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(54) Title: VIRAL REPLICON

(57) Abstract

The present invention provides viral replicons comprising RNA encoding i) at least one anti-sense gene; and ii) flanking viral sequence or sequences such that in the absence of a virus or viral components the replicon is non-coding, and in the presence of a virus or viral components the replicon is activated by viral replicase converting the non-coding negative-sense RNA into positive-sense coding RNA, allowing expression of the gene. Also provided are DNA expression cassettes encoding viral replicons according to the present invention, constructs, plant cells and plants transfected and transformed with same.

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Viral Replicon

This invention concerns a viral replicon for controlling plant viral infection, and allowing visualisation of virus infected cells.

Virus diseases cause serious losses of yield and quality in many crop species. Whilst molecular biologists have developed a wide range of approaches to non-conventional control of plant viruses, one of the most efficient is a natural process encoded by the plant called the hypersensitive response (HR). The hypersensitive response is usually conferred by single dominant genes within the plant and confers resistance by restricting the virus to the initial site of infection usually by a necrotic response. There are many examples where plant breeders have exploited such genes; however, for many crop-virus combinations no HR gene exists and also some resistance genes have been shown to be overcome by the virus under field conditions. This is primarily due to the fact that in many virus-host systems, confinement of the virus by the necrotic response is a competition between the rate of virus replication and how fast the onset of HR occurs.

The present invention provides a system for giving plants resistance or enhanced resistance to viral infection.

According to the present invention there is provided a viral replicon comprising RNA encoding

i) at least one anti-sense gene; and

ii) flanking viral sequence or sequences

such that in the absence of a virus or viral components the replicon is non-coding, and in the presence of a virus or viral components the replicon is activated by viral replicase converting the non-coding negative-sense RNA into positive-sense coding RNA, allowing expression of the gene or genes.

By "viral components" is meant those components of a virus sufficient to activate the replicon.

Hence in the absence of virus, the gene would be in the form of non-coding negative-sense RNA. Upon infection, viral replicase would transcribe the negative-sense RNA into positive-sense coding RNA allowing expression of the gene. In this way the activation of a resistance response may be linked directly to the replication of the virus, since replication is an early event in the infectious cycle. Also, as replication levels increase, so the resistance response will increase.

At least one gene may encode a cell-autonomous cytotoxic protein; at least one cytotoxic protein may be *Bacillus amyloliquifaciens* RNAse (Barnase). Alternatively, a gene may encode any other protein which would direct instant death of the cell within which it is expressed.

At least one gene may encode an enzymatic marker; at least one enzymatic marker may be the gene for luciferase. Hence for example an infected cell may be made to glow when infected by a virus due to the catalytic activity of luciferase.

At least one gene may encode a visual marker.

At least one gene may confer viral resistance; at least one gene may encode a protein or nucleic acid sequence which confers viral resistance.

Hence the replicon may contain a gene for a protein which confers viral resistance or it may contain genes for both an enzymatic marker and a cytotoxic protein.

The viral flanking sequence or sequences may be selected from the group of a subgenomic promoter, a 5' terminal sequence, and a 3' terminal sequence.

The subgenomic promoter may be the potato virus S subgenomic promoter.

The replicon may be activated by at least one of the group of pea early browning virus (PEBV), potatoo virus X (PVX), potato virus S (PVS) and potato virus M (PVM).

The subgenomic promoter region of the gene involved in early replication of the virus may comprise sequences which will interact with viral replicases to trans-activate the negative-sense templates to coding mRNA upon viral infection.

Hence by the inclusion of a viral flanking sequence or sequences in the viral replicon, the replicon may be activated by the presence of a particular virus or viruses and as such may confer protection against it or them. Obviously, the viruses which activate the replicon may be any plant viruses and not just restricted to the above list.

The viral replicon may be encoded by a DNA expression cassette.

By "DNA expression cassette" is meant a DNA sequence which is transcribed, the transcription product in the case of the present invention comprising a viral replicon. Hence a DNA expression cassette works in an analogous way to a gene, although it differs in that the transcription products are not translated.

The DNA expression cassette may comprise at least one promoter and a sequence encoding a viral replicon.

At least one promoter may be the CaMV35S promoter.

At least one promoter may cause the constitutive transcription of the viral replicon.

Hence in the absence of virus, the expression cassette would transcribe the anti-sense gene or genes into non-coding negative-sense mRNA. Upon infection, viral replicase would transcribe the negative-sense mRNA into positive-sense coding mRNA allowing expression of the gene or genes. Hence the cassette may ensure protection of a cell or plant from viral infection.

Potato virus S and M are members of the carlavirus group and potato virus X is a member of the potexvirus group. Both groups of viruses generate within infected tissue two 3' co-terminal subgenomic mRNAs. The nucleotide sequences of these mRNAs have been investigated and homologous regions identified. These sequences

have been proposed to be involved in the production of positive-sense subgenomic mRNA by serving as cis-acting element involved in replicase recognition of the negative-sense template.

A DNA expression cassette according to the present invention encoding a viral replicon may comprise at least one promoter, at least one anti-sense gene and a viral subgenomic promoter, the subgenomic promoter containing a hexamer motif selected from one of the group of:

5' TTAGGT and

5' TTAAGT

or any anologue or homologue thereof. Examples of viruses containing subgenomic promoters having these hexamer motifs are as follows:-

LSV 25K	5' AGC-TTAGGT TAT-GC
PVS 25K	5' AGC-TTAGGT AACAGC
PVM 25K	5' AGC-TTAGGT ATT--GC
LSV CP	5' ACCTTTAGGT TTAC-AG
CLV CP	5' ACC-TTAGGT TCAC--G
PVS CP	5' ACCTTTAGGT TCAC-AG
PVM CP	5' -CCTTTAGGT -CATAAG
HelVSCP	5' -CCTTTAGGT TTACACG

Carla Consensus 5' TTAGGT

Potex Consensus 5' TTAAGT

Hyphens are used as spacers in order to demonstrate sequence homology and do not indicate nucleotides.

The viral subgenomic promoter may comprise the potato virus S subgenomic promoter having the sequence of SEQ ID NO: 1:

```
5'   GAGCTCACAA GAGATTTGGT GGAAGCCGTA GCAACATTGG GGCCGTTGAA
51   GCACCTTTAG GTTCACAGGT AAGAGTTCGA AGAAACTGTC CCACAGAGAC
101  CATGG
```

Hence a DNA expression cassette according to the present invention encoding a viral replicon may comprise the 35Sx2 promoter linked to the anti-sense luciferase gene linked to a flanking viral sequence; the flanking viral sequence may comprise a subgenomic promoter selected from the group of the pea early browning virus 12K subgenomic promoter and the pea early browning virus 3' UTR-S.

By "35Sx2" promoter is meant the 35Sx2 promoter which comprises two tandem copies of the CaMV35S promoter.

Alternatively, a DNA expression cassette according to the present invention encoding a viral replicon may comprose the 35Sx2 promoter linked to the anti-sense barnase gene linked to a flanking viral sequence; the flanking viral sequence may comprise a subgenomic promoter selected from the group of the pea early browning virus 12K subgenomic promoter and the pea early browning virus 3' UTR-S.

According to a further aspect of the present invention there is provided a genetic construct encoding a viral replicon or DNA expression cassette encoding a viral replicon according to the present invention.

Such a construct may comprise a plasmid; the plasmid may contain a DNA expression cassette.

According to further aspects of the present invention there are provided plant cells transformed or transfected with a construct according to the present invention, together with a plant comprising cells transformed with a construct according to the present invention.

A plant according to the present invention may be any plant or horticultural crop, generally of commercial interest, for example tobacco, flowers such as e.g. roses, potato plant, tomato plant or mushroom and the like.

The invention will be further apparent from the following description with reference to the several Figures of the accompanying drawings and experimental details.

Of the drawings:

Figure 1 shows a diagram of pAnti2-Luc construct used in the experiment of Figure 4.

Figure 2 shows a viral replicon linked to a marker gene driven by

CaMV35S promoter used for activation experiments in healthy and virus infected tissue via a transient expression system.

Figure 3 shows a graph demonstrating the activation of a viral replicon in infected plant tissue.

Figure 4 shows a viral replicon linked to the cell cytotoxic gene barnase used for cell ablation experiments in healthy and virus infected tissue via a transient expression system.

Figure 5 shows a shematic representation of a the viral replicon may function within a transgenic plant to protect against viral infection.

Figure 6 shows PEBV replicons designed with subgenomic (p12K-LUC) and terminal sequences (pSUTR-LUC) and a graph demonstrating activation of replicon in the presence of an infectious clone of PEBV RNA 1 (pCaN1).

EXPERIMENTAL DETAILS

1. Construction of a replicon

pAnti2-Luc construct, shown in detail in Figure 1 contains the marker gene luciferase linked to the subgenomic promoter (SGP) for the coat protein mRNA of the carlavirus potato virus S (SGP for PVS designated VTE in some constructs). The SGP-luciferase is linked in such a manner to the CaMV35S promoter that negative-sense non-coding mRNA is generated *in planta*.

The construct pAnti2-Luc was generated as follws:-

The upstream region of the coat protein (the PVS-SGP) was present on clone p7CP and was amplified by PCR using the 5' primer

5' GCTCTAGAGC TCACAAGAGA T

and the 3' primer

5' CGGATCCATG GTCTCTGTGG GACAGT

covering the ATG start codon.

The viral sequence in and around the ATG has been changed to incorporate an NcoI restriction site, representing a change from the viral sequence of AA to CC. The primers also contain add-on restriction sites to enable the PCR generated product to be cloned into the appropriate vector. The 5'-primer includes an XbaI site whilst the 3'-primer contains a BamH1 site downstream from the ATG. The PCR product containing the PVS-SGP was digested with XbaI and BamH1 and cloned into pBluescript SK which had been digested with the corresponding enzymes. The clones

containing the PCR product were then sequenced using single stranded DNA rescued via helper phage to check for any PCR generated errors. Sequencing was done using the dideoxynucleotide termination method. The resultant plasmid containing the PVS-SGP was designated pVTE. The nucleotide sequence of VTE which represents the subgenomic promoter (SGP) was generated by PCR from potato virus S and comprises the sequence of SEQ ID NO: 1:

```
5'   GAGCTCACAA GAGATTTGGT GGAAGCCGTA GCAACATTGG GGCCGTTGAA
51   GCACCTTTAG GTTCACAGGT AAGAGTTCGA AGAAACTGTC CCACAGAGAC
101  CATGG
```

The position of the consensus sequence (residues 57-62) is indicated in bold. The position of the ATG start codon which is fused to the ATG start codon of the luciferase marker gene, via an NcoI site, is underlined.

pVTE was digested with NcoI and BamH1 and ligated to a luciferase gene isolated from pRTL2 as a NcoI/BamH1 fragment. The resultant plasmid containing the PVS SGP linked to the marker gene luciferase was designated pVTE- Luc.

A double CaMV35S promoter (35Sx2) isolated from pWP83 as a Kpn1/Hind III fragment was then ligated into pVTE-Luc digested with Kpn1/Hind III. The resultant plasmid containing the PVS SGP-Luc-CaMV35S was designated pAnti2-Luc (see Figure 1).

Constructs were also designed and constructed, as described above, for the subgenomic promoter for the 12K gene of the tobravirus, pea early browning virus

(PEBV), and also for the 3' untranslated region (3'UTR) of RNA 2 of PEBV. In the case of the 12K SGP, PCR primers were used to isolate the region corresponding to nucleotides 5897-6302 of RNA1, and fuse it in an antisense orientation to an antisense luciferase gene under the control of CaMV35S promoter. In the case of the 3'UTR (nucleotides 3113 to 3374 of RNA 2), the viral sequences have been arranged so that positive-sense RNA will be generated fused to the end of an antisense RNA luciferase sequence, as in this situation it is positive-sense that the viral replicase first recognises. These plasmids have been designated, p12K-LUC in the case of the 12K SGP, and pSUTR in the case of the 3'UTR of RNA 2 (see Figure 6).

2. Transfection of a replicon into plant cells

(i) Preparation of DNA coated tungsten microprojectiles:

A tungsten suspension was prepared by adding particles (M10: average diameter 10μm) to absolute ethanol (50mg per ml of ethanol) and stored at -20°C. Before firings particles were washed with sterile distilled water and 25μl aliquots placed into microcentrifuge tubes. To each tube 10ug of plasmid DNA was added with 25μl of a 1.0M solution of calcium chloride, and 10μl of a 0.1M solution of spermidine (free base). The suspension was allowed to stand for 10 minutes.

(ii) Particle bombardment:

25μl of supernatant from the DNA-tungsten suspension, which had been allowed to settle for 10 minutes, was removed and approximately 2μl of the tungsten-DNA preparation was placed onto the front surface of the macroprojectile. The macroprojectile was placed into the barrel of the particle gun, with the DNA side facing

down. A blank charge (Remington 1) was placed into the barrel behind the macroprojectile and the firing assembly placed over the barrel with the stopping plate in position. Finally a leaf sample was placed into the firing chamber and the assembly closed. The vacuum pump was switched on until 275mm Hg was reached before the particle gun was fired. Following bombardment the plates were incubated at 26°C overnight under fluorescent light.

3. Method of detection of marker gene

Healthy and infected tissue were extracted in LEB (luciferase extraction buffer: 100mM KPO_4, 1mM DTT). Debris was removed by a 10 second pulse in a microcentrifuge and supernatant removed to a fresh tube. 50µl of the supernatant was assayed in a Luminometer in the presence of 100µl ATP (500mM Hepes pH 7.8, 1M $MgCl_2$, 20mM ATP (in 10mM KPO_4 pH 7.5)) and 100µl luciferin 0.5mM.

4. Results

To date results have been obtained which demonstrate that a 'silent' plant virus replicon containing a subgenomic promoter may be activated within infected tissue. A plasmid containing just the luciferase gene without any plant promoter or viral RNA subgenomic promoter (pSyn-Luc), see Figure 3, was used in healthy and infected potato leaves to obtain background readings, shown in lanes 1 and 2 respectively of the graph of Figure 3. When pAnti2-Luc was fired into healthy tisue (lane 3) similar results were obtained to those with the promoterless construct, as the plant promoter 35S will drive negative sense RNA for the luciferase gene. As the plants are healthy no viral replicase

is available to convert the negative sense RNA into positive therefore no expression of luciferase above background occurs. However, when Anti2-Luc was fired into potato leaves infected with potato virus S (lane 4), the luciferase values obtained (experiments were performed in triplicate) were 1.5 to 2 fold higher than the values obtained for the same construct in healthy leaves. These results therefore demonstrate that a silent marker gene may be activeted within infected tissue when it is linked to a viral subgenomic promoter, as illustrated schematically in Figure 2.

Results have also been obtained with the tobravirus pea early browning virus PEBV), using the 12K SGP present on RNA1 and the 3'UTR of RNA2. In these experiments, p12K-LUC was fired into tobacco leaves in the presence or absence of an infectious clone of RNA1 which contains the viral replicase gene (pCaN1). When p12K-LUC was fired into tobacco cells in the presence of pCaN1, luciferase values were 2.5 fold higher than those obtained from tobacco cells fired with p12K-LUC alone (see Figure 6). Transgenic plants were generated by Agrobacterium mediated transformation, in which the construct p12K-LUC was transferred into the host genome. Leaves from these plants were then used in firing experiments to test for activation of luciferase. In this case, a plasmid was used which only expresses the replicase gene of PEBV, this plasmid has been designated pCa201KN. When the plasmid was fired into leaves from transgenic lines containing the p12K-LUC construct there was a 3 fold activation of luciferase. In addition, these experiments also demonstrated activation of a luciferase gene when linked to a terminal sequence of a virus, in this case the 3'UTR of RNA2 of PEBV linked in a sense of orientation to the antisense luciferase gene. When this plasmid, designated pSUTR-LUC was delivered into tobacco cells in the presence of pCaN1, there was a 3 fold activation of expression of the luciferase gene, compared with

cells fired with pSUTR-LUC alone (see Figure 6). These results clearly demonstrate that activation of a gene may be obtained within virus infected cells, when the gene is expressed in a non-coding antisense orientation, but linked to either a viral subgenomic promoter or a 5' or 3' terminal sequence of a virus.

5. Conclusion

Replacement of the marker gene luciferase with a cytotoxic gene (eg. barnase) linked to either a subgenomic promoter or 5'/3' terminal sequence of a virus promises to give similar results, ie no expression of the barnase gene, and therefore protein, in healthy cells but high expression in infected cells. Expression of barnase in infected cells will result in cell death and prevent the spread of the infection within a healthy plant leaf, demonstrated schematically in Figure 4. Figure 5 shows a schematic diagram of this system in a transgenic plant.

It will be apreciated that it is not intended to limit the invention to the above example only, many variations, such as might readily occur to one skilled in the art, being possible, without departing from the scope thereof as defined by the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(D) STATE: Leicestershire
(E) COUNTRY: Great Britain
(F) POSTAL CODE (ZIP): LE2 6HG

(ii) TITLE OF INVENTION: Viral Replicon

(iii) NUMBER OF SEQUENCES: 1

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: GB 9308317.8
(B) FILING DATE: 22-APR-1993

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 105 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "cDNA representing positive sense RNA strand of Potato Virus S Subgenomic Promoter"

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

GAGCTCACAA GAGATTTGGT GGAAGCCGTA GCAACATTGG GGCCGTTGAA GCACCTTTAG 60

GTTCACAGGT AAGAGTTCGA AGAAACTGTC CCACAGAGAC CATGG 105

CLAIMS

1. A viral replicon comprising RNA encoding

i) at least one anti-sense gene; and

ii) flanking viral sequence or sequences

such that in the absence of a virus or viral components the replicon is non-coding, and in the presence of a virus or viral components the replicon is activated by viral replicase converting the non-coding negative-sense RNA into positive-sense coding RNA, allowing expression of the gene or genes.

2. A viral replicon according to claim 1 wherein at least one gene encodes a cell-autonomous cytotoxic protein.

3. A viral replicon according to claim 2 wherein at least one cytotoxic protein is barnase.

4. A viral replicon according to any one of claims 1 to 3 wherein at least one gene encodes an enzymatic marker.

5. A viral replicon according to claim 4 wherein at least one gene is the gene for luciferase.

6. A viral replicon according to any one of claims 1 - 5 wherein at least one gene encodes a visual marker.

7. A viral replicon according to any one of claims 1 - 6 wherein at least one gene confers viral resistance.

8. A viral replicon according to claim 7 wherein at least one gene encodes a protein or nucleic acid sequence which confers viral resistance.

9. A viral replicon according to any one of claims 1 - 8 wherein the viral flanking sequence or sequences are selected from the group of a subgenomic promoter, a 5' terminal sequence, and a 3' terminal sequence.

10. A viral replicon according to claim 9 wherein the subgenomic promoter is the potato virus S subgenomic promoter.

11. A viral replicon according to any one of claims 1 - 10 wherein it is activated by at least one of the group of pea early browning virus, potato virus X, potato virus S and potato virus M.

12. A viral replicon according to any one of claims 1 - 11 wherein it is encoded by a DNA expression cassette.

13. A viral replicon according to claim 12 wherein the DNA expression cassette comprises at least one promoter and a sequence encoding the viral replicon.

14. A viral replicon according to claim 13 wherein at least one promoter is the CaMV35S promoter.

15. A viral replicon according to any one of claims 12 - 14 wherein the DNA expression cassette comprises at least one promoter, at least one anti-sense gene and a viral subgenomic promoter, the subgenomic promoter containing a hexamer motif selected from one of the group of 5' TTAGGT and 5' TTAAGT.

16. A viral replicon according to claim 15 wherein the viral subgenomic promoter comprises the sequence of SEQ ID NO: 1

```
5'   GAGCTCACAA GAGATTTGGT GGAAGCCGTA GCAACATTGG GGCCGTTGAA
51   GCACCTTTAG GTTCACAGGT AAGAGTTCGA AGAAACTGTC CCACAGAGAC
101  CATGG
```

17. A viral replicon according to any one of claims 12 - 16 wherein it comprises the 35Sx2 promoter linked to the anti-sense luciferase gene linked to a flanking viral sequence.

18. A viral replicon according to claim 17 wherein the flanking viral sequence comprises a subgenomic promoter selected from one of the group of the pea early browning virus 12K subgenomic promoter and the pea early browning virus 3' UTR-S.

19. A viral replicon according to any one of claims 14 - 16 wherein it comprises the 35Sx2 promoter linked to the anti-sense barnase gene linked to a flanking viral sequence.

20. A viral replicon according to claim 19 wherein the flanking viral sequence comprises a subgenomic promoter selected from one of the group of the pea early

browning virus 12K subgenomic promoter and the pea early browning virus 3' UTR-S.

21. A genetic construct encoding a viral replicon according to any one of claims 1 - 20.

22. A genetic construct according to claim 21 wherein it comprises a plasmid.

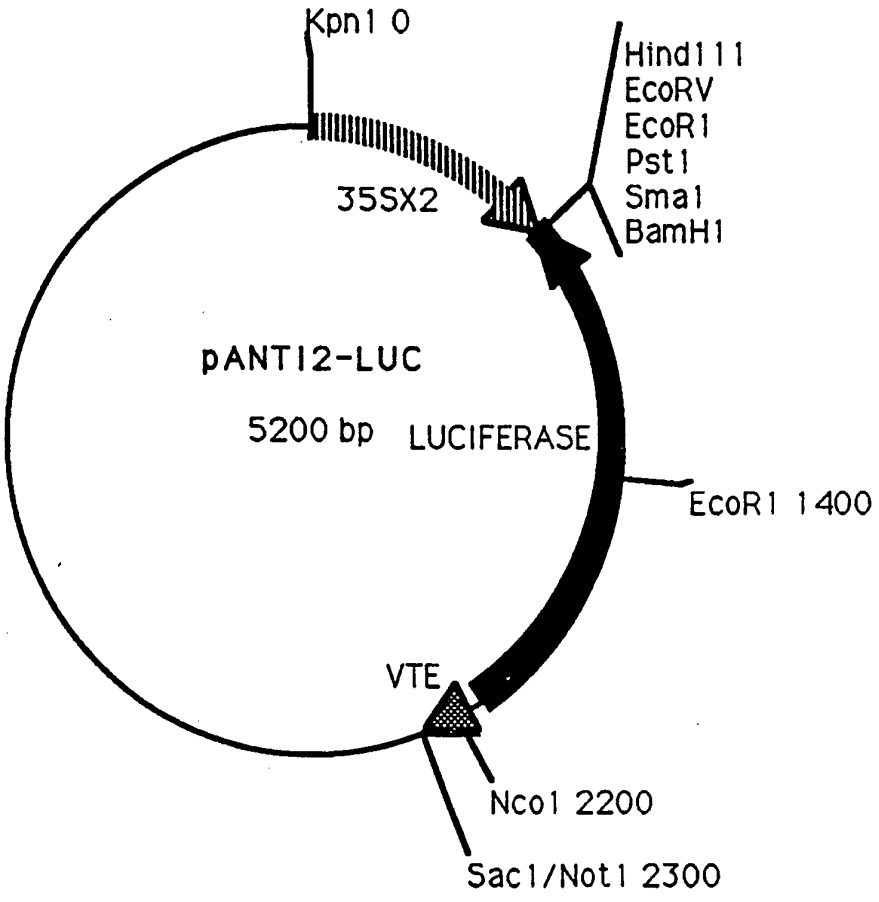
23. A genetic construct according to claim 22 wherein the plasmid contains a DNA expression cassette.

24. A plant cell transfected with a construct encoding a viral replicon according to any one of claims 21 - 23.

25. A plant cell transformed with a construct encoding a viral replicon according to any one of claims 21 - 23.

26. A plant comprising cells transformed with a construct encoding a viral replicon according to any one of claims 21 - 23.

FIGURE 1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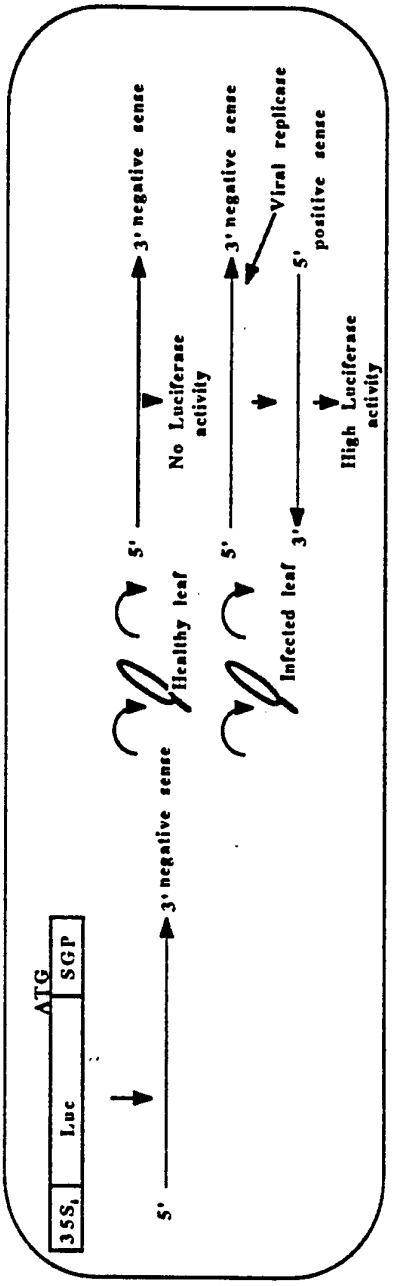


FIGURE 2

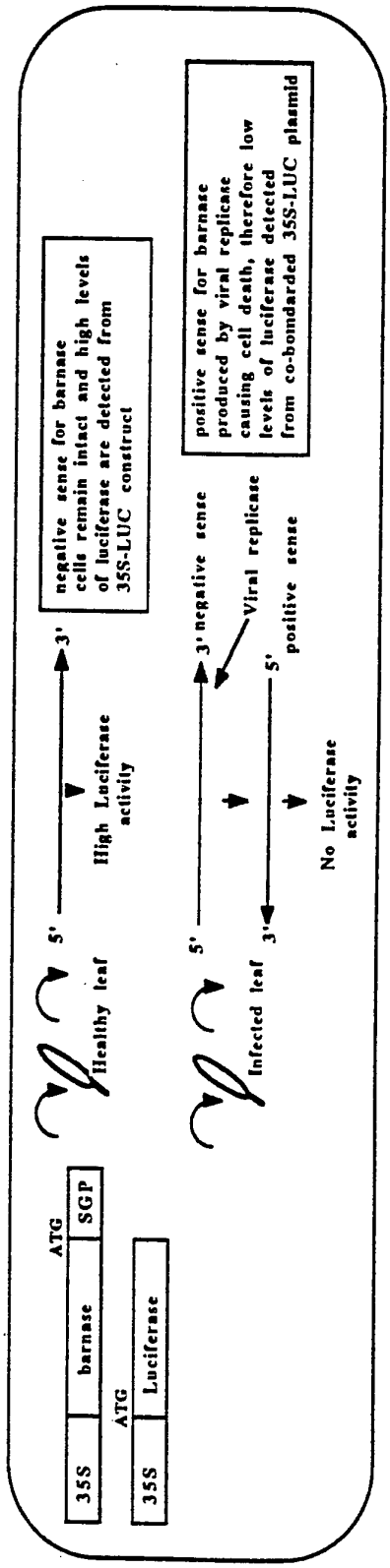
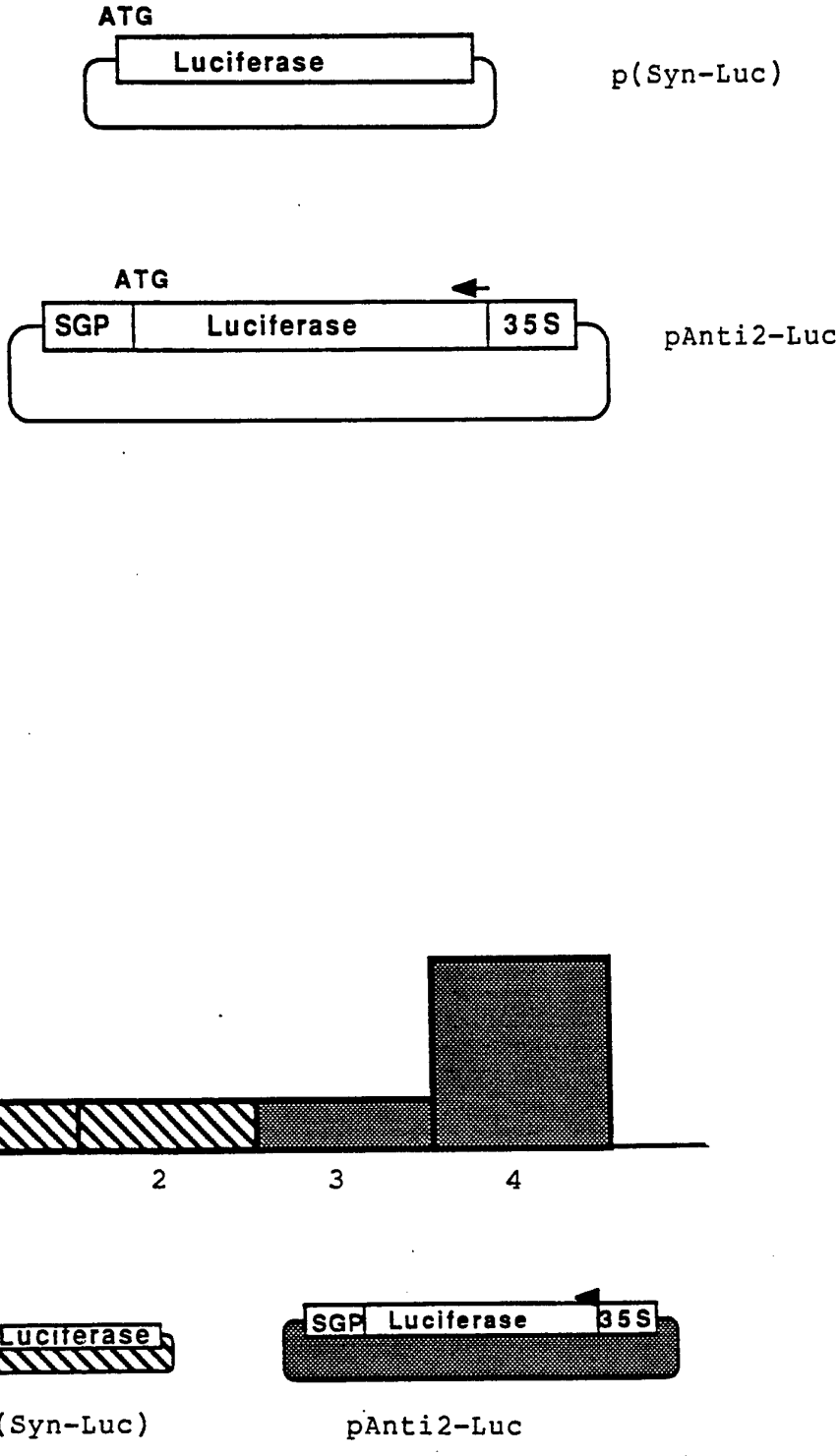


FIGURE 4

FIGURE 3


ATG
Luciferase
p(Syn-Luc)
ATG
SGP
Luciferase
35S
pAnti2-Luc
Luciferase Activity
1
2
3
4
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pAnti2-Luc

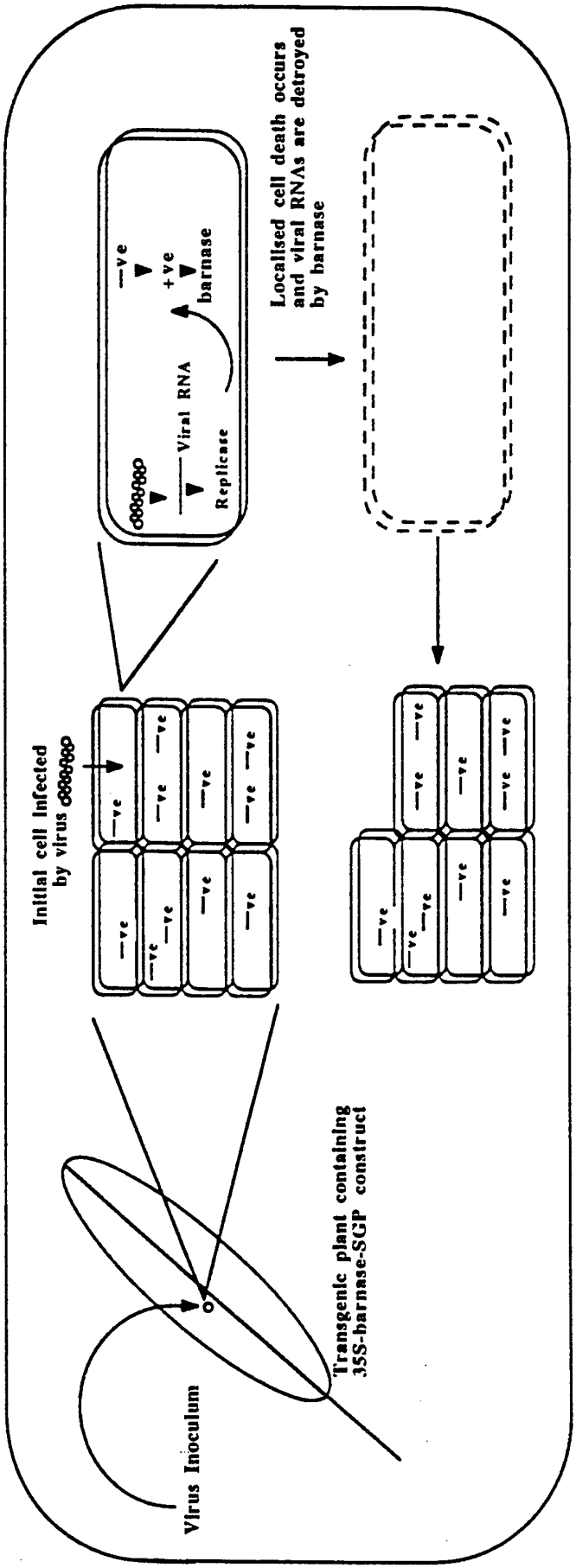
Virus Inoculum
Transgenic plant containing 35S-barnase-SGP construct
Initial cell infected by virus
-ve
Viral RNA
Replicase
+ve barnase
Localised cell death occurs and viral RNAs are detroyed by barnase

FIGURE 5

FIGURE 6

Constructs used in PEBV experiments

