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The transmission by nematodes of tobraviruses is not determined exclusively by the virus coat protein

Stuart A. MacFarlane[1], Derek J. F. Brown[1] and John F. Bol[2]
[1] *Scottish Crop Research Institute, Invergowrie, Dundee, DD2 5DA, Scotland, UK;* [2] *Gorlaeus Laboratories, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands*

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Abstract

The coat protein gene of the nematode non-transmissible, SP5 isolate of pea early-browning tobravirus was replaced with that of the highly nematode transmissible, PPK20 isolate of tobacco rattle tobravirus. Plants were infected with the recombinant virus when mechanically inoculated and the virus invaded the plants systemically. However, although the PPK20 isolate of TRV was transmitted by nematodes from these plants, the recombinant virus was not transmitted. Therefore, the virus coat protein is not the exclusive determinant of nematode transmission.

Introduction

Tobraviruses are bipartite, rod-shaped viruses which include tobacco rattle (TRV), the type member of the group, pea early-browning (PEBV) and pepper ringspot (PRV). They are transmitted by root-feeding nematodes in the genera *Trichodorus* and *Paratrichodorus* (trichodorids), and to a much lesser extent through infected seed. The viruses cause several economically important diseases, the most important of which occur in Europe in potato and flower-bulb crops [Harrison and Robinson, 1986].

The association between serologically distinct isolates of TRV and their natural vector species is highly specific [Ploeg *et al.*, 1992a]. By making reciprocal pseudo-recombinants between transmissible and non-transmissible isolates of TRV, Ploeg *et al.* [1993] showed that transmissibility segregated with RNA2. Although both virus isolates used in this study were PRN-serotypic, sequencing showed that the viral coat proteins differed at 12% of amino acid residues [J. F. Bol, unpublished]. Moreover, the proteins could be differentiated by their reaction with a specific monoclonal antibody, suggesting differences in the surface properties of the particles of the two isolates [Legorburu *et al.*, 1992]. Thus, the serological properties of the viruses were correlated with specificity of vector transmission.

The type-isolate (SP5) of English PEBV was collected from Sporle, Norfolk, England in 1964 and its natural nematode vector was identified as *Trichodorus primitivus* [Harrison, 1966]. Subsequently, this isolate was sequenced and full-length cDNA clones of both genomic RNAs were constructed, from which infectious transcripts could be prepared *in vitro* [MacFarlane *et al.*, 1991; 1992]. In addition we constructed an infectious, recombinant cDNA clone of PEBV RNA2 in which the coat protein gene of the SP5 isolate was replaced with the coat protein gene of TRV PPK20 (MacFarlane *et al.*, 1994]. Here we report on experiments to determine the transmissibility of virus derived from these clones by nematodes.

Materials and methods

Virus isolates

Isolate TRV PPK20 was from the SCRI tobravirus collection and is highly transmissible by *Paratrichodorus pachydermus* [Ploeg *et al.*, 1992a]. Isolate PEBV SP5 was derived from full-length infectious cDNA clones (pCaN1 and pCaN2) inoculated to *Nicotiana ben-*

thamiana as described by MacFarlane *et al.*, [1992]. A recombinant PEBV isolate, PEBV SP5(CPK20), in which the SP5 coat protein gene was replaced by that of TRV PPK20, was derived from infectious cDNA clones pCaN1 and pCaN2CPK20 as described by MacFarlane *et al.* [1994].

Acquisition and transmission tests

The transmissibility of PEBV SP5, PEBV SP5(CPK20) and TRV PPK20 was examined using a mixed population of *P. pachydermus* and *T. primitivus* nematodes, present in the ratio 2:1, from Woodhill, Scotland and which had previously been shown not to be associated with any tobravirus [Ploeg *et al.*, 1992b]. The virus acquisition and transmission experiments were done following the procedures described by Brown *et al.* [1989] using *Nicotiana benthamiana* source and bait plants grown singly in 25 cm^3 plastic pots. Groups of 60 nematodes were allowed access for a period of 4 wks to *N. benthamiana* virus source plants infected with the appropriate virus isolate. Subsequently, the nematodes were extracted from the soil of the source plants and groups of about 20 individuals were transferred to pots containing healthy *N. benthamiana* bait plants, again for a 4 wk period. Thereafter, the roots of the bait plants were washed free of adhering soil and nematodes and comminuted in a mortar and pestle and the resultant suspension rubbed onto the leaves of *Chenopodium quinoa* and *C. amaranticolor* indicator plants. RNA was extracted from leaves of plants showing symptoms of virus infection and was examined by northern blotting using virus specific cDNA probes [MacFarlane *et al.*, 1991] to confirm the identity of the transmitted virus.

Infectivity assay

N. clevelandii plants infected with TRV PPK20, PEBV SP5 (derived from infectious cDNA clones) or PEBV SP5(CPK20) were homogenized in tap water and mechanically inoculated to other plants. Virus was purified at 17 days post inoculation as described before [MacFarlane *et al.*, 1989]. Virus concentration was determined by absorbance at 260 nm and 50 μl aliquots of different dilutions of each virus were inoculated to *C. amaranticolor*. Three leaves per plant were inoculated, each with a different virus, and each dilution of virus was inoculated to 5 plants. The infectivity of each virus was assessed by counting the number of lesions appearing on the inoculated leaves after 3 to 5 days.

Root inoculation

Healthy 8 week old *N. benthamiana* were removed from pots, their roots washed free from adhering compost and patted dry on paper towels. A homogenate of virus infected root material was mixed with corundum abrasive powder and used to mechanically inoculate the roots of the healthy plants. After inoculation the roots were rinsed in tap water and the plants re-potted in sterile compost and grown for 4 wks. Subsequently, the inoculated root systems were washed free from adhering compost and tested for the presence of virus by inoculation to *C. amaranticolor* plants.

Results

Vector transmission tests

In the first experiment TRV PPK20 virus was recovered from all 16 bait plants exposed to groups of 20 nematodes whereas PEBV SP5 and PEBV SP5(CPK20) viruses were not recovered from any of 10 and 12 bait plants respectively. In a second experiment TRV PPK20 was recovered from 9 of 11 bait plants whereas the PEBV SP5(CPK20) was not recovered from any of 22 bait plants (Table 1). These results confirm that *P. pachydermus* is an efficient vector of TRV PPK20 and is not a vector of PEBV SP5. The recombinant PEBV SP5(CPK20) virus, incorporating the coat protein gene of TRV PPK20, was not transmitted by *P. pachydermus*. Also, *T. primitivus* was confirmed as not being a vector of the laboratory isolate of PEBV SP5 used in these experiments or PEBV SP5(CPK20).

Infectivity assay of recombinant virus

To test whether the recombinant PEBV SP5(CPK20) virus was reduced in viability compared the two parental viruses, PEBV SP5 and TRV PPK20, different dilutions of purified virus were inoculated to the common local lesion host *C. amaranticolor*. All three viruses produced a few lesions at the lowest dilution tested (10 ng/ml). At 100ng/ml PEBV SP5, TRV PPK20 and PEBV SP5(CPK20) produced *c.* 10, 30 and 60 lesions per leaf. At higher concentrations the relative infectivities of the viruses were maintained, with PEBV SP5(CPK20) producing around 3 times as many lesions as TRV PPK20 and PEBV SP5 (data not shown).

Table 1. Comparison of the transmission by *Paratrichodorus pachydermus* and *Trichodorus primitivus* of a recombinant pea early-browning tobravirus [SP5((CPK20)K20)] isolate incorporating the coat protein gene from a tobacco rattle tobravirus isolate [PPK20] with the wild type PPK20 and SP5 virus isolates

Virus RNA1	RNA2 (coat protein gene)	Acquisition Plant Nematodes (mean No./plant)		Bait Plant Nematodes (mean No./plant)		Virus in roots
		Added	Recovered	Added	Recovered	
			Experiment One			
SP5	SP5(CPSP5)	*ca.* 60	*ca.* 40	*ca.* 20	10.3	0/10
SP5	SP5(CPK20)	*ca.* 60	*ca.* 40	*ca.* 20	11.5	0/12
PPK20	PPK20(CPK20)	*ca.* 60	*ca.* 40	*ca.* 20	10.4	16/16
			Experiment Two			
SP5	SP5(CPK20)	*ca.* 60	*ca.* 40	*ca.* 20	9.8	0/22
PPK20	PPK20(CPK20)	*ca.* 60	*ca.* 40	*ca.* 20	9.9	9/11

Examination of virus in infected roots

Non-transmission of PEBV SP5 and PEBV SP5(CPK20) viruses could result from an inability of the viruses to move systemically from the inoculated leaves to the roots of the source plants. Virus would then not be available to the vector nematodes during the period when they had access to the roots. However, *C. amaranticolor* plants inoculated with suspensions of comminuted roots of source plants infected with these viruses and with TRV PPK20 produced similar numbers of local lesions, indicating that similar amounts of each virus were present in the source plant roots (Table 2). Thus, movement of the recombinant virus from inoculated leaves, and accumulation of virus in the roots was not noticeably different from that of the parental viruses.

Examination of virus by root inoculation

Transmission would also be prevented if the virus fails to infect the roots of the bait plant that the vector nematode feeds upon. To test this possibility, the roots of eight, healthy *N. benthamiana* plants were mechanically inoculated with *c.* 50 μg/ml of purified TRV PPK20, PEBV SP5 and PEBV SP5(CPK20) virus. After 16 days the roots of these plants were tested for the presence of viruses by inoculation onto the leaves of *C. amaranticolor* indicator plants. All of the inoculated plants were found to be heavily infected with virus showing that the roots of these plants were susceptible to infection by the viruses (at least by mechanical inoculation) and showing, in agreement with findings above, that all three viruses were maintained at high levels in the roots of infected plants.

Table 2. Mean, one standard deviation and minimum and maximum number of lesions, in brackets, per leaf of *Chenopodium amaranticolor* after mechanical inoculation with tobacco rattle isolate PPK20, pea early-browning virus isolate SP5, derived from full-length infectious cDNA clones [pCaN1/pCaN2], and a recombinant isolate of SP5 in which the coat protein gene had been replaced with that of PPK20 [pCaN1/pCaN2(CPK20)]. Virus was obtained from the roots of *Nicotiana benthamiana* plants previously mechanically inoculated on their leaves with these virus isolates

Virus isolate	*Nicotiana benthamiana* virus source plants		
	1	2	3
CaN1/CaN2(CPK20)	618 ± 60 (510–660)	683 ± 111 (450–82)	632 ± 80 (520–740)
CaN1/CaN2	599 ± 92 (410–710)	657 ± 93 (520–810)	616 ± 92 (470–740)
PPK20	555 ± 128 (380–790)	577 ± 79 (470–660)	645 ± 97 (510–810)

Discussion

The results obtained in our tests reveal that replacing the coat protein of a vector non-transmissible tobravirus with that of another, transmissible virus is not sufficient to confer transmission by nematodes. Therefore, the tobravirus coat protein is not the sole determinant of nematode transmission and at least one other gene product is required for successful vector transmission. This apparent requirement for a second gene product in the transmission process may be anal-

ogous to the involvement of aphid transmission factor with the caulimoviruses, or of the helper component with potyviruses [Pirone, 1991]. The genetic location of the tobravirus transmission factor(s) has not been determined. In previous experiments RNA2 was proposed to determine transmissibility, however, it is possible that an RNA1-encoded gene could act in combination with the virus coat protein to promote transmission. In addition to the replicase and cell-to-cell movement proteins, both PEBV and TRV RNA1 encode a small cysteine-rich, zinc-finger-like protein [MacFarlane *et al.*, 1989]. Previous authors have suggested that this protein plays a role in nematode transmission [Boccara *et al.*, 1986] although no data are available to support or disprove this hypothesis.

Our inability to transmit the SP5 isolate of PEBV may reflect differences between the *T. primitivus* populations from the Sporle and Woodhill sites. Alternatively, the current virus stock might have acquired a mutation somewhere in the virus genome. PEBV SP5 has been cultured for over 20 years in herbaceous plants by mechanical transmission. In experiments, serial passaging has been shown to result in the rapid production of virus mutations which prevented aphid transmission of potyviruses [Evans and Zettler, 1970], and passaging of TRV in this way has resulted in the formation of isolates with deletions in RNA2 [Harrison and Woods, 1966]. Sequencing studies and mutagenesis experiments have shown that non-transmissibility of a potyvirus can result from minor alternations to the amino acid composition of either the virus coat protein or the helper component [Atreya *et al.*, 1990; 1992]. Such experiments have not been done with tobraviruses but it is possible that a similar situation occurs when these viruses are mechanically propagated. Also, vector transmission of tobraviruses might be affected by more extensive rearrangements of the virus genome resulting from recombination occurring *in planta* [Robinson *et al.*, 1987; Goulden *et al.*, 1991].

The tobravirus RNA2 exhibits substantial variation between different isolates, perhaps because of its dispensability for virus multiplication and systemic spread (Fig. 1). The smallest RNA2 molecule sequenced to date is that of PRV (1799 nt) and the largest is that of TRV TCM (3389 nt) [Bergh *et al.*, 1985; Angenent *et al.*, 1986]. This variability reflects the different genes, in addition to the coat protein, which are present on RNA2. The RNA2 of PEBV SP5 encodes three genes in addition to the coat protein gene [Goulden *et al.*, 1990] whereas TRV TCM RNA2

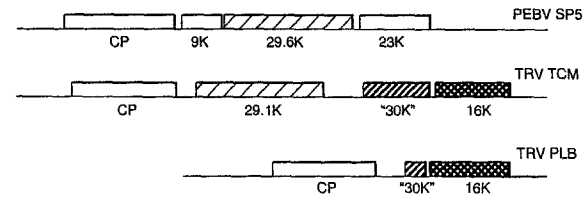

Fig. 1. Genome organisation of RNA2 of pea early-browning tobravirus strain SP5 and tobacco rattle tobravirus strains TCM and PLB. Boxes indicate the positions of open reading frames and the molecular weights are in kilodaltons. Open reading frames (ORFs) with similar hatching contain amino acid sequence homology, the ORFs denoted by '30K' contain only part of the 30K gene and the coat protein gene is denoted as CP.

encodes two additional genes, one being homologous to one of the PEBV RNA2 genes and the second being a duplication of the RNA1-encoded zinc-finger protein gene. The RNA2 of TRV PLB encodes only the coat protein gene and a duplication of the RNA1 zinc-finger protein gene [Angenent *et al.*, 1989]. TRV PLB is not transmissible (D. J. F. Brown, unpublished results) which lends support to our suggestion that genes in addition to the coat protein gene are involved in nematode transmission of tobraviruses.

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