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Molecular characterization and detection of plum bark necrosis stem pitting-associated virus

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Summary

The complete RNA genome of plum bark necrosis stem pitting-associated virus (PBNSPaV) was cloned and sequenced and was determined to be 14, 214 nts long. The genome structure revealed seven major open reading frames (ORFs), and nontranslated regions at the 5′ and 3′ ends. PBNSPaV represents the simplest genome organization in the genus *Ampelovirus*, family *Closteroviridae*. The ORFs 1a and 1b encode, respectively, a large polyprotein with a molecular mass (M_r) of 259.6 kDa containing conserved domains characteristic of a papain-like protease, methyltransferase and helicase (ORF1a) and a 64.1-kDa protein of eight conserved motifs characteristic of viral RNA-dependent RNA polymerase (RdRp) (ORF1b). ORF1b is presumably expressed via a +1 ribosomal frameshift mechanism. ORF2 encodes a small 6.3-kDa hydrophobic protein of unknown function. ORF3 encodes a 57.4-kDa protein, a homologue of the HSP70 family of heat shock proteins. ORF4 encodes a 61.6-kDa protein with unknown function. ORF5 encodes a 35.9-kDa capsid protein (CP). Lastly, ORF6 encodes a 25.2-kDa minor capsid protein (CPm). Phylogenetic analyses performed on sequences of the HSP70h RdRp and CP support classification of the virus in the genus *Ampelovirus*. A real-time TaqMan® RT-PCR assay and a one-step RT-PCR were developed for PBNSPaV detection and compared using three different sample preparation methods.

Introduction

Plum bark necrosis stem pitting (PBNSP) disease was first observed in 1986 affecting trees of 'Black Beaut' plum (*Prunus salicina* Lindl) in Dinuba, CA. The trunks and scaffold branches exuded dark-colored gum balls, severe necrosis of bark tissues, and necrotic pitting on the woody cylinders. The infectious nature of PBNSP was confirmed by graft transmission assays [28]. Similar bark and stem symptoms were observed in Apulia (southern Italy) on 'Tirynthos' apricot (*P. armeniaca*) trees [7]. The putative causal agent in both instances was identified as a closterovirus and designated as plum bark necrosis stem pitting-associated virus (PBNSPaV)

The GenBank/EMBL/DDBJ accession number of the sequence reported in this paper is EF546442.
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[1, 16]. PBNSPaV was further identified in other *Prunus* species and cultivars: almond cv. 'Filippo Ceo', peach cvs. 'Springcrest' and 'May Crest', plum cv. 'Black Beaut', apricot cv. 'Castlebrite', and sweet cherry cv. 'Ferrovia' in Italy [3, 4]. In Morocco, stem-pitted trees of uncultivated almonds (seedling-derived) tested positive for PBNSPaV by RT-PCR assays [6]. PBNSPaV infection has also been reported to occur in plum in Jordan [25] and in plum, prune and cherry in Turkey [27]. With PBNSPaV, only a portion of the HSP70h gene has been sequenced, i.e. 673 bp of an American isolate (accession number AF195501) and 590 bp of the Italian isolate (AJ305307).

Several diagnostic methods have been developed for PBNSPaV. A dot blot hybridization assay detecting either total nucleic acid or double-stranded RNA was developed by Abou Ghanem-Sabanadozovic et al. [1]. A major disadvantage of this method was its reduced sensitivity and lack of high-throughput detection. A RT-PCR was also developed using primers designed for an Italian isolate of the virus [1]. This system failed to detect the virus early in the growing season [3, 4]. To overcome this problem, Amendoni et al. [3, 4] developed a nested RT-PCR; however, this method prolongs the detection process and increases the risk of cross-contamination among samples. A different PBNSPaV RT-PCR system was also developed by Marini et al. [16] using the sequences from an American isolate, but this set of primers was not able to amplify all of the known isolates of the virus due to a high variability within the forward primer region. Therefore, because of the above-mentioned problems for the existing methods, the development of a new detection method for PBNSPaV is highly desirable. The successful use of real-time RT-PCR using TaqMan® probe (TaqMan® RT-PCR) for the detection of several plant viruses [17] encouraged us to develop the method for the detection of PBNSPaV. This real-time RT-PCR is a robust and quantitative method and does not require gel electrophoresis for detecting PCR products.

In this paper, we report the full genome sequence of PBNSPaV, its phylogentic relationship with other members of the family *Closteroviridae*, development of a real-time TaqMan® RT-PCR assay and conventional one-step RT-PCR for its detection.

Materials and methods

Virus source and double-stranded RNA (dsRNA) isolation

The virus source was an advanced plum selection (*Prunus domestica*), designated here as PL186, whip-grafted with multiple dormant shoots onto Marianna 2624 rootstock. Immediately thereafter, new shoot growths developed poorly and bore small, chlorotic, distorted leaves. dsRNA was extracted from 30 g of bark scraping as described previously [19]. Double-stranded RNA was further purified by enzymatic digestions according to the method of Saldarelli et al. [23]. An aliquot of dsRNA solution was visualized in a 1% agarose gel stained in ethidium bromide solution.

cDNA synthesis and cloning

Complementary DNA (cDNA) was synthesized using purified dsRNA as a template [23]. The SuperScript™ choice system (cDNA synthesis kit; Invitrogen Life Technologies, Carlsbad, USA) was used to construct a cDNA library from denatured viral dsRNA per manufacturer's instructions. *Taq* DNA polymerase was used to add (A) to the ends of synthesized cDNAs [29], and dsDNAs were ligated into a pGEM-Teasy vector (Promega, Madison-USA) with the UltraClone ligation and transformation kit (Lucigen Corp., Middleton USA). Plasmids were transformed into *Escherichia coli* 10G Supreme electro-competent cells (Lucigen crop. Middleton-USA).

Sequences and sequence analysis

The nucleotide sequences of select clones were determined using a 3730×1 DNA Analyzer at the genomics facility at the University of California, Davis. Gaps between sequences were bridged using specific primers flanking the gap region. Reverse transcriptase polymerase chain reaction (RT-PCR)

was done using Phusion High Fidelity DNA polymerase (Finnzymes, Espoo Finland) per manufacturer's instructions. The fragments obtained were cloned and sequenced as above. Sequences were analyzed and assembled using Sequence Analysis and Molecular Biology Data Management software from Invitrogen, Vector NTI Advance™ 10 (InforMax, North Bethesda-USA). Identification of major open reading frames (ORFs), translated protein sequences and conserved domains were done with ORF finder, BLASTN, and BLASTX [National Center for Biotechnology Information (NCBI) http://www.ncbi.nlm.nih.gov/]. Alignment analysis was done with ClustalW (European Bioinformatics Institute (EBI) http://www.ebi.ac.uk/websites).

Sequencing of the 5′ and 3′ termini

The clone walking strategy was used for sequence extensions in both directions of the viral genome. The exact 5′ terminal sequence was determined using the RNA-ligase-mediated rapid amplification of cDNA ends (RLM-RACE) strategy (FirstChoice® RLM-RACE kit, Ambion, Austin, USA). The 3′ terminal sequence was determined with the rapid amplification of cDNA ends (RACE) (5′ RACE kit Invitrogen) with dsRNAs as templates and designed forward virus-specific primers. Two approaches were used to confirm the sequences at the 3′ end. First, the RLM-RACE method to amplify single-stranded RNAs [15] was used, and this involved premixing 10 µl dsRNA and 1 µl Oligo 1 primer, heating the mixture at 90 °C for 2 min, followed by chilling in an ice bath for 5 min. Ligation was done in a 50-µl volume containing 5 µl of the 10 × T4 RNA ligase buffer (New England Biolabs, Beverly-USA), 1.5 µl of T4 RNA ligase enzyme (20 Units/µl), 5 µl dimethyl sulfoxide (DSMO), and 27.5 µl milli-Q, sterile H_2O. The mixture was then incubated at 17 °C for 17 hours. After phenol-chloroform extraction, ethanol precipitation and centrifugation, the product was suspended in 10 µl of water and used in a reverse transcriptase reaction with Oligo 2 primer and 20 mM methyl mercuric hydroxide. PCR was done using the Oligo 2 primer and an appropriate gene-specific primer corresponding to the sequences closest to the 3′ end of known sequences. Second, we used the 3′ RACE described in the FirstChoice® RLM-RACE kit per manufacturer's instructions. The resulting PCR products were cloned as previously described. Ten clones per cDNA were sequenced in both directions to compare the consistency of sequence data.

Phylogenetic analysis

Amino acid sequences were used to compare phylogenetic relationships of PBNSPaV's ORFs for RdRP (ORF1b), HSP70 and CP with other closterovirus sequences. Multiple alignments of amino acid sequences were made with the default options of Clustal X 1.8, a Windows interface for the Clustal W multiple sequence alignment program [26]. Phylogenetic analysis was done using the minimum evolution method of phylogenetic inference with 1000 bootstrap replicates [22]. Phylogenetic trees were constructed using the 3.1 version of the Molecular Evolutionary Genetics Analysis software MEGA version 3.1 [14]. Accession numbers of the viruses used in the Alignment and phylogenetic analysis are: beet pseudo yellows virus (BPYV, AY330918, AY330919); beet yellows virus (BYV, NC_001598); beet yellow stunt virus (BYSV, U51931); blackberry yellow vein-associated virus (BYVaV, NC_006962, NC_006963); citrus tristeza virus (CTV, NC_001661); cucurbit yellow stunting disorder virus (CYSDV, NC_004809, NC_004810); grapevine leafroll-associated virus 1 (GLRaV-1, AF195822); GLRaV-2 (NC_007448); GLRaV-3 (NC_004667); GRLaV-5 (AF233934); GRLaV-9 (AY297819); grapevine rootstock stem lesion-associated virus (GRSLaV, NC_004724); lettuce infectious yellows virus (LIYV, NC_003617, NC_003618); little cherry virus 2 (LChV 2, NC_005065); mint virus 1 (MV 1, NC_006944); pineapple mealybug wilt-associated virus 1 (PMWaV 1, AF414119); PMWaV 2 (AF283103); potato yellow vein virus (PYVV, NC_006062, NC_006063); strawberry pallidosis-associated virus (SPaV, NC_005895, NC_005896); sweet potato chlorotic stunt virus (SPCSV, NC_004123, NC_004124); tomato chlorosis virus (ToCV, NC_007340, NC_007341).

Northern hybridization

The dsRNAs were denatured with glyoxal and DSMO, separated on a 1 % agarose gel and transferred to Hybond N+ membrane (Amersham Biosciences, Piscataway USA) [24]. The α^{32}P-labeled RNA probe corresponding to the 5′-proximal region of ORF1a (sequence between nt 1248 and 1628) was prepared using MAXIscript™ *in vitro* transcription kit (Ambion, Austin, USA) per manufacturer's instructions. Pre-hybridization and hybridization steps were done as described [11].

Virus detection

Seven PBNSPaV-infected trees were used in this study, including three plum tree cultivars (PL186, Black Beaut, and Frank Ann) and four GF305 peach seedlings that were graft inoculated with an isolate from PL186 and kept in the greenhouse. Three methods for sample preparation were used; the starting material in each method was 0.3 g of cambial scraping: Method 1, using RNeasy Plant Mini kit supplied by Qiagen (Valencia, CA, U.S.A.) and following the manufacturer's protocol, method 2, using the glycine-based buffer (GES) described previously [20], and method 3, using spotting on Hybond N^+ nylon membrane (Pharmicia Biotec, New Jersey, U.S.A.) as described before [18].

The Primers and TaqMan® probe for the specific detection of PBNSPaV were designed in the 100% conserved region within the hHSP70 gene after comparative alignments of newly obtained sequences with the sequences available in the GenBank (accession numbers AF195501 and AJ305307). For the design of the primers and probe, the Primer Express™ software from Applied Biosystems (AB, Foster City, CA, U.S.A.) was used. The primer sequences were: forward primer, PBNaSPV-55-F: 5′ GCTAATAAGAGCAACGGTTGCA 3′ and reverse primer, PBNaSPV-175-R: 5′ CCAAACC CTCTTCGGTAGTCACA 3′ with an amplicon size of 121 bp. The TaqMan® probe PBNaSPV-103-P was a dual-labeled 3′TAMRA-5′FAM Minor Groove-binding Probe (TaqMan® MGB™ probe) with the sequence 5′ CCGACTATTCTGGGGTTG CGAAAAGATG 3′. Real-time TaqMan® RT-PCR reactions (total volume of 12 µl) were set up in a 96-well reaction plate using the PCR core reagent kit (AB). The reactions contained 1 µl of RNA and were carried out in a Biorad I-Cycler (Hercules, CA, U.S.A.) in a one-step reaction as described previously [17]. The data were analyzed quantitatively by measuring the threshold cycles (*C*T) in a Microsoft Excel program and graphically by an amplification plot. The threshold cycle (*C*T) is the cycle at which a significant increase in fluorescence occurs; hence a *C*T value below 40 indicates a positive result in our setup used for virus detection.

For conventional RT-PCR, Primers were designed based on a multiple sequenced alignment used previously for designing the TaqMan® primers and probe. Primer sequences were: forward primer, PBNSPaV det-F: 5′ TACCGAAGAGGGTTTGG ATG 3′ and reverse primer, PBNSPaV det-R: 5′ AGTCGCACCACCAGTCTTCT 3′. RT-PCR amplification was performed in a 25-µl final volume using 2 µl of purified RNA as a template. The one-step RT-PCR was prepared according to Rowhani et al. [20] with an annealing temperature of 56 °C.

Results and discussion

dsRNA isolation

A high M_r dsRNA of approximately 15 kb was consistently recovered in extracts derived from cortical scraping of PBNSPaV-infected tissues, but not from healthy tissues. Several low-M_r dsRNAs were also co-purified (Fig. 1a). A specific probe transcribed from the 5′-promixal region of ORF1a was used in northern hybridization assays; only the full-length RNA genome was detected (Fig. 1b).

Sequence analysis and genome organization

The nucleotide sequence of PBNSPaV genomic RNA was compiled by assembling multiple sequence overlaps of cDNA clones of at least four cloned sequences per region of overlap. The full PBNSPaV genomic RNA was 14214 nt (accession no. EF546442) and consisted of seven ORFs (Fig. 2). The 5′ terminal genome sequence was determined

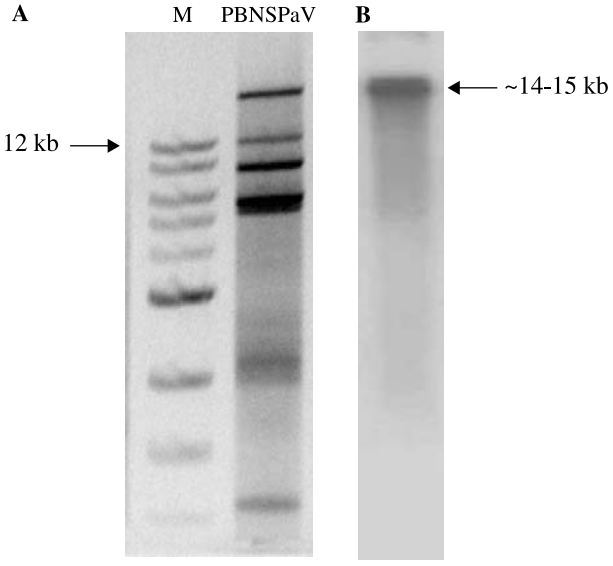


Fig. 1. Analysis of PBNSPaV in dsRNA profile and Northern hybridization analysis. **A** Viral dsRNA profile in a 1% agarose gel, *M* 1 kb plus DNA molecular marker, *PBNSPaV* dsRNA extracted from an PBNSPaV-infected plant. **B** Northern hybridization blot detecting the high M_r dsRNA of PBNSPaV using α^{32}P-labeled RNA probe corresponding to the 5′-proximal region of ORF1a

and the first nucleotide was a ''G''. The 5′ UTR was 301 nt long and had a high A/T content (186 A/Ts) with no significant similarity with other viruses.

ORF1a potentially encodes a large polypeptide of 2343 amino acid residues with a M_r of 259.6 kDa. The predicted amino acid sequence of ORF1a revealed the presence of a putative leader papain-like protease (L-PRO) characteristic [2]. PBNSPaV alignments with GLRaV3 and PMWaV2 comparing 103 aa located at positions 601–703 showed the presence of catalytic cysteine (position 636) and histidine (position 679, data not shown) residues. The region of ORF1a immediately downstream of the L-PRO domain was identified as a methyltransferase (MTR) domain based on homology with other closteroviruses and the presence of seven conserved motifs [21]. The C-terminal portion of ORF1a was identified as a helicase (HEL) domain, consistent with seven conserved motifs of positive-stranded RNA viruses in superfamily I [10, 13]. ORF1b overlaps with ORF1a by 107 nt and encodes a putative protein of 561 amino acid residues and M_r of 64.1 kDa. ORF1b contains eight

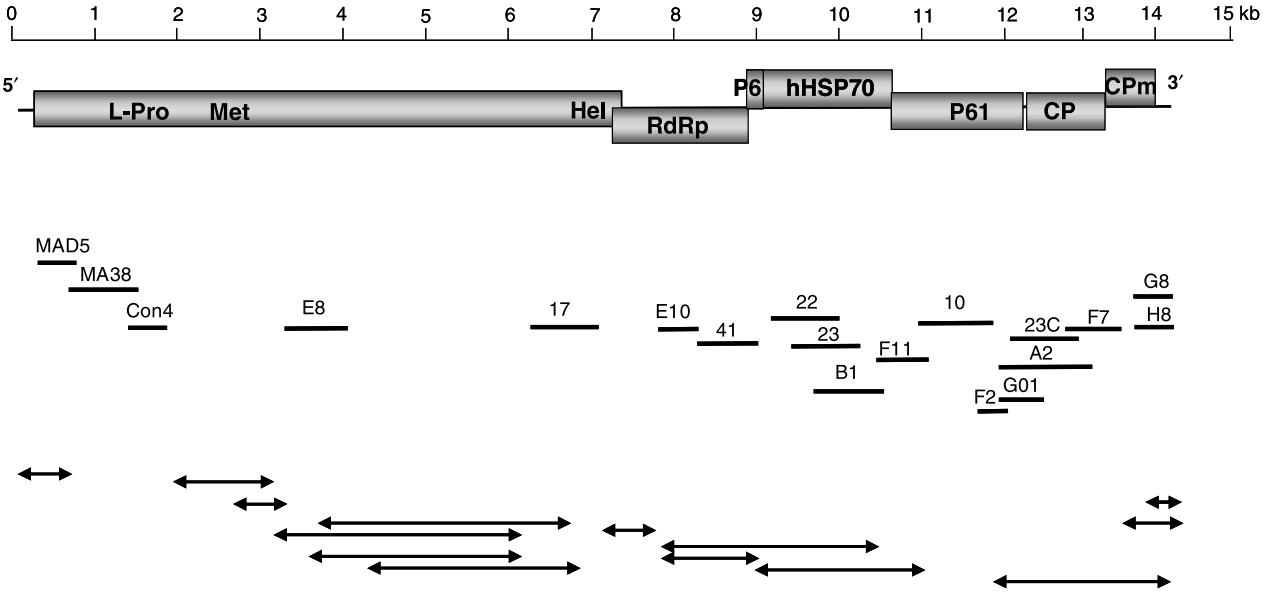


Fig. 2. Putative genome organization of PBNSPaV and schematic presentation of overlapping cDNA clones. Bars represent clones obtained from the cDNA library. Arrows represent DNA clones resulting from direct RT-PCR using virus-specific primers, RLM-RACE and RACE PCR kits. *L-Pro* Leader papain-like protease; *Met* methyltransferase; *Hel* helicase; *RdRp* RNA-dependent RNA polymerase; *P6* protein of unknown function; *HSP70h* heat shock protein 70 homolog protein; *P61* protein of unknown function; *CP* capsid protein; *CPm* minor capsid protein

conserved motifs characteristic of viral RNA-dependent RNA polymerase (RdRp) [12] and probably is expressed via a +1 ribosomal frame shift, a characteristic reported for some members of the family *Closteroviridae* [9]. ORF2 overlaps ORF1b by 25 nt and potentially encodes a protein of 57

Table 1. Comparison of amino acid sequences of various gene products of PBNSPaV and six closteroviruses. Percentages were determined using the AlignX function in the Vector NTI advance 10 program (Invitrogen)

Virus	Amino acid identities (expressed in percentages)						
	MET*	HEL**	RdRp	HSP70h	P61	CP	CPm
CTV	23	31	32	29	14	14	10
LIYV	27	27	31	26	16	14	12
GLRaV3	31	30	40	30	14	18	16
GLRaV9	14	34	42	44	31	35	15
PMWaV1	15	33	35	44	29	31	15
PMWaV2	15	28	33	31	15	26	12

* Amino acid sequence from MET I to MET IV domains. ** Amino acid sequence from HEL I to HEL VI domains

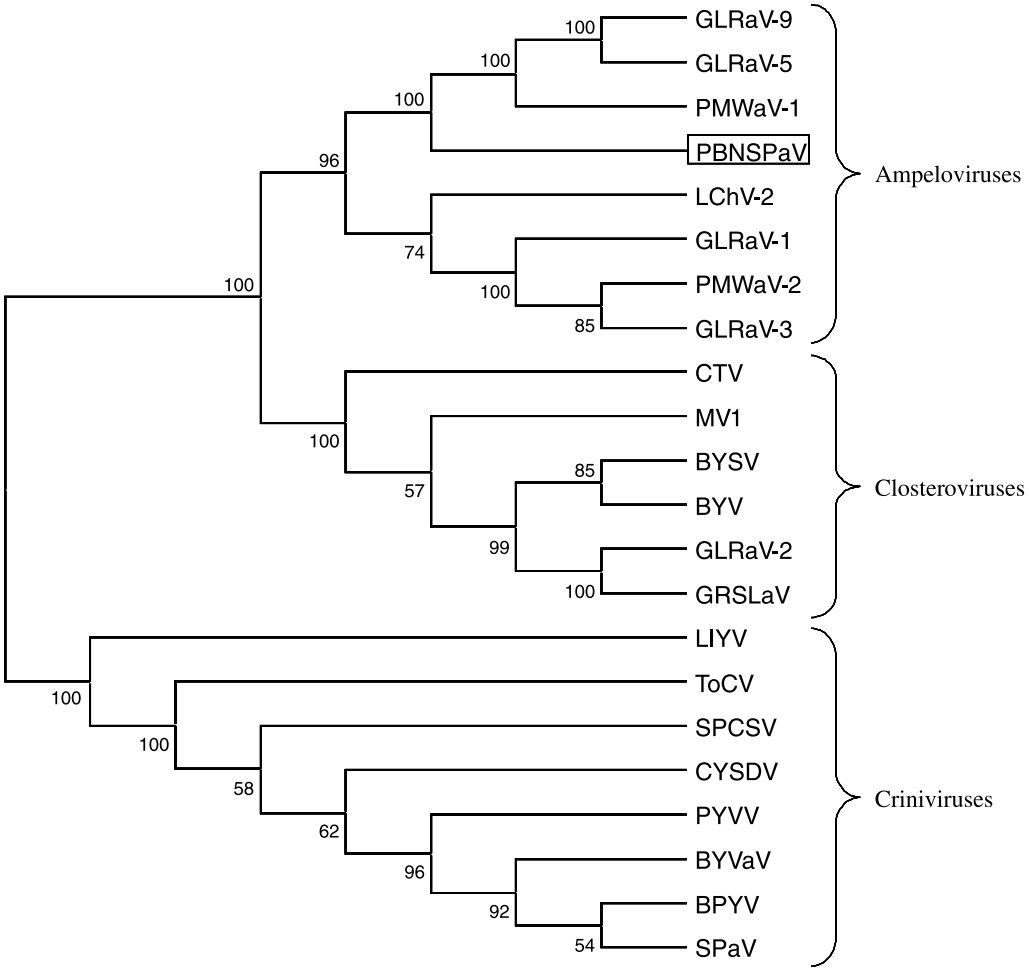
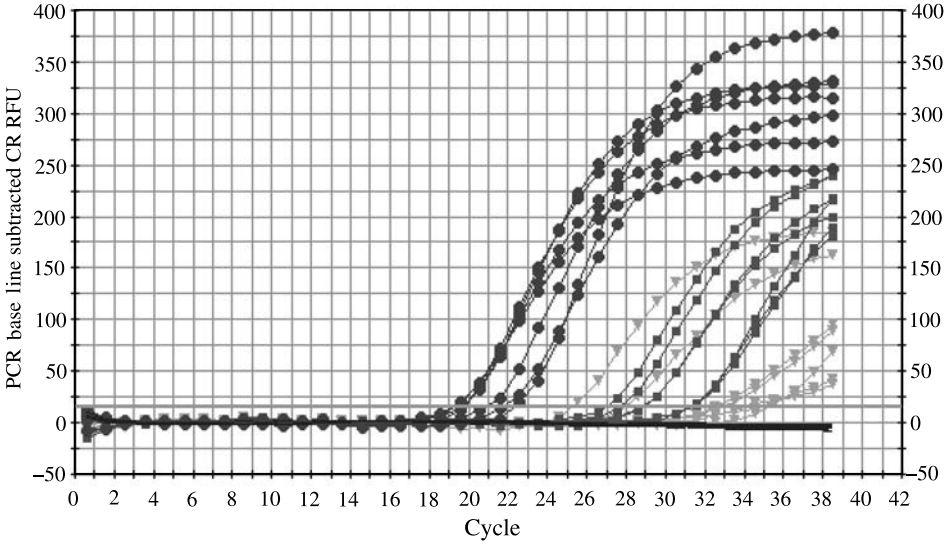


Fig. 3. Phylogenetic analysis of the HSP70h showing the relationship of PBNSPaV and other members of family *Closteroviridae* based on their amino acid sequences. Bootstrap values are shown as percentage values

amino acid residues with a M_r of 6.3 kDa (P6). Hydrophobic residues (A, I, L, F and V) were dominant in its composition. This protein lacked significant sequence similarity to other protein sequences in the database. ORF 3 starts seven nucleotides downstream of ORF2 and potentially encodes a protein of 529 amino acids residues and a M_r of 57.4 kDa. Blast searches showed that this product is a homologue of the cellular heat shock protein 70 (HSP70h), the hallmark gene of the family *Closteroviridae*. The HSP70h of PBNSPaV contains motifs conserved among cellular HSP70 homologs [5]. The PBNSPaV sequences determined in this paper showed 96–97% identity at the nucleotide level with the previously sequenced portion of the PBNSPaV-HSP70h gene [1, 16]. ORF 4, which overlaps ORF3 by 13 nt, potentially encodes a protein of 546 amino acid residues and a M_r of

A



B

Sample	CT Values		
	RNA	Spotting	GES
Black bueat.	22.3	28.7	27.9
3. GF305	19.2	31.3	32.6
12. GF305	21.7	26.9	32.9
6. GF305	19.6	31.4	35.2
4. GF305	20.9	28.8	34
Frank Ann	19.3	31.3	25.2
PL186	19.7	27.7	34.8
Helthy plum	NA	NA	NA
Healthy GF305	NA	NA	NA
Water	NA	NA	NA
Mix	NA	NA	NA

Fig. 4. Comparison of the effects of different sample preparation methods in TaqMan RT-PCR. **A** Real-time PCR graph showing the reaction progress in each step. Purified total RNA (●), samples prepared in GES buffer (▼), spotted nylon membrane discs (■). Healthy controls, water control and master mix are represented by (–). **B** tables showing the *C*T value (threshold cycle) for the corresponding reactions. *NA* Not applicable (value not attained = negative)

61.6 kDa (P61). Following a UTR of 63 nt, ORF 5 encodes the CP (325 amino acids; M_r of 35.9 kDa). Multiple amino acid sequences alignment of PBNSPaV-CP with the corresponding proteins of other closteroviruses identified three hallmark residues (S, R, and D), that are universally conserved in the CPs of filamentous plant viruses [8] (data not shown). Lastly, ORF 6 overlaps ORF 5 by 3 nt and potentially encodes a protein of 223 amino acid residues and a M_r of 25.2 kDa. This protein resembles the CPm and is similar in size to those of GLRaV-5 and GLRaV-9 (23.1 kDa and 23.2 kDa, respectively) but different from that of GLRaV-3 (53 kDa), the type member of the genus *Ampelovirus*. Amino acid sequence alignment of PBNSPaV CPm with those of GLRaV-5 and GLRaV-9 revealed the invariant consensus of the CP hallmark amino acid residues S, R, and D at positions 59, 72 and 101, respectively (data not shown). Percentages of amino acids identity of different ORFs for PBNSPaV with the corresponding proteins of viruses in the genera *Closterovirus,* CTV; *Crinivirus,* LIYV; and *Ampelovirus,* GLRaV3, GLRaV 9, PMWaV1 and PMWaV2, are shown in Table 1. The highest identity percentages were found in both RdRp and HSP70h and the lowest was in CPm. Further sequence analysis revealed that PBNSPaV consisted of seven ORFs and represented the simplest genome organization so far known among members in the genus *Ampelovirus*. The 3′ UTR of PBNSPaV consisted of 231 nucleotides, terminating with GGA. No significant similarities were found between the 3′ UTR of PBNSPaV and other closteroviruses.

Phylogenetic analysis

Phylogenetic analysis of the amino acid sequences of conserved proteins of HSP70h (Fig. 3) with various members of the family *Closterviridae* revealed that PBNSPaV consistently clustered together with viruses in the genus *Ampelovirus*. PBNSPaV showed a closer relationships to PMWaV-1, GLRaV-5 and GLRaV-9. Similar results were obtained for the phylogenetic analysis of RdRp and CP genes (data not shown). Our sequence study and phylogenetic analyses presented in this study suggest that PBNSPaV is a new member of the genus *Ampelovirus*; family *Closteroviridae*.

Virus detection

Based on three PBNaSPV hHSP70 sequences available in the GenBank, a set of forward/reverse primers (PBNaSPV-55-F/PBNaSPV-175-R) and a TaqMan® probe (PBNaSPV-103-T) were designed for real-time RT-PCR. An additional set of primers for the conventional RT-PCR was designed in parallel. A collection of seven samples (three different virus sources) were tested by real-time TaqMan® RT-PCR and conventional RT-PCR. The detection using both methods was repeated three times to insure the consistency of the results. All samples tested positive using the real-time Taq-

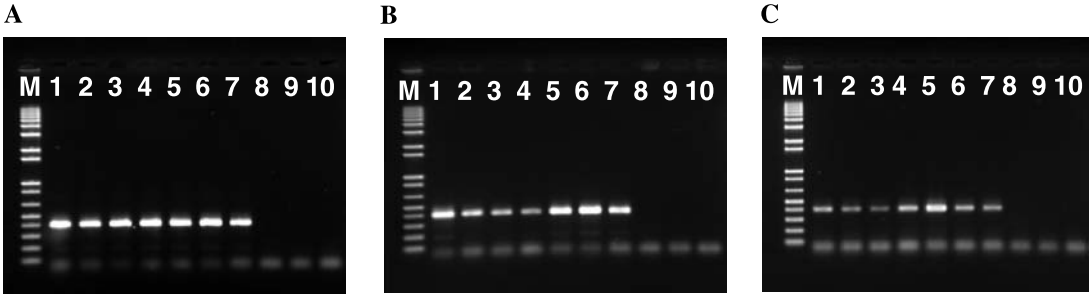


Fig. 5. DNA fragments (400 bp) generated by conventional RT-PCR with primers PBNSPaV det-F and PBNSPaV det-R from the total RNA extractions (**A**), spotted Hybond H^+ Nylon membrane discs (**B**), and GES homogenate (**C**). Fragments were examined by electrophoresis in 1.5% agarose gels. *1*: Black Beaut, *2–5*: GF305, *6*: Frank Ann, *7*: PL186, *8*: Helthy plum, *9*: Healthy GF305, *10*: water control, and *M*: 1 kb Plus DNA ladder (Invitrogen)

Man® RT-PCR regardless of the sample preparation method. However, total RNA extracted with the Qiagen RNAeasy kit produced more specific results and lower *C*T values when spotted Nylon Hybond membrane discs were used (Fig. 4). Conventional one-step RT-PCR yielded the expected fragment size (400 bp). The results presented in this paper clearly show that TaqMan® RT-PCR and conventional RT-PCR are robust and reliable methods for the detection of PBNaSPV, whether using purified RNA, spotted Nylon Hybond membrane discs or GES homogenate as the starting template.

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