European Journal of Plant Pathology **103:** 721–724, 1997.

A bymovirus PCR primer and partial nucleotide sequence provides further evidence for the recognition of rice necrosis mosaic virus as a bymovirus

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Accepted 18 July 1997

Abstract

Rice necrosis mosaic virus (RNMV) has physico-chemical and biological properties typical of the *Bymovirus* genus. However, to date no sequence data has been reported for RNMV. In this paper we report the use of a PCR primer, designed from an area of high homology between bymoviruses and related macluraviruses within the NIb gene, which generated a 1.4kb fragment from the 3' terminal region of RNA 1 of RNMV. The sequence data generated from this region confirmed the classification of RNMV as a member of the *Bymovirus* genus.

Rice necrosis mosaic virus (RNMV) has been classified as a member of the *Bymovirus* genus of the *Potyviridae* (Usugi et al., 1989), based on its biological characteristics and particle morphology. It is transmitted by the fungus *Polymyxa graminis* and has flexuous rod-shaped particles with two modal lengths of 275nm and 550nm (Inouye and Fujii, 1977). It was isolated in Japan from rice, the only known host, showing mosaic symptoms characterised by spindle shaped yellow flecks and streaks on the lower leaves. RNMV is serologically weakly related to barley yellow mosaic virus (BaYMV) and wheat yellow mosaic virus (WYMV) (Usugi and Saito, 1976). To date, no sequence data has been reported for RNMV.

In order to provide a rapid method to detect all bymoviruses which could also be utilised to provide sequence information on new or existing viruses, it was decided to design a universal bymovirus PCR primer. Such a technique has been proved to be a powerful tool in the identification of plant viruses (Rybicki and Hughes, 1990; Langeveld et al., 1991; Badge et al., 1996). Little homology exists between the bymovirus coat protein genes, hence the nuclear inclusion body b (NIb) gene was utilised to design a primer specific to the bymoviruses and that would be unlikely to cross react to the potyviruses. As sequence data in this region was available for only three distinct bymovirus, it was decided to compare this region to a newly proposed genus, the macluraviruses (Badge et al., 1997), which have been shown to have high homology with the bymoviruses within the NIb gene. All positive single-stranded RNA viruses share a consensus sequence (GDD) which is thought to represent the RNA-dependent RNA-polymerase binding site (Dougherty and Carrington, 1988). The bymoviruses and macluraviruses are unique in that they share an extended consensus around this motif (FVCN**GDD**NK). The nucleotide sequence is particularly well conserved here and is degenerate at only 6 positions out of 24 (Figure 1). This sequence was therefore used as a primer for amplification of the 3′ terminus of RNMV RNA 1, and incorporated five of the six degenerate positions. The bymo-, macluravirus specific primer was denoted 'NGDD'.

The primer was tested on total nucleic acid from tissue infected with bymoviruses. Two isolates of barley mild mosaic virus (BaMMV-Ka1 and BaMMV-Na1; Kashiwazaki et al., 1992) and a BaYMV isolate (BaYMV-II-1; Kashiwazaki et al., 1989) were propagated in mechanically inoculated barley plants (cv. Ishukushirazu for BaMMV or cv. New Golden for BaYMV). RNMV was propagated in rice plants (cv. Akebono) sown in naturally infested soil collected in Okayama, Japan. Total nucleic acid was extracted from approximately 10mg of leaf tissue with a Sepa Gene nucleic acid

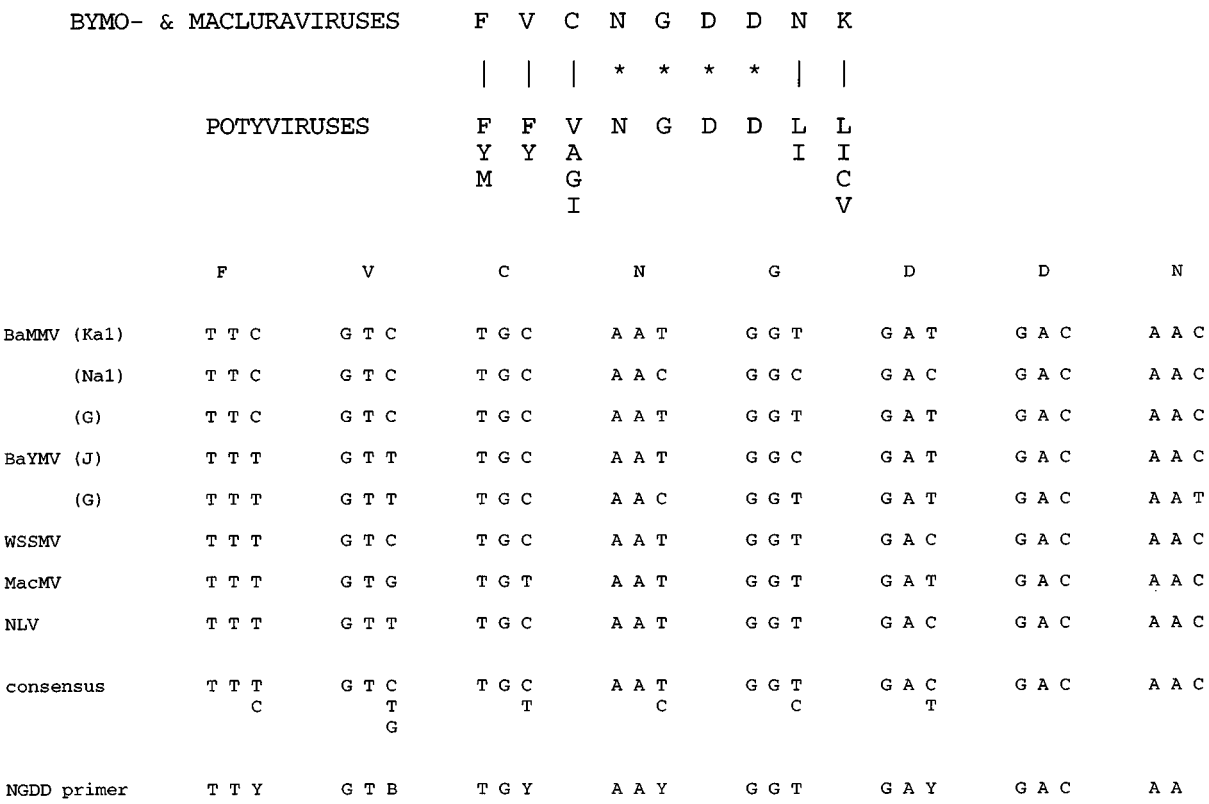

Figure 1. Diagram to show the design of the 'NGDD' PCR primer. Alignment of the amino acid sequence around the 'FVCNGDDNK' motif in the nuclear inclusion body b (NIb) gene shows that there is consensus among the bymoviruses and macluraviruses, but variation among the potyviruses, with the nucleotide sequences also being conserved in this region. The 'NGDD' primer based on this consensus sequence is indicated (Y=C/T, B=C/T/G). Three strains of barley mild mosaic virus (BaMMV) were used: Japanese isolates Na 1 and Ka 1 (Kashiwazaki et al., 1992) and German (G) isolate (Schlichter et al., 1993); two strains of barley yellow mosaic virus (BaYMV): Japanese (J) (Kashiwazaki et al., 1990) and German (G) (Peerenboom et al., 1992) and a third bymovirus WSSMV (Sohn et al., 1994). The macluravirus sequences are from MacMV and NLV (Badge et al., 1997).

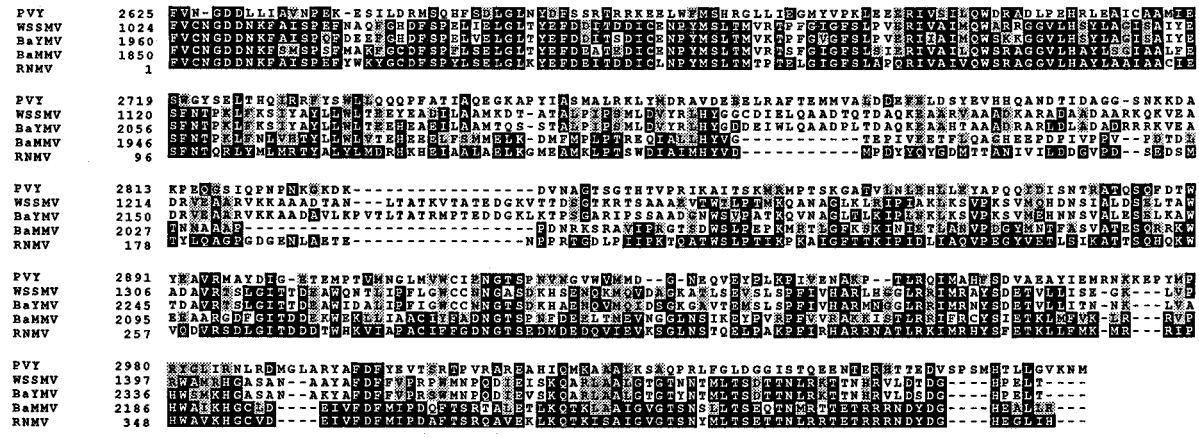

Figure 2. CLUSTAL W alignment of the partial ORF of RNMV (GenBank: U95205) with three bymoviruses sequenced to date, barley mild mosaic virus (BaMMV: GenBank D1049), barley yellow mosaic virus (BaYMV: GenBank D01091), wheat spindle steak mosaic virus (WSSMV: GenBank X73883) and a potyvirus, potato virus Y (PVY: GenBank A08776). Gaps (–) have been introduced for maximum alignment and the program BOXSHADE was used to create boxing. Residues identical to RNMV are boxed with a black background, chemically similar residues are boxed in grey.

extraction kit (Sanko Junyaku, Japan) (Kashiwazaki and Hibino, 1996). RT-PCR was performed as previously described by Badge et al., (1996) using oligo d(T)Not I (5′-CAATTCGCGGCCGC$(T)_{15}$-3′) primed first-strand cDNA and 'NGDD' primers (5′-TTYGTBTGYAAYGGTGAYGACAA-3′: Y=C/T, B=C/T/G). This generated products of the expected size (1.5 kb) from two isolates of BaMMV and one isolate of BaYMV. A similar result was obtained with the positive control, pNLVdT1, a cDNA clone from narcissus latent virus, known to contain the 'FVC-NGDDN' and oligo-d(T)Not I primers, generating the expected 1.6 kb product. RNA extracted from uninfected barley did not generate a product (results not presented).

With a RNMV-infected sample a PCR product of 1.4 kb was obtained, whereas no product was obtained from a uninfected rice sample. The RNMV product was cloned using pCR-SCRIPT (Stratagene) and three independent clones were selected (pRNMV4, pRNMV1A29 and pRNMV2A18). Northern hybridisation on RNA extracted from purified RNMV particles (as described by Usugi and Saito, 1976) was probed with an *EcoR I* fragment from pRNMV4, revealed a 8 kb band on RNMV RNA only. The RNA isolated from purified RNMV particles gave two bands of 8kb (RNA 1) and 4kb (RNA 2) on a ethidium bromide-stained gel (results not presented).

The nucleotide sequences of pRNMV4, pRNMV1A29 and pRNMV2A18 were determined on one strand by using the dideoxynucleotide chain termination method (Sanger et al., 1977; Pharmacia T^7 kit) and on the opposite strand by an ABI automatic sequencer (dye-terminator reactions) using internal primers. Nucleotide sequences were aligned and translated using Gene Jockey II (Biosoft, Cambridge). These clones yielded sequence for the 3′ terminal 1431 nucleotides (nt) of RNMV RNA1, excluding the poly (A) tail. There were only two discrepancies between the clones, at *c.* position 587 nt two clones had the codon CCT encoding Pro (P), whilst one clone contained CTT encoding Leu (L) and at *c.* position 790 nt, 2 clones contained CTC Leu (L) and one clone TTC Phe (F). Analysis showed that a large single open reading frame (ORF) of 421 amino acids (aas) was present, with a stop codon at position 1264 nt and hence a 3′ untranslated region (UTR) of 167 nt.

From the nucleotide sequence the putative amino acid sequence was deduced. Figure 2 shows an alignment of the RNMV amino acid sequence obtained and the corresponding sequence for other bymoviruses sequenced to date, barley mild mosaic virus (BaMMV: Kashiwazaki et al., 1992), barley yellow mosaic virus (BaYMV: Kashiwazaki et al., 1990), wheat spindle streak mosaic virus (WSSMV: Sohn et al., 1994) and a typical potyvirus, potato virus Y (PVY: Robaglia et al., 1989). These sequences represent the C-terminal region of a large polyprotein which contains a part of the NIb protein and the entire coat protein. A consensus amino acid sequence (V-X-X-Q/A,S,G or V) required for a potyviral cleavage site has been determined by mutational analysis and *in vitro* translation studies (Shukla et al., 1994). RNMV conforms to part of this consensus and has the residues QA at position 180-182 aas which would generate a coat protein of 27.1 kDa. This is a similar size to the coat protein size estimated from sequence data for BaYMV (*c.* 32kDa), BaMMV (*c.* 28 kDa) and WSSMV (*c.* 31.9 kDa) and is in agreement with the observed coat protein size for RNMV (33kDa) (Usugi et al., 1989).

Table 1. Pairwise percent amino acid sequence identities between the complete coat protein of RNMV, a typical potyvirus, PVY and three bymoviruses, BaMMV, BaYMV and WSSMV. Sequences used are those represented in Figure 2

	BaYMV	BaMMV	WSSMV	PVY
RNMV	34	54	32	12
BaYMV		31	72	12
BaMMV			30	14
WSSMV				12

The sequence data from the C-termini of the large polyprotein encoded by RNMV RNA allowed confirmation of its classification that supports the serological and biological evidence. Shukla and Ward (1988) have shown that distinct species of the potyvirus genus have coat protein sequence similarities within the range of 38% to 71% (average 54%). Table 1 shows that RNMV has a percentage similarity to the other members of the *Bymovirus* genus of 32% to 54% in the coat protein, and should therefore be classified as a distinct species of the *Bymovirus* genus.

The use of the 'NGDD' primer in the present investigation has provided further information about RNMV and may also be useful to distinguish between potyviruses and bymo- or macluraviruses in mixed infections. This primer could be further used to detect novel bymo- or macluraviruses. However, the possibility of absence of the universal primer sequence in these viruses should not be ruled out.

Acknowledgements

We thank Dr. Takashi Idei, Agriculture and Forestry Department of Okayama Prefecture for supplying RNMV-infested soil. J.B. was funded by a BBSRC research studentship. Gary Foster holds a BBSRC Advanced Research Fellowship.

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