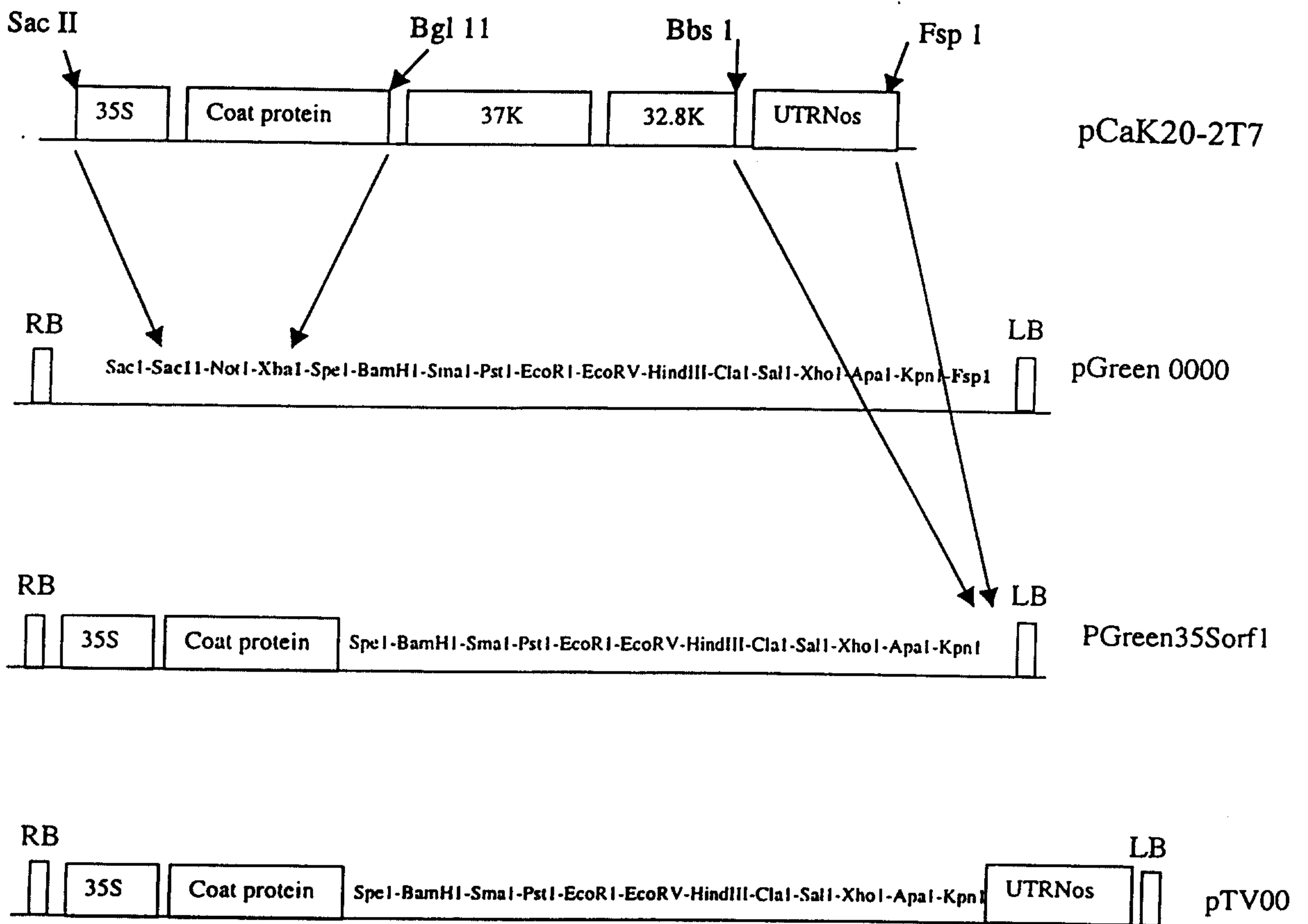

FIGURE 1

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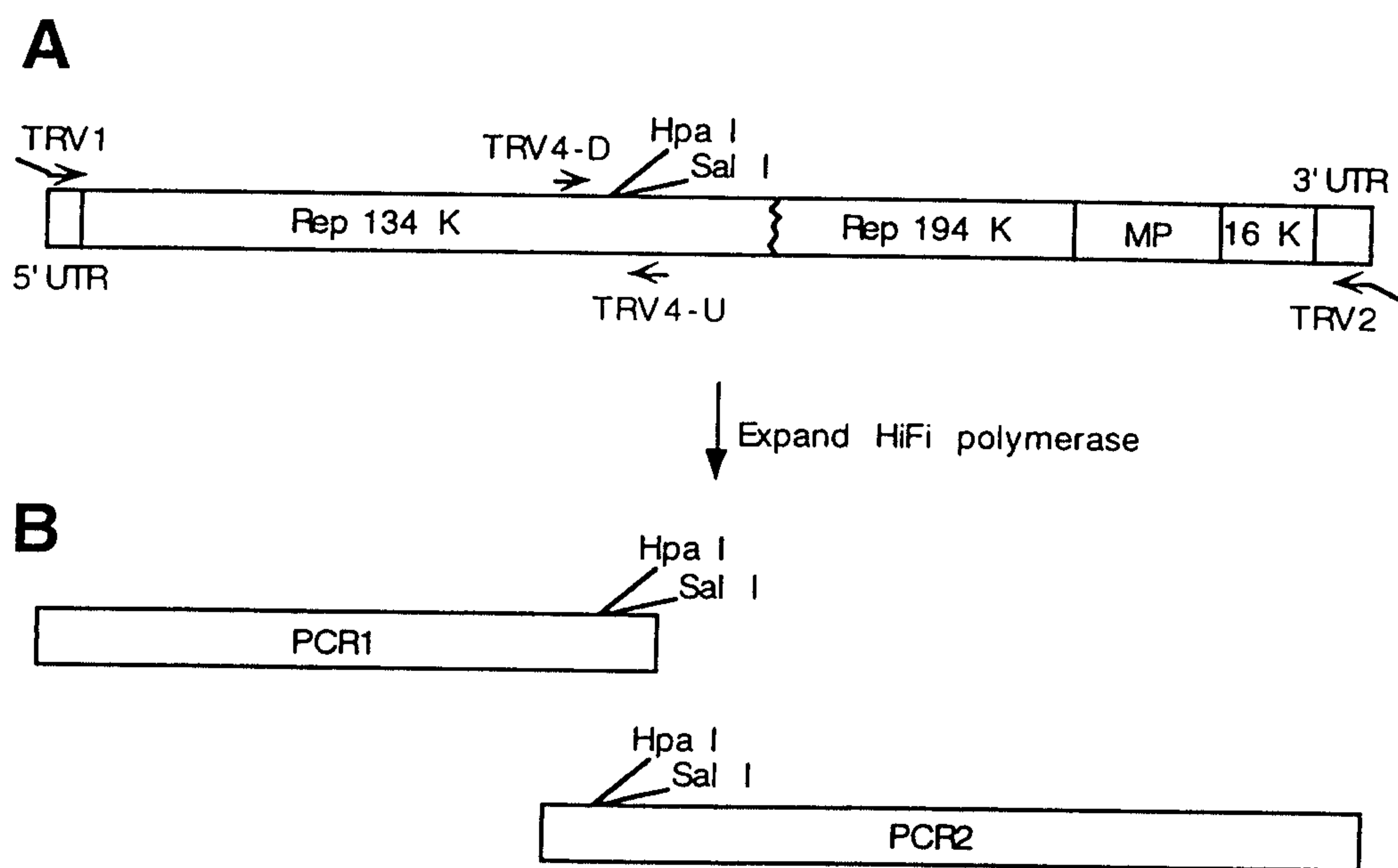

FIGURE 2

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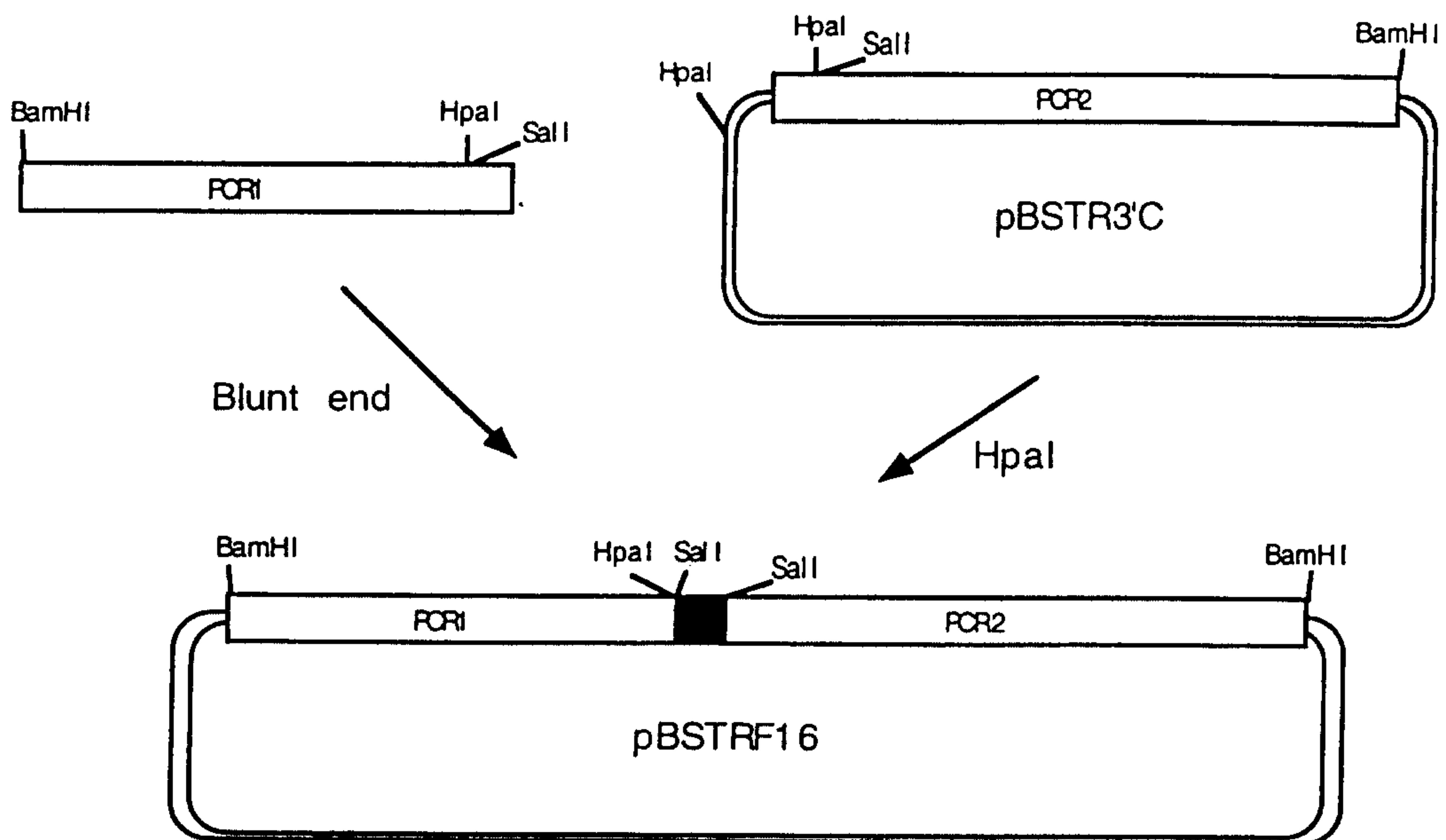

FIGURE 3

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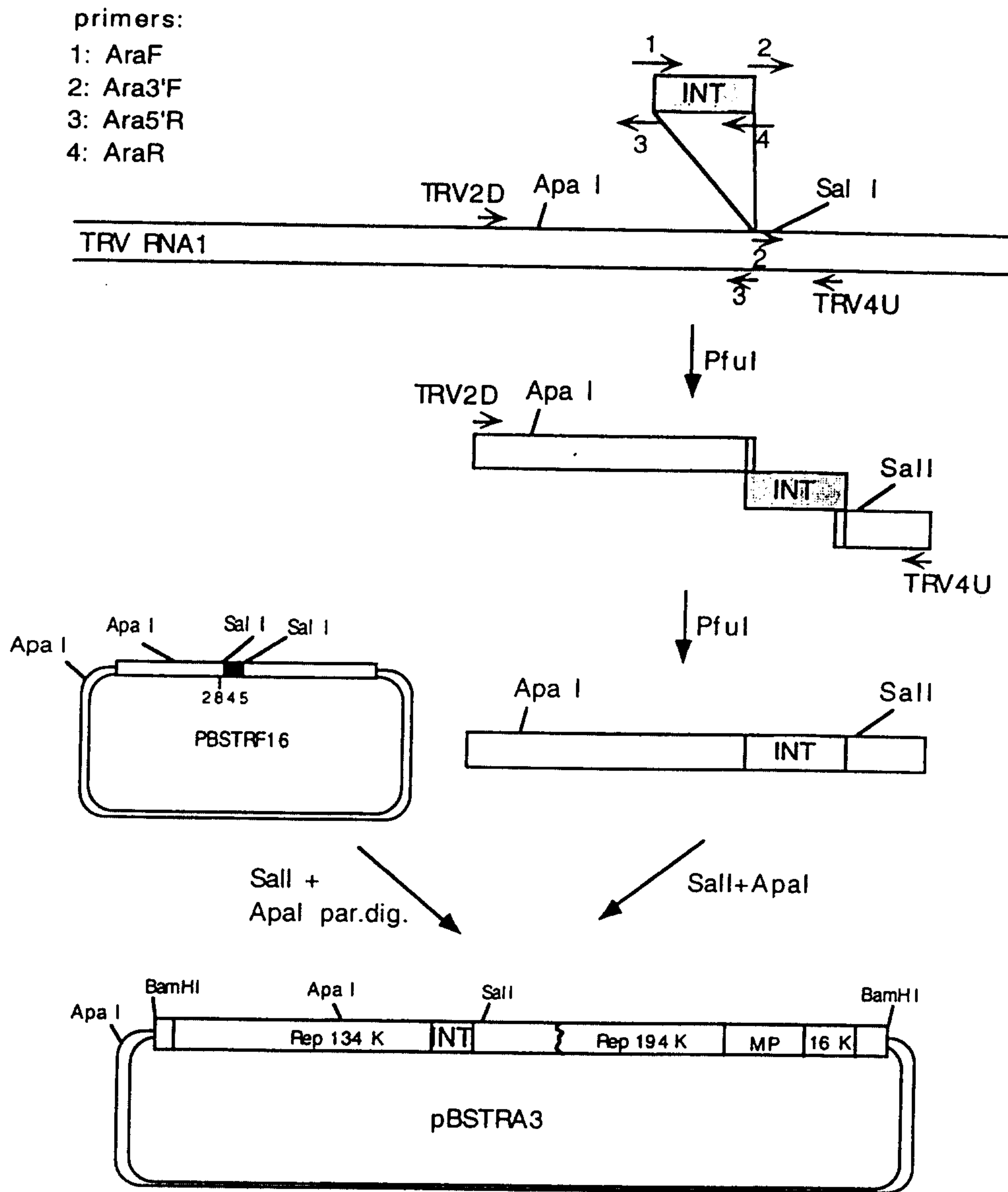

FIGURE 4

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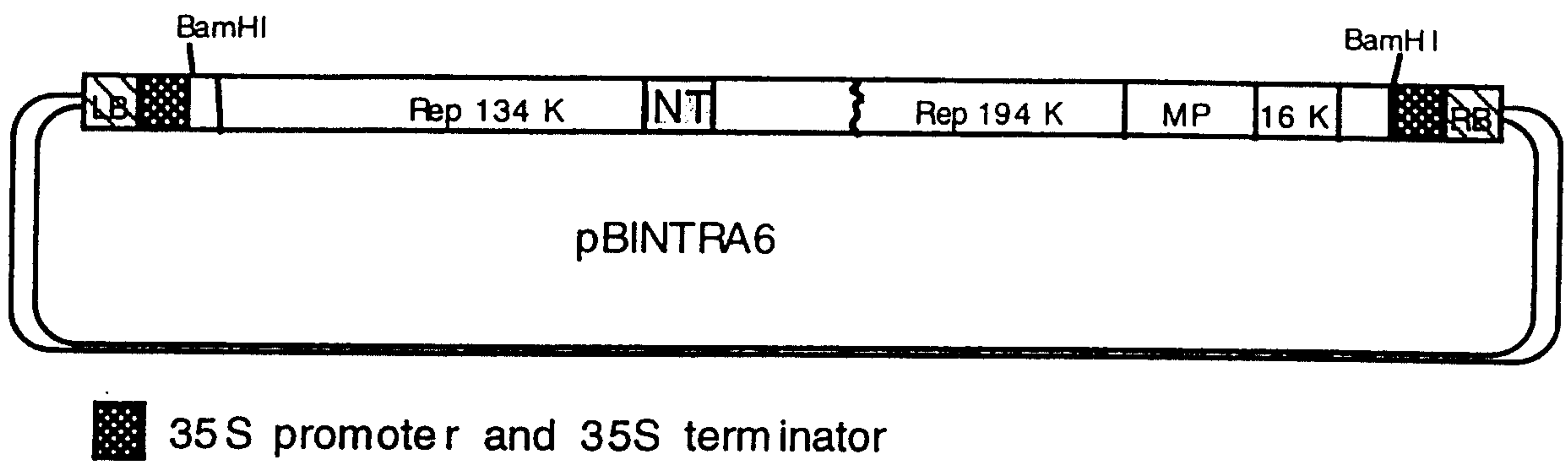

FIGURE 5

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pTV00 Construction: