Molecular biology of the tobravirus genome

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The three viruses that make up the tobravirus genus, tobacco rattle virus (TRV), pea early-browning virus (PEBV) and pepper ringspot virus (PRV), collectively are able to infect an extremely wide range of plant species and, with the exception of PRV which until now has only been detected in South America, they occur widely in the world. In Scotland, TRV is mostly associated with a disease of potato known as spraing, whereas PEBV is found primarily as a disease of peas occurring in Eastern England. SCRI has been, and continues to be, a major centre for research into these viruses, where we are studying several of the properties which are unique to this group of viruses and which contribute to their success as plant pathogens.

Tobraviruses are one of only two types of plant viruses (the other being nepoviruses) which are moved (transmitted) from plant to plant by nematodes. Part of our research programme aims to understand what special features these viruses possess to allow transmission to occur. In addition, we are studying how different virus isolates maintain their highly specific relationships with usually only one or two nematode species. Detailed investigation of the virus genes specifying nematode transmission involves *in vitro* mutagenesis of a cDNA copy of the virus genome, and inoculation of plants with synthetic virus RNAs carrying the different mutations. To carry out this work, full-length, infectious cDNA clones of RNA2 of four viruses (PEBV TpA56, TRV PaY4, TRV PpK20 and TRV TpO1) have been constructed. Virus derived from the cDNA clones of the first three viruses can be transmitted in the glasshouse by nematodes but transmission of cloned TRV TpO1 has not yet been confirmed (although the wild-type virus stock is transmissible). Transmission tests of all four viruses using three different nematodes species has identified a complex pattern of interactions (Fig. 1). Comparison of the nucleotide sequence of each of these viruses has revealed interesting similarities in their genes, and some notable differences. Unlike many of the previously sequenced TRV isolates, which have been maintained in the glasshouse for a long time and have lost the ability to be transmitted by nematodes, these four nematode-transmissible isolates encode at least two genes in addition to the coat protein gene (Fig. 2). Downstream of the coat protein gene in PEBV TpA56 and TRV TpO1 there is a possible gene for a 9K protein. Results of previous experiments suggested that the PEBV 9K protein might be involved in the nematode transmission process. Identification of a gene encoding a similar protein in a second tobravirus increases the likelihood that it is functional, and additional experiments are underway to clarify whether the PEBV 9K gene does indeed have a rôle in transmission. Next to the PEBV 9K gene, there is a third gene encoding the 29K protein. Mutagenesis experiments indicated that this protein plays an essential rôle in

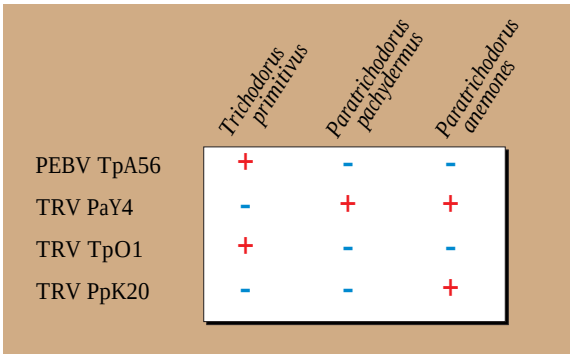

Figure 1 Transmissibility of tobravirus isolates by three different nematode species.

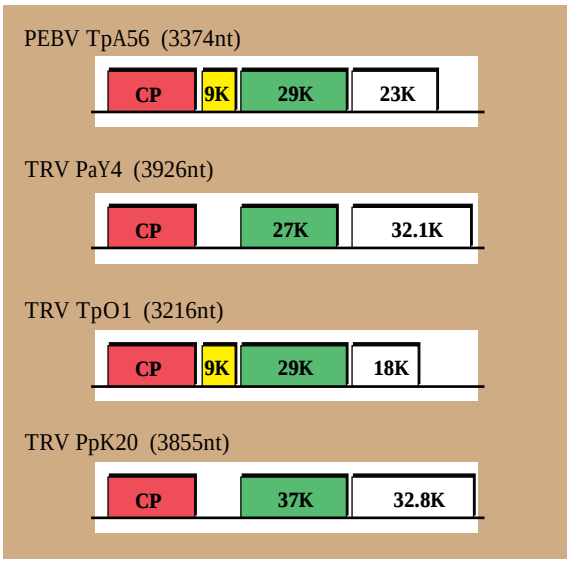

Figure 2 Genome organization of four nematode-transmissible tobravirus isolates.

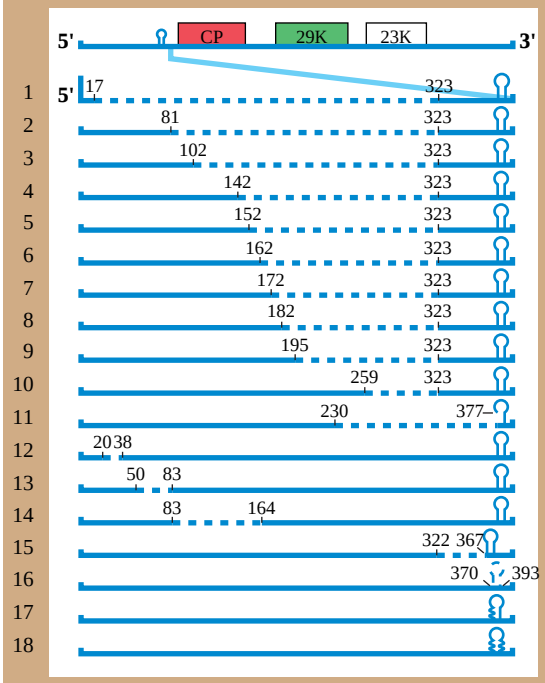

Figure 3 Depiction of mutations introduced into PEBV RNA2 to study sequences necessary for RNA replication and coat protein expression.

nematode transmission. Proteins having many sequence similarities to the PEBV 29K protein are encoded by each of the other three viruses (TRV PpK20 37K, TRV TpO1 29K, TRV PaY4 27K) and have been shown or are expected to be essential for nematode transmission. The last gene encoded by each virus is much more variable in sequence, and possibly also in function. Previously, experiments showed that a frameshift mutation of the PEBV 23K gene prevented transmission, whereas a small, internal deletion of the gene reduced transmission to a low level. We have now constructed and analysed a mutant in which the entire 23K gene has been deleted. This mutant can be nematode-transmitted but at a much reduced frequency. It is not clear how the 23K protein functions, as the deletion mutant appears to spread and accumulate within the plant in the same way as does the wild-type virus. Experiments using antibodies raised against the 23K protein have shown that it is expressed in both leaves and roots of infected plants. Interestingly, the 23K protein appears to be glycosylated, which may be important for it to function correctly in the transmission process. The equivalent genes of the other virus isolates that are being studied (TRV PpK20 33K, TRV TpO1 18K, TRV PaY4 32K) have no amino acid sequence similarities. Deletion of the TRV PpK20 33K gene did not diminish the frequency of nematode transmission, and recent experiments revealed that deletion of the TRV PaY4 18K gene also does not affect transmission by *Paratrichodorus anemones* nematodes. Our tests have shown that TRV PaY4 is transmitted by both *P. anemones* and *P. pachydermus*, whereas TRV PpK20 is transmitted only by *P. pachydermus*. One of our future aims is to investigate which parts of the TRV PaY4 coat protein and/or 27K protein enable this virus to interact with two, different vector nematodes.

Tobraviruses control the expression of the coat protein gene in an unusual way. All of the genes on RNA2, including the 5' proximal, coat protein gene, are translated from subgenomic RNAs. This arrangement appears to be unique to tobraviruses, for in all other viruses examined to date, the 5' proximal gene is expressed directly from the full-length, genomic RNA. It was previously shown that sequences at the 5' terminus of RNA2 (the 5' non-coding region, 5' NCR) are involved in the specific recognition, and subsequent multiplication, of RNA2 by the viral polymerase protein. This region also contains as yet undefined

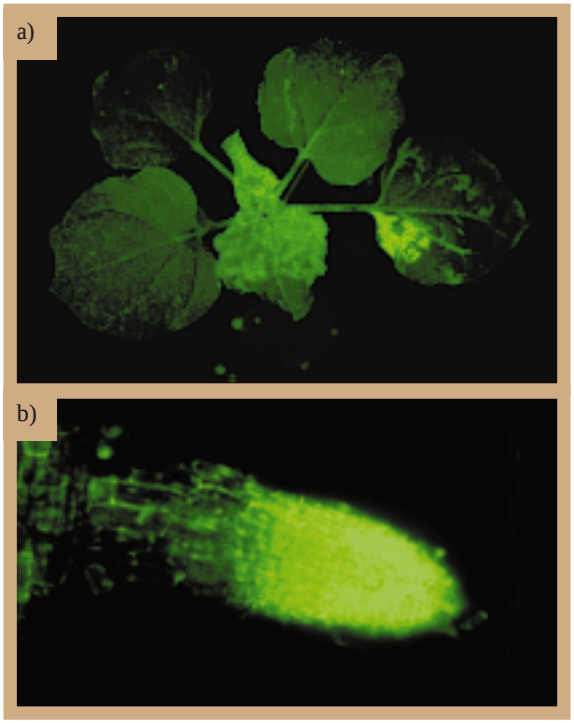

Figure 4 Expression of green fluorescent protein by a modified tobacco rattle virus. GFP fluorescence in (a) systemically infected leaves and (b) lateral root tip of *N.benthamiana*.

sequences that are required for production of the coat protein subgenomic RNA (sgRNA). A series of PEBV RNA2 mutants has been constructed which have deletions or base changes in the 5' NCR (Fig. 3). The mutants were tested for the ability to replicate in whole plants and in protoplasts, and for the capacity to express viral coat protein and the 29K and 23K nematode-transmission proteins. These experiments indicated that sequences present in the first 81 nucleotides of RNA2 are essential for it to be replicated, although deletion of some of the downstream sequences (between bases 81 to 170) reduces the efficiency of RNA2 replication. It also has been confirmed that the presence of a putative stem-loop structure located immediately upstream of the start site of the coat protein sgRNA, is essential for coat protein expression but is not involved in expression of the 29K and 23K proteins.

Plant viruses have become useful tools in agricultural biotechnology, for example by providing active sequences (cauliflower mosaic virus 35S promoter, TMV Ω translational enhancer, tobacco etch virus proteinase) or as high copy number, extrachromosomal gene expression systems (TMV and PVX). TRV possesses a number of properties which makes it a promising candidate as a gene vector. Firstly, although it has two genomic RNAs, RNA1 provides all the functions for virus multiplication, gene expression and movement in the plant. Thus, RNA2 can be extensively modified without affecting the viability of the virus. Secondly, our previous work has shown that the highly active coat protein promoter (for synthesis of the sgRNA) is separate from sequences involved in replication of the virus, and that the PEBV promoter is active when inserted into TRV and *vice versa*. A TRV-based gene expression vector has been constructed in which both of the non-structural (37K and 33K) genes have been deleted from RNA2 of TRV PpK20. In addition, a fragment carrying the PEBV coat protein promoter has been introduced immediately downstream of the TRV coat protein gene. As a first step in assessing the potential of this system, the gene for the green fluorescent protein (GFP), from the jellyfish *Aequorea victoria*, has been inserted next to the PEBV promoter. Inoculation of this construct (together with RNA1) onto plants results in infection by the virus throughout the leaf and root systems, and the concomitant expression of GFP. The GFP is expressed at a sufficiently high level that the green fluorescence is visible by eye when the plant is illuminated by UV light (Fig. 4). In contrast to some other plant viruses, the tobraviruses have evolved a very efficient mechanism for movement into and spread throughout the root system (a reflection of their strategy of nematode transmission). By studying the pattern of GFP fluorescence using the confocal laser microscope, we have shown that TRV moves to the root tip and emerges from the plant vascular system at the position where the phloem tissue is unloading solutes. This work is likely to progress in several directions. The practical use of this modified virus as a gene delivery system will involve the replacement of the GFP gene with other genes encoding, for example, proteins involved in the protection of plants against pests or pathogens. In addition, an exciting area for future scientific study will be the investigation of the virus genes which allow tobraviruses to move so efficiently into roots but which are lacking in many other types of plant virus.