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READ-THROUGH PROTEIN GENE OF POTATO MOP-TOP FUROVIRUS IS ASSOCIATED WITH ACQUISITION AND TRANSMISSION OF THE VIRUS BY *SPONGOSPORA SUBTERRANEA* F.SP. *SUBTERRANEA*

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Abstract

Spontaneous deletion was detected in read-through domain of potato mop-top furovirus (PMTV) RNA 3 of isolate T by reverse transcription-polymerase chain reaction (RT-PCR). PCR amplification of read-through of PMTV-T and comparison with PMTV-S, a full-length isolate, indicated ~700 nucleotides difference in size but no difference in coat protein gene. Sequence analysis of the 5'-end of the read-through gene of PMTV-T relative to that of PMTV-S occurs in 3'-half of the gene and is associated with loss of transmission by the fungus vector, *Spongospora subterranea* f. sp. *subterranea*. A mono-fungal *S. subterranea* f. sp. *subterranea* was unable to acquire and transmit PMTV-T which had ~700 nucleotides deletion in 5'-half of read-through domain of PMTV RNA 3, while PMTV-S with full-length read-through gene was efficiently acquired and transmitted by the same fungus cultures. These results indicated that read-through gene encoded on RNA 3 is involved in acquisition and transmission of PMTV by its fungal vector, *S. subterranea* f. sp. *subterranea*.

Introduction

Potato mop-top virus (PMTV) has fragile tubular rod-shaped particles of two predominant lengths (100-150 nm and 250-300 nm), a tripartite, single-stranded RNA genome (Harrison & Jones, 1970; Scott *et al.*, 1994) and is a species of the genus Furovirus (Brunt, 1995). PMTV occurs in potato growing regions of Northern and Central Europe, the Andean region of South America, China, Japan (Jones, 1988), Canada (J. MacDonald, personal communication) and everywhere in the world where its fungal vector is available. PMTV was considered to be transmitted by the plasmodiophorid fungus *Spongospora subterranea* f. sp. *subterranea* (Jones & Harrison, 1969) and confirmed by acquisition and transmission experiments using a virus-free fungus culture that was derived from single cystosorus (Arif *et al.*, 1995).

The smallest genomic RNA (RNA 3) contains a single open reading frame (ORF) for coat protein (20K) terminated by an amber codon, followed by an in-phase coding region for an additional 47K read-through protein (Kashiwazaki *et al.*, 1995). RNA 2 contains four open reading frames (ORFs) which encodes protein of 51K, 13K, 21K and 8K. The first three proteins resemble the triple gene block (TGB) of some other plant viruses particularly barley stripe mosaic hordeivirus (Reavy *et al.*, 1993; Scott *et al.*, 1994). The 8K cysteine-rich protein is of unknown function. PMTV has some

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common features with other furoviruses in that it contains a coat protein/ read-through gene (CP/RT) and TGB proteins. It resembles beet necrotic yellow vein furovirus (BNYVV) and differs from soil-borne wheat mosaic furovirus (SBWMV) in having a TGB and RNA 2+3 together which are functionally equivalent to the RNA 2 of BNYVV. The RNA 3 of PMTV is not directly equivalent to RNA 2 of SBWMV as it lacks a 3'-terminal cysteine-rich protein (Jupin *et al.*, 1991; Reavy *et al.*, 1993; Shirako & Wilson, 1993; Scott *et al.*, 1994; Kashiwazaki *et al.*, 1995).

In other furoviruses such as SBWMV and BNYVV, RNA 1 and RNA 2 are involved in viral multiplication and infection in all biological hosts (Shirako & Brakke, 1984a; Kuszala *et al.*, 1986; Koenig *et al.*, 1986; Lemarie *et al.*, 1988; Tamada *et al.*, 1989). In BNYVV, RNA 3 plays a role in multiplication and translocation of the virus within the host (Lemaire *et al.*, 1988) and in symptom expression (Jupin *et al.*, 1992). RNA 4 is responsible for efficient transmission of the virus by *P. betae* (Tamada & Abe, 1989). Thus, BNYVV RNAs 1-4 are always present in naturally infected sugarbeet roots but infection of leaves of *Chenopodium quinoa* or *Tetragona expensa* by mechanical inoculation requires only RNAs 1 and 2 (Koenig *et al.*, 1986; Burgermeister *et al.*, 1986; Kuszala *et al.*, 1986; Tamada & Abe, 1989; Quillet *et al.*, 1989; Brunt & Richards, 1989). RNA 3 and RNA 4 can be eliminated intentionally from the isolate or they may undergo extensive internal deletion in the course of prolonged propagation (Bouzoubaa *et al.*, 1985; 1991; Koenig *et al.*, 1986; Tamada *et al.*, 1989; Brunt & Richards, 1989). A similar effect has been reported in SBWMV (Shirako & Brakke, 1984b; Chen *et al.*, 1994, 1995) and peanut clump furovirus (PCV) (Manohar *et al.*, 1993). SBWMV undergoes spontaneous deletions in RNA 2 upon repeated mechanical inoculation or cultivation of the virus at elevated temperatures (Shirako & Brakke, 1984b; Chen *et al.*, 1994, 1995). Recent work demonstrated that these deletions occur in the coat protein/ read-through coding region of SBWMV RNA 2 (Chen *et al.*, 1994, 1995). Tamada & Abe (1989) reported that RNA 4 is required for efficient transmission of BNYVV by *Polymyxa betae*. Later, it was shown that 75K read-though product encoded by the second ORF of RNA 2 is essential for transmission of BNYVV by *P. betae* (Tamada & Kusume, 1991; Tamada *et al.*, 1996) and virus assembly (Schmitt *et al.*, 1992). In this study, we report the occurrence of spontaneous deletion in read-through domain of PMTV RNA 3 and present evidence that this deletion affects acquisition and transmission of the virus by its fungus vector, *S. subterranea* f. sp. *subterranea*.

Materials and Methods

Virus isolates: source, propagation and maintenance: Three PMTV isolates were used in these studies; isolate T which was obtained from Scotland and its properties described by Harrison & Jones (1970) were maintained by manual inoculation to *Nicotiana debneyi* or *N. benthamiana* for more than 30 years. PMTV-R was obtained from a field-infected potato tuber (Torrance *et al.*, 1993) whereas PMTV-S was obtained by growing *N. debneyi* plants in air-dried soil (Arif *et al.*, 1994). PMTV-S and R produce severe symptoms on *N. debneyi, N. benthamiana, Chenopodium amaranticolor* and *C. quinoa* by mechanical inoculation of sap from infected plants.

PMTV-T does not produce severe symptoms on any of the above plant species but the virus did produce severe symptoms when it was originally isolated from potato cv. Kerr's Pink (Jones & Harrison, 1969).

Virus purification and RNA extraction: Virus particles of PMTV-isolate S were purified from mechanically inoculated *N. benthamiana* plants using the procedure described by Torrance *et al.*, (1993) and RNA was extracted from purified virus particles as described by Scott *et al.*, (1994). Virus particles were diluted in Tris-borate-ethylenediamine tetra-acetic acid (TE) buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA containing 0.05 M NaCl, 0.5% SDS and 0.1 mg/ml proteinase K, and incubating the mixture at 37°C for 2 hr, followed by phenol: chloroform (1:1 v/v) extraction and precipitation with ethanol. Total cell RNA was extracted from inoculated *N. benthamiana* leaves as described by Verwoerd *et al.*, (1989). RNA was precipitated with 2 vol of ethanol at -20°C, recovered by centrifugation, air dried and dissvolved in 30 μl of nuclease-free water and stored at -70°C.

Reverse transcription and polymerase chain reaction: Two micrograms of viral RNA or 5 μg of total cell RNA was used as a template for first-strand cDNA synthesis using a 22-mer oligonucleotide (TRT-252) complimentary to the 3' terminal sequence of RNA 3 (μl 2296-2315). First strand cDNA synthesis was carried out in 10 μl reaction volume using method as already described (Arif *et al.*, 1994). The total cDNA product was amplified by PCR using TRT-352 (5'-GCCAACGCTATGTCCTGATAGC-3') and TRT-66 (5'-TACGCTGGGCTGGTGCATAG-3') primers. The PCR reaction contained 200 μM each dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl and 10 mM Tris-HCl, pH 8.3 in a 50 μl reaction volume with 2.5 units of Taq DNA polymerase (AmpliTaq$^{(R)}$, Perkin Elmer or Boehringer Mannheim). PCR amplification was done for 30 cycles, each cycle was 94° for 30 sec. (denaturing), 55° for 30 sec. (annealing) and 72° for 1 min. (synthesis) in a thermal cycler (Perkin Elmer GeneAmp PCR System 9600). Five microlitres of each PCR reaction were loaded onto a 1% agarose gel and separated by electrophoresis in TE buffer containing 0.5 μg/ml ethidium bromide.

Cloning and sequencing of PCR products: DNA fragments generated by PCR amplification were separated by electrophoresis in agarose gels and purified by electroelution. The fragments were cloned with a TA Cloning kit (Invitrogen Coporation) and sequenced using Taq Dye Primer Cycle Sequencing kit in an automated DNA sequencer (model 373, Applied Biosystem, Inc.). Sequence analysis was performed using the Applied Biosystems Sequence Navigator Software. Sequence comparisons were done by using UWGCG software package (Devereux *et al.*, 1984) on the Seqnet computer at Daresbury.

Fungus transmission and assessment of virus and vector: The single cystosoral culture of *S. subterranea f. sp. subterranea* was derived from virus-free isolate N (Arif *et al.*, 1995) and maintained in tomato plants growing in sterile quartz sand with nutrient solution (Merz, 1989) in an automatic irrigation system. Carborundum-dusted leaves of *in vitro* cultured (Arif *et al.*, 1995) *N. debneyi* seedlings (3-5 wk old) were manually inoculated with PMTV isolates (T and S) and roots were exposed to virus-free, single cystosorus derived *S. subterranea* f. sp. *subterranea*. *S. subterranea* f. sp. *subterranea* infection in *N. debneyi* roots was assessed and scored as: 0= No zoosporangia seen;

1 = a few root hairs infected with zoosporangia in only few of the root pieces (trace infection); 2 = several root hairs in most root pieces infected with zoosporangia (slight infection); 3 = zoosporangia present in all samples inspected (moderate infection); 4 = zoosporangia present in all samples inspected (heavy infection). The mean of 10 randomly selected lateral pieces 2-4 mm long was used to give an estimate of *S. subterranea* f. sp. *subterranea* infection for each treatment. PMTV-infection was determined by TAS-ELISA (Torrance, 1992). Samples were considered to be positive when the A_{405} values exceeded the mean of the virus-free sample by at least a factor of two. Control treatments were: non-infected seedlings, virus-free seedlings inoculated with N culture, and *Spongospora*-free PMTV-infected *N. benthamiana* or *N. debneyi* plants.

Results

Detection of deletion in the read-through protein of PMTV-T RNA 3: Potato mop-top virus isolate S (PMTV-S) was obtained from viruliferous soil and propagated by manual inoculation on *N. benthamiana* or *N. debneyi*. RT-PCR approach was devised using primers to amplify full-length read-through protein (RT) gene (Fig. 1). PCR product of size ~2.2kb was identified in isolate S which is larger than the size of isolate T (~1.4kb). Figure 2 shows RT-PCR analysis of the read-through of PMTV-T. A PCR product of the predicted size (~1.4kb) was detected. Compartive analysis of read-through gene of PMTV-S with PMTV-T confirmed a difference in size of ~700 bp (Fig 3). The coat protein (CP) genes of these isolates were 98% homologous and there was no difference in their reactivities with the monoclonal antibodies (L. Torrance, personal communication). The CP genes of isolates S, R and T were amplified by RT-PCR using primers A838 (5'-CGGGATCCTTATGCACCAGCCCAGCGT-3') which is

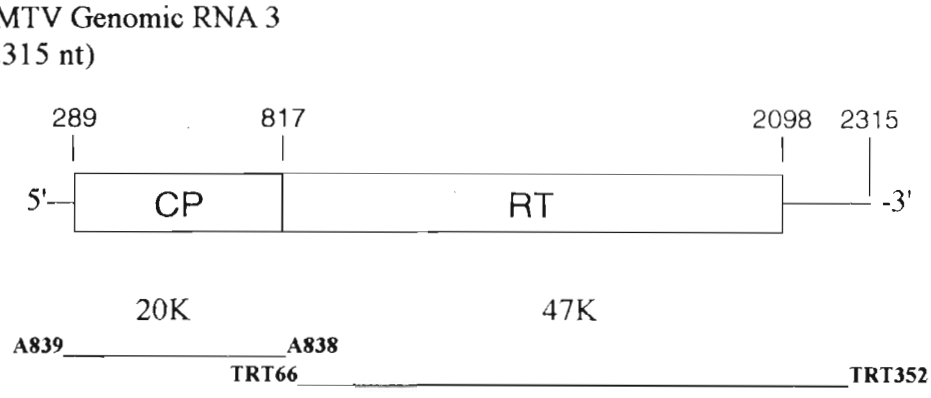

Fig.1. Schematic representation of PMTV RNA 3 showing position of coat protein (CP) and read-through protein genes (RT) with their nucleotide positions. The position of oligonucleotides primers A839 and A838 used for cDNA synthesis and amplification of CP gene and TRT-352 and TRT-66 for RT gene by reverse transcription-polymerase chain reaction (RT-PCR).

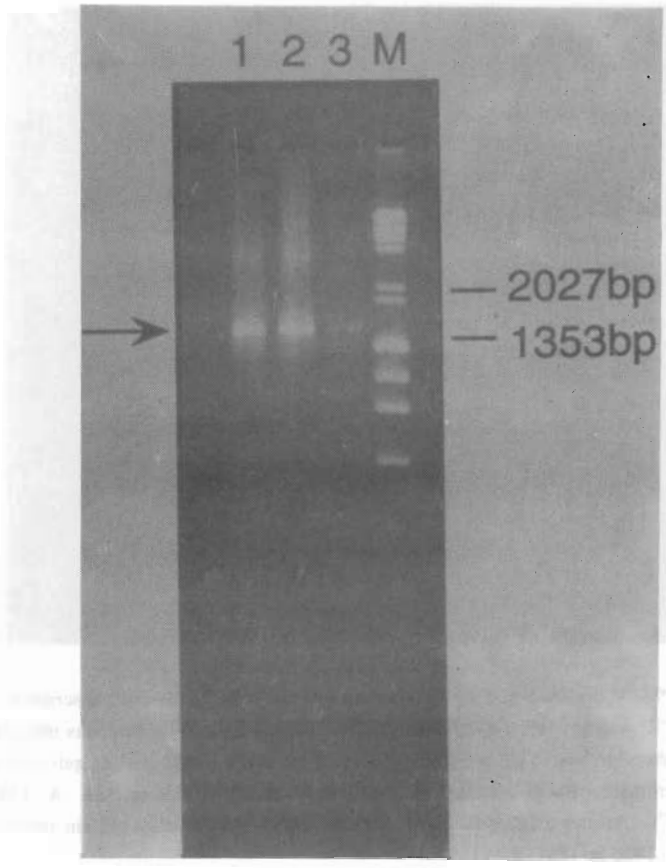

Fig.2. Detection of PMTV (isolate T) RNA 3 read-through gene by reverse transcription-polymerase chain reaction (RT-PCR) with primers TRT-352 and TRT-66 in total RNA from the virus-infected *Nicotiana benthamiana* or *N. debneyi* leaves. PCR products were electrophoresed in 1% agarose gels and stained with 0.5 μg/ml ethidium bromide. Lanes contain: M, DRIgest III molecular size marker, 1-3 PCR products from isolate T. Arrow indicate the position of the PMTV read-through protein specific ~1400 bp

complementary to the 3'-end of the CP gene (nucleotides 801-818), and A839 (5'-TCGGATCCTCTCGGGATACCACCCTT-3') which is the same as the 5' untranslated region immediately upstream of the CP gene (nucleotides 268-284; Fig. 1). There was no difference in the size of PCR products corresponding to the CP genes of isolates S, R and T (Arif *et al.*, 1994; unpublised results). All three isolates tested and four PMTV-isolates from different geographical origins indicated no size difference in the CP gene region in PMTV-T and PMTV-S (unpublished results).

Sequence analysis of the read-through gene of PMTV-S: The PCR products of PMTV-S produced, using primers TRT-352 and TRT-66, were cloned into the vector pCR (Invitrogen Corporation). The sequence of 673-nts of 5' end of the RT gene of PMTV-

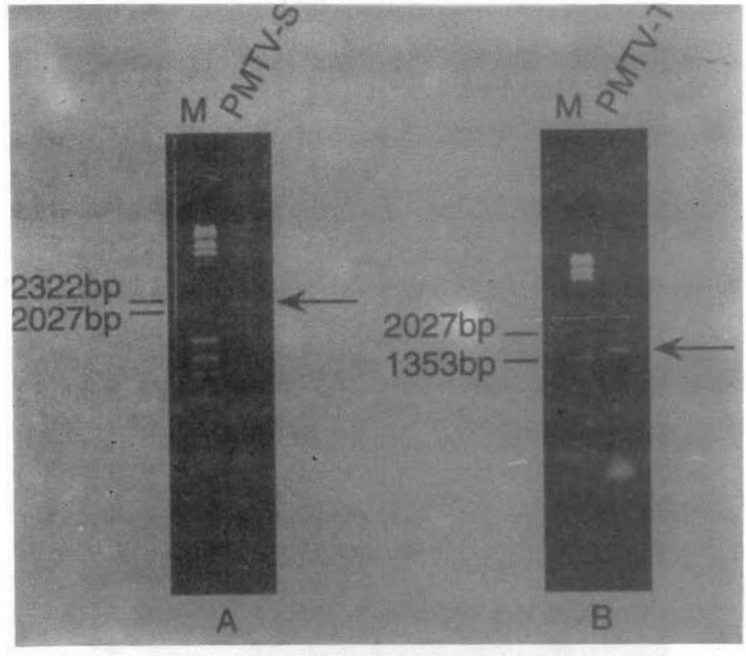

Fig.3. Detection of PMTV (isolate S and T) RNA 3 read-through gene by reverse transcription-polymerase chain reaction (RT-PCR) with primers TRT-352 and TRT-66 in total RNA from the virus-infected *Nicotiana benthamiana* or *N. debneyi* leaves. PCR products were electrophoresed in 1% agarose gels and stained with 0.5 μg/ml ethidium bromide. Lanes contain: M, DRIgest III molecular size marker. A, PMTV-S serial passage 1. B, PMTV-T. Arrows indicate the position of the PMTV read-through protein specific ~2200 bp bands (PMTV-S) and ~1400 bp (PMTV-T).

S was obtained. Comparison of this sequence with that of PMTV-T shows 97.623% sequence identity. The deduced amino acid sequence of PMTV-S was compared with that from PMTV-T and again there was very high sequence identity (97.768%) (results not shown) These results indicate that the difference of ~700 bp in the read-through gene of PMTV-T relative to that of PMTV-S must occur in the 3' half of the gene. Further work is in progress on sequencing of 3'-end of read-through gene of PMTV-S and sequence comparison with read-through gene of PMTV-T which will elucidate the deletion sites in read-through gene of PMTV-T that results in the failure of the virus acquisition and transmission by *S. subterranea* f. sp. *subterranea*.

Transmission of PMTV-isolates by *Spongospora subterranea* f. sp. *subterranea*: Successful acquisition and transmission of PMTV-isolates (S and R) were described in previous study (Arif *et al.*, 1995) by *S. subterranea* f. sp. *subterranea* culture derived from single cystosorus. In this paper the acquisition and transmission of PMTV-isolate (PMTV-T, read-through deleted mutant) by a virus-free single cystosorus culture of *S. subterranea* was tested and compared with PMTV-S12. *N. debneyi* plants were generated through micropropagation (Arif *et al.*, 1995) and transferred into sterilized

Table 1. Acquisition and transmission of PMTV in *Nicotiana debneyi* plants by virus-free *Spongospora subterranea* culture that was derived from single cystosorus.

Treatment	Acquisition A_{405}[a]		Transmission		
	Root	Leaves	Estimate of root infection by Ss[b]	F[c]	A405[d]
PMTV-S12+Ss	1.048	2.079	2.8	20/22	1.257[1]
PMTV-T+Ss	1.516	2.136	2.8	0/13	0.052[2]
Controls: Ss only	0.075	0.070	2.8	0/9	0.085[3]
PMTV-S12 only	0.065	0.071	0	0/8	0.167[4]
ELISA controls:					
PMTV-S12[e]	2.199	2.204	-	-	2.117
PMTV-T	2.149	2.029	-	-	1.825
Non-infected	0.061	0.046	-	-	0.129

[a]A405 values are the mean of 4 to 9 replicate Nicotiana debneyi plants; each plant tested over three wells. A405 values are those obtained after overnight incubation of substrate.

[b]Mean scores of Spongospora subterranea-infection on 10 randomly selected lateral root pieces 2-4 mm long.

[c]Frequency of transmission: number of plants infected/ number of plants tested.

[d]A405 values are the mean of 1=22, 2=13, 3,4=9 N. debneyi plants and are those obtained after overnight incubation of substrate.

[e]A405 value of root sample systemically infected with PMTV-S12 added to the pot as control was >2.000.

quartz sand and watered with nutrient solution (Merz, 1989) in an automatic watering system. The *N. debneyi* plants were either inoculated with PMTV-T or PMTV-S12 and virus-free *S. subterranea* was used to infect the roots on the same day. Virus and vector multiplication was assessed as previously described (Arif *et al.*, 1995). After 4-5 wk, the leaves and roots of *N. debneyi* plants were tested by ELISA for PMTV, and root infection of plants by *S. subteranea* was assessed by light microscopy. Virus was readily detected in both roots and leaves of *N. debneyi* plants and samples of 10 replicated root pieces showed moderate infection with *S. subterranea* (Arif *et al.*, 1995). Roots of these infected plants were chopped and placed on healthy *N. debneyi* plants. Results described in Table 1 indicated that PMTV-S12 was readily acquired and transmitted in plants exposed to virus- free *S. subterranea* f. sp. *subterranea* infected roots, but PMTV-T was not transmitted by *S. subterranea* f. sp. *subterranea* to bait plants exposed to the infected roots (Table 1). No virus was detected in bait plants exposed to virus-free fungus-infected, or fungus-free virus-infected control treatments (Table 1).

Discussion

These studies provide circumstantial evidence that the RT domain encoded by PMTV RNA 3 contains determinants that play an important role in acquisition and transmission of PMTV by its natural fungus vector *S. subterranea* f. sp. *subterranea*. The mono-fungal culture of *S. subterranea* f. sp. *subterranea* was unable to acquire and transmit the isolate (PMTV-T) which had ~700 nucleotides less in the RT domain, whereas the other (PMTV-S) with full-length RT was efficiently acquired and transmitted by the same fungal culture. These results similar with the evidence presented by Tamada & Kusume (1991) that 75K RT protein domain of BNYVV RNA 2 is responsible for transmission by *Polymyxa betae*. Two BNYVV RNA 2 deletion mutants appeared spontaneously during serial passage of virus on leaves and contained deletions of ~500 and ~600 nucleotides. The deletions in these mutants has been shown to fall within the C-terminal portion of the RT domain and these deleted isolates were not transmissible by *P. betae* (Tamada & Kusume, 1991).

The PMTV RNA 3 RT domain seems to be relatively stable, as the size of RT of PMTV-isolates S and R remained the same over 20 sequential passages. In contrast, deletions occured in SBWMV RNA 2 molecule rapidly and even in a single mechanical transfer of the virus from naturally infected wheat plants. The virus particles containing deletions became dominant in the culture only after a few repeated mechanical inoculations and no full-length RNA 2 remained only after 5 passages (20 wk) (Chen *et al.*, 1994). The apparent stability of PMTV may play an important role in virus persistence and survival. PMTV can persist in the vector for up to 12 years in agricultural soils which were not cropped with potatoes (Jones, 1988). However, there is no such published information available for long term field survival of SBWMV.

PMTV-S produced strong symptoms on *N. debneyi* and *N. benthamiana* and no change was recorded after one year propagation by serial mechanical passages but symptoms produced by PMTV-T on the same hosts were slightly mild. However, when isolate T was first isolated from potato, it also produced strong symptoms on *N. debneyi* and *N. benthamiana* (Jones & Harrison, 1969). Tamada & Kusume (1991) observed that symptoms produced by the deletion mutants of BNYVV in *Beta macrocarpa* were milder than those produced by wild-type virus. By contrast, SBWMV deletion mutants caused more severe symptoms in wheat than the original wild-type virus (Shirako & Brakke, 1984b; Chen *et al.*, 1994).

The involvement of CP+RT genes in virus transmission by vector has also been reported in other vector-borne plant viruses. Coat protein read-through domain of luteoviruses undergo internal deletions in the course of propagation and deleted isolates are no longer transmitted by aphids (Bahner *et al.*, 1990; Jolly & Mayo, 1994; Wang *et al.*, 1995; Brault *et al.*, 1995). McLean *et al.*, (1994) assessed the role of cucumber necrosis tombusvirus (CNV) coat protein gene in the specificity of transmission by *Olpidium bornovanus* by constructing full-length infectious clones containing reciprocal exchanges between the coat protein gene of CNV and cherry strain of tomato bushy stunt tombusvirus (TBSV-Ch). Virions containing TBSV-Ch genome with the CNV coat protein were efficiently transmitted, but those containing modified CNV genome

with TBSV-Ch coat protein gene were not. However, both viruses have different transmission mechanisms. CNV is acquired by zoospores *in vitro* and carried externally on the surface of the zoospores and resting spores (Dias, 1970; Adams, 1991) and CNV genome has no CP+RT gene. By contrast, PMTV and other furoviruses are acquired by fungi only *in vivo* from virus infected root cells by an unknown mechanism (possibly phagocytosis or pinocytosis) and carried internally in the zoospores (Shirako & Wilson, 1994) and have CP+RT genes. Further work is required to confirm these preliminary results by sequence analysis CP/RT domain of more fungally-transmissible and non-transmissible PMTV isolates. An alternative approach would be to use full-length clones of PMTV and tobacco rattle tobravirus (TRV) and perform a reciprocal exchange between the RT gene of PMTV and that of the TRV. If RT domain is the only and sole transmission determinant, then virus-free *S. subterranea* could acquire and transmit a tobravirus infectious clones with RT domain of PMTV.

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