

A naturally occurring deleted form of RNA 2 of *Potato mop-top virus*

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A spontaneous deletion in RNA 2 of *Potato mop-top virus* (PMTV) was identified by RT-PCR. The deletion occurred reproducibly during manual passage of two isolates of PMTV and during fungal inoculation of plants with viruliferous soil. The borders of the deletion were conserved in all instances and sequence analyses showed that a contiguous segment of 2113 nucleotides was deleted internally from the genomic RNA 2, leaving the 5′- and 3′-terminal sequences. RT-PCR experiments also showed that the deletion was present in preparations of PMTV particles.

The genome of *Potato mop-top virus* (PMTV) comprises three species of single-stranded, positive-sense RNA. RNA 1 encodes the replicase (B. Reavy, unpublished results); RNA 2 contains four overlapping open reading frames (ORFs), three of which encode proteins that share sequence similarity to the triple-gene-block (TGB) movement proteins of other viruses, and a small cysteine rich protein (8 kDa) (Scott *et al.*, 1994). RNA 3 contains ORFs encoding the capsid protein and a larger protein produced by translational readthrough of the capsid protein termination codon (Kashiwazaki *et al.*, 1995). PMTV is soil-borne, being transmitted by the plasmodiophorid fungus *Spongospora subterranea* f. sp. *subterranea* (Jones & Harrison, 1969; Arif *et al.*, 1995). Formerly a member of the genus *Furovirus*, PMTV has been re-classified as the type member of the genus *Pomovirus* (Torrance & Mayo, 1997; Pringle, 1998). Studies with laboratory (PMTV-T) and field (PMTV-S) isolates of PMTV have revealed a deletion in the coat protein readthrough domain of RNA 3 of PMTV-T compared to PMTV-S (Reavy *et al.*, 1998). Spontaneous deletions also occur in the genomes of other soil-borne, tubular, rod-shaped viruses, for example, in RNA 2 of *Soil-borne wheat mosaic virus* (Shirako

& Brakke, 1984), in RNA 3 and RNA 4 of *Beet necrotic yellow vein virus* (BNYVV) (Bouzoubaa *et al.*, 1985, 1991; Koenig *et al.*, 1986), and in RNA 2 of *Beet soil-borne virus* (Koenig *et al.*, 1997).

Attempts to obtain large (> 2.7 kbp) cDNA fragments of PMTV RNA 2 by an RT-PCR method consistently produced bands of smaller than expected size. This paper reports investigations to establish the nature of the smaller bands, and shows that a spontaneous deletion occurs in PMTV RNA 2 molecules.

Total RNA extracts of PMTV-T-infected *Nicotiana benthamiana* leaves at 4, 9 and 14 days post-inoculation (p.i.) were prepared by a modification of the method of Verwoerd *et al.* (1989) as described by Barker *et al.* (1993). RNA was extracted from leaf discs (0.5–1.0 g), either from systemically infected leaves, or inoculated leaves on day 4 p.i. In the first experiments, RT-PCR was done using three primer sets (Fig. 1a). 1. Primers F1 [5′ CGCTCGAGTTTAGGTGACACTATAG GTATTTCAACTCTACCTAG3′, representing the 5′-terminal 19 nucleotides of RNA 2 with attached nucleotides (underlined) to create a *Xho*I site and an SP6 RNA polymerase promoter sequence] and R2702 (5′ CCGAATTCCTGTAA-GCACTAACAC 3′, the complement of RNA 2 nucleotides 2679–2702, with the underlined nucleotide changed from G to T to create an *Eco*RI site) amplify > 91% of RNA 2 including all of the open reading frames. 2. Primers F1 and R2007 (5′ AACTGGCAAGAACAATTGGGAG 3′, the complement of RNA 2 nucleotides 1986–2007) amplify the 5′ 1.9 kb region of RNA 2. 3. Primers F1482 (5′ CATTGTGTTCTCTGAAAG-CACTCG 3′, representing nucleotides 1482–1504 of RNA 2) and R2702 amplify the 1.2 kb 3′ terminal region of RNA 2. The RT-PCR was done essentially as described by Arif *et al.* (1994) using primer R2702 or R2007 to prepare cDNA. The PCR was done in a Perkin Elmer PE9600 instrument using the following conditions: 95 °C for 5 min, then 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s, followed by 70 °C for 10 min. Only faint bands of ~ 2.7 kbp were obtained using primers F1 and R2702 (Fig. 2a; lanes 1, 2 and 3), and bands of about 600 bp (Δ 1) and 1100 bp (Δ 2) were visible on gel electrophoresis of the products from the samples extracted 9 and 14 days p.i. (Fig. 2a; lanes 2 and 3). However, the 5′ and 3′ segments could be amplified from these samples using

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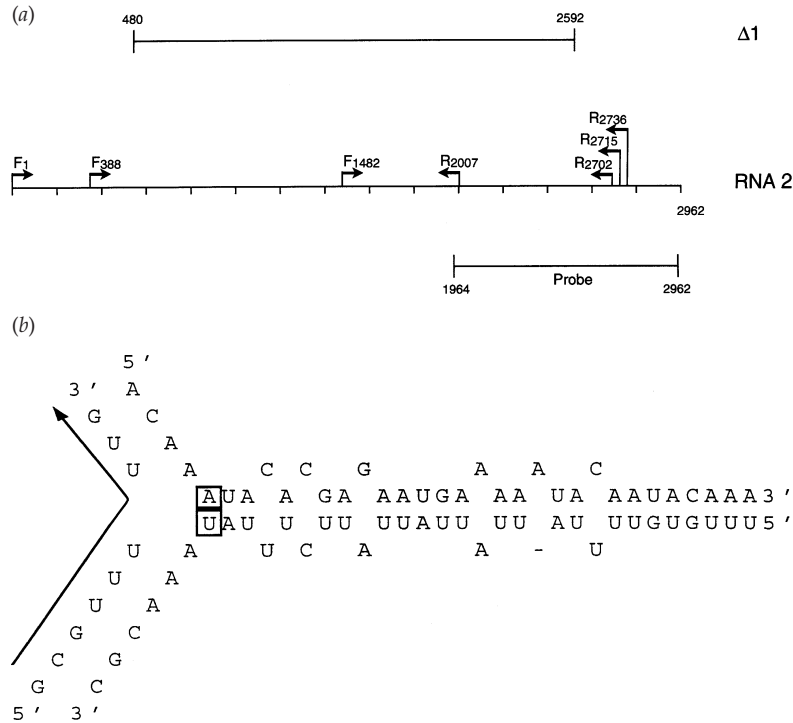


Fig. 1. (a) Diagram of PMTV RNA 2 showing the location of the primers, probe and $\Delta 1$. (b) Model for deletion formation. The complement of PMTV RNA 2 nucleotides 2563–2592 (upper strand) and of nucleotides 480–507 (lower strand) as they would occur in a minus-strand template are shown forming a stem structure with adjacent nucleotides forming a fork. The complement of nucleotide 480 (U) and of nucleotide 2592 (A) are in boxes. The growing plus-strand is shown to the left (5'-3' from bottom to top) with the arrow indicating the direction of transcription. The bend in the arrow indicates the point of template switching which deletes nucleotides 480–2592.

primer combinations F1/R2007 and F1482/R2707, which indicates that full-length RNA 2 molecules were present (Fig. 2a; lanes 6–10). The nucleotide sequence of the $\Delta 1$ product was obtained and analysis revealed that the fragment contained sequence that was identical to the 5' and 3' termini of PMTV RNA 2, and that a deletion occurred removing nucleotides 480–2592 inclusive, or 479–2591 inclusive depending on the assignment of the U residues at the junction of the deletion. The sequence of the $\Delta 2$ product was found to be identical to the 5' portion of the RNA 2 up to nucleotides 1140–1150, with no apparent deletion, and may have been the result of mispriming of primer R2702 at nucleotide 1175. To try to eliminate mispriming, the RT-PCR was repeated using F1 and either of two different downstream primers, R2715 (5' CACTAACACTTAACGTAGTGTC 3', the complement of RNA 2 nucleotides 2694–2715), or R2736 (5' CTCCAATGGTTCGATTTAAGTG 3', the complement of nucleotides 2715–2736), and an increased annealing temperature of 60 °C. The $\Delta 1$ product only was obtained with both downstream primers, and Fig. 2(b) shows the result obtained with combination F1/R2736.

A Northern blot was done with the same RNA extracts prepared at 4 or 9 days p.i. as shown in Fig. 2(a), together with a sample prepared at 10 days p.i. The extracts were hybridized with a ^{32}P -labelled cDNA probe complementary to nucleotides 1964–2962 of RNA 2 (Fig. 1a). The results showed only the presence of genomic RNA in these samples (Fig. 2c; lanes 1, 2 and 3). Thus, in these experiments, the deleted form of RNA 2 was the predominant species identified by RT-PCR but was present in infected tissues at low concentrations relative to the

full-length RNA 2. In previous work, smaller bands (0.4–0.9 kb) were found in Northern blots of total RNA extracts from PMTV-infected leaves (Arli *et al.*, 1996; unpublished results), and the presence of the smaller bands coincided with decreased amounts of genomic length RNA. PMTV is erratically distributed in host tissues, and it is possible that the abundance of deleted molecules in the culture varies with sample location or time after infection.

To exclude the possibility that the deletion occurred during cDNA synthesis, RT-PCR was performed on transcripts of PMTV RNA 2 derived from a full-length cDNA clone of RNA 2 cloned downstream of the SP6 RNA polymerase promoter (B. Reavy, unpublished results). Transcripts were made *in vitro* following the manufacturer's instructions (MEGAscript, Ambion), and then the template DNA was removed by treatment with DNase I. The cDNA was prepared using primer R2715, and the PCR was done with the increased annealing temperature of 60 °C to minimize mispriming, with primer combinations F1/R2702 or F1/R2715. Only one band of approx. 2.7 kbp was obtained using either primer set (Fig. 3a).

To test the reproducibility of the results, the experiments were repeated using primers F1 and R2702 on extracts of PMTV-T-infected *N. benthamiana* leaves taken every 2 days between 4 and 20 days p.i. and the $\Delta 1$ product was visible in all samples (data not shown). The PCR was repeated using upstream primer F388 [5' CGGGATCCGAAGTAGACCA-CACAGAGTG 3', representing nucleotides 388–407 of RNA 2 with attached nucleotides (underlined) to create a *Bam*HI site] and primer R2702 on the cDNA prepared from systemically infected leaves 12 days p.i. PCR products of about 2.7 kbp and

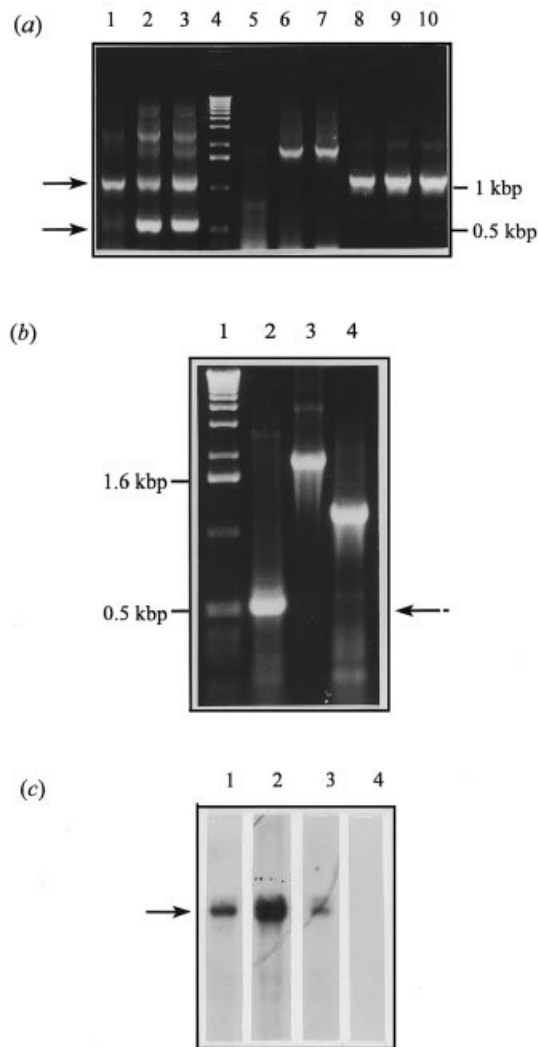


Fig. 2. (a) Products obtained by RT-PCR of RNA extracts of PMTV-infected *N. benthamiana* leaves. RNA samples were prepared 4 (lanes 1, 5 and 8), 9 (lanes 2, 6 and 9) or 14 (lanes 3, 7 and 10) days p.i., and were amplified either with primers F1 and R2702 (lanes 1–3), F1 and R2007 (lanes 5–7) or F1482 and R2702 (lanes 8–10); lane 4, molecular size markers (1 kb DNA ladder, Gibco BRL). The positions of the 1 kbp and 0.5 kbp markers are indicated on the right, and those of $\Delta 1$ and $\Delta 2$ by arrows. (b) Products obtained by RT-PCR of RNA extracts of PMTV-infected *N. benthamiana* leaves. Samples were amplified using primers F1 and R2736 (lane 2) or F1 and R2007 (lane 3) or F1482 and R2736 (lane 4). Lane 1, molecular size markers (1 kb DNA ladder, Gibco BRL). The arrow indicates the position of $\Delta 1$. (c) Northern blot of RNA extracts prepared from PMTV-infected *N. benthamiana* leaves 4, 9 and 10 days p.i. (lanes 1–3 respectively), and non-infected tissue (lane 4). The blot was hybridized with a ^{32}P -labelled cDNA probe complementary to nucleotides 1964–2962 of RNA 2. The arrow indicates the position of full-length molecules of RNA 2.

600 bp were obtained with F1/R2702 and products of 2.3 kbp and 200 bp with F388/R2702 (data not shown). Nucleotide sequencing of the 600 and 200 bp products again identified the deletion in RNA 2 of nucleotides 480–2592. Also, this result showed that the sequence of nucleotides flanking the deletion was reproducible over three independent PCR experiments.

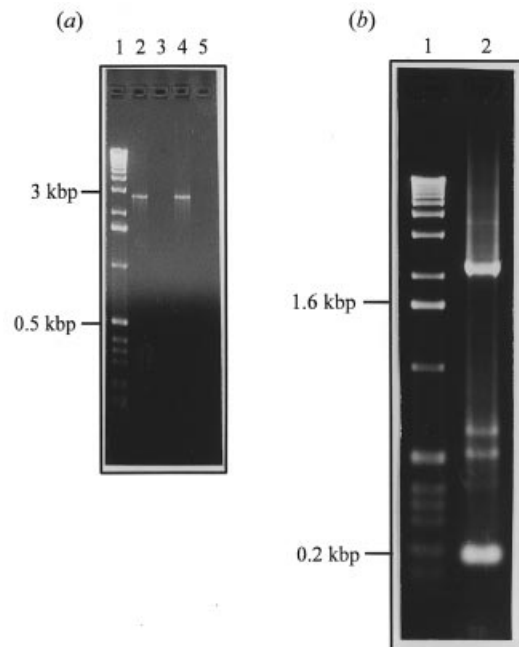


Fig. 3. (a) Products obtained after RT-PCR of RNA 2 transcripts. Lane 1, molecular size markers (1 kb DNA ladder, Gibco BRL); lanes 2 and 3, products obtained using primers F1 and R2702; lanes 4 and 5 products obtained using primers F1 and R2715. The samples in lanes 3 and 5 are control reactions and did not contain reverse transcriptase. (b) Products obtained after RT-PCR of PMTV particles; lane 1, molecular size markers (1 kb DNA ladder, Gibco BRL); lane 2, products obtained using primers F388 and R2715. The positions of the markers are indicated on the left.

We performed two further experiments to determine whether the production of a deletion in RNA 2 was peculiar to the laboratory isolate PMTV-T which has been maintained by manual inoculation for more than 30 years. In the first experiment *N. benthamiana* plants were infected with a more recently acquired isolate (PMTV-S; Arif *et al.*, 1994) that had been maintained by manual inoculation. In the second experiment, tomato cv. Kondine Red plants were inoculated with PMTV by exposure of roots to virus-carrying *S. subterranea* spore balls. Total RNA extracts were made and assayed by RT-PCR as above. A product of approx. 600 bp was obtained with primers F1 and R2702 from cDNA prepared from both the PMTV-S-infected *N. benthamiana* leaves, and the naturally infected tomato leaves (data not shown). Nucleotide sequence analysis of the 600 bp product from the infected tomato plants again revealed the same RNA 2 deletion (nucleotides 480–2592).

RT-PCR was done on purified virus particles to investigate whether the deleted forms are encapsidated. PMTV particles were obtained from infected *N. benthamiana* leaves essentially as described by Torrance *et al.* (1993) except that after treatment with chloroform the particles were precipitated by 8% (w/v) polyethylene glycol and 0.2 M NaCl followed by one cycle of differential centrifugation over a 3 ml 25% w/v sucrose cushion. The virus preparation was stored at $-20\text{ }^{\circ}\text{C}$

before use. RT-PCR was done as described above with primers F388 and R2715 either directly on the preparation, or after immunocapture of particles by incubation of the preparation for 1.5 h at 4 °C in PCR tubes (Greiner) pre-coated with anti-PMTV antibodies. A 200 bp product of the size expected for ΔI was obtained in both immunocapture and direct RT-PCR (Fig. 3b) experiments, and sequence analysis revealed the same RNA 2 deletion (nt 480–2592).

Our experiments show that a contiguous segment of PMTV RNA 2 sequence has been deleted rather than several short fragments, giving a discrete band in the RT-PCR which comprises 5' and 3' sequence moieties. Sequence deletions have been found previously in several RNA plant viruses (reviewed in Simon & Bujarski, 1994) and have been attributed to errors in RNA replication such as a 'copy choice' mechanism where the RNA polymerase dissociates and primes elsewhere on the template during RNA synthesis (King *et al.*, 1987). Mechanisms requiring either double-stranded intermolecular duplexes as occurs in *Brome mosaic virus* or sequence motifs resembling the 5' ends of genomic or subgenomic RNAs in the case of *Turnip crinkle virus* have been described (Simon & Bujarski, 1994). Examination of the PMTV RNA 2 sequences flanking the deletion did not reveal any extensive areas of sequence identity between them. However, a model can be proposed where the deletion occurs during synthesis of plus-strand RNA 2 from minus-strand RNA templates. The complement of nucleotides 2563–2592 and of nucleotides 480–507 in the minus-strand can base-pair to form a stem 29 nucleotides long with five mismatches and one unpaired nucleotide. This would bring nucleotides 480 and 2592 into direct proximity at the base of the stem and the deletion would be produced by template switching from nucleotide 479 to 2593 (Fig. 1b).

Some deleted molecules have been shown to interfere with replication of genomic RNA and have been called DI RNAs (Simon & Bujarski, 1994; Zaccomer *et al.*, 1995). Among the fungus-transmitted rod-shaped viruses, deletions are commonly found in the readthrough domain of the capsid protein, and such deletions have been correlated with lack of fungus transmissibility (Tamada *et al.*, 1996). Artificial DI RNAs derived from BNYVV RNA have been produced, and the RNA-2-derived molecules were shown to inhibit replication of genomic RNA 1 and 2 (Hehn *et al.*, 1994). It is not known whether the ΔI deleted form of RNA 2 identified here is a true DI in that it inhibits genomic replication. Although the deletion occurs spontaneously, it was present in cultures obtained by fungal inoculation as well as in a laboratory culture maintained by manual inoculation for many years, and is encapsidated.

Deletion of nucleotides 480–2592 in RNA 2 removes the entire TGB sequence (apart from the 5' 110 nucleotides of ORF 1) but results in an in-frame fusion of the 5' end of ORF 1 with the 3' end of ORF 4. The TGB, by analogy with other viruses, is thought to encode proteins responsible for virus movement. PMTV is known to be erratically distributed in plants and can

be found in some stems but not others from the same plant (Torrance *et al.*, 1992). Furthermore, PMTV is gradually self-eliminating from potato stocks if sources of re-infection are removed (Calvert, 1968; Cooper *et al.*, 1976). Deletion of the TGB sequence from the virus culture may explain this behaviour.

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References

- Arif, M., Torrance, L. & Reavy, B. (1994). Improved efficiency of detection of potato mop-top furovirus in potato tubers and in the roots and leaves of soil-bait plants. *Potato Research* **37**, 373–381.
- Arif, M., Torrance, L. & Reavy, B. (1995). Acquisition and transmission of potato mop-top furovirus by a culture of *Spongospora subterranea* f. sp. *subterranea* derived from a single cystosorus. *Annals of Applied Biology* **126**, 493–503.
- Arli, M., Reavy, B. & Torrance, L. (1996). Studies on potato mop-top virus replication. In *Proceedings of the Third Symposium of the International Working Group on Plant Viruses with Fungal Vectors*, pp. 57–60. Edited by J. L. Sherwood & C. M. Rush. Denver, CO: American Society of Sugar Beet Technologists.
- Barker, H., Webster, K. D. & Reavy, B. (1993). Detection of potato virus Y in potato tubers: a comparison of polymerase chain reaction and enzyme-linked immunosorbent assay. *Potato Research* **36**, 13–20.
- Bouzoubaa, S., Guilley, H., Jonard, G., Richards, K. & Putz, C. (1985). Nucleotide sequence analysis of RNA 3 and RNA 4 of beet necrotic yellow vein virus, isolates F2 and G1. *Journal of General Virology* **66**, 1553–1564.
- Bouzoubaa, S., Niesbach-Klößgen, U., Jupin, I., Guilley, H., Richards, K. & Jonard, G. (1991). Shortened forms of beet necrotic yellow vein virus RNA-3 and -4: internal deletions and a subgenomic RNA. *Journal of General Virology* **72**, 259–266.
- Calvert, E. L. (1968). The reaction of potato varieties to potato mop-top virus. *Records of Agricultural Research Ministry of Agriculture Northern Ireland* **17**, 31–40.
- Cooper, J. I., Jones, R. A. C. & Harrison, B. D. (1976). Field and glasshouse experiments in the control of potato mop-top virus. *Annals of Applied Biology* **83**, 215–230.
- Hehn, A., Bouzoubaa, S., Jonard, G., Guilley, H. & Richards, K. E. (1994). Artificial defective interfering RNAs derived from RNA2 of beet necrotic yellow vein virus. *Archives of Virology* **135**, 143–151.
- Jones, R. A. C. & Harrison, B. D. (1969). The behaviour of potato mop-top virus in soil, and evidence for its transmission by *Spongospora subterranea* (Wallr.) Lagerh. *Annals of Applied Biology* **63**, 1–17.
- Kashiwazaki, S., Scott, K. P., Reavy, B. & Harrison, B. D. (1995). Sequence analysis and gene content of potato mop-top virus RNA 3: further evidence of heterogeneity in the genome organization of furoviruses. *Virology* **206**, 701–706.
- King, A. M. Q., Ortlepp, S. A., Newman, J. W. I. & McCahon, D. (1987). Genetic recombination in RNA viruses. In *The Molecular Biology of Positive Strand RNA Viruses*, pp. 129–152. FEMS Symposium no. 32. Edited by D. J. Rowlands, M. A. Mayo & B. W. J. Mahy. London: Academic Press.
- Koenig, R., Burgermeister, W., Weich, H., Sebald, W. & Kothe, C. (1986). Uniform RNA patterns of beet necrotic yellow vein virus in

- sugar beet roots, but not in leaves from several plant species. *Journal of General Virology* **67**, 2043–2046.
- Koenig, R., Commandeur, U., Loss, S., Beier, C., Kaufmann, A. & Leseman, D.-E. (1997).** Beet soil-borne virus RNA2: similarities and dissimilarities to the coat protein gene-carrying RNAs of other furoviruses. *Journal of General Virology* **78**, 469–477.
- Pringle, C. R. (1998).** The universal system of virus taxonomy of the International Committee on Virus Taxonomy (ICTV), including new proposals ratified since publication of the Sixth ICTV Report in 1995. *Archives of Virology* **143**, 203–210.
- Reavy, B., Arif, M., Cowan, G. H. & Torrance, L. (1998).** Association of sequences in the coat protein/read-through domain of potato mop-top virus with transmission by *Spongospora subterranea*. *Journal of General Virology* **79**, 2343–2347.
- Scott, K. P., Kashiwazaki, S., Reavy, B. & Harrison, B. D. (1994).** The nucleotide sequence of potato mop-top virus RNA 2: a novel type of genome organization for a furovirus. *Journal of General Virology* **75**, 3561–3568.
- Shirako, Y. & Brakke, M. K. (1984).** Spontaneous deletion mutation of soil-borne wheat mosaic virus RNA II. *Journal of General Virology* **65**, 855–858.
- Simon, A. E. & Bujarski, J. J. (1994).** RNA–RNA recombination and evolution in virus-infected plants. *Annual Review of Phytopathology* **32**, 337–362.
- Tamada, T., Schmitt, C., Saito, M., Guilley, H., Richards, K. & Jonard, G. (1996).** High resolution analysis of the readthrough domain of beet necrotic yellow vein virus readthrough protein: a KTER motif is important for efficient transmission of the virus by *Polymyxa betae*. *Journal of General Virology* **77**, 1359–1367.
- Torrance, L. & Mayo, M. A. (1997).** Proposed re-classification of furoviruses. *Virology Division News. Archives of Virology* **142**, 435–439.
- Torrance, L., Cowan, G. H., Scott, K. P., Pereira, L. G., Roberts, I. M., Reavy, B. & Harrison, B. D. (1992).** Detection and diagnosis of potato mop-top virus. *Annual Report of Scottish Crop Research Institute for 1991*, pp. 80–82.
- Torrance, L., Cowan, G. H. & Pereira, L. G. (1993).** Monoclonal antibodies specific for potato mop-top virus and some properties of the coat protein. *Annals of Applied Biology* **122**, 311–322.
- Verwoerd, T. C., Dekker, B. M. M. & Hoekema, A. (1989).** A small scale procedure for the rapid isolation of plant RNAs. *Nucleic Acids Research* **17**, 2362.
- Zaccomer, B., Haenni, A.-L. & Macaya, G. (1995).** The remarkable variety of plant RNA virus genomes. *Journal of General Virology* **76**, 231–247.

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