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(71) Applicant (*for all designated States except US*): SCOTTISH CROP RESEARCH INSTITUTE [GB/GB]; Invergowrie, Dundee DD2 5DA (GB).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): TALIANSKI, Mikhail Emmanuilovitch [RU/GB]; 2F–L, 14 Baxter Park Terrace, Dundee DD4 6NW (GB). RIABOV, Evgueni Vitalievich [RU/GB]; 7 Provost McGowan Place, Dundee DD2 1DS (GB). ROBINSON, David, John [GB/GB]; 15 Canisp Crescent, Dundee DD2 4TP (GB). WILSON, Thomas, Michael, Aubrey [GB/GB]; The Coach House, 4 Balruddery Meadows, Invergowrie, Dundee DD2 5LJ (GB).

(74) Agent: MURGITROYD & COMPANY; 373 Scotland Street, Glasgow G5 8QA (GB).

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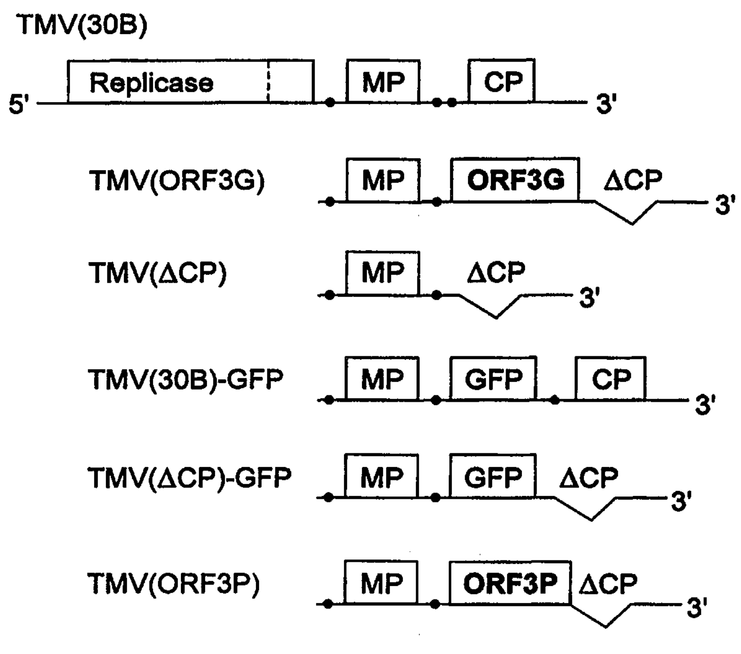


(57) Abstract

There is described a polynucleotide transporter protein able to promote the movement of a single stranded polynucleotide through the vascular system of a plant. The polynucleotide transporter protein described is the ORF3 protein of an *Umbravirus*, or is a functional equivalent thereof. The single stranded polynucleotide will usually encode a protein or polypeptide of interest, and use of the transporter protein enables the target protein or polypeptide to be expressed in tissues which are remote from the site of infection or production. ORF3 proteins from the *Umbraviruses* Groundnut rosette virus, tobacco mottle virus and pea enation mosaic virus 2 have each been shown to demonstrate the polynucleotide transporting utility.

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**"Polynucleotide Transporter Protein"**

The present invention relates to a novel protein and to the use of this protein, its functional equivalents or portions thereof, to transport a polynucleotide in the vascular system of a plant.

A rapidly growing body of evidence suggests that intercellular communications are fundamental for many general biological processes and phenomena in plants such as control of plant growth and development (1, 2), systemic acquired resistance to infection (3) and systemic gene silencing (2, 4, 5). It is believed that the signals involved in these processes are specific nucleic acids and proteins that can move from cell to cell through plasmodesmata, the intercellular cytoplasmic channels (6, 7), and through the plant's long distance transport system, the phloem (1, 4, 5). An example of such trafficking of plant endogenous macromolecules from cell to cell is the recent finding that the maize *knotted 1 (kn1)* homeobox gene encodes a nuclear-functional transcriptional regulator, KN1, which moves between cells through plasmodesmata (1). Interestingly, KN1 also facilitates transport of its own mRNA. The sequence specificity of post-transcriptional gene silencing implies that the signals involved in systemic transmission of the silencing state are polynucleotides that can enter the vasculature of the plant, move long distances and exit from the phloem (2, 4, 5). Recently, Xoconostle-Cázares et al. (Science (1999), Vol. 283, 94-97) have demonstrated that a plant endogenous protein CmPP16 moves from cell to cell, mediates the transport of sense and antisense RNA, and moves together with its own mRNA into the sieve elements delivering RNA into the long-distance translocation stream.

It is suggested that plant viruses move from cell to cell and over long distances by exploiting and modifying these preexisting pathways for macromolecular movement (1, 8). During the last 10 years much information has been obtained on the role of specialized virus-encoded movement proteins (MP) in promoting the cell-to-cell spread of virus infection through plasmodesmata (reviewed in ref. 6-8). Several types of MP have been identified. Some viruses, such as tobacco mosaic virus (TMV), encode single MPs that modify plasmodesmata and facilitate transport of the MPs themselves and of polynucleotides through the modified channel (9-11). Some other groups of viruses encode MPs that form plasmodesmata-associated tubules through which the virus moves (12-14). Several viruses, such as potato virus X (PVX), contain a set of movement genes called the triple gene block which encodes three proteins that together with coat protein (CP) are proposed to function co-ordinately to transport virus RNA through plasmodesmata (15-17).

Much less is known about the molecular details of long distance virus movement. It is not clear how viruses enter, move through or exit the vascular system. Minor veins are generally sheathed by bundle sheath (BS) cells and contain various cell types including vascular parenchyma (VP) cells, companion (C) cells and enucleate sieve elements (SE) (reviewed in ref. 18). Thus, transport of a virus to and within vascular tissue implies movement from mesophyll cells to BS cells, from BS cells to VP and C cells and entry to SE. The exit from vascular tissue probably occurs in the reverse order. It has been observed that the plasmodesmata between these types of cells differ from those interconnecting mesophyll cells (18). Analysis of virus-host systems in which systemic movement is impaired has provided evidence of the need for specific virus factors, different from the cell-to-cell MP, for trafficking through these types of plasmodesmata (8, 18). With only a few exceptions (19), the coat protein (CP) is essential for efficient long distance transport of plant viruses, because even in the rare cases where the *CP* gene is partially or wholly dispensable for systemic spread, the time required for systemic infection is often increased in its absence (20, 21). Although the precise role of CP in promoting movement via phloem remains to be determined, it might simply relate to its capacity to form virus particles. Several viruses also encode proteins that provide additional functions needed for systemic spread of infection. Mutations inactivating the p19 protein of tomato bushy stunt virus and the 2b protein of cucumber mosaic virus (CMV) prevented long distance movement of these viruses in some hosts but not in others (21, 22). A mutation in a central region of the helper component proteinase (HC-Pro) of tobacco etch virus also prevented systemic spread (23). Additionally, some virus-encoded replication proteins appear to have specific roles in long distance transport (24-26).

However, recently, experimental evidence has been reported that at least some of these proteins - such as 2b and HC-Pro proteins - have only indirect functions in movement such as suppressing post-transcriptional gene silencing (Anandalakshmi et al. (1998) Proc. Natl. Acad. Sci. USA, 95, 13079-13084; Brigneti et al. (1998) EMBO Journal, 17, 6739-6746; Kasschau and Carrington, (1998) Cell, 95, 461-470). It has been suggested that these proteins act by blocking a potential host-defence mechanism that restricts systemic spread rather than by promoting the process of long-distance transport itself.

Members of the genus *Umbravirus* are unusual since they do not code for a CP but nonetheless accumulate and spread systemically very efficiently within infected plants (27, 28). *Umbraviruses* utilise the coat protein of a co-infecting helper virus for encapsidation and transmission between plants. Typical helper viruses include members of the family *Luteoviridae*.

Members of the genus *Umbravirus* include bean yellow vein-banding virus (BYVBV), carrot mottle virus (CMoV), carrot mottle mimic virus (CMoMV), groundnut rosette virus (GRV), lettuce speckles mottle virus (LSMV), pea enation mosaic virus-2 (PEMV-2) and tobacco mottle virus (TMoV). Other viruses have been identified as being putative members of the genus *Umbravirus* and include sunflower crinkle virus (SCV), sunflower yellow blotch virus (SYBV), tobacco bushy top virus (TBTV) and tobacco yellow vein virus (TYVV).

The genomes of three different *Umbraviruses* have been sequenced and published. RNA 2 of pea enation mosaic virus (PEMV-2) is now classified as an *Umbravirus* and its genome sequence was reported by Demler et al., in

J. Gen. Virol (1993), Vol 74, pages 1-14. The genome sequence of groundnut rosette virus was reported by Taliansky et al., J. Gen. Virol. (1996), Vol 77, pages 2335-2345 and the genome sequence of carrot mottle mimic virus was published by Gibbs et al., in Virology (1996), Vol 224, pages 310-313. (This last paper refers to "an Australian isolate of carrot mottle virus", but this isolate was subsequently shown to be a distinct species and named carrot mottle mimic virus by Gibbs et al., Molecular Plant Pathology On-Line (1996) [http://www.bspp.org.uk/mppol/1996/1111gibbs]). The genome of TMoV has also been partially sequenced (see Example 4, Fig.10 and SEQ ID No 13).

Comparison of the genome organisation of these *Umbraviruses* has demonstrated significant similarity and is discussed herein with particular reference to groundnut rosette virus.

The RNA genome of GRV contains four open reading frames (ORFs). The two ORFs at the 5′-end of the RNA (ORF1 and ORF2) are expressed by a -1 frameshift to give a single protein, which appears to be an RNA-dependent RNA polymerase. The other two ORFs overlap each other in different reading frames. ORF4 encodes the 28 kDa cell-to-cell MP that contains stretches of similarity with several other viral MPs (28). Database searches with the sequence of the 27 kDa ORF3 protein revealed no significant similarity with any other viral or non-viral proteins, except the corresponding proteins encoded by the other *Umbraviruses* CMoMV and PEMV-2 of known sequence, there being a 42-50% homology (28) between these three ORF3 proteins. Further work with TMoV, which is also an *Umbravirus*, shows that the ORF3 protein has 34% homology with that of GRV. To date there has been no indication of the possible function of the ORF3 protein of the *Umbraviruses*. We have now performed a functional analysis of the GRV ORF3 protein which suggests that it is a novel *trans*-acting long distance movement factor, which can facilitate systemic transport of an unrelated single-stranded polynucleotide in non-virion form. ORF3 proteins from other *Umbraviruses* are expected to operate in a similar manner.

In summary, we have found that the ORF3 protein of *Umbraviruses* comprises two conserved domains: a highly basic domain which is a putative nucleic acid binding site, and a hydrophobic domain.

The present invention provides the use of the ORF3 protein from *an Umbravirus*, or a functional equivalent thereof, to transport a pre-determined single stranded polynucleotide through the vascular system of a plant.

In a preferred embodiment, the ORF3 *Umbravirus* protein exhibits *trans*-activity by transporting a single stranded polynucleotide which is non-native in that *Umbravirus*.

The term "functional equivalent" as used herein includes modified versions of the ORF3 protein of *Umbraviruses* which exhibit substantially the same level, or an improved level, of the biological activity (namely transport of a single-stranded polynucleotide molecule through the vascular system of a plant) compared to the naturally occurring protein. Modifications to the *Umbravirus* ORF3 protein which fall within this definition include (but are not limited to) versions of *Umbravirus* ORF3 having one or more of the following modifications: amino acid deletions, amino acid insertions and/or amino acid substitutions. Also included with this definition are modifications where whole domains of the protein are rearranged, deleted or substituted by alternative polypeptides, provided always that the biological activity level is retained or increased. The term "functional equivalent" also includes portions of the *Umbravirus* ORF3 protein, provided again that the function (biological activity level) is maintained or increased. For example the functional equivalent or modified version of the ORF3 protein may retain at least 50% (preferably at least 60%, more usually at least 80% or more, such as 90% or 95%) homology with the wild-type sequence of such a protein.

The single-stranded (ss) polynucleotide to be transported may be either RNA or DNA, although RNA is preferred since this avoids the need for a transcription step. Optionally the RNA to be transported is positive sense ssRNA (for example mRNA). We anticipate that single-stranded polynucleotide of about 10 kb or more may be transported and to date we have been able to cause transport of a single-stranded polynucleotide of 6.7 kb. Generally the single-stranded polynucleotide will encode a polypeptide or protein (these terms are used interchangeably herein) of interest.

Advantageously the polynucleotide is characteristic of a viral genome, especially a single-stranded positive sense viral genome. By "characteristic of a viral genome" we include single-stranded polynucleotides which are associated with MPs (ie virus-encoded movement proteins) responsible for cell-to-cell movement. Examples of cell-to-cell MPs include (but are not limited to) those found in plant viruses, for example as referred to by Lucas in Curr. Opin. Cell

Biol. (1995), Vol 7, pages 673-680; by Citovsky in Plant Physiol, (1993) Vol 102, pages 1071-1076; or by Carrington et al., The Plant Cell (1996), Vol 8, pages 1669-1681. Alternatively the cell-to-cell MPs may be of plant origin, for example KN1 or the MPs discussed in references 1, 2, 4 and 5. Particular mention may be made of the *Begomovirus* MPs (BV1 and BC1); ORF4 of *Umbraviruses*; P1 of CaMV; and the MPs of TMV and TMV-like viruses (eg RCNMV, CMV and AMV); and homologous proteins in related viruses.

Thus, the present invention envisages providing an MP (optionally by provision of an MP-encoding polynucleotide) which will associate with and further facilitate transport throughout the plant of the single-stranded polynucleotide encoding a polypeptide or protein of interest.

In a further aspect the present invention provides the use of an ORF3 protein from an *Umbravirus*, or a functional equivalent thereof, to transport a complex comprising a single-stranded polynucleotide associated with a cell-to-cell MP, in the vascular system of a plant.

Thus, the cell-to-cell MP will associate with the single-stranded polynucleotide of interest and will transport that polynucleotide originally from the cell of its manufacture or introduction in a cell-to-cell manner to reach a cell adjacent to the vascular system of the plant. Potential mechanisms of this cell-to-cell movement are discussed above, but the present invention is not limited to any particular mode of cell-to-cell transport. The important feature with respect to the present invention is that the single-stranded polynucleotide becomes located in cells adjacent to the vascular system, enabling the ORF3 *Umbravirus* protein, its functional equivalent or portion thereof, to facilitate rapid systemic transport of the polynucleotide via the vascular system.

The ORF3 protein may be derived from any currently known, or subsequently discovered or reclassified, *Umbravirus*. Mention may be made of known *Umbraviruses* which include bean yellow vein-banding virus (BYVBV), carrot mottle virus (CMoV), carrot mottle mimic virus (CMoMV), groundnut rosette virus (GRV), lettuce speckles mottle virus (LSMV), pea enation mosaic virus-2 (PEMV-2) and tobacco mottle virus (TMoV); and also of putative *Umbraviruses* which include sunflower crinkle virus (SCV), sunflower yellow blotch virus (SYBV), tobacco bushy top virus (TBTV) and tobacco yellow vein virus (TYVV). Particular mention may be made of the best studied *Umbraviruses* carrot mottle mimic virus (CMoMV), pea enation mosaic virus-2 (PEMV-2), groundnut rosette virus (GRV) and tobacco mottle virus (TMoV). Homology of the ORF3 of each of these viruses is acknowledged in the literature, as discussed above.

In one embodiment of the invention the ORF3 *Umbravirus* protein is the 27 kDa ORF3 protein of groundnut rosette virus (GRV). In alternative embodiments the ORF3 *Umbravirus* protein is the ORF3 protein of RNA 2 of pea enation mosaic virus (PEMV-2) or is the ORF3 protein of tobacco mottle virus (TMoV).

The advantage of the invention is that the ORF3 *Umbravirus* protein encoded by the polynucleotide herein described will cause the single stranded polynucleotide encoding for the polypeptide or protein of interest to be systemically spread throughout the whole host plant or the host plant cells. Thus, widespread transfection of that polynucleotide sequence encoding the polypeptide or protein of interest will be achieved and thus the yield of the polypeptide or protein of interest will be enhanced.

In summary, our finding that the GRV ORF3 facilitates long distance nucleic acid movement through vascular tissues is based on the following:

1. Long distance movement facilitated by the ORF3 appears to be very rapid: it takes just 4-5 days to reach the upper uninoculated leaves. To the best of our knowledge, only phloem-associated movement may be so rapid.

2. Direct localisation of TMV(ORF3G) in the phloem-associated cells such as bundle sheath and companion cells and in the sieve elements using immunogold labelling techniques with antibodies against the GRV ORF3 protein.

3. The pattern of unloading of TMV(ΔCP)-GFP from vascular tissues in uninoculated leaves in the presence of TMV(ORF3G) resembles the normal unloading pattern of a virus from phloem (Roberts, A.G., Santa Cruz, S., Roberts, I. M., Prior, D.A.M., Turgeon, R. and Oparka, K.J (1997) *The Plant Cell* 9, 1381-1396).

Viewed from a further aspect, the present invention provides a recombinant polynucleotide comprising a polynucleotide sequence which encodes the ORF3 protein of an *Umbravirus*, or a functional equivalent thereof. Preferably the ORF3 protein encoded is derived from GRV, CMoMV, TMoV or PEMV-2, that is it has at least a 50% homology, preferably 60% homology, to the amino acid sequence of the native version thereof. More usually the ORF3 protein will exhibit 80% (more preferably 90% or even 95%) homology with the native ORF3 protein of GRV, CMoMV, TMoV or PEMV-2.

Fig.10 and SEQ ID No 13 set out the novel polynucleotide sequence of the ORF3 protein of TMoV. Thus, in a further aspect, the present invention provides a polynucleotide having the nucleotide sequence of SEQ ID No 13, or at least 90%, more particularly 95% (preferably 98%) homology thereto.

In one embodiment the recombinant polynucleotide according to the invention may also comprise a polynucleotide sequence encoding a polypeptide or protein of interest. The polypeptide or protein of interest may be of microbial (especially bacterial), viral, plant, animal or synthetic origin. The polypeptide or protein of interest may be native or non-native to the host plant. Examples include surface antigens of viruses, growth factors, peptide hormones and the like.

In an alternative embodiment the recombinant polynucleotide according to the invention may also comprise a polynucleotide sequence encoding for a cell-to-cell MP.

Optionally, the recombinant polynucleotide may comprise a polynucleotide sequence encoding the ORF3 protein of an *Umbravirus* (preferably GRV, CMoMV, TMoV or PEMV-2), or a functional equivalent thereof (preferably GRV, CMoMV, TMoV or PEMV-2); a polynucleotide sequence encoding for a protein or polypeptide of interest; and a polynucleotide sequence encoding for a cell-to-cell MP.

Alternatively, the recombinant polynucleotide may comprise a polynucleotide sequence encoding the ORF3 protein of an *Umbravirus* (or a functional equivalent thereof) and the ORF4 cell-to-cell MP of the same *Umbravirus* (or a functional equivalent thereof), and a sequence encoding for a protein or polypeptide of interest.

The recombinant polynucleotide of the invention may be in any form (for example DNA or RNA double or single stranded) but generally double-stranded DNA is most convenient. However, it may also be convenient to present the recombinant polynucleotide in the form of a viral vector and single-stranded positive-sense RNA vectors (for example those based on TMV or potato virus X) are suitable.

There is a substantial body of knowledge concerning the techniques required for the art of genetic engineering and reference is made to Maniatis et al, "Molecular Cloning, A Laboratory Manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 1982, and Old and Primrose, "Principles of Genetic Engineering", fifth edition, 1994.

Where a polynucleotide encoding a protein or polypeptide of interest (whether or not that polynucleotide is part of the recombinant polynucleotide encoding the ORF3 protein) is introduced into the host plant in the form of DNA (eg cDNA), it is conveniently the transcribed mRNA form thereof that will be the single-stranded polynucleotide transported through the vascular system as described by the present invention.

Where a polynucleotide encoding a protein or polypeptide or interest (whether or not that polynucleotide is part of the recombinant polynucleotide encoding the ORF3 protein) is introduced into the host plant in the form of RNA (eg as in the form of an RNA viral vector), the replicated version of that RNA will be the single stranded polynucleotide transported through the vascular system as described by the present invention.

The recombinant genetic construct according to the present invention may itself be part of a vector (for example an expression vector). Conveniently the recombinant polynucleotide may be formed by simply inserting a construct comprising the polynucleotide sequence(s) of interest in-frame into such a viral genome based vector (especially of a plant virus). The introduced polynucleotide sequence(s) may even replace the coat protein sequence of the virus. Suitable viral vectors are well-known in the art. Alternatively the recombinant polynucleotide according to the present invention may be incorporated into the genome of a host forming a transgenic organism, especially a transgenic plant. Any vectors or transgenic organisms comprising a recombinant polynucleotide as described herein form a further aspect of the present invention.

Thus, viewed in a yet further aspect the present invention provides a recombinant expression system able to express the ORF3 protein of an *Umbravirus* (preferably the 27 kDa ORF3 protein of GRV, or its equivalent in CMoMV, TMoV or PEMV-2), or a functional equivalent thereof. Optionally, the recombinant expression system may also have the ability to express one or more proteins or polypeptides of interest and/or the ability to express a cell-to-cell MP. Vectors including such recombinant expression systems, especially those vectors based upon plant viruses, are also encompassed by the present invention.

The term "expression system" is used herein to refer to a genetic sequence which includes a protein-encoding region and is operably linked to all of the genetic signals necessary to achieve expression of that region. Optionally, the expression system may also include regulatory elements, such as a promoter or enhancer to increase transcription and/or translation of the protein encoding region or to provide control over expression. The regulatory elements may be located upstream or downstream of the protein encoding region or within the protein encoding region itself. Where two or more protein encoding regions are present these may use common regulatory element(s) or have separate regulatory element(s).

In an alternative embodiment it is envisaged that co-transfection of a host cell (especially a plant host cell) with two or more distinct recombinant expression systems could be used to achieve widespread transmission of the polynucleotide encoding the polypeptide or protein of interest. Thus, a first expression system or vector comprising a recombinant polynucleotide encoding the ORF3 protein of an *Umbravirus* (especially GRV) may be used in combination with a second expression system comprising a recombinant polynucleotide encoding the protein or polypeptide of interest. Either of these recombinant polynucleotides may additionally encode for a suitable cell-to-cell MP. Alternatively the cell-to-cell MP may be encoded by a third expression system, requiring triple inoculation of the host cell. Alternatively the host cell could be transgenically engineered to express the cell-to-cell MP.

Viewed from a further aspect the present invention comprises a transgenic organism, especially a transgenic plant wherein a polynucleotide sequence encoding the ORF3 protein of an *Umbravirus*, or a functional equivalent thereof, is stably incorporated into the genome of the host organism. In this embodiment the protein or polypeptide of interest may be introduced into the host transgenic plant as a separate construct. The cell-to-cell MP may either be encoded on the same construct as the protein or polypeptide of interest or may be present on a separate construct.

In an alternative embodiment the host cell is transgenically engineered to express both the ORF3 protein of an *Umbravirus*, or a functional equivalent thereof, and also a cell-to-cell MP. The resulting transgenic organism could then simply be transfected at a single site with a construct encoding the protein or polypeptide of interest. The combined action of the cell-to-cell MP and the ORF3 protein will ensure rapid transmission of the transfected construct and thus expression of the protein or polypeptide or interest throughout the organism.

Suitable host cells include plant cells, whether present in cell culture or as part of plantlets, plant parts (including seeds) or whole plants. Host cells particularly worthy of mention include: for GRV the natural host plant is groundnut *(Arachis hypogaea)*, but GRV has also been transmitted to several other species of Leguminosae *(Glycine max, Indiogofera nummularifolia, Macrotyloma uniflorus, Phaseolus vulgaris, Stylosanthes gracilis, S. guayensis, S. mucronata, S. juncea, S. sundaica, Tephrosia purpurea, Trifolium incarnatum, Trifolium repens* and *Vigna* *gracilis)* and to species in the Amaranthaceae *(Gomphrena globosa)*, Chenopodiaceae *(Chenopodium amaranticolor, C. murale, C.quinoa, Spinacia oleracea)* and Solanaceae *(Nicotiana benthamiana, N. clevelandii, N. debneyi, N. occidentalis)*. PEMV-2 infects many legumes and also a few species in other familes, for example *P. sativum, V. faba, Chenopodium album, C. amaranticolor, C. quinoa, Nicotiana clevelandii, and N. tabacum*. Other *Umbraviruses* infect at least their natural host. For example carrot mottle mimic virus infects carrot plants; tobacco mottle virus infects tobacco plants; bean yellow vein-banding virus infects bean plants, and so on.

In a further aspect, the present invention provides a method of producing a target protein or polypeptide, said method comprising:

introducing into a host plant cell one or more polynucleotides able to express:

a) an ORF3 protein of an *Umbravirus* or a functional equivalent thereof; and

b) a cell-to-cell movement protein; and

c) the target protein or polypeptide.

The ORF3 protein is desirably chosen from GRV, PEMV-2, TMoV or CMoMV. The cell-to-cell movement protein may conveniently be the ORF4 protein of GRV, PEMV-2, TMoV or CMoMV.

The present invention will now be further described with reference to the following (non-limiting) examples and figures in which:

**FIGURE LEGENDS**

Fig.1. Schematic representation of TMV-based vector,

TMV(30B) and its derivatives expressing GRV ORF3, PEMV-2 ORF3 and GFP with and without deletion of the *CP* gene. Boxes represent open reading frames, lines represent untranslated sequences. MP, movement protein; CP, coat protein; GFP, green fluorescent protein; ORF3G, GRV ORF3 protein; ORF3P, PEMV-2 ORF3 protein; •, subgenomic promoters. Deleted sequences are indicated.

Fig. 2. Symptoms of *Nicotiana benthamiana* plants infected with (a) TMV(30B), (b) TMV(ΔCP) and (c) TMV(ORF3G).

Fig. 3. Representative Northern blot analysis of viral RNAs from inoculated (i) and uninoculated (u) leaves of *Nicotiana benthamiana* plants infected with TMV(30B), TMV(ORF3G) and TMV(ΔCP), as indicated. Exposure time for autoradiography (2 hours and 24 hours) is indicated and the position of TMV genomic RNA is marked.

Fig. 4. *Nicotiana benthamiana* plants photographed under long-wavelength UV light 8 days (a,b) and 12 days (c,d,e) after infection with (a,c) TMV(30B)-GFP, (b) TMV(ΔCP)-GFP, (d,e) TMV(ΔCP)-GFP + TMV(ORF3G). Inoculated (I) and systemically infected (S) leaves are indicated.

Fig. 5. Schematic representation of the GRV ORF3 construct used for transformation of *N. benthamiana*. GRV ORF3 sequence was inserted in the pROK2Ω vector between the 5'-end leader sequence of tobacco mosaic virus genomic RNA (Ω leader), located downstream from the 35S promoter of cauliflower mosaic virus (CaMV 35S), and the transcriptional terminator from *Agrobacterium tumefaciens* nopaline synthase gene (NOS ter) to give pROK2Ω.GRV3. The NPII gene for neomycin phosphotransferase II was used as the selectable marker

gene.

Fig. 6. Symptoms in *Nicotiana benthamiana* plants infected with (a) TMV(30B), (b) TMV(ΔCP) and (c) TMV(ORF3P).

Fig. 7. Representative Northern blot analysis of viral RNAs from inoculated (i) and uninoculated (u) leaves of *Nicotiana benthamiana* plants infected with TMV(ΔCP) TMV(ORF3P) and TMV(30B), as indicated. Exposure time for autoradiography is 24 hours.

Fig. 8. Symptoms in *Nicotiana clevelandii* plants infected with (a) TMV(30B), (b) TMV(ΔCP) and (c) TMV(ORF3P).

Fig. 9. Representative Northern blot analysis of viral RNAs from inoculated (i) and uninoculated (u) leaves of *Nicotiana clevelandii* plants infected with TMV(ΔCP), TMV(30B) and TMV(ORF3P), as indicated. Exposure time for autoradiography is 24 hours.

Fig.10. Nucleotide sequence of TMoV ORF3 and, below, the amino acid sequence encoded by this ORF.

**Example 1**

**MATERIALS AND METHODS**

**Plasmids, Generation of Chimeric cDNA Constructs and Mutants.** Chimeric TMV constructs were made using the TMV-based vector pTMV(30B), (Fig. 1, see also ref.1). This vector contains multiple cloning sites and an additional copy of the subgenomic promoter for the CP mRNA inserted between the genes for the MP (30 kDa protein) and the CP (Fig. 1). Plasmid pTXS.GFP (29) containing jellyfish green fluorescent protein (GFP) cDNA was used as a template for PCR amplification of the *GFP* gene sequence. GRV cDNA clone grmp2 (28) was used for PCR amplification of GRV ORF3 sequences. Using standard DNA manipulation techniques (30) the following constructs were generated:

pTMV(ORF3G) (Fig. 1). A single nucleotide substitution (T→C) was introduced into the plasmid grmp2 (28) to change the initiation codon (AUG) of the ORF4 located inside the GRV ORF3 to (ACG) by overlap extension PCR (31) using a pair of complementary mutagenic primers, one of which was 5'-GTCAAGTGTAATAAACGTCTTCGCAAGTG-3' (SEQ ID No 1). This mutation is predicted to eliminate the ORF4, but does not change the amino acid sequence encoded by the ORF3. Then the fragment containing GRV ORF3 was amplified using oligonucleotides 5'-CATGATCGATATGGACACCACCC-3' (SEQ ID No 2) with a *Cla*I site preceding 13 nucleotides (nt) identical to those of the 5'-end of GRV ORF3 as a forward primer and 5'-CATGCTCGAGTTACGTCGCTTTGC-3' (SEQ ID No 3) with a *Xho*I site preceding 14 nt complementary to those of the GRV RNA sequence downstream of ORF3 as a reverse primer. The amplified fragment was cloned between the *Pme*I and *Xho*I sites of pTMV(30B). Then, the *Pml*I-*Hpa*I fragment (nucleotides 5833 to 6465 of the pTMV(30B) sequence) carrying the native subgenomic promoter for the *CP* gene and the 5'-part of this gene was excised from the resulting plasmid to give pTMV(ORF3G) (Fig. 1).

pTMV(ΔCP) (Fig. 1). The *Pml*I-*Hpa*I fragment (nucleotides 5833-6465) carrying the native subgenomic promoter for the *CP* gene and the 5' part of this gene was excised from pTMV(30B) to give pTMV(ΔCP).

pTMV(30B)-GFP (Fig. 1). The *GFP* gene was amplified using oligonucleotides 5'-GATCGTCGACATGAGTAAAGGAGAAG-3'(SEQ ID No 4) with a *Sal*I site preceding 16 nt identical to those of the 5'-end of the *GFP* gene as a forward primer and 5'-GATCCTCGAGTTACGTCGCTTTGC-3'(SEQ ID No 5) with a *Xho*I site preceding 14 nt complementary to those of the 3'-end of the *GFP* gene as a reverse primer. The amplified product was cloned into *Xho*I site of pTMV(30B) to give pTMV(30B)-GFP.

pTMV(ΔCP)-GFP. The *Xho*I - *Hpa*I fragment (nucleotides 5782 to 6465 of the pTMV(30B) sequence) of pTMV(30B)-GFP carrying the subgenomic promoter and the 5' part of the *CP* gene, was excised to give pTMV(ΔCP)-GFP.

All the viruses derived from these constructs, designated by eliminating the prefix p in the names of the progenitor plasmids, were tested in *Nicotiana benthamiana* protoplasts. All replicated, but, in agreement with previous reports (32, 33), the viruses lacking CP accumulated to significantly lower levels (data not shown).

***In vitro* Transcription, Inoculation of plants and**

**Isolation of Protoplasts.** Plasmids were linearized by digestion with *Kpn*I, and *in vitro* transcripts were synthesized with T7 RNA polymerase using an mCAP RNA capping kit (Stratagene). The transcripts were inoculated directly to leaves of 3- to 4-week-old *N. benthamiana* plants by rubbing corundum-dusted leaves with the transcription products derived from 0.2 μg plasmid template.

Biological assays of nucleic acid extracts from inoculated and uninoculated leaves of *N. benthamiana* were conducted on *Nicotiana tabacum* L. cv. Xanthi nc, a local lesion host of TMV. Viral infectivity was determined as the average number of local lesions per half leaf.

Mesophyll protoplasts were isolated from fully expanded mature uninoculated leaves of plants infected with TMV(ORF3G) and TMV(30B) as described (34).

**Analysis of RNA.** Total RNA was isolated from leaf tissue or protoplasts as described (35). For northern blot analysis, total RNA preparations were denatured with formaldehyde and formamide. Electrophoresis was in 1.5% agarose gels (30). RNA was transferred to Hybond N membrane by the capillary method with 20xSSC (3M sodium chloride and 0.3M sodium citrate, pH 7.0) and immobilized by UV crosslinking. For dot blot hybridization analysis, samples of RNA were spotted onto Hybond N nylon membrane and immobilized by UV crosslinking. Hybridization was done as described (30) with [$^{32}$P] RNA probes complementary to sequences of the TMV replicase gene [nucleotides 445 to 2675 of pTMV(30B)]. Quantitative analysis of dot blots was done by densitometry of the autoradiographic images, using a Bio Image Intelligent Quantifier Version 2.5.0.

A dilution series of TMV RNA was used as concentration standard.

**Detection of GFP Fluorescence in Plants**. Plants were illuminated with long-wavelength UV light and photographed as described previously (29, 36). GFP fluorescence in plant tissues was viewed with a Bio-Rad MRC 1000 confocal laser scanning microscope. The methods were as described previously (29, 36).

**RESULTS**

**Symptom Induction by TMV(ORF3G), a Hybrid TMV with Replacement of the *CP* Gene with GRV ORF3.** The inability of GRV to form conventional virus particles creates technical difficulties in isolation of viral RNA and hence in generation of full-length cDNA clones to produce infective transcripts. This limits the potential of using a reverse genetics approach for functional analysis of GRV-encoded proteins. Therefore, we employed a gene replacement strategy to generate hybrids between TMV and GRV. The CP is not required for cell-to-cell movement of TMV but is essential for its long distance movement. The *CP* gene of TMV was deleted and replaced by ORF3 of GRV in the TMV-based vector, TMV(30B), to give the hybrid TMV(ORF3G) (Fig. 1). TMV(30B), and TMV(30B) with a deleted *CP* gene [TMV(ΔCP)], were used as controls (Fig. 1).

TMV(ΔCP) induced mild chlorotic spots in inoculated *N. benthamiana* leaves by 5 days post-inoculation (DPI), but no systemic symptoms were observed in these plants even five weeks after inoculation. In contrast, TMV(30B) induced very severe systemic symptoms, first observed at 5 DPI (Fig. 2). The infected plants were stunted, and showed strong mosaic and deformation of leaves. TMV(ORF3G) also induced systemic symptoms on *N. benthamiana* plants. At approximately 7 DPI expanding leaves at the top of the plant began to show some deformation followed by mild mosaic and rugosity at 10-12 DPI (Fig. 2). These results suggest that despite lacking the *CP* gene TMV(ORF3G) spreads systemically.

**Accumulation of TMV(ORF3G) RNA in Inoculated and Systemically Infected Leaves.** To verify that TMV(ORF3G) RNA moves systemically, inoculated and upper uninoculated leaves were harvested and analyzed by inoculation of nucleic acid extracts onto the hypersensitive host, *N. tabacum* L. cv Xanthi nc. As expected, TMV(30B) RNA accumulated both in inoculated and in uninoculated systemically infected leaves (Table 1). Both TMV(ΔCP) and TMV (ORF3G) RNAs also accumulated in inoculated leaves, but only TMV(ORF3G) spread systemically (Table 1).

Table 1. Accumulation of viral RNA in *N. benthamiana* plants inoculated with chimeric TMV-based viruses.

| Inoculum* | Infectivity as average number of lesions per half-leaf of N.tabacum cv.Xanthia nc[†] | | | | | |
|---|---|---|---|---|---|---|
| | 3 DPI | | 4 DPI | | 14 DPI | |
| | i[‡] | u[§] | i | u | i | u |
| TMV(30B) | 46±11 | 0 | 128±7 | 59±4 | 111±19 | 189±31 |
| TMV(ΔCP) | 9±4 | 0 | 24±5 | 0 | 31±7 | 0 |
| TMV(ORF3G) | 8±3 | 0 | 12±6 | 12±3 | 22±4 | 24±5 |
| TMV(30B)-GFP | 42±6 | nt | 62±4 | nt | 75±13 | nt |
| TMV(ΔCP)-GFP | 7±2 | nt | 12±5 | nt | 15±8 | nt |

* Nucleic acid extracts from *N. benthamiana* plants infected with chimeric viruses obtained after different intervals post inoculation (3 DPI, 4 DPI, 14 DPI) were used as inocula for tests on *N. tabacum* cv. Xanthi nc. Samples consisted of material obtained from 0.1g of tissue.

† Data are mean ± standard deviation from three independent experiments with three replicate plants in each.

‡ i, nucleic acid extracts were obtained from inoculated leaves.

§ u, nucleic acid extracts were obtained from uninoculated leaves.

nt = not tested.

It should be noted, however, that levels of accumulation of both viruses lacking CP [TMV(ΔCP) and TMV(ORF3G)] were significantly lower compared with those of TMV(30B) probably because of reduced stability of unprotected RNA. However, in spite of the low level of accumulation, TMV(ORF3G) was first detected in uninoculated leaves 4 DPI, the same time as TMV(30B) (Table 1), implying that both viruses move long distances at the same speed. TMV(ΔCP) was not detected in uninoculated leaves even 30 DPI.

Additional experiments conducted on extracts from stem nodes showed that TMV(ΔCP) RNA was detected only in the nodes attached to inoculated leaves, whereas TMV(30B) and TMV(ORF3G) RNAs were present in all the nodes, including those at the shoot apex (data not shown). Northern blot analysis of RNA samples isolated from the inoculated and uninoculated leaves confirmed the results of the biological assays, indicating that despite poor accumulation TMV(ORF3G) RNA spread systemically in *N. benthamiana* plants (Fig. 3). Northern blot analysis was conducted using a cRNA probe corresponding to nucleotides 445 to 2675 of TMV RNA (as indicated above for the dot blot analysis) transcribed from a corresponding plasmid using [$^{32}$P] ATP.

To test directly whether TMV(ORF3G) is able not only to move rapidly to uninoculated leaves but also to exit from the vascular system and spread into mesophyll tissues, mesophyll protoplasts from uninoculated systemically infected leaves were isolated. RNA extracted from these protoplasts was analyzed by dot-blot hybridization. As shown in Table 2, viral RNA was detected in protoplasts isolated from leaves systemically infected with either TMV(30B) or TMV(ORF3G). However, the amount of the TMV(ORF3G) RNA was approximately 11-fold lower than that of TMV(30B) RNA. Quantitation of viral RNA isolated from entire leaf tissues revealed a similar ratio (about 1:13) between the levels of accumulation of TMV(ORF3G) RNA and TMV(30B) RNA. These results suggest that TMV(ORF3G) is able not only to move from inoculated to uninoculated leaves but also can exit from the vascular system.

**Table 2** The presence of viral RNA in mesophyll cells of the leaves systemically infected with TMV(ORF3G).

| Inoculum | Amount of viral RNA in* | |
|---|---|---|
| | Leaf tissues $\mu$g/g of leaf | Mesophyll protoplasts ng/$10^6$ protoplasts |
| TMV(ORF3G) | 3±0.2 | 28±6 |
| TMV(30B) | 38±4 | 320±30 |

*The viral RNA was quantitated by dot blot hybridization using a dilution series of TMV RNA as concentration standard. Data are mean ± standard deviation from three independent experiments with three replicate plants in each.

**Complementation of the Long Distance Movement Defect of the TMV CP Deletion Mutant by TMV(ORF3G).** GFP is often used as a non-invasive reporter to monitor viral infections (29, 36, 37). The *GFP* gene was inserted into the genomes of TMV(30B) and TMV(ΔCP) to give TMV(30B)-GFP and TMV(ΔCP)-GFP, respectively (Fig. 1). In inoculated leaves of *N. benthamiana* TMV(ΔCP)-GFP caused the development of green fluorescent foci, which were clearly visible under long-wavelength UV light starting on the third DPI. Similar foci appeared at the same time after inoculation in leaves inoculated with TMV(30B)-GFP. However, the rate of enlargement of fluorescent foci induced by TMV(ΔCP)-GFP was significantly higher compared with those induced by TMV(30B)-GFP (Fig. 4). In contrast, biological assays conducted on nucleic acid extracts from inoculated leaves showed that TMV(30B)-GFP RNA accumulated to much higher levels than TMV(ΔCP)-GFP RNA (Table 1). Thus it seems, that in spite of the low rates of RNA accumulation, TMV(ΔCP)-GFP moves from cell to cell in inoculated leaves more efficiently than TMV(30B)-GFP. One explanation for this difference might be that the gene encoding the cell-to-cell MP (30 kDa protein) is less highly expressed in TMV(30B)-GFP, for example because of its more distant position from the 3′-end of the RNA. Another possibility is that, in the presence of CP, formation of virus particles might diminish cell-to-cell movement and cause a switch to long distance transport.

Following the development of fluorescent foci in the inoculated leaves, subsequent systemic infection by TMV(30B)-GFP led to the appearance of green fluorescence in the uninoculated leaves (Fig. 4). In contrast, as expected, systemic infection by TMV(ΔCP)-GFP did not occur and fluorescence in the uninoculated leaves was never observed.

Experiments on complementation of the long distance movement defect of TMV(ΔCP)-GFP by TMV(ORF3G) were conducted. TMV(ΔCP)-GFP was coinoculated with TMV(ORF3G) onto *N. benthamiana*. The majority of the doubly infected plants showed systemic symptoms characteristic of TMV(ORF3G) and developed green fluorescent spots induced by TMV(ΔCP)-GFP in both inoculated and uninoculated leaves (Fig. 4), implying systemic spread of the TMV(ΔCP)-GFP in the presence of TMV(ORF3G). In inoculated leaves, fluorescent spots included by TMV(ΔCP)-GFP in the presence or absence of TMV(ORF3G) were practically indistinguishable, but in uninoculated leaves the fluorescence appeared only in the case of mixed TMV(ΔCP)-GFP + TMV(ORF3G) infection. The first indication of entry of TMV(ΔCP)-GFP into an uninoculated leaf in this case was the appearance of fluorescent flecks along veins on the lamina, indicating that the virus was being unloaded at discrete foci. After the appearance of these fluorescent flecks, some leaf veins became more clearly delineated by fluorescence (Fig. 4E), and with time the mesophyll tissues neighboring the flecks also became labeled (Fig. 4D, 4E). Confocal laser scanning microscopy confirmed these observations and showed that up to 90% of mesophyll cells in the fluorescent area were infected with TMV(ΔCP)-GFP. The time of appearance of GFP fluorescence (about 8 DPI) and the pattern of virus unloading in uninoculated leaves observed in mixed TMV(ΔCP)-GFP + TMV(ORF3G) infections were similar to those observed for TMV(30B)-GFP (Figs. 4A and 4C) and correspond to the usual manner of vascular-associated long distance virus movement described for other viruses (29,36). Because TMV(ΔCP)-GFP was unable to move long distance alone, these results suggest that TMV(ORF3G) can complement long distance movement of TMV(ΔCP)-GFP. However, the number of initial fluorescent flecks in uninoculated leaves generated as a result of complementation of TMV(ΔCP)-GFP by TMV(ORF3G), and the extent of their spread, were usually lower than in the case of TMV(30B)-GFP infection and varied significantly from leaf to leaf (Fig. 4C vs 4D and 3E), probably reflecting differences in efficiencies of complementation which might depend on numerous factors including interference between virus variants. TMV(ORF3G) does not depend on TMV(ΔCP)-GFP for replication and spread and therefore may sometimes outcompete it, decreasing the efficiency of the complementation. To confirm that the effect is based on complementation rather than on recombination, the progeny virus that accumulated in the uninoculated leaves was analyzed by back inoculation first to a local lesion host of TMV, *N. tabacum* cv Xanthi NN. Subsequent transfer of virus from individual lesions to a systemic host, *N. benthamiana* produced one of two phenotypes characteristic of each the original viruses: either systemic symptoms and no fluorescence [TMV(ORF3G)] or no systemic symptoms and fluorescence in inoculated but not in uninoculated leaves [TMV(ΔCP)-GFP]. No plants displayed fluorescence in uninoculated leaves as would be expected if recombination had occurred. Confocal laser scanning microscopy confirmed that TMV(ΔCP)-GFP moved in the presence of TMV(ORF3G) to uninoculated leaves and showed that up to 90% of mesophyll cells in the area of fluorescent foci were infected with TMV(ΔCP)-GFP. These results clearly show that GRV ORF3 protein expressed from TMV(ORF3G) can mediate long distance movement of RNA of the unrelated virus, TMV.

**DISCUSSION**

Previous investigations revealed that cell-to-cell movement and long distance transport of plant viruses are distinct processes with different requirements (reviewed in ref. 8). Recently, it has been shown that the GRV ORF4 protein facilitates cell-to-cell movement (37). Here, we demonstrate that another GRV nonstructural protein, encoded by ORF3, provides a specific function that is both *cis*-active and *trans*-

active in vascular-associated long distance transport. The CP is critical for and directly involved in phloem-dependent spread of TMV (38-45). Therefore, functional replacement of the TMV CP by GRV ORF3 protein suggests that the ORF3 protein plays a direct role in the long distance movement rather than that it suppresses host response systems restricting systemic spread, as has been suggested for other factors (8). Recently, it has been found that CP is not required for TMV to penetrate from BS cells into VP cells, the presumed first step in the process of phloem-dependent movement, but is required for further movement into the C cell / SE complex. Thus, results presented here suggest that the GRV ORF3 protein may control entry to the vascular system at the level of the C cell / SE complex (45), and perhaps also exit from phloem to mesophyll cells in uninoculated systemically infected leaves.

ORF3 has been found in all three umbraviruses (GRV, pea enation mosaic virus 2 and carrot mottle mimic virus) sequenced to date (28, 46, 47). The deduced amino acid sequences of the corresponding proteins are also conserved (28). Analysis of amino acid sequences of the ORF3 proteins using the programs PileUp and PEPTIDESTRUCTURE revealed that the most conservative central region consists of a rather basic and highly hydrophilic domain, which seems to be exposed on the protein surface (amino acids 108-130), and a hydrophobic part (amino acids 151-180). One can speculate that the basic hydrophilic domain may possesses RNA-binding capacity. However, a database search with the sequences of these proteins revealed no significant similarity with any other known viral or non-viral proteins (28).

Thus the GRV ORF3 protein represents a novel class of *trans*-acting long distance movement factors. To the best of our knowledge, this is the first example of a nonstructural viral protein facilitating long distance movement of unrelated viral RNA. However, a prerequisite for the ORF3-directed long distance spread is effective cell-to-cell movement of the dependent RNA. GRV ORF3 could not functionally replace CP in the long distance movement of PVX RNA, because in this hybrid virus CP was also required for the cell to cell movement (37).

Another interesting feature of the GRV ORF3 protein is that because of the inability of GRV to form virus particles this protein may be adapted to transport RNA in non-virion form. This process may more closely resemble long distance transport of endogenous plant macromolecules. Plant virus evolution may have apparently involved the acquisition of cellular genes (48), and it is possible that the putative plant long distance movement factors that are necessary for normal plant growth and development were the progenitors to the GRV ORF3 protein. However, GRV ORF3 overlaps almost completely with ORF4, and this arrangement seems typical in umbraviruses (28). The ORF4 protein is a cell-to-cell movement protein that has clear similarities in sequence with the MPs of other plant viruses (28), and all these MPs probably share a common origin. The ORF3 sequence, however, seems unique to the umbraviruses and has most likely arisen as a result of "overprinting" (49) on ORF4 to give a functional, and perhaps structural, analogue of the hypothetical cellular long distance transport factor. Thus, umbraviruses may have evolved from a virus that had conventional cell-to-cell *MP* and *CP* genes. Once the ancestral umbravirus had developed an ORF3, and acquired the ability for its RNA to be packaged by helper virus CP and thereby transmitted by the vector of the helper virus its own CP became expendable. On a practical level, expression in transgenic plants of the ORF3 protein may constitute a powerful approach to the modulation of plant transport processes and it may also be valuable in the design, environmental containment and complementation of plant virus vectors to produce pharmaceutical or industrial proteins.

Different types of viral nucleic acids including RNAs of potyviruses, cucumoviruses, tobraviruses etc. as well as DNAs of geminiviruses, caulimoviruses etc. are being tested for their ability to be transported long-distances by GRV ORF3.

Localization of GRV ORF3 protein in different cells of the vascular system is also being monitored.

**Example 2**

**Generation of Transgenic Plants Expressing GRV ORF3**

**Generation of a construct.** A single nucleotide substitution was introduced into a plasmid grmp2 (28) to change the initiation codon AUG of the ORF4 located inside the GRV ORF3 to ACG by overlap extension PCR using a pair of complementary mutagenic primers, one of which was 5′-GTCAAGTGTAATAAACGTCTTCGCAAGTG-3′ (SEQ ID No 1). This mutation is predicted to eliminate the ORF4 but does not change the amino acid sequence encoded by the ORF3. Then the fragment containing the ORF3 was amplified using oligonucleotides 5′-GTACTCTAGATGGACACCACCC-3′ (SEQ ID No 6) with an *XbaI* site preceding 13 nucleotides (nt) identical to those of the 5′-end of GRV ORF3 as a forward primer and 5′-

CATGGGTACCTTACGTCGCTTTGCGG-3′(SEQ ID No 7) with a *Kpn*I site preceding 16 nt complementary to those of the GRV RNA sequence downstream of ORF3. The amplified fragment was cloned between the *Xho*I and *Kpn*I sites of pROK2Ω, a modified pROK2, a binary plant transformation vector based on pBin19 to give pROK2ΩGRV3 (Fig. 5). Pieces of *Nicotiana benthamiana* stem tissue were transformed as described by Benvenuto et al. (1991). Transgenic shoots were regenerated on a selection medium containing kanamycin (100 μg/ml). Rooted plantlets were transferred to sterilized compost and, after an adaptation period in a climate room at a humidity of 70%, were maintained in a glasshouse.

The presence of entire GRV ORF3 sequences in all transgenic plants was confirmed by PCR amplification with primers specific to termini of the inserted sequences. Reverse transcription-PCR analysis demonstrated expression of GRV ORF3 in transgenic plants.

The transgenic plants may be used for analysis of long-distance movement of different nucleic acids, including viral RNAs and DNAs of viruses belonging to different groups, for example as described below.

**Innoculation of Transgenic Plants**

PVX was shown to require both the triple gene block (TGB)-encoded movement proteins and the CP for cell-to-cell and long-distance movement (15-17). However, when GRV ORF4 was substituted for the PVX CP gene, the hybrid virus was able to move normally from cell to cell in inoculated leaves but not long distances (37). To study possible complementation of long-distance movement of chimeric PVX RNA in transgenic plants expressing GRV ORF3, we used hybrid PVX.4.GFP.ΔCP generated earlier by Ryabov *et al.* (37) which contained GRV ORF4 in place of its own CP and the GFP gene as a molecular reporter.

$S_1$ progeny plants of two independently transformed lines (GRV3-2, GRV3-5) expressing GRV ORF3, were inoculated with PVX.4.GFP.ΔCP. Nontransformed plants were used as a control. The ability of the virus to move long distances was tested by confocal laser scanning microscopy. In all transformed and nontransformed plants green fluorescence developed in the inoculated leaves, indicating that the virus accumulated, spread from cell to cell and expressed GFP in these leaves. However fluorescence in noninoculated leaves was detected only in plants of the transformed lines. Approximately 60% of plants of each transgenic line (GRV3-2 or GRV3-5), infected with PVX.4.GFP.ΔCP, developed sporadic fluorescent spots in noninoculated leaves 7-8 DPI, indicating that PVX.4.GFP.ΔCP could spread systemically in plants expressing GRV ORF3. Northern blot analysis of RNA isolated from green fluorescent noninoculated leaves confirmed that long distance movement of PVX.4.GFP.ΔCP indeed took place in transgenic plants expressing GRV ORF3. These results demonstrate that the GRV ORF3-encoded protein is a *trans*-acting movement factor that faciliates long distance movement of foreign RNA molecules. Moreover, experiments with transgenically expressed GRV ORF3 demonstrated that the ORF3 protein was able to mediate long-distance movement not only of TMV RNA but also of PVX-derived chimeric RNA.

**Example 3**

**PEMV-2 ORF3 protein facilitates long-distance movement of TMV RNA in *N. benthamiana* and *N.clevelandii* plants**

**MATERIALS AND METHODS**

**Generation of Chimeric cDNA Constructs**. The plasmids, pTMV(30B) and pTMV(ΔCP) were described in Example 1 (Fig. 1). A cDNA fragment containing PEMV-2 ORF3 was generated by reverse-transcription-PCR using PEMV-2 RNA as a template and oligonucleotide 5′-GCATGTCGACATCACCCGTAGTGAGAG-3′ (SEQ ID No 8), with a *Xho*I site preceding 18 nt complementary to those of the PEMV-2 RNA sequence downstream of ORF3, as a primer for synthesis of first strand cDNA and as a reverse primer for PCR, and oligonucleotide 5′-GGCCTTAATTAAATGGCGGTAGGGAAATATATGAC-3′(SEQ ID No 9), with a *Pac*I site preceding 23 nt identical to those of the 5′-end of PEMV-2 ORF3, as a forward primer for PCR. The amplified fragment was cloned between the *Pac*I and *Xho*I sites of pTMV(ΔCP) o give pTMV(ORF3P).

**Analysis of RNA.** Electrophoresis of RNA and Northern blot analysis were performed as described in Example 1 using [$^{32}$P] cDNA probes complementary to sequences of the TMV replicase gene (nucleotides 270 to 4254 of TMV RNA; TMV probe) and to sequences of the PEMV-2 ORF3 (nucleotides 2763 to 3474 of PEMV-2 RNA; PEMV probe), labelled with [$^{32}$P] using a Random Primer DNA labelling kit.

**RESULTS AND DISCUSSION**

As mentioned in Example 1, in contrast to TMV(ΔCP) which is unable to spread systemically, TMV(ORF3G) induced systemic symptoms in *N. benthamiana* plants.

TMV(ORF3P) was also shown to induce systemic symptoms (Fig. 6), and the symptoms were even more severe and appeared earlier (4-5 DPI) than those induced by TMV(ORF3G) (see Example 1). Northern blot analysis using two different probes specific to the TMV replicase gene and to PEMV-2 ORF3 confirmed the chimeric nature of TMV(ORF3P) RNA and indicated that this RNA was able to move long distances rapidly, suggesting that the PEMV-2 ORF3 protein mediated long-distance transport of foreign (TMV) RNA in infected *N. benthamiana* plants (Fig. 7).

The capability for long-distance movement of TMV(ORF3P) was then tested in another plant species, *N. clevelandii*, which is also a host for TMV and PEMV-2. As expected, TMV(ΔCP) did not induce any systemic symptoms in *N. clevelandii* even 5 weeks post-inoculation. In contrast, TMV(30B) induced in *N. clevelandii* very severe symptoms including strong stunting and deformation of leaves (Fig. 8). TMV(ORF3P) also induced systemic symptoms in *N. clevelandii* plants, although they were milder and appeared later (at approximately 10-12 days post-inoculation) than those induced by TMV(30B) (Fig. 8). These results suggest that TMV(ORF3P) spreads systemically not only in *N. benthamiana* plants but also in *N. clevelandii*. To confirm this suggestion, RNA was isolated from inoculated and upper uninoculated leaves of *N. clevelandii* and analysed by Northern blot hybridization. As expected, TMV(30B) RNA accumulated both in inoculated and uninoculated systemically infected leaves to high levels (Fig. 9). Both TMV(ΔCP) and TMV(ORF3P) also accumulated in inoculated leaves, but only to low levels compared with those of TMV(30B). Moreover, they were significantly degraded, migrating in electrophoresis as a "low molecular weight smear" rather than as bands corresponding in size to TMV RNA (Fig. 9). However, in spite of low levels of accumulation in inoculated leaves, TMV(ORF3P) RNA was clearly detected in upper systemically infected leaves (Fig. 9). TMV(ΔCP) was never detected in uninoculated leaves. These results confirm the suggestion that PEMV-2 ORF3 protein mediates long-distance movement of TMV RNA in *N. clevelandii* plants.

Thus, results presented here taken together with data on amino acid sequence similarity between the ORF3s of all Umbraviruses sequenced to date (see Example 1) show that the ability to facilitate long-distance movement of RNA molecules is characteristic not only of the GRV ORF3 protein but represents a general property of umbraviral proteins encoded by ORF3. Moreover, experiments with TMV(ORF3P) indicated that ORF3 protein may operate as a long-distance RNA transporter not only in *N. benthamiana* plants but also in other plant species, for example, in *N. clevelandii*.

## Example 4

## TMoV ORF3 protein facilitates long-distance movement of TMV RNA in *N. benthamiana*

### MATERIALS AND METHODS

**Preparation of double stranded (ds)RNA from TMoV-infected plants, sequencing and cloning of cDNA.** DsRNA was prepared from a 100g portion of TMoV-infected *N. benthamiana* leaf tissue by the method described earlier (28). The first series of cDNA clones were produced using the dsRNA denatured with methylmercuric hydroxide as template, and random deoxyribonucleotide hexamers (Boehringer) as primer for synthesis of first-strand cDNA, as described by Taliansky *et al.* (28) Following sequence analysis of clones of this first series, a second series was generated using the synthetic oligonucleotide primer 5'-CTACCGCTGGTTGATTC-3' (SEQ ID No 10) designed to match the sequence of 17 nt corresponding to the 3'-proximal part of the gene that encodes a putative TMoV RNA-dependent RNA polymerase. This primer was used for first-strand cDNA synthesis on the denatured dsRNA template. DNA fragments obtained after synthesis of second-strand cDNA were cloned and sequenced as described previously (28). Database searches with the nucleotide and amino acid sequences so obtained revealed that clone pORF3T-12 contained sequences showing similarities with the complete ORF3s of other umbraviruses.

**Generation of chimeric cDNA Constructs.** Plasmid pTMV(ΔCP) was described in Example 1 (Fig.1). A cDNA fragment containing TMoV ORF3 was generated by PCR using pORF3T-12 as template and oligonucleotide 5'-GCATCTCGAGCTAGTATTTGTTCCCATCACAG-3' (SEQ ID No 11), with a *Xho*I site preceding 22 nt complementary to those of the TMoV RNA sequence downstream of ORF3, as reverse primer for PCR, and oligonucleotide 5'-GGCCTTAATTAATGGGCAAGTGTTGTAAATGTCAAC-3' (SEQ ID No 12), with a *Pac*I site preceding 24 nt identical to those of the 5'-end of TMoV ORF3, as forward primer. The amplified fragment was cloned between the *Pac*I and *Xho*I sites of pTMV(ΔCP) to give pTMV(ORF3T). This plasmid was transcribed into RNA as described in Example 1, and the transcripts were used to inoculate plants.

**RESULTS AND DISCUSSION**

Fig. 10 and SEQ ID No 13 shows the complete nucleotide sequence of TMoV ORF3. Amino acid sequence comparisons showed that the putative product of TMoV ORF3 (26 KDa) has significant homology with other umbraviral ORF3 proteins. For example, TMoV ORF3 protein displays 34% and 35% similarity, respectively, with the corresponding proteins encoded by GRV RNA and PEMV-2 RNA. The central part of the TMoV ORF3-encoded protein, in particular, is similar to those of ORF3 proteins encoded by all other umbraviruses sequenced to date. This part consists of a very basic and highly hydrophilic domain (amino acids 93 to 116), and a hydrophobic part (amino acids 136 to 164).

As mentioned in Examples 1 and 3, in contrast to TMV(ΔCP) which is unable to spread systemically, TMV(ORF3G) and TMV(ORF3P) induced systemic symptoms in *N. benthamiana* plants. TMV(ORF3T) was also shown to induce systemic symptoms, and the symptoms were as severe as in the case of TMV(ORF3P) and appeared 4-5 DPI. These results indicate that the chimeric RNA [TMV(ORF3T)] was able to move long distances rapidly, suggesting that the TMoV ORF3 protein mediated long-distance transport of heterologous viral (TMV) RNA in infected *N. benthamiana* plants.

Thus, these results, taken together with data on amino acid sequence similarity between the ORF3s of all umbraviruses sequenced to date (GRV, PEMV-2, TMoV, CMoMV), strongly confirm that the ability to facilitate long-distance movement of RNA molecules is characteristic not only of the GRV ORF3 protein but represents a general property of proteins encoded by ORF3 of umbraviruses.

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11160.

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## Claims

1. The use of the ORF3 protein from an *Umbravirus*, or a functional equivalent thereof, to transport a pre-determined single stranded polynucleotide through the vascular system of a plant.

2. Use as claimed in Claim 1 wherein the ORF3 protein or functional equivalent thereof is derived from GRV, PEMV-2, TMoV or CMoMV.

3. Use as claimed in either one of Claims 1 and 2 wherein the pre-determined single stranded polynucleotide is non-native to said *Umbravirus*.

4. Use as claimed in any one of Claims 1 to 3 wherein the single stranded polynucleotide is positive sense single stranded RNA.

5. Use as claimed in any one of Claims 1 to 4 wherein the single stranded polynucleotide is transported as a complex which comprises the polynucleotide associated with viral-encoded cell-to-cell movement proteins.

6. Use as claimed in any one of Claims 1 to 5 wherein the single stranded polynucleotide is a viral vector encoding a protein or polypeptide of interest.

7. A recombinant polynucleotide comprising a polynucleotide which encodes the ORF3 protein of an *Umbravirus* or a functional equivalent thereof.

8. A recombinant polynucleotide which comprises the nucleotide sequence as set out in SEQ ID No 13 or

which encodes a protein having the amino acid sequence as set out in SEQ ID No 14.

9. A recombinant polynucleotide as claimed in Claim 7 which lacks a functional internal initiation codon for ORF4.

10. A recombinant polynucleotide as claimed in any one of Claims 7 to 9 wherein the ORF3 protein or a functional equivalent thereof encoded has at least 50% homology with the amino acid sequence of the ORF3 from at least one of GRV, PEMV-2, TMoV or CMoMV.

11. A recombinant polynucleotide as claimed in any one of Claims 7 to 10 which further comprises:
   a) a polynucleotide sequence encoding a protein or polypeptide of interest; and/or
   b) a polynucleotide sequence encoding a cell-to-cell movement protein.

12. A recombinant polynucleotide as claimed in Claim 11 wherein the cell-to-cell movement protein is ORF4 of the same *Umbravirus*.

13. A plant viral vector which comprises a recombinant polynucleotide as claimed in any one of Claims 7 to 12.

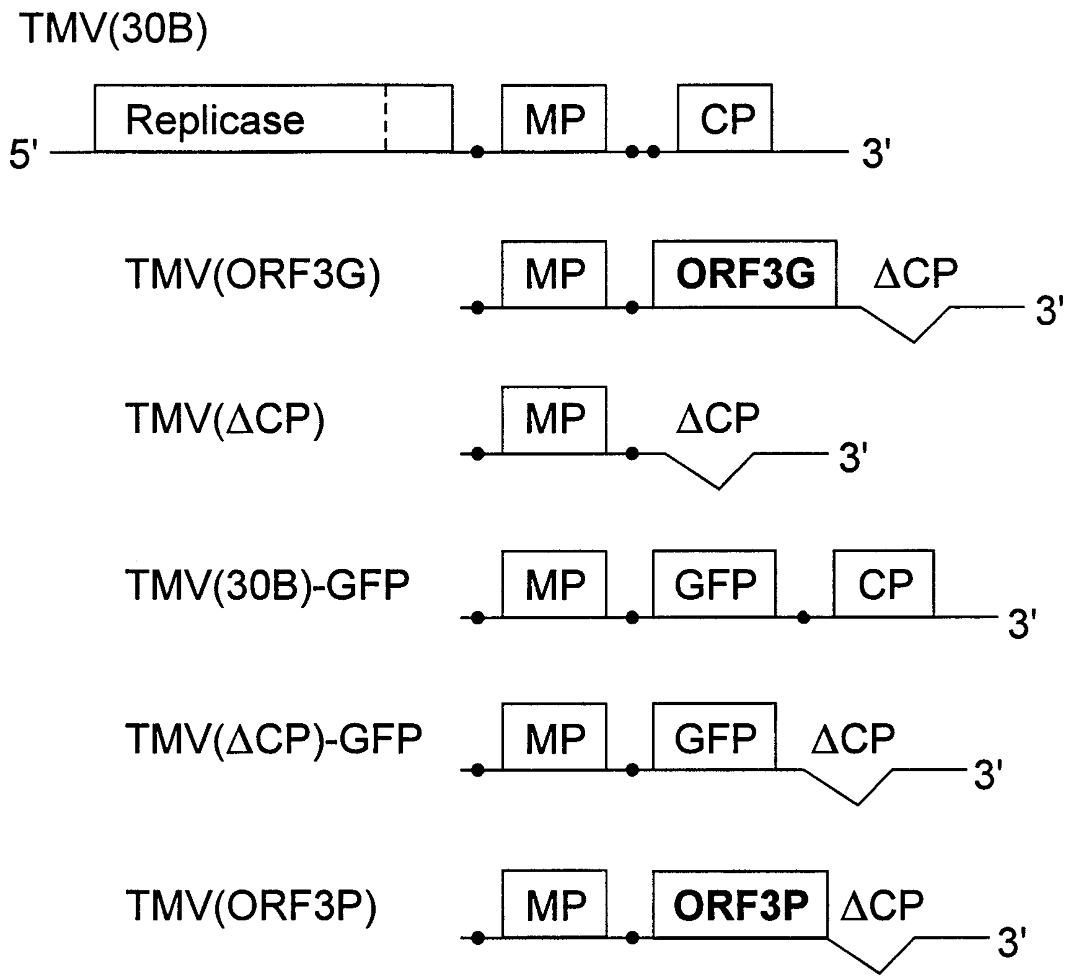
14. A plant viral vector as claimed in Claim 13 having a single stranded positive sense RNA genome.

15. A transgenic plant having a recombinant polynucleotide as claimed in any one of Claims 7 to 12 stably integrated into its genome.

16. A transgenic plant as claimed in Claim 15 wherein the recombinant polynucleotide encodes an ORF3 protein of an Umbravirus or a functional equivalent thereof, and a cell-to-cell movement protein.

17. A method of producing a target protein or polypeptide, said method comprising: introducing into a host plant cell one or more polynucleotides able to express:

a) an ORF3 protein of an *Umbravirus* or a functional equivalent thereof; and
b) a cell-to-cell movement protein; and
c) the target protein or polypeptide.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ΔCP
3'
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MP
GFP
CP
3'
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3'

*Fig. 1*

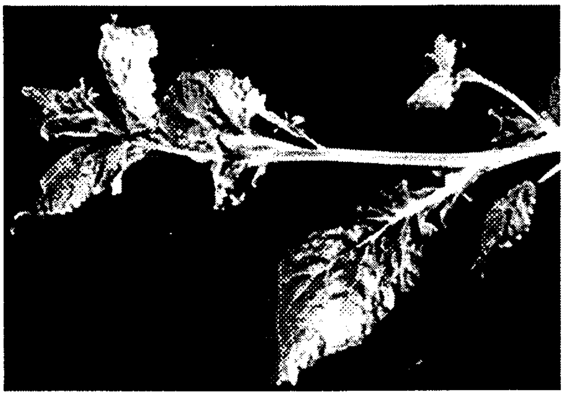


Fig. 2c

Fig. 2b

Fig. 2a

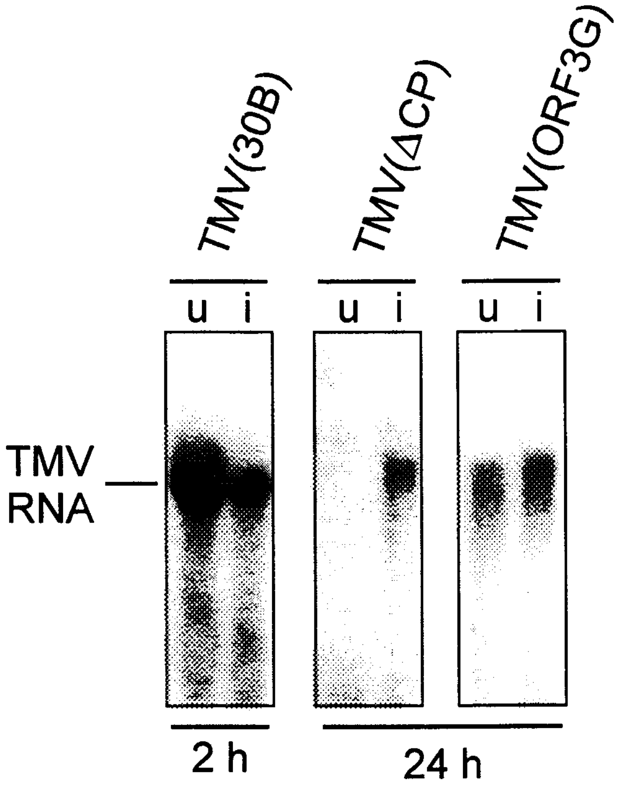



*Fig. 3*


Fig. 4a

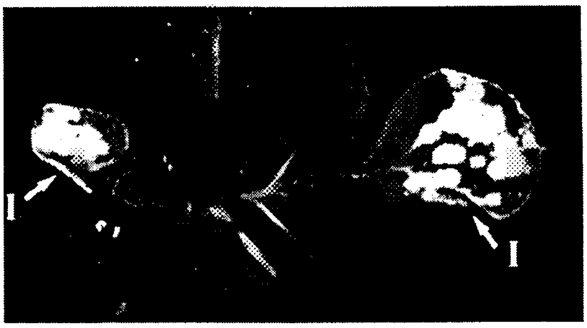
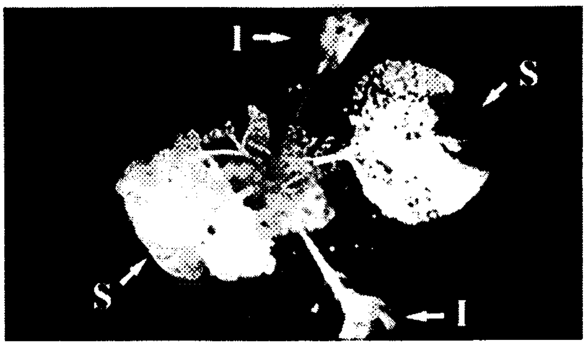



Fig. 4b

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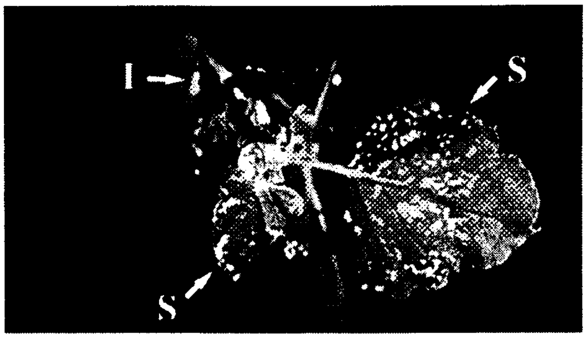



Fig. 4d


Fig. 4e

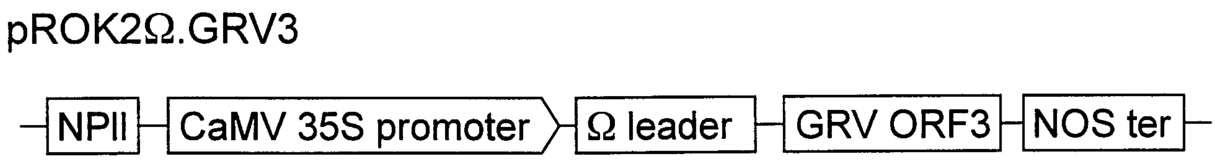



*Fig. 5*

Fig. 6a

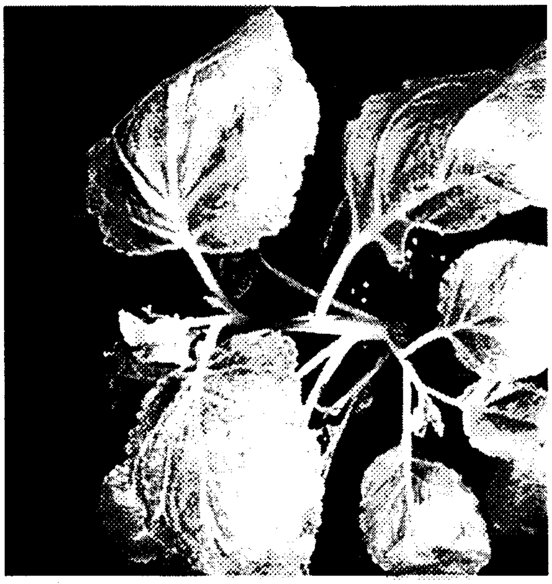
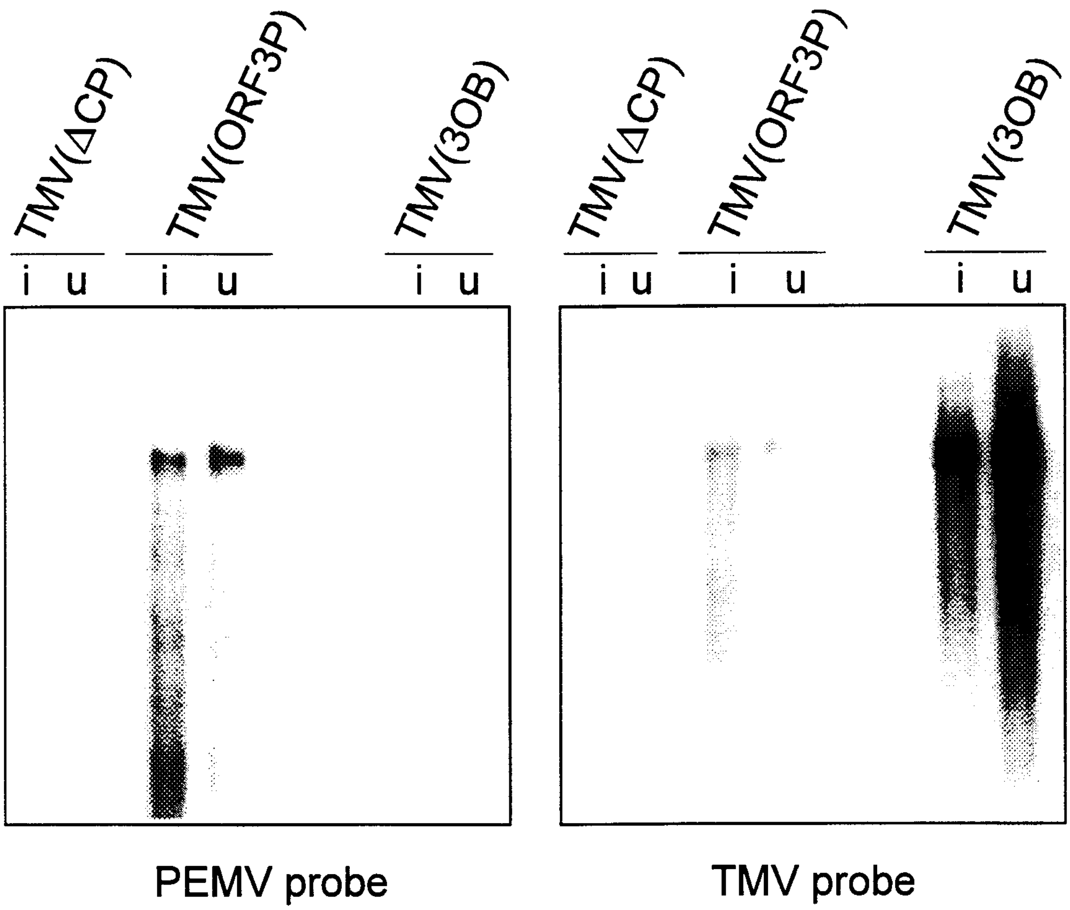


Fig. 6b

Fig. 6c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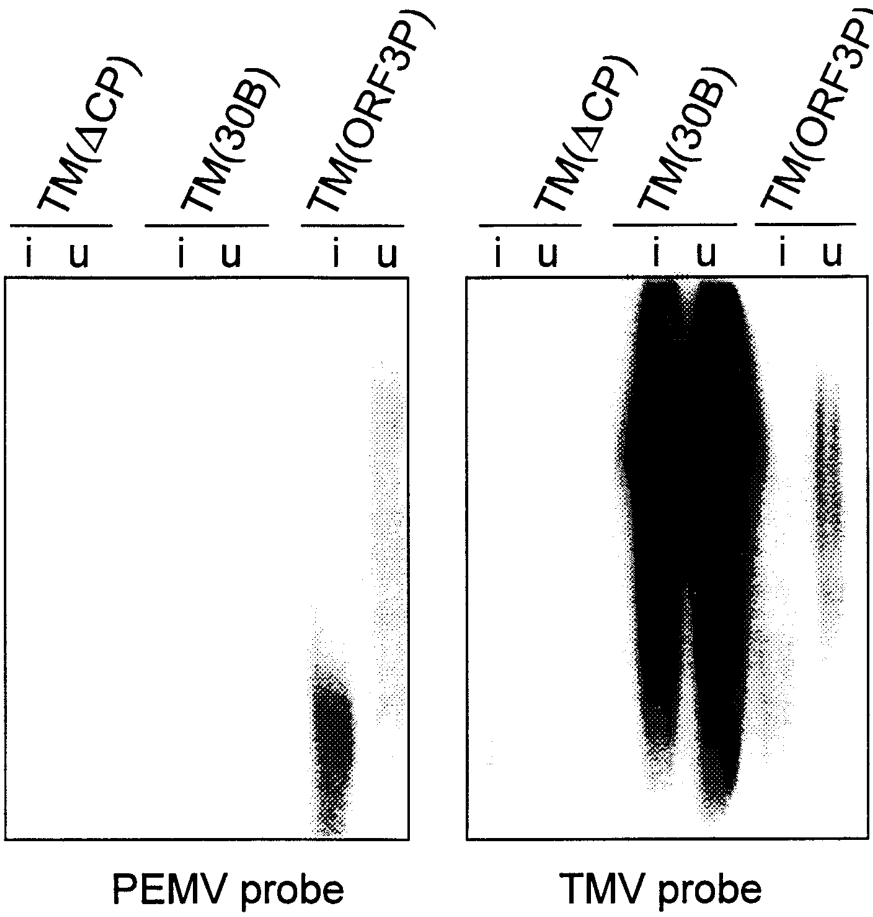
i u i u i u
PEMV probe
TMV probe

*Fig. 7*


Fig. 8a


Fig. 8b


Fig. 8c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TM(ORF3P)
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PEMV probe
TMV probe

*Fig. 9*

ATGGCAAGTGTTGTAAATGTCAACCTCAATAGTAAGAGCGGACTGCAAACAAGAACTACT
M A S V V N V N L N S K S G L Q T R T T

GGACGCCCTCTACGGGGAGGTAACTACGAAAGAACTCCAGGAAAGCAATCTAGGAGTTCT
G R P L R G G N Y E R T P G K Q S R S S

CACCCCCGTCAGAGGAAGTGCGCGGGTAACACTCACCCCGCTGCTACCCCCAGACACTCA
H P R Q R K C A G N T H P A A T P R H S

AAGCCGGCTGTCCAAGGTACTAAGGAAGTACCGCCCCACTCGCCACACAGGCGGAATGTT
K P A V Q G T K E V P P H S P H R R N V

GTTCATAGAGAGGGTGGTTATTGTTTTGACGCCCCACGTGCCGGACGATTATCCCGGCGC
V H R E G G Y C F D A P R A G R L S R R

GGTGGAGGTTTACATTCACGACAACCTACTCCCCAATCTCAACTCAATGGGCGAGAGGGT
G G G L H S R Q P T P Q S Q L N G R E G

GAGAGTAGAGCTCAATGGAGGGCCGAAGTTGATGGCCTTTTATCCCCACTACTCGATACC
E S R A Q W R A E V D G L L S P L L D T

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I V G H G W N P T K V F L R G I Q L G R

GTCTACACTCGGAACAAGCGGGGCGAGCCTATTCTCAATGTATCTGATGTGGCACCCAAA
V Y T R N K R G E P I L N V S D V A P K

CGTGGAGAGCAGGTCTCACAACTACCTGCCACAAAGCCCCAGATTACACCCAGTCTGCCG
R G E Q V S Q L P A T K P Q I T P S L P

ACACAGGGTCCAGCAAACGCTACACCTATTGGACCACCGGCAGAAGTACCTCAGCGGCGC
T Q G P A N A T P I G P P A E V P Q R R

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*Fig. 10*

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<150> UK9816096.3
<151> 1998-07-24

<160> 14

<170> PatentIn Ver. 2.1

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Thr Arg Thr Thr Gly Arg Pro Leu Arg Gly Gly Asn Tyr Glu Arg Thr
20 25 30

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Pro Gly Lys Gln Ser Arg Ser Ser His Pro Arg Gln Arg Lys Cys Ala
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Gly Asn Thr His Pro Ala Ala Thr Pro Arg His Ser Lys Pro Ala Val
50 55 60

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Gln Gly Thr Lys Glu Val Pro Pro His Ser Pro His Arg Arg Asn Val
65 70 75 80

gtt cat aga gag ggt ggt tat tgt ttt gac gcc cca cgt gcc gga cga 288
Val His Arg Glu Gly Gly Tyr Cys Phe Asp Ala Pro Arg Ala Gly Arg
85 90 95

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Leu Ser Arg Arg Gly Gly Gly Leu His Ser Arg Gln Pro Thr Pro Gln
100 105 110

tct caa ctc aat ggg cga gag ggt gag agt aga gct caa tgg agg gcc 384
Ser Gln Leu Asn Gly Arg Glu Gly Glu Ser Arg Ala Gln Trp Arg Ala
115 120 125

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Glu Val Asp Gly Leu Leu Ser Pro Leu Leu Asp Thr Ile Val Gly His
130 135 140

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Gly Trp Asn Pro Thr Lys Val Phe Leu Arg Gly Ile Gln Leu Gly Arg
145 150 155 160

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Val Tyr Thr Arg Asn Lys Arg Gly Glu Pro Ile Leu Asn Val Ser Asp
165 170 175

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Val Ala Pro Lys Arg Gly Glu Gln Val Ser Gln Leu Pro Ala Thr Lys
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Pro Gln Ile Thr Pro Ser Leu Pro Thr Gln Gly Pro Ala Asn Ala Thr
195 200 205

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Pro Ile Gly Pro Pro Ala Glu Val Pro Gln Arg Arg Asp Val Glu Gln
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225 230

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35 40 45

Gly Asn Thr His Pro Ala Ala Thr Pro Arg His Ser Lys Pro Ala Val
50 55 60

Gln Gly Thr Lys Glu Val Pro Pro His Ser Pro His Arg Arg Asn Val
65 70 75 80

Val His Arg Glu Gly Gly Tyr Cys Phe Asp Ala Pro Arg Ala Gly Arg
85 90 95

Leu Ser Arg Arg Gly Gly Gly Leu His Ser Arg Gln Pro Thr Pro Gln
100 105 110

Ser Gln Leu Asn Gly Arg Glu Gly Glu Ser Arg Ala Gln Trp Arg Ala
115 120 125

Glu Val Asp Gly Leu Leu Ser Pro Leu Leu Asp Thr Ile Val Gly His
130 135 140

Gly Trp Asn Pro Thr Lys Val Phe Leu Arg Gly Ile Gln Leu Gly Arg
145 150 155 160

Val Tyr Thr Arg Asn Lys Arg Gly Glu Pro Ile Leu Asn Val Ser Asp
165 170 175

Val Ala Pro Lys Arg Gly Glu Gln Val Ser Gln Leu Pro Ala Thr Lys
180 185 190

Pro Gln Ile Thr Pro Ser Leu Pro Thr Gln Gly Pro Ala Asn Ala Thr
195 200 205

Pro Ile Gly Pro Pro Ala Glu Val Pro Gln Arg Arg Asp Val Glu Gln
210 215 220

Val Cys Thr Thr Cys Asp Gly Asn Lys Tyr
225 230

# INTERNATIONAL SEARCH REPORT

Inter: ial Application No
PCT/GB 99/02424

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC 7 C12N15/33 C12N15/82 C07K14/08 C12N15/83 A01H5/00

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

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

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

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

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | RYABOV, E.V., ET AL. : "intracellular location of two groundnut rosette umbravirus proteins delivered by PVX and TMV vectors" VIROLOGY, vol. 242, March 1998 (1998-03), pages 303-313, XP002124271 cited in the application the whole document --- -/-- | 7,8 |

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

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 30 November 1999 | 15/12/1999 |

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

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication,where appropriate, of the relevant passages | Relevant to claim No. |
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| A | TALIANSKY,M.E., ET AL. : "complete nucleotide sequence and organization of the RNA genome of groundnut rosette umbravirus" JOURNAL OF GENERAL VIROLOGY, vol. 77, 1996, pages 2335-2345, XP002124272 cited in the application the whole document --- | 1-17 |
| A | WO 95 21248 A (SCRIPPS RESEARCH INST ;BEACHY ROGER N (US); FITCHEN JOHN H (US)) 10 August 1995 (1995-08-10) page 2, line 10 - line 11 --- | 1-17 |
| A | DEMLER,S.A., ET AL. : "the chimeric nature of the genome of Pea enation mosaic virus: the independent replication of RNA 2" JOURNAL OF GENERAL VIROLOGY, vol. 74, 1993, pages 1-14, XP002124273 cited in the application abstract; page 6,12,13; Fig. 2 + 3 --- | 1-17 |
| A | TALIANSKY, M.E., ET AL. : "role of cucumovirus capsid protein in long-distance movement within the infected plant" JOURNAL OF VIROLOGY, vol. 69, no. 2, February 1995 (1995-02), pages 916-922, XP002124274 the whole document --- | 1-17 |
| A | SKAF,J., ET AL.: "the coat protein is dispensable for the establishment of systemic infections by pea enation mosaic enamovirus" MOLECULAR PLANT MICROBE INTERACTIONS, vol. 10, no. 7, 1997, pages 929-932, XP000856484 the whole document --- | 1-17 |
| P,X | RYABOV,E.V., ET AL. : "a plant virus-encoded protein facilitates long-distance movement of heterologous viral RNA" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 96, February 1999 (1999-02), pages 1212-1217, XP002124275 the whole document ----- | 1-8,10, 11,13-17 |

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Information on patent family members

Inter nal Application No

PCT/GB 99/02424

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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| | | US 5955647 A | 21-09-1999 |