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Characterization and vector relation of a serologically distinct isolate of tobacco rattle tobravirus (TRV) transmitted by *Trichodorus similis* in northern Greece

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Abstract

A virus isolated from diseased tobacco plants growing in Macedonia, northern Greece, had host range and physico-chemical properties typical of a tobravirus. Although it was serologically unrelated to any of the ten tobravirus isolates tested, it reacted in spot hybridization tests with a probe derived from RNA-1 of tobacco rattle virus (TRV) strain SYM. Therefore, the isolate probably represents a previously undescribed serotype of TRV. Male, female and juvenile *Trichodorus similis* nematodes recovered from the rhizosphere of the diseased tobacco plants transmitted TRV in each of three laboratory experiments. In two of these experiments 50% and 54%, respectively, of the nematodes transmitted virus to *Petunia hybrida* bait plants, whereas only 18% transmitted virus to *Nicotiana tabacum* plants in a third test. Ultrathin sections of the feeding apparatus of individual nematodes, which had transmitted virus, were examined by electron microscopy. Virus particles were observed, retained as a monolayer in the apices of the oesophageal lumen and as a group of particles within a matrix in the open part of the lumen.

Introduction

Tobraviruses, transmitted by members of the Trichodoridae, cause economically important diseases in a range of crops throughout Europe [Brown, 1989]. An unidentified *Trichodorus* species was recovered from soil collected from fallow land in Ilia, Greece, but was not reported to be associated with a tobravirus [Koliopanos and Kalyviotis-Gazelas, 1973].

In 1986, a virus was found associated with patches of diseased tobacco plants growing in Macedonia, northern Greece. The patches increased in size each year, which is characteristic of infection by soil-borne viruses in association with a natural vector [Taylor *et al.*, 1994]. Soil samples collected from the rhizosphere of diseased plants from several tobacco fields were found to contain trichodorid nematodes. Results of investigations of the isolate recovered from diseased tobacco plants, which proved to be tobacco rattle tobravirus (TRV), and its association with vector trichodorid nematodes are reported here.

Materials and methods

Virus isolates and test plants

Virus was recovered from leaves of stunted tobacco plants growing near Katerini, Mount Olympus, Macedonia, northern Greece, by mechanically inoculating expressed sap onto leaves of *Chenopodium amaranticolor* test plants. After 5 days leaves showing local lesions were harvested and expressed sap was used to inoculate *Nicotiana tabacum* cv. Samsun test plants to propagate the virus. Virus particles were purified by the method of Semancik [1966]. In host-range tests, done in Scotland and Greece and involving 24 plant species, the presence of virus in mechanically inoculated plants was checked by rubbing sap obtained from the plants onto the leaves of *C. amaranticolor* and *C. quinoa* plants. The leaves of the plants used in the host-range study were tested 7 days after being inoculated with virus and non-inoculated tip leaves after 2 to 4 weeks. All test plants were grown in aphid-proof glasshouses at 15–25 °C.

Serological tests

Antiserum to the virus was produced by injecting a rabbit intravenously with 1 mg purified virus particles followed 10 and 30 days later by subcutaneous injection of further 1 mg samples of virus particles emulsified with an equal volume of Freund's complete adjuvant. Antiserum was obtained 2 weeks after the final injection.

$F(ab')_2$ – ELISA was done as described by Ploeg *et al.* [1992] using antisera prepared against TRV strains OR3, PRN, RQ and SYM [Robinson and Harrison, 1985a], TcB2 [Ploeg *et al.*, 1992], TsNL [described as TS by Ploeg *et al.*, 1991] and TsB from roots of a *P. hybrida* bait plant infected by *T. similis* from Belgium, and PEBV strains BBYB, E116 and SP5 [Robinson and Harrison, 1985a, b]. Antiserum to strain TsNL and one that reacted with strain OR3 were obtained from C. J. Asjes, the Netherlands, antiserum to the Greek isolate was prepared at the Benaki Institute, and the other antisera were prepared at SCRI.

Spot hybridisation

Blots of sap samples were prepared and hybridized as described by Robinson and Romero [1991]. The probe was made by nick-translation of plasmid 25B [Boccara *et al.*, 1986], which contains cDNA representing 2kb close to the 3′ end of TRV strain SYM RNA-1.

Sampling, nematode extraction and vector transmission tests

Soil samples were collected at 10 to 50 cm depth from the rhizosphere of diseased tobacco plants growing at Katerini and sent by mail to SCRI. Upon receipt the samples were placed in a cold room at 4 °C. Trichodorid nematodes were extracted by a decanting and sieving method [Brown and Boag, 1988] and bait tested individually in 0.5 cm^3 plastic capsules following the method of Brown *et al.* [1989]. After 10 days the contents of each capsule were washed into a counting dish, the nematode recovered and identified, and the plant placed in a compost block in which it grew for a further three to four weeks. The bait-plant roots were then washed free of adhering compost, comminuted in a mortar and pestle and the resulting suspension rubbed, by finger, onto the leaves of *Chenopodium quinoa* and *C. amaranticolor* test plants. After 5 to 10 days leaves of test plants showing symptoms of virus infection (necrotic or chlorotic local lesions) were harvested, comminuted as before, and rubbed onto leaves of *Nicotiana clevelandii*. After a further 10 to 14 days, virus recovered from the *N. clevelandii* plants was identified by $F(ab')_2$ – ELISA, using IgG and $F(ab')_2$ fragments prepared from the antiserum against the Greek TRV isolate.

Nematode identification

Identification of the nematodes recovered from the plastic capsules was made from water-mounted specimens examined at 630 fold magnification. Thereafter, most of these nematodes, and others recovered from the soil samples but not used in the virus transmission study, were heat-killed and fixed in a hot (80 °C) formalin/glycerol mixture (1%/1% in water), processed and mounted in anhydrous glycerol on glass microscope slides. These specimens were used for morphometric analysis and several adult specimens, which had transmitted TRV to bait plants, were held in the formalin/glycerol mixture for subsequent examination by electron microscopy of identify the presence and location of retained virus particles.

Electron microscopy

Individual adult nematodes, identified as having transmitted virus to bait seedlings, were processed for examination by electron microscopy [Robertson and Henry, 1986]. Specimens were fixed in 3% glutaraldehyde in phosphate buffer, pH 7.2, post-fixed in 1% osmium tetroxide in buffer and dehydrated in ethanol, followed by infiltration in Emix resin which was polymerised at 70 °C. Ultrathin sections were cut on a microtome, stained with uranyl acetate and lead citrate and examined in a JEOL electron microscope of 80kV.

Results

Virus host range and symptomatology

All 24 species of plants mechanically inoculated with virus became infected, 12 systemically (Table 1). Symptoms were evident in the inoculated leaves of 19 species and 10 species produced systemic symptoms. Of the legumes tested, *Phaseolus aureus* and *Vicia faba*, but not *Phaseolus vulgaris*, were infected systemically.

Physico-chemical properties

Particles of the virus sedimented as two components, contained two RNA species and, in the electron microscope, had the typical appearance of tobravirus

Table 1. Symptom expression in test plants mechanically inoculated with a virus isolate from Katerini, Greece

Test plant species	Symptoms*	
	Local	Systemic
Chenopodium amaranticolor	CL or NL	–
quinoa	NL	–
Beta vulgaris	R	–
Cucurbita pepo	NL	–
Cucumis sativus	CR, NR	–
melo	CL, NL	–
Datura stramonium	NL	M
Gomphrena globosa	NL	–
Lactuca sativa	(SI)	–
Lycopersicon esculentum	(SI)	M
Momordica balsamina	NL	–
Nicotiana clevelandi	(SI)	(SI)
glutinosa	LP, NL, NR	LP, NL, NR, SNS
tabacum (Samsun)	LP, NL, NR	LP, NL, NR, SNS
(White Burley)	LP, NL, NR	LP, NL, NR, SNS
Petunia hybrida	NL, NR	CB, CH
Phaseolus vulgaris	NL	–
aureus	VN	(SI)
Solanum melongena	CR, NR	–
tuberosum	VN	SBS
Tropaeolum sp.	(SI)	M, NL
Vicia faba	NL, NR	N
Vigna sinensis	NL	–
Zinnia elegans	(SI)	M

* CB, colour breaking; CH, general chlorosis; CL, chlorotic lesions; CR, chlorotic rings; LP, line patterns; M, mottle; N, necrosis of the plant; NL, necrotic lesions; NR, necrotic rings; R, rings; SBS, stem black streaks; SNS, stem necrotic streaks; (SI), symptomless infection; VN, vein necrosis; –, not systemically infected.

particles. The properties listed in Table 2 are typical of tobraviruses, although the 28 kD particle protein is rather larger than that of most tobraviruses [22–24 kD; Robinson, 1995].

Spot hybridisation and serological relationships

In spot hybridization tests, sap extracts from plants infected with the Greek virus reacted with a probe derived from RNA-1 of TRV strain SYM. This test is specific for TRV [Robinson, 1995]. In $F(ab')_2$-ELISA tests, antiserum to the Greek Greek TRV isolate did not react with tobravirus strains representing five common serotypes of TRV and three of PEBV, nor did antisera to these strains react with the Greek isolate. Thus, it represents a previously undescribed serotype of TRV.

Nematode identification

Trichodorid specimens recovered from samples from Greece were all morphologically similar and identified as *Trichodorus similis* Seinhorst, 1963. Selected morphometrics of eight male and 18 female specimens

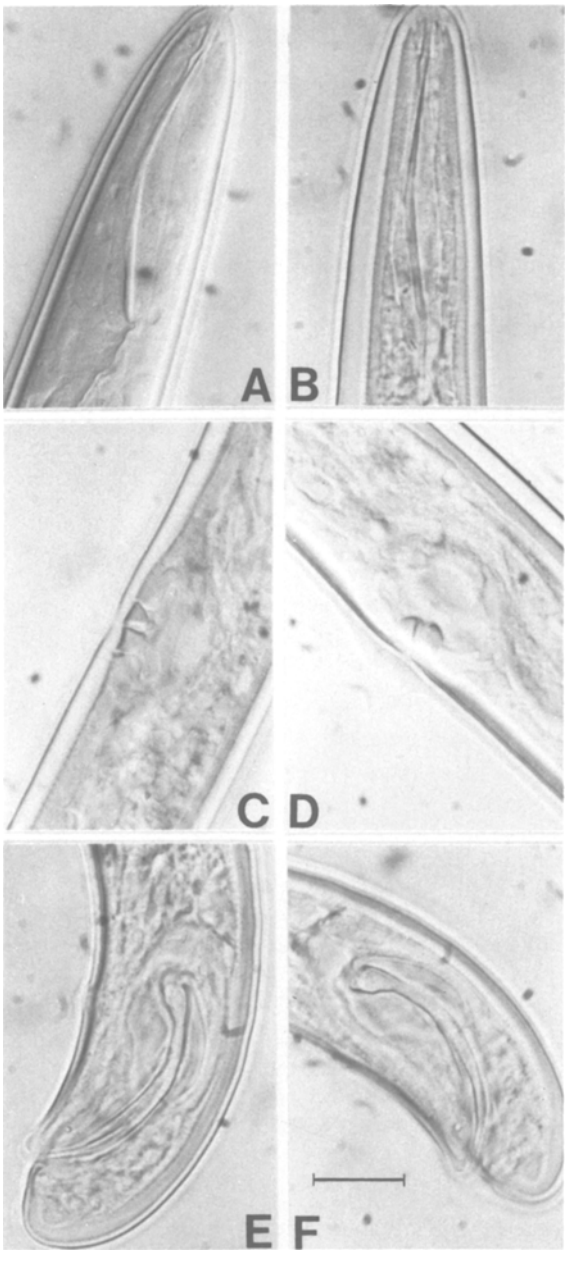

Fig. 1. Photomicrographs of *Trichodorus similis* from Katerini, Greece: A & B, anterior region of females; C & D, vulva region; E & F, posterior region of males. Scale bar represents 15 μm.

from Greece and those reported for the type specimens of *T. similis* are shown in Table 3. Photomicrographs of the female vulva and male spicule regions of specimens from Greece are given in Fig. 1. These feature are similar to those reported in the description of *T. similis*.

Virus transmission

Female and juvenile nematodes transmitted virus in each of three tests and males transmitted virus in two of the three tests (Table 4). In total, the proportion of female nematodes that transmitted virus was twice that of males, with the proportion of juvenile nematodes that transmitted virus being intermediate. Half the nematodes transmitted virus in two tests in which *Petunia hybrida* bait-plants were used whereas in a third test in which *Nicotiana tabacum* White Burley were used only about one fifth of the nematodes transmitted virus. Virus isolates recovered from all of the bait plants reacted positively in ELISA with an antiserum prepared against the Greek TRV isolate. However, when the Greek isolate was compared with two other TRV isolates that were recovered from naturally viruliferous *T. similis*, TsNL from the Netherlands and TsB from Belgium, all three were found to be serologically distinct (Table 5).

Presence and location of specifically retained virus particles

Electron microscope examination of ultra-thin sections of the feeding apparatus of nematodes which had transmitted TRV revealed virus particles in the food canal in the stylet and oesophageal regions (Fig. 2). In the oesophagus, virus particles were also found trapped in each of the tri-radiate limbs of the pump chamber. The tubular virus particles lay lengthways against the wall of the food canal and were not observed attached by their ends to the surface of the canal. Some particles were also found in groups in the central lumen and appeared to be associated with an amorphous material.

Discussion

The virus isolated from diseased tobacco plants in northern Greece was identified as an isolate of TRV on the basis of its hybridization with a cDNA probe to TRV RNA-1. Except for the size of its particle protein and its ability to infect systemically two legume species its properties were typical of TRV. It showed no serological relationship to any of the other tobraviruses tested which is further evidence of the serological diversity of tobravirus isolates [Robinson, 1989, 1995].

The virus was associated naturally with *T. similis* nematodes, and serologically similar isolates were

Table 2. Properties of a virus isolate from Katerini, Greece and of its particles

Thermal Inactivation Point (10 min)*		75 °C
Dilution End Point*		10^{-8}
Longevity *in vitro* (20 °C*		>70 days
Sedimentation coefficient $S^{\circ}_{20,w}$	L particles	290 S
	S particles	184 S
Protein: one species		28000 Daltons
Nucleic Acid: RNA, single stranded	RNA-1	2.2×10^6 Daltons**
	RNA-2	1.2×10^6 Daltons**

* As determined in *Nicotiana tabacum* cv. Samsun crude sap.
** RNA molecular weights obtained in 2.4% acrylamide gel, under non-denaturing conditions.

Table 3. Selected morphometrics* of *Trichodorus similis* from Katerini, Greece, and type specimens from Wageningen, The Netherlands (from Seinhorst, 1963)

Character	*Trichodorus similis* populations			
	Katerini, Greece		Wageningen, The Netherlands Type specimens (Seinhorst, 1963)	
	Males	Females	Males	Females
n	8	18	8	10
Body length	831 ± 46 (772–920)	834 ± 69 (726–1010)	760–870	750–830
a	30 ± 2.6 (27–35)	28 ± 2.4 (25–33)	24–30	21–27
b	6.0 ± 0.6 (5.0–6.7)	5.9 ± 0.7 (4.2–7.1)	5.2–6.3	5.0–6.0
c	102 ± 28 (72–160)	–	63–79	–
V%	–	55 ± 1.1 (53–58)	–	51–58
Onchiostyle	41 ± 1.6 (39–43)	41 ± 2.3 (38–46)	38–42	41–43
Spicule length	38 ± 3.8 (33–45)	–	36–40	–

* In micrometers; mean ± one standard deviation (minimum – maximum)

obtained from individual *T. similis* and from diseased tobacco plants. Therefore, there can be little doubt that *T. similis* is the natural vector.

In the laboratory tests, the nematodes transmitted TRV more frequently to *P. hybrida* than to *N. tabacum* White Burley seedlings. Extrapolation of the laboratory results to the field suggests that about 20% of *T. similis* present in a population are potential vectors of TRV to tobacco plants. This represents a substantial reservoir of virus-carrying nematodes available to infect field-grown plants.

The identification of *T. similis* in association with TRV and causing disease in tobacco crops in Macedonia, northern Greece, provides further evidence of the widespread distribution of this vector and its associated virus in Europe. *Trichodorus similis* occurs in Denmark, Germany, Norway, Poland, Sweden and Russia [Alphey and Taylor, 1986]. Most importantly, it

Table 4. Transmission of naturally acquired tobacco rattle virus by individual *Trichodorus similis* from a tobacco crop at Katerini, Greece

Experiment	*Trichodorus similis*			
	Males	Females	Juveniles	Proportion transmitting
One*	1/2***	2/3	3/7	0.50
Two*	1/2	3/5	3/6	0.54
Three**	0/5	3/12	4/22	0.18
Total (proportion)	2/9 (0.22)	8/20 (0.40)	10/35 (0.29)	

* *Petunia hybrida* used as bait plants.
** *Nicotiana tabacum* White Burley used as bait plants.
*** Numerator, number of nematodes that transmitted virus; denominator, the number of nematodes tested.

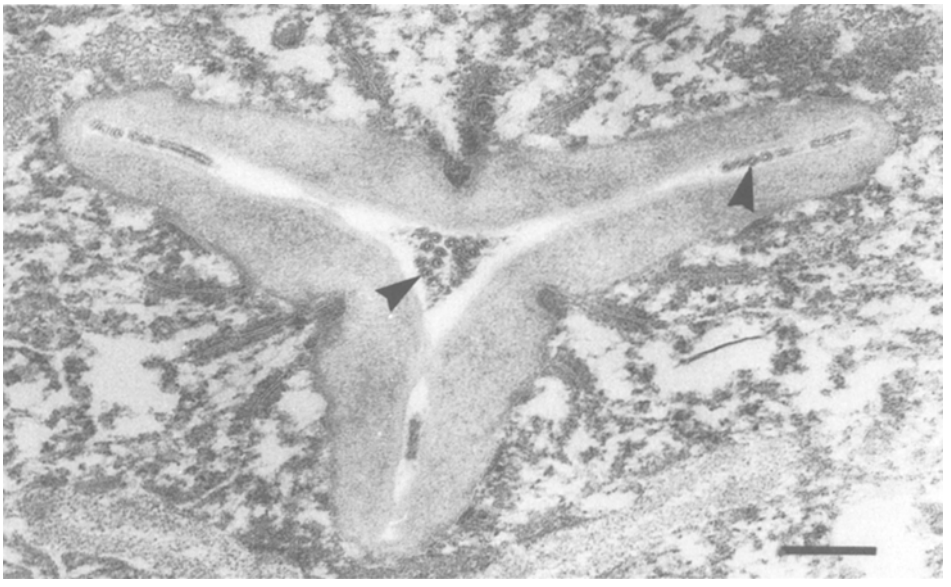

Fig. 2. Transverse section through the anterior oesophagus of a *Trichodorus similis* nematode from Katerini, Greece showing tobacco rattle virus particles trapped in the apices as a monolayer and within the open part of the lumen as a group of particles in a matrix. Scale bar represents 200 nm.

has been found transmitting TRV to potato crops, causing 'spraing' disease, in Belgium [Pelsmaeker, 1987; Pelsmaeker and Coomans, 1987], France [Van Hoof *et al.*, 1991], and the Netherlands [Cremer and Kooistra, 1964]; to gladiolus, causing notched leaf disease in the Netherlands [Cremer and Schenk, 1967] and in Dutch bulb-fields [Ploeg *et al.*, 1991]. In Britain *T. similis* recovered from a grass pasture transmitted TRV in laboratory tests [Ploeg *et al.*, 1992]. In southern Europe *T. similis* has been found in Italy [Roca and Lamberti, 1984, 1985] and Bulgaria [Choleva, 1988], but no association with TRV was demonstrated in either of these countries. The occurrence of *T. similis* in association with TRV in the Macedonian region of Greece suggests that this vector and its associated virus may occur together in Bulgaria and other adjacent countries.

Particles of TRV are specifically absorbed to the cuticular lining of the oesophagus in *Paratrichodorus pachydermus* [Taylor and Robertson, 1970] and we observed similar sites of virus particle retention in *T. similis*. Therefore, the sites of virus retention appear to be similar in the virus-vector genera *Trichodorus* and *Paratrichodorus*. Taylor and Robertson [1970]

Table 5. Serological comparisons using $F(ab')_2$ ELISA of three isolates of tobacco rattle tobravirus naturally associated with and transmitted by *Trichodorus similis*

Virus isolate*	Antiserum	
	TsGR	TsNL
TsGR	2.179**	0.259
TsNL	0.212	2.430
TsB	0.225	0.178
Healthy control	0.360	0.210

* Isolates of tobacco rattle tobravirus naturally associated with and transmitted by *Trichodorus similis* (Ts), from Greece (GR), Netherlands (NL) and Belgium (B).
** Mean absorbance values from three tests.

reported that TRV particles were attached by their ends to the surface of the food canal wall in *P. pachydermus*, which may involve flexible 'protruding' peptides at the C-terminus of the virus coat protein 'linking' the particles to the lining of the nematodes oesophagus [Mayo *et al.*, 1995]. However, in *T. similis* virus particles were observed to lie lengthways against the food canal wall, which may indicate a specific difference in the mechanism of retention of TRV by these two genera.

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