Complete nucleotide sequence of Pelargonium zonate spot virus and its relationship with the family Bromoviridae

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The complete sequence of the Pelargonium zonate spot virus (PZSV) genome was determined. It comprises 8477 nt, distributed in three positive-strand RNA species encoding four proteins. RNA-1 is 3383 nt long, with an ORF that encodes a polypeptide with a molecular mass of 108419 Da (denoted protein 1a). This protein contains the conserved sequence motifs I–III of type I methyltransferases and the seven consensus motifs of the helicases of superfamily 1. RNA-2 is 2435 nt long and encodes a major polypeptide with a molecular mass of 78944 Da (denoted protein 2a), which shows identity to the RNA-dependent RNA polymerases of positive-strand RNA viruses. RNA-3 is 2659 nt long and contains two major ORFs. The first ORF is located in the 5′ portion of the genome and sequence comparison of the putative translation product revealed similarities with the 30K superfamily of virus movement proteins. The second ORF is located in the 3′ half and encodes the viral coat protein, which is expressed via a subgenomic RNA, RNA-4. The transcription initiation site of RNA-4 maps to the intergenic region of RNA-3. The organization of the PZSV genome, including the primary structure of terminal non-coding regions, strongly suggests that this virus belongs to the family Bromoviridae. The overall biological and genomic characteristics of PZSV indicate affinities in diverging directions with one or other of the virus species in this family, thus enabling it to be considered as a possible representative of a new genus within the family Bromoviridae.

INTRODUCTION

Pelargonium zonate spot virus (PZSV) is a virus with quasi-spherical particles of 25–35 nm in diameter. PZSV sediments as three components in sucrose density gradients and contains two molecules of linear, single-strand RNA (Gallitelli, 1982). PZSV was isolated originally from Pelargonium zonale (Quacquarelli & Gallitelli, 1979) and is the causal agent of a tomato disease characterized by concentric chlorotic/necrotic rings and line patterns of leaf stems and fruits, reported in Italy (Gallitelli, 1982; Vovlas et al., 1986), Spain (Luis-Arteaga & Cambra, 2000) and France (Gebre-Selassie et al., 2002). In nature, PZSV also infects globe artichoke, Capsea bursa-pastoris, Chrysantherum segetum, Diplotaxis erucoides, Picris echoides and Sonchus oleraceus. PZSV is seed-transmitted in D. erucoides and from this weed is transmitted to tomatoes (Vovlas et al., 1989). This mode of transmission was reported for members of the genus Ilarvirus, i.e. Tobacco streak virus (TSV) (Sdoodee & Teakle, 1987), Prune dwarf virus (PDV) and Prunus necrotic ringspot virus (PNRSV) (Greber et al., 1992). Thus, PZSV resembles ilarviruses in particle morphology, some physico-chemical properties and epidemiology, but it is serologically unrelated to Apple mosaic virus (ApMV), PDV, PNRSV, TSV and Tulare apple mosaic virus as well as to other viruses within the family Bromoviridae (Gallitelli et al., 1983). When preliminarily characterized, PZSV appeared to differ significantly from ilarviruses in having only two RNA species, nor does it require the addition of a coat protein (CP) for RNA infectivity, subgenomic RNA (RNA-4) or any of the ultrastructural modifications usually induced in host cells (Gallitelli et al., 1983). For these reasons, the ICTV, in their last report, included PZSV in the list of unassigned viruses (Calisher et al., 2000).

We have now determined the nucleotide sequence of the PZSV genome and show that, contrary to previous reports (Gallitelli et al., 1983), it comprises three positive-strand RNA species with an organization that is similar to that of virus species within the family Bromoviridae. These results provide the basis for which the taxonomic status of PZSV is discussed.

The nucleotide sequence data reported in this paper have been deposited in the EMBL database under accession numbers AJ272327 (RNA-1), AJ272328 (RNA-2) and AJ272329 (RNA-3).
METHODS

Viruses purification and RNA analysis. A tomato isolate of PZSV (PZSV-T) (Gallitelli, 1982) was propagated in Nicotiana glutinosa and purified from systemically infected leaves 10 days after inoculation by the method described by Gallitelli et al. (1985), with minor modifications. Leaf tissue was homogenized in 2 vol. (v/w) 50 mM sodium acetate, pH 6-0, containing 0·1 % (v/v) 2-mercaptoethanol. The extract was filtered through cheesecloth and clarified by adjusting the pH to 5-0 with acetic acid. After low-speed centrifugation, the supernatant was adjusted to pH 6-0 with 0·1 N NaOH and virus particles were concentrated by precipitation with 10 % PEG-8000 and 200 mM NaCl, followed by one cycle of centrifugal precipitation. The final pellet was resuspended in 50 mM NaCl, containing 30 % glycerol and trace amounts of sodium azide, and stored as small aliquots at −20 °C until used.

Viral RNA was extracted as described by Finetti-Sialer et al. (1997) and analysed by 1-2 % agarose gel electrophoresis in TBE (Sambrook et al., 1989). For Northern blot analysis, [32P]UTP-labelled negative-sense riboprobes were generated from cloned fragments (see below) using the Sp6/77 transcription kit (Roche), following the manufacturer’s instructions. Pre-hybridization, hybridization and detection were carried out as described previously (Gallitelli et al., 1985).

A preparation of PZSV RNA was also subjected to one cycle of oligo(dT)–cellulose chromatography, according to the protocol of Milner & Jackson (1979).

Translation of viral RNA was carried out using the Rabbit Reticulocyte Lysate system (Amersham Pharmacia) using 2 μg RNA and approximately 15 μCi [35S]methionine (Amersham Pharmacia). Translation products were analysed by PAGE, as described by Burgyan et al. (1986).

cDNA synthesis, cloning and sequencing. PZSV RNA was fractionated by two runs of electrophoresis in low-melting-point agarose, as described by Gallitelli et al. (1985). For cDNA cloning, 1 μg RNA was polyadenylated with 4 U Escherichia coli poly(A) polymerase (Gibco-BRL), annealed to an oligo(dT)23 primer and reverse-transcribed using the cDNA Synthesis Module (Amersham Pharmacia), according to the manufacturers’ protocols. The double-strand cDNA was ligated into SmaI-digested, de-phosphorylated pUC18 and was used to transform competent DH5α (HE) strain DH5α (HE) strain DH5α (HE) strain DH5α (HE). Plasmids containing sizeable inserts were used for double-strand DNA sequencing on both strands using the Thermo Sequenase Cycle Sequencing Kit (Amersham Pharmacia) and [32P]ATP as label. The 3′-terminal sequence of the genomic RNAs was determined from at least four different clones for each RNA species, each containing an artificially added poly(A) tail.

The 5′-end regions of RNA-1 and RNA-2 were cloned separately following a modified RACE protocol (Grieco et al., 1990). Primer 5′-CCATATTGACCTGACTGCG-3′, which is complementary to nt 63–82 of RNA-1, and primer 5′-GCTCTGAGATTATCTCTGATA-3′, which is complementary to nt 135–152 of RNA-2, were used. The 5′ terminus of PZSV RNA-3 was sequenced using M-MLV RNA-dependent RNA polymerase (RdRp) (Gibco-BRL), according to the protocol described by Fichot & Girard (1990), using the internal primer 5′-CTATGGAATGCGAAGATGCA-3′, which is complementary to nt 37–54. The 5′-terminal nucleotide of each genomic RNA was determined according to the method of DeBorde et al. (1986).

To map the 5′ end of the subgenomic RNA, the synthetic primer 5′-GCTCTGAGATCCGCTGATGCGCTG-3′, which is complementary to nt 1794–1865 of PZSV RNA-3, was labelled with 10 μCi polynucleotide kinase and 32P[γ-ATP (3000 Ci mM−1) and was used extending RNA-4 as template. The size of the subgenomic RNA was determined by comparison to plasmid DNA containing the primer sequence as a size ladder. The subgenomic RNA 5′ termini were sequenced directly from the viral RNA, as with RNA-3, using the above primer.

Sequence data were assembled using the DNA program STRIDER (Marck, 1988). Nucleotide and deduced amino acid sequences were compared using TREEPU and CLUSTAL (GCG) (Anon., 1994). Putative translation products were compared with the GenBank non-redundant sequence database. Tentative phylogenetic trees were constructed and bootstrap analyses were made using the programs of the PHYLIP package (Felsenstein, 1989). The following sequences have been used in this study (EMBL accession numbers are given in parentheses): AMV, Alfalfa mosaic virus (L00163, K02702 and K02703); APMV (AF174584, AF174585 and U15608); BMV, Barley stripe mosaic virus (J04342 and M16576); BMV, Broad bean mosaic virus (M66138, M66719 and M66091); BMV, Brome mosaic virus (X58456, X58457 and X58458); CCMV, Cowpea chlorotic mottle virus (M66139, M28817 and M28818); CIRLV, Citrus leaf rugose virus (U23715, U17726 and U17390); CMV-Ny, Cucumber mosaic virus (D00356, D00355 and D10538); CMV-NT9 (D28778, D28779 and D28780); CMV-Q (X02733, X00985 and J02059); EVM, Elm mottle virus (U57047, U34050 and U57048); OL2–1, Olive latent virus 2 (X94346, X94347 and X76993); PDV (U57648, AF277662 and L28145); PSV, Peanut stunt virus (D11126, D11127 and U15730); RBDV, Raspberry bushy dwarf virus (S15157, S55890 and Q12923–2); SpLV, Spinach latent virus (J019129, U93193 and U93194); TMV, Tobacco mosaic virus (A019133); TAV, Tomato aspermy virus (D10044, D10663 and D10015); TUS (U80934, U75538 and X00435).

Cloning and expression of the putative PZSV CP gene. Two deoxyprimers were designed to amplify the coding region of the 23 kDa (23k) protein. The forward primer 5′-GGATCCAGGCGC-CCCCCTACGAGCACG-3′ was homologous to nt 1619–1636 of the second ORF of RNA-3 and contained a BamHI site (underlined). The reverse primer 5′-TGGCACTACAGATGTTATGCTCTG-3′ was complementary to nt 2223–2245 of the same ORF of RNA-3 and contained a SalI site (underlined). RT-PCR was carried out according to the method described by Finetti-Sialer et al. (1999) and the amplified product was digested and cloned into the expression vector pGEX-6P-1 (Amersham Pharmacia). Recombinant clones were transferred to E. coli strain BL21 to express the PZSV 23K product as a fusion protein with glutathione S-transferase (GST). Protein expression was induced for 3 h with 2 mM IPTG at 37 °C, after which the samples were subjected to Western blot analysis (Sambrook et al., 1989) using a polyclonal antiserum raised against PZSV virions (Quacquarelli & Gallitelli, 1979).

Construction of infectious PZSV transcripts and inoculation assays. cDNAs of PZSV RNAs were obtained by RT-PCR using Thermoscript (Invitrogen) and Expand Long (Roche) enzymes, according to the manufacturers’ protocols. Two overlapping RT-PCR products encompassing RNA-1 were generated using two sets of oligonucleotide pairs. One set was constituted by a primer (5′-end primer) homologous to nt 1–20 (5′-GGTTTTGAGTCTGATTTTGTTGTA-3′) and a primer (3′-end primer) complementary to nt 1619–1636 of the second ORF of RNA-3 and contained a SalI site (underlined). RT-PCR was carried out according to the method described by Finetti-Sialer et al. (1999) and the amplified product was digested and cloned into the expression vector pGEX-6P-1 (Amersham Pharmacia). Recombinant clones were transferred to E. coli strain BL21 to express the PZSV 23K product as a fusion protein with glutathione S-transferase (GST). Protein expression was induced for 3 h with 2 mM IPTG at 37 °C, after which the samples were subjected to Western blot analysis (Sambrook et al., 1989) using a polyclonal antiserum raised against PZSV virions (Quacquarelli & Gallitelli, 1979).
sequence used for RNA-1. A primer complementary to nt 2634–2659 (5'-CTTTTTGGTCTCTGTTAGGAAAAGG-3') and a primer homologous to nt 1–22 (5'-GTTTGAACCTTAGAAAATGCATG-3'), anchored at the T7 promoter, were used to generate RT-PCR products corresponding to full-length cDNAs of RNA-3. All full-length PCR products were gel-purified using the QIAquick Gel Extraction kit (Qiagen) and used for transcription. For long-term storage, full-length cDNAs were ligated into the plasmid vector pCR-XL-TOPO (Invitrogen) and used to transform E. coli strain TOP10, following the manufacturer’s instructions. Capped transcripts representing RNA-1, -2 and -3 were obtained directly with the mMessage mMachine (Ambion) and, after treatment with RQ1 RNase-free DNase (Promega), were used in different combinations for plant inoculation. Two leaves from each of two N. glutinosa plants were rubbed with 20 μl of inoculum containing 5 μg of each transcript in 10 mM Tris/HCl, pH 7-4, and 1 mM EDTA in the following combinations: RNA-1 + RNA-2 + RNA-3; RNA-1 + RNA-2; RNA-1 + RNA-3; and RNA-2 + RNA-3. Another two plants for each inoculum combination were mock-inoculated with buffer alone.

Total RNA was extracted from 100 mg of inoculated and systemic leaves of the different tests using TriPure Isolation reagent (Roche), according to the manufacturer’s instructions, and subjected to treatment with RNase-free DNase for 1 h at 37°C, phenol-extracted and ethanol-precipitated. The dried pellet was resuspended in RNase-free water and used in RT-PCR. RNA-1 was amplified with a primer complementary to nt 63–82 (5'-CCATTAGAACTGAGTGCCTG-3') and the 5'-end primer (see above). RNA-2 was amplified with an oligonucleotide complementary to positions 63–82 (5'-GGCTGTTGATTATGAACTGATG-3') and the 5'-end primer used for RNA-1. RNA-3 was amplified with an oligonucleotide complementary to nt 2640–2659 (5'-GCTCATTTATGAACTGATG-3') and a primer homologous to nt 1542–1562 (5'-GTGTTAGTAAATTGAGGATTTGCATG-3').

RESULTS AND DISCUSSION

Characterization of viral RNA

Electrophoretic analysis of nucleic acid extracted from virus particles showed five predominant RNA species with an apparent size of 3·3 (RNA-1), 2·5 (RNA-3), 2·3 (RNA-2), 1·1 (RNA-4) and 0·7 kb (RNA-5) (Fig. 1A, lane 1). In Northern blot analysis, RNA species extracted from purified virions and fractionated by two runs of electrophoresis in low-melting-point agarose were clearly differentiated by hybridization with riboprobes derived from different parts of the PZSV genome (Fig. 1B, lanes 2–4). The 594 nt fragment riboprobe derived from the 3'-half of RNA-3 also was amplified with bands corresponding to RNA-3 and RNA-5 (Fig. 1C, lane 6), whereas these bands were not recognized by the 3a riboprobe derived from the 5' proximal half of RNA-3 (Fig. 1C, lane 5). The amount of each RNA species was found to vary depending on the propagation host. For instance, in tomato, RNA-3 was abundant and masked RNA-2 completely, whereas RNA-4 and RNA-5 were barely visible (data not shown). Independently from the propagation host, the amount of RNA-4 and RNA-5 was generally lower than that of the other RNA species and, to address whether this was due to the method of purification or to inefficient packaging, we analysed RNA profiles obtained from infected leaves of N. glutinosa and viral particles purified from the same host. Fig. 1(D) shows that the amount of RNA-4 and RNA-5 was much lower in infected leaves (Fig. 1D, lanes T10 and T7) than in virus particles (Fig. 1D, lane P).

PZSV viral RNA also failed to bind to an oligo(dT) column, thus suggesting that the RNA species lack 3'-terminal poly(A) tails.

In rabbit reticulocyte lysates, unfractionated preparation of PZSV RNA. (B) PZSV RNA fractionated by two runs of electrophoresis in low-melting-point agarose and probed with cRNAs specific for RNA-1 (a 746 nt fragment, derived from the 3' proximal half of PZSV RNA-1) (lane 2), RNA-2 (a 398 nt fragment, derived from the central part of PZSV RNA-2) (lane 3) and RNA-3 (a 594 nt fragment, derived from the 3’proximal half of PZSV RNA-3) (lane 4). (C) An unfractionated RNA preparation probed with a 298 nt fragment, derived from 5'-proximal half of PZSV RNA-3 (lane 5) and the riboprobe specific for PZSV RNA-3, defined above (lane 6). The latter probe also recognizes RNA-4 and RNA-5 as they are co-terminal with RNA-3. (D) Northern blot hybridization of virion and total RNA profiles obtained from purified particles (lane P) and infected leaves (lanes T10 and T7) with a 32P-labelled riboprobe that recognizes RNA-3, -4 and -5 (see above). Lanes P, T10 and T7 were loaded with 0·5, 10 and 7 μg RNA, respectively. It was necessary to overload lanes T10 and T7 in order to make visible bands corresponding to RNA-4 and RNA-5. Numbers on the left indicate the size of the standards (kb); arrowheads mark the positions of the PZSV RNAs; lines mark the positions of RNA-3, -4 and -5. Migration is from top to bottom.

Nucleotide sequences and coding regions

Synthesis of cDNA from viral RNA was obtained using in vitro-polyadenylated RNA. Approximately 500 recombinant...
clones were obtained, with inserts ranging in size from 250 nt to more than 2500 nt. Selected clones were sequenced to characterize the viral genome.

As shown in Fig. 3, the genome of PZSV consists of three major RNA species, RNA-1, RNA-2 and RNA-3, whose nucleotide sequence was completely determined and deposited in the EMBL database under the following accession numbers: AJ272327 (RNA-1), AJ272328 (RNA-2) and AJ272329 (RNA-3). RNA-1 is 3383 nt long and contains a major open reading frame (ORF-1) (Fig. 4A) beginning at AUG (nt 79–81) and ending at UGA (nt 2965–2967). The sequence context of the first in-frame initiation codon (CAUAAUGGCUGC) seems in a favourable translation context with an A at position +2 and a G at position +4 (Lütcke et al., 1987). ORF-1 encodes a putative polypeptide of 962 aa with a molecular mass of 108 419 Da (108K), denoted protein 1a, which has a size comparable to that of the 110K translation product. The N-terminal domain (aa 78–270) of protein 1a contains conserved sequence motifs I–III of type I methyltransferases of positive-strand RNA viruses (Koonin & Dolja, 1993). The C-terminal domain contains the tobamovirus lineage (Koonin & Dolja, 1993) of the seven consensus motifs of the helicases of superfamily 1, including the motifs A–B of the purine NTP-binding pattern (aa 691–728) (Koonin & Dolja, 1993). Pairwise alignments of the conserved sequence motifs of protein 1a with the comparable signatures of other members of the family Bromoviridae are shown in Table 1. A second putative ORF of about 300 nt is present in the +2 position of ORF-1.

RNA-2 is 2435 nt long. Its major ORF (ORF-2) (Fig. 4B) starts at AUG (nt 82–84, in the context AUAAAUGGCUG) and stops at UGA (nt 2143–2145). The predicted polypeptide (protein 2a) has 687 aa with a molecular mass of 78 944 Da (79K), i.e. a size consistent with the translation product of 82K. This protein contains the consensus motifs I–VIII (aa 355–590) of the tobamovirus lineage of the supergroup 3 of the RdRps of positive-strand RNA viruses (Koonin & Dolja, 1993), including the GDD motif (aa 525–527). The PZSV RdRp signature showed a close relationship with comparable domains present in the 2a protein of other members of the family Bromoviridae (Table 1). Analysis of the three triplet codon phases of positive-strand RNA-2 of PZSV did not reveal any cucumovirus- or ilarvirus-like 2b ORFs. However, we have generated a probe against the two small putative ORFs that are present in the +2 position of ORF-2 (Fig. 4B). This probe hybridized only with RNA-2 in Northern blot analyses of RNA obtained from infected leaves and purified virions (data not shown).

RNA-3 is 2659 nt long and contains two major ORFs (Fig. 4C), the first of which (ORF-3) begins at AUG (nt 335–337, in the context GAAUAUGUCUC) and ends at UGA (nt 1262–1264). The putative polypeptide encoded by ORF-3 (protein 3a) consists of 310 aa with an estimated molecular mass of 33 735 Da (34K) and is comparable in size to the 34K translation product. This protein contains the LXDX50-70G motif and the SIS tail (aa 233–235) found near the C termini of most members of the 30K superfamily of virus movement proteins (MPs) (Melcher, 2000). Amino acid sequence comparison revealed 36–48 % similarity with the putative MPs of virus species of the family Bromoviridae (Table 1).

The second ORF (ORF-4) starts at AUG (nt 1619–1621, in the context GCAUAAUGCCCCC) and ends at UGA (nt 2243–2245). ORF-4 encodes a putative product of 209 aa...
with a predicted molecular mass of 23,070 Da (23K), which presumably corresponds to the 24K translation product. This putative CP showed 36–49% similarity with the CPs of other species of the family *Bromoviridae* (Table 1). The ORF-4 product was expressed as a GST fusion protein in *E. coli* and was recognized specifically in Western blot analyses by an antiserum to PZSV (Fig. 5, lanes 1 and 2), thus confirming that ORF-4 encodes the putative PZSV CP. There was no cross-reaction with the wild-type pGEX-6P-1 plasmid, which expresses GST alone (Fig. 5, lane 3).

There are three putative additional ORFs of about 200 nt each in the +3 position of ORF-4 (Fig. 4, panel C).

The two major ORFs of RNA-3 are separated by an intergenic region (IR) of 354 nt, which includes an 11 nt sequence (GGUUCAAUUCC) resembling the internal control region (ICR-2) of eukaryotic tRNA gene promoters. This sequence starts at nt 1406 and ends at nt 1416 and is identical to that found in the IR of BMV RNA-3 (Pogue *et al.*, 1992). RNA-4 is 1118 nt long and is monocistronic. It has 100% sequence identity with the 3′-proximal half of RNA-3 on which it maps, downstream of the ICR-2-like sequence (nt 1542) in the IR of this RNA and was, therefore, identified as a likely subgenomic RNA for the expression of the PZSV CP. By analogy with other viruses of the family *Bromoviridae*, the sequences required for the synthesis of subgenomic RNA are likely contained upstream and downstream of the transcription initiation site (TIS). The region between the ICR-2-like motif and downstream of the TIS is particularly rich in A and U residues, thus resembling the sequences thought to be important as promoters of subgenomic mRNA production (Marsh *et al.*, 1988). This tract includes an UUAGUUAAUU block, 58 nt upstream of the TIS, which is similar to the UUAUUAAUU block thought to act as an enhancer for the transcription of subgenomic RNAs. A GC/GUUA block (the symbol \ precedes the first nucleotide of RNA-4) identical to one of the four sequence blocks of promoter sequences of plant viruses is also evident (Marsh *et al.*, 1988; Buck, 1996).

**Fig. 4.** Diagram of the three triplet codon phases of positive-strand PZSV RNA-1 (A), RNA-2 (B) and RNA-3 (C). The AUG triplet and the stop codons are shown as short and long vertical lines, respectively. Open boxes represent major ORFs; grey boxes represent minor putative ORFs.
Table 1. Pairwise comparison of amino acid sequence similarities between PZSV and virus species of the family Bromoviridae

Pairwise comparisons of amino acid sequence similarities between PZSV and virus species of the family Bromoviridae were carried out for the methyltransferase (MET), helicase (HEL), RdRp motifs, and putative MPs and CPs. See Methods for the viruses used and for their associated accession numbers.

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Fig. 5. Western blot analysis using an antiserum to PZSV. Lane V, total protein from a purified preparation of PZSV particles; arrows point to the recognized monomer (23 kDa) and a possible dimer of about 47 kDa. Lane M, size protein standards (kDa). Lanes 1 and 2, total protein from E. coli strain BL21 containing the plasmid pGEX-6P-PZSVcpc; arrowheads indicate the PZSVcp–GST fusion protein recognized by the PZSV antiserum. Lane 3, total protein from E. coli strain BL21 containing the plasmid pGEX-6P-1, used as negative control. Migration is from top to bottom.

RNA-5 is 712 nt long and has 100% sequence identity with the 3′-proximal half of RNA-4. Thus, it may represent a degradation product derived from subgenomic RNA-4.

5′ and 3′ non-coding regions (NCRs)

The 5′-terminal NCRs of PZSV RNA-1, -2 and -3 are 78, 81 and 334 nt long, respectively. The 5′-proximal 78 nt displayed the following sequence identities: RNA-1/RNA-2, 93.6%; RNA-1/RNA-3, 48%; and RNA-2/RNA-3, 46%. The 5′ ends of PZSV RNA extracted from virions showed two major run-off products when analysed by oligonucleotide-primed run-off reverse transcription, as indicated by two intense bands across the four lanes (data not shown). The upper band is likely to reflect that it is capped, whereas the lower band corresponds to the initiation of transcription site.

ICR-2-like sequences (GGUUCAAAUCC) are present in the 5′ NCRs of both RNA-1 and RNA-2 (nt 25–36) but ICR-like motifs were not readily identified in the 5′ NCR of RNA-3.

The 3′-terminal NCRs of RNA-1, -2 and -3 are 416, 289 and 414 nt long, respectively, with the following identities in the 3′-proximal 260 nt: RNA-1/RNA-2, 88.5%; RNA-1/RNA-3, 89.5%; and RNA-2/RNA-3, 94%. In the last 80 nt, the match was 100%. All three RNAs contained a terminal CCA-box, which may serve as an RNA initiation signal for the viral RdRp (Buck, 1996; Yoshinari & Dreher, 2000) and RNA-1 and RNA-3 also contain an AAAAG extension after the CCA-box. Computer-assisted analyses showed that the 3′ end of each RNA has the potential to form tRNA-like structures (data not shown).

Infectivity of in vitro transcripts representing PZSV RNA-1, -2 and -3

Within 2 weeks after inoculation, the two plants challenged with a mixture of T7 polymerase run-off transcripts of PZSV RNA-1, -2 and -3 showed chlorotic/necrotic local lesions, followed by clear symptoms of systemic infection that were similar to those induced by the wild-type virus, although they developed less rapidly. RT-PCR from total RNA extracted from inoculated and systemic leaves of the two plants yielded amplicons of the expected size for RNA-1, RNA-2, RNA-3 and RNA-4, and RNA-5 and was shown to contain all PZSV RNAs, as determined by gel electrophoresis (data not shown). Plants inoculated with RNA-1 + RNA-2, RNA-1 + RNA-3 and RNA-2 + RNA-3 or mock-inoculated did not show either local or systemic symptoms. RT-PCR from total RNA extracted from the two plants challenged with a mixture of RNA-1 + RNA-2 transcripts yielded RNA-1- and RNA-2-specific amplicons from inoculated leaves only (Fig. 6, lanes 7 and 9). The two RNAs were still detectable 22 days after inoculation, suggesting that they probably replicate in inoculated leaves but cannot move systematically.
The observed survival of RNA-1 and RNA-2 in the inoculated leaves is now the subject of further investigation. Total RNA from plants inoculated with RNA-1 + RNA-3 and RNA-2 + RNA-3 or mock-inoculated did not yield any amplification products.

**Phylogenetic analysis**

Tentative phylogenetic trees constructed by comparing the helicase and methyltransferase signatures of the virus species of the *Bromoviridae* are shown in Fig. 7. Helicase signatures generated a tree with two clearly distinct clusters, one comprising ilarviruses and alfamoviruses and the other comprising PZSV and the genera *Bromovirus*, *Cucumovirus*, *Oleavirus* and *Idaeovirus*. Methyltransferase signatures yielded a tree with four clusters: (i) the genus *Bromovirus*; (ii) PZSV and the genera *Cucumovirus* and *Oleavirus*; (iii) the genera *Ilarvirus* and *Alfamovirus*; and (iv) the genus *Idaeovirus*. RdRp signatures produced a tentative phylogenetic tree (Fig. 7C) in which OLV-2 was clearly separate, while the other viruses, including PZSV, showed a tendency to form a continuum rather than to cluster. Alignment of the MPs produced a dendrogram (Fig. 7D) in which two main clusters are clearly discernible. One cluster includes alfamoviruses and ilarviruses, whereas the other separates into two branches, one of which includes the genus *Cucumovirus* and the other PZSV and the genera *Bromovirus*, *Idaeovirus* and *Oleavirus*. The dendrogram relative to CPs (Fig. 7E) exhibited a sharper distinction of PZSV from other virus species of the family *Bromoviridae*.

**Concluding remarks**

The genomic organization of PZSV (Fig. 3) is typical of members of the family *Bromoviridae* and consists of a total of 8477 nt distributed in three positive-sense RNA species encoding four proteins. On the whole, PZSV resembles members of the genus *Ilarvirus* in particle size, morphology and particle instability, physico-chemical and hydrodynamic properties, number of genomic RNAs, mode of transmission (seed, pollen and thrips) and, to some extent, cytopathology. However, there are a number of biological and molecular characteristics that separate PZSV from ilarviruses. A striking characteristic of this virus is the unusually large RNA-3, which, contrary to what is known for ilarviruses and other members of the family *Bromoviridae*, is larger than RNA-2. The standard convention is to denote RNAs in decreasing order of size but, given the homologies with other members of the family *Bromoviridae*, we have preferred to refer to the smallest genomic RNA as RNA-2 to facilitate sequence comparisons. RNA-3 is the most abundant RNA species of those encapsidated by PZSV particles and in gel electrophoretic analysis it usually masks RNA-2, which had originally led to the hypothesis that PZSV had a bipartite genome (Gallitelli *et al.*, 1983). However, the results of experiments with infectious transcripts provided in this paper suggest that all three PZSV RNAs are required for systemic infection of *N. glutinosa*, thus clearly establishing that the genome of PZSV is tripartite. Another intriguing feature of PZSV is that virions contain a low amount of RNA-4, the subgenomic RNA for the synthesis of the viral CP. This was apparently not due to the method of purification or to inefficient packaging, as the amount of RNA-4 was also extremely low in plant-infected tissue. Unlike ilarviruses and alfamoviruses, PZSV does not require either a CP or subgenomic RNA-4 in addition to the three genomic RNAs for initiation of infection and the CP gene does not contain the zinc finger-type domain. In its primary structure, the 3′-terminal tract of PZSV RNA does not contain the AUGC motif that occurs in the 3′-terminal sequences of alfamovirus and ilarvirus RNAs; this motif is thought to play a role in binding RNA to the CP (Bol, 1999). Viruses of the genera *Alfamovirus* and *Ilarvirus* have 3′ structures consisting of a series of stem–loops that are flanked by the single-strand tetranucleotide AUGC (Bol, 1999). Unlike these viruses, the 3′-terminal sequences of the PZSV NCRs include a CCA-box and have the potential to form a tRNA-like structure. The dispensability of subgenomic RNA-4 to initiate infection might explain its poor packaging into the virus particles, which, in turn, might be the reason why the amount of CP translated in the system that we used was far less than other products of translation. It is also possible that the CP is translated efficiently but is poorly detected in the system that we used, as its putative methionine content (two residues) is lower than that of MPs (10 residues). Finally, it cannot be excluded that RNA-4 could have a secondary structure that interferes with translation.

It is also noteworthy that the PZSV genome does not seem to encode a 2b gene similar to cucumoviruses and ilarviruses; however, there are small ORFs in RNA-1 and RNA-3 (Fig. 4) which could potentially encode a protein with a similar role to the cucumovirus 2b protein. These small ORFs are being analysed in our laboratory.

![Detection of amplified fragments specific for PZSV RNA-1, -2 and -3](http://vir.sgmjournals.org)}
The similarities found between PZSV and the other known members of the Bromoviridae in the overall organization and expression of genome products warrant the inclusion of this virus in this family. However, PZSV differs enough from other bromoviruses to substantiate the suggestion that it may be the representative of a novel genus of the family. With the information available, the evolutionary pattern of PZSV is difficult to establish. As a plant pathogen, PZSV is a quite recent discovery and there are no sequences available for other strains or isolates, as the virus has been detected only in Europe where its natural host range seems also to be restricted to few species in four different botanical families. While the trees obtained from ORFs establish a clear cut between the CP encoded by PZSV and that of the

**Fig. 7.** Phylogenetic trees inferred by parsimony analysis of the (A) methyltransferase, (B) helicase and (C) RdRp signatures and of the (D) MPs and (E) CPs of virus species of the family Bromoviridae. The number on each branch is the result of bootstrap analysis. BSMV was used as the outgroup in trees (A), (B) and (C) and TMV in trees (D) and (E). See Methods for the viruses used and for their associated accession numbers.
other genera within the Bromoviridae, the remaining gene products of the virus seem less similar to AMV and ilarviruses than to members of the genera Bromovirus, Cucumovirus, Oleanivirus and Idaeovirus. Therefore, it is possible that PZSV originated from ancestors common to these viruses but acquired its CP gene from another source.

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