Molecular Identification of Potyviruses Infecting Bulbous Ornamentals by the Analysis of Coat Protein (CP) Sequences

K.T.K. Pham, M.J.D. de Kock, M.E.C. Lemmers and A.F.L.M. Derks
Crop Protection, Applied Plant Research, P.O. Box 85
2160 AB Lisse
The Netherlands

Keywords: potyviruses, bulbous ornamentals, coat protein sequences

Abstract

Potyviruses (genus *Potyvirus*, family *Potyviridae*) are transmitted by aphids in a non-persistent manner and cause significant losses in many crops including bulbous ornamentals. Host range, symptoms, physical and biochemical properties of many potyviruses in bulbous ornamentals are reported, but, especially for viruses infecting ornamentals of minor economical importance, sequence data are still lacking. We used molecular techniques for the identification, characterization and detection of these viruses. Leaf material of several ornamental crops showing virus-like symptoms were tested in indirect ELISA, using monoclonal antibodies specific for potyviruses. Generic potyvirus primers were used in an RT-PCR to amplify the 3' terminal region of these viruses. The fragments encode the viral coat protein (CP) gene and comprise the 3'-untranslated region (3'-UTR). Nucleotide sequences of the obtained fragments were determined and compared with potyvirus sequences present in the NCBI database using the BLAST algorithm. We have characterized some potyviruses previously accepted by the International Committee on Taxonomy of Viruses (ICTV), including *Freesia mosaic virus* (FreMV), *Gloriosa stripe mosaic virus* (GSMV), *Hippeastrum mosaic virus* (HiMV), *Hyacinth mosaic virus* (HyaMV), *Iris mild mosaic virus* (IMMV) and *Nerine yellow stripe virus* (NeYSV). Additionally, the identities of other potyviruses infecting ornamentals such as *Anemone, Galtonia, Muscari, Ornithogalum, Allium, Stenomesson* and *Veltheimia* have been determined. These viruses, however, have not yet been reported by the ICTV. The virus-specific sequence information generated in this research project can subsequently be used to develop PCR-based detection methods.

INTRODUCTION

The genus *Potyvirus* is the largest plant virus genus, and includes more than 200 definitive and tentative species. Potyviruses are transmitted by aphids in a non-persistent manner and cause significant losses in many crops. Potyviruses have particles of 700 to 900 nm in length harboring a ssRNA genome of approximately 10 kb in length. The single open reading frame (ORF) encodes a large polyprotein which is processed into 10 smaller proteins, including the coat protein (CP). The coat protein encoding region is located at the 3'end. More than 200 distinct potyviruses were reported and accepted in the NCBI database; the complete genome sequence is only available for about 55 species. The remaining potyviruses are currently only characterized based on the coat protein encoding sequences. Host range, symptoms, physical and biochemical properties of many potyviruses in bulbous ornamentals were reported, but sequence data are still lacking, especially of viruses infecting ornamentals of minor economical importance. In this study potyviruses infecting bulbous ornamentals were identified based on their reaction to a monoclonal antibody specific to the potyvirus group and their coat protein sequence.

We have used serological and molecular techniques for the identification, characterization and detection of these viruses. CP nucleotide sequences were determined and compared with potyvirus sequences present in the NCBI database using the BLAST algorithm. For species demarcation we used the criteria described by Adams et al. (2005) and listed in the current ICTV Report which states: "potyvirus species are characterized

by a CP amino acid sequence identity less than about 80% and/or a nucleotide sequence identity less than about 85% over the whole genome" (Fauquet et al., 2005).

MATERIALS AND METHODS

Virus Sources

Leaf samples of different bulbous ornamentals showing characteristic symptoms of virus infection were used for this study. Most samples are from our own collection, especially samples of viruses previously reported by the ICTV (Fauquet et al., 2005). Samples were tested in indirect ELISA using antibodies detecting a large group of members of the genus *Potyvirus* (Agdia Inc., IN, USA). RNA was extracted for further molecular analysis from the samples with positive reactions.

RNA Extraction and RT-PCR with Degenerate Primers

RNA was extracted from leaf samples using the plant tissue protocol (10 mg) of the Purescript RNA isolation Kit (Gentra Systems Inc., Minneapolis, MN, USA). The potyvirus viral RNA was detected by amplifying the 3' terminal region by RT-PCR with a potyvirus-specific degenerate forward primers: S-primer (Chen et al., 2001) or primer U880 (Langeveld et al., 1991), in combination with an oligo-dT reverse primer.

First strand cDNA synthesis was performed on 1 to 2 µg RNA in 20 µl volume at 37°C for 1 hour, with 200 ng oligo-dT primer and 200 units M-MLV reverse transcriptase (Invitrogen, California, USA). For PCR, 2 µl first strand cDNA solution was added to 23 µl amplification mixture containing 12.5 µl PCR Mastermix (Promega, Madison, USA), and 100 ng of each primer (Oligo-dT and either S-primer or U880). PCR reactions were done by first incubating for 4 min at 94°C, followed by 40 cycles each of 0.5 min at 94°C, 1 min at 48°C and 2 min at 72°C, with a final extra 10 min at 72°C. PCR products were examined by electrophoresis in 1% agarose gels.

Cloning, Sequencing, and Pairwise Sequence Analysis

The PCR products were ligated to pCR2.1 TOPO vector from the TA-TOPO Cloning kit (Invitrogen, California, USA). Clones containing an insert op appropriate size were identified by using *EcoRI* restriction digestion and selected for sequencing the insert. Nucleotide and protein sequences were analyzed using software program BLASTn or BLASTp of the National Center for Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS AND DISCUSSION

The results are separated in two sections: (1) characterization of potyviruses that were previously accepted by the ICTV and (2) characterization of potyviruses that are not yet accepted by the ICTV.

Characterization of Potyviruses Accepted by the ICTV

1. *Freesia mosaic virus* (FreMV). This virus (ICTV code: 00.057.0.01.078) was first identified in *Freesia refracta* in Lisse, The Netherlands by Van Koot et al. (1954). Nucleotide sequence and pairwise sequence analyses showed that the FreMV isolate (1020nt, EF203688) from cultivar Golden Yellow shared more than 98% identity with *Spiranthes mosaic virus 2* (SpiMV-2) from *Spiranthes* (AY685219) (Guaragna et al., 2006) and from *Freesia* (AM748701) (Kumar et al., 2009). However, SpiMV-2 is not accepted yet by ICTV. Based on the high sequence homology between FreMV and SpiMV-2 it can be concluded that SpiMV-2 is not a distinct virus species but a synonym of FreMV.

2. *Gloriosa stripe mosaic virus* (GSMV). GSMV (ICTV code: 00.057.0.01.026) was first reported in *Gloriosa* in Germany by Koenig and Lesemann (1974). Nucleotide sequence of the GSMV isolate CP in our collection (1600 nt; EU042761) showed more than 81% identity with *Glory lily mosaic virus* from Chinese Taipei (EU250360). Based on the

criteria for discrimination of virus species, we showed that *Glory lily mosaic virus* is synonymous to *Gloriosa stripe mosaic virus*.

3. *Hippeastrum mosaic virus* (HiMV). HiMV (ICTV code: 00.057.0.01.031) was first identified in *Hippeastrum hybridum* in Baarn, the Netherlands by Kunkel (1922). The synonym of *Hippeastrum mosaic virus* is *Amarylis potyvirus*. The nucleotide sequence identity between our HiMV isolate (EF203685) and an *Amarylis potyvirus* isolate from Taiwan (AY566239) is 83%. However, the identity is 96% with accession number AY590143, a sequence derived from a virus called *Amazon lily mosaic virus* (Hu et al., 2004). Probably this is an incorrect name because the other sequence of *Amazon lily mosaic virus* (AB158523) is totally different (Fuji et al., 2004). Therefore, we can conclude that HiMV occurs in *Amazon lily* (*Eucharis*) and that *Amazon lily mosaic virus* is a distinct potyvirus and not synonymous to HiMV.

4. *Hyacinth mosaic virus* (HyaMV). HyaMV (ICTV code: 00.057.0.01.079) was identified in *Hyacinthus orientalis* in Bulgaria and U.S.A. (Atanasoff, 1928; Smith and Brierley, 1944) and has been shown to infect hosts belonging to the family of *Hyacinthaceae*. In the investigated material, we have identified four different, highly homologous HyaMV isolates from the following hosts: *Hyacinth* (EF203679), *Hymenocallis* (EF203681), *Veltheimia* (EF203680) and *Muscari* (EU042754). Sequences were deposited in the GenBank database at indicated accession numbers.

5. *Iris mild mosaic virus* (IMMV). IMMV (ICTV code: 00.057.0.01.033) was first identified in *Iris* spp. in Lisse, the Netherlands by Van Slogteren (1958). Two IMMV isolates were previously identified in New Zealand and were deposited in the NCBI database (DQ436918 and DQ436919) before our IMMV isolate (EF203682). The identity between our isolate and DQ436918 is 98% whereas the identity is only 86% when compared with isolate DQ436919; all within the recommended criteria for being strains of IMMV. This is a good example where pairwise sequence analysis of the predicted CP amino acid sequences would help further to distinguish (or not) these isolates.

6. *Nerine yellow stripe virus* (NeYSV). NeYSV (ICTV code: 00.057.0.01.081) was first identified in *Nerine sarniensis* in England and reported by Brunt et al. (1970). The synonymous name of *Nerine yellow stripe virus* is *Nerine potyvirus* (Plant Virus Online). We have analyzed and determined sequences for three different NeYSV isolates from *Nerine* (EF362621), *Hymenocallis* (EF362622), and *Stenomesson* (EU042758). One NeYSV isolate from *Vallota speciosa* was previously identified in New Zealand (DQ407932); which has 85-90% identity with these isolates.

Characterization of Potyviruses Not Yet Accepted by the ICTV

1. Potyviruses in *Anemone*. Based on the sequences of two different and distinct amplicons we can conclude that this specific *Anemone* sample was infected by two different potyviruses. One of the two sequences (EU042756) had very high identity (97%) with *Ranunculus mild mosaic virus* (RanMMV) of sequences from Israel (EF445546) and Italy (DQ152191). However, the ICTV currently reported only *Ranunculus mottle virus* (RanMoV) as a tentative species (ICTV code: 00.057.0.81.075), and not *Ranunculus mild mosaic virus*. Unfortunately, no sequence or serological information is available of RanMoV. Therefore, we propose that RanMMV and RanMV are synonymous and propose to rename RanMMV as RanMoV.

The other distinct potyvirus sequence identified in *Anemone* did not result in any significant BLAST hit (>85% nucleotide identity) and was therefore designated *Anemone mosaic virus*, AneMV (EU042755). Based on pairwise sequence alignment, AneMV is most related to *Bean common mosaic virus*. AneMV has been previously proposed for a virus disease of *Anemone coronaria* in which infected plants exhibit mottled leaves, and broken and distorted flowers (Hollings, 1957).

2. *Potyviruses* in *Muscari*. Based on the sequences of two different and distinct amplicons obtained from the tested *Muscari* sample, we can conclude that the sample was infected with two distinct potyviruses. One of them was identified as *Hyacinth mosaic virus* (HyaMV) (EU042754) (see above). The other virus sequence had no significant

(>80%) nucleotide identity with any other potyvirus in the NCBI database (less than 73% identity with HyaMV) and was therefore tentatively named *Muscari mosaic virus* (MMV) (EU042752). A virus designated as MMV was previously identified and described by Navalinskiene and Samuitiene (2001). Unfortunately, molecular data was not obtained in that study and the genetic relationship between both MMV isolates is currently unknown.

3. *Potyvirus* in *Veltheimia*. Based on the sequences of two different and distinct amplicons obtained from the tested *Veltheimia* sample, we can conclude that this sample was infected with two distinct potyviruses. One of them was HyaMV (EF203680) (see above). The other virus sequence had no significant (>80%) nucleotide identity with any other potyvirus in the NCBI database and was tentatively named *Veltheimia mosaic virus* (EF203686). This virus is not closely related to *HyaMV* and showed less than 75% identity with *Ornithogalum virus 3* (OV3). *Veltheimia mosaic virus* was additionally identified in *Eucomis* (EU042763).

4. *Potyvirus* in *Stenomesson*. Based on the sequences of two different and distinct amplicons obtained from the tested the *Stenomesson* sample, we can conclude that this sample was infected with two distinct potyviruses. One of them was *Nerine yellow stripe virus* (NeYSV) (EU042758). The other virus sequence had no significant (>80%) nucleotide identity with any other potyvirus and was named *Stenomesson mosaic virus* (EU042757). This virus is not closely related with *Nerine yellow stripe virus*.

5. *Potyviruses* in *Allium* (Ornamental Onion). A virus disease in an ornamental onion species was associated initially with 'Onion mosaic'. ELISA revealed the presence of *Shallot latent virus* (genus *Carlavirus,* SLV) and unknown potyvirus(es) (A.F.L.M. Derks, pers. commun.). Only one of the potyviruses reported to be able to infect *Alliaceae* was identified in Ornamental onion, and appeared to be *Leek yellow stripe virus* (LYSV). Sequence analysis also identified a distinct potyvirus. The coat protein nucleotide sequence of this virus showed no identity with other potyviruses known to occur in *Allium* species like *Leek yellow stripe virus* (LYSV), *Shallot yellow stripe virus* (SYSV) or *Onion yellow dwarf virus* (OYDV) (Tsuneyoshi et al., 1998; Van der Vlugt et al., 1999). Therefore, we propose to name this new virus *Ornamental onion stripe mosaic virus* (OOSMV) (EU042750) based on typical symptoms in potyvirus infected ornamental onion plants.

6. *Potyvirus* in *Ornithogalum* and *Galtonia*. A potyvirus-specific 1315 bp amplicon was obtained from an *Ornithogalum* sample that was collected in 1998. The deduced Nib+CP amino acid sequence of this potyvirus was surprisingly not homologous to *Ornithogalum mosaic virus* (OrMV), *Ornithogalum virus 2* (OrV2) or *Ornithogalum virus 3* (OrV3) (Fuji et al., 2003). Additionally, it shared less than 73% sequence identity with *Freesia mosaic virus*. We propose *Ornithogalum virus 4* (OrV4) as tentative name for this new virus (EU042753).

Additionally, a partial 0.7 kb CP nucleotide sequence of the potyvirus isolated from *Galtonia* (tentatively named *Galtonia mosaic virus*, EU042751) has 85-99% identity with different *Ornithogalum virus 3* isolates. Therefore, we can conclude that *Galtonia* is a susceptible host for *Ornithogalum virus 3*. In 2008 Matsumoto et al. proposed the name *Ornithogalum necrotic mosaic virus* (OrNMV) for *Ornithogalum virus 3*.

We have observed that many of the bulbous crop samples that we tested were infected by at least two distinct potyviruses. A summary is listed in Table 1.

CONCLUSIONS

In this study, using a 'generic' potyvirus monoclonal antibody and generic potyvirus primers in RT-PCR, we have identified a number of different potyviruses infecting bulbous ornamentals. Analysis of the RT-PCR coat protein amplicon sequences appears to be a fast method to help identifying known and unknown potyviruses.

By this method we identified viruses for which alternative names have been reported previously. For example, SpiMV-2 should be a synonym of FreMV. We have also found well known potyviruses in ornamental crops for which any alternate host status was unknown. For example, HyaMV was identified in host plants other than *Hyacinth*,

including *Hymenocallis, Veltheimia* and *Muscari*. Some of the ornamental crop samples were infected with a mixture of at least two different potyviruses. Using molecular cloning techniques we were able to identify several new, previously unknown potyviruses, including for example, the potyviruses identified in *Anemone*, *Muscari, Stenomesson, Veltheimia, Allium* and *Ornithogalum*. These findings illustrate the need for the continuation of appropriate virus nomenclature and renaming of confusing virus names.

In addition, the obtained sequence information can now be used to design specific PCR tests for quality control and for epidemiological studies.

Literature Cited

Atanasoff, D. 1928. Mosaic disease of flower bulb plants. Bull. Soc. Bot. Bulgarie 2:51.

Adams, M.J, Antoniw, J.F. and Fauquet, C.M. 2005. Molecular criteria for genus and species discrimination within the family Potyviridae. Arch. Virol. 150:459-479.

Brunt, A.A., Hollings, M. and Stone, O.M. 1970. Rep. Glasshouse Crops Res. Inst. 1969, p. 138.

Chen, J., Chen, J.P. and Adams, M.J. 2001. A universal PCR primer to detect members of the Potyviridae and its use to examine the taxonomic status of several members of the family. Arch. Virol. 146:757-766.

Derks, A.F.L.M., Vink-van den Abeele, J.L. 1980. *Hyacinth mosaic virus*: symptoms in hyacinths, serological detection, and relationships with other potyviruses. Acta Hort. 109:495-502.

Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U. and Ball, L.A. (eds.). 2005. 8th Reports of the International Committee on Taxonomy of Viruses. Academic Press, New York.

Fuji, S., Inoue, M., Yamamoto, H., Furuya, H., Naito, H. and Matsumoto, T. 2003. Nucleotide sequences of the coat protein gene of potyviruses infecting *Ornithogalum thyrsoides*. Arch Virol.148:613-21.

Fuji, S., Terami, F., Furuya, H., Naito, H. and Fukumoto, F. 2004. Nucleotide sequence of the coat protein genes of Alstroemeria mosaic virus and Amazon lily mosaic virus, a tentative species of genus potyvirus. Arch Virol. 149:1843-1849.

Guaragna, M.A., Ndum, O. and Jordan, R. 2006. Detection and Characterization of Two Previously Undescribed Potyviruses in the Terrestrial Orchid *Spiranthes cernua*. Acta Hort. 722:209-217.

Hollings, M. 1957. Anemone mosaic, a virus disease. Ann. appl. Biol.45 (I): 44-61.

Hu, W.-C. and Chang, Y.-C. 2004. A new mosaic disease of Amazon lily in Taiwan. Plant Pathology 53:240.

Koenig, R. and Lesemann, D. 1974. A potyvirus from *Gloriosa rothschildiana*. Phytopath. Z. 80:136-142.

Kumar, Y., Hallan, V. and Zaidi, A.A. 2009. Freesia mosaic virus from Palampur. Plant Pathol. 58:404-404.

Kunkel, L.O. 1922. Ameboid bodies associated with Hippeastrum mosaic. Science 55:73.

Langeveld, S.A., Dore, J.-M., Memelink, J., Derks, A.F.L.M., van der Vlugt, C.I.M., Asjes, C.J. and Bol, J.F. 1991. Identification of potyviruses using the polymerase chain reaction with degenerate primers. Journal of General Virology 72:1531-1541.

Matsumoto, T., Yamamoto, H., Fuji, S. and Inoue, M. 2008. Characterization of a novel potyvirus tentatively named Ornithogalum virus 3, successfully isolated from O. thyrsoides co-infected with two other potyviruses by single-aphid inoculation. J. Gen. Plant Pathol. 74:76-80.

Navalinskiene, M. and Samutiene, M. 2001. Viruses affecting some bulb and corm flower crops. Biologija 4:40-42.

Smith, F.F. and Brierley, P. 1944. Ornithogalum mosaic Phytopathology 34: 497.

Tsuneyoshi, T., Matsumi, T., Natsuaki, K.T. and Sumi, S. 1998. Nucleotide sequence analysis of virus isolates indicates the presence of three potyvirus species in *Allium* plants. Arch. Virol. 143:97-113.

Van der Vlugt, R.A.A., Steffens, P., Cuperus, C., Barg, E., Lesemann, D.E., Bos, L. and Vetten, H.J. 1999. Further evidence that Shallot yellow stripe virus (SYSV) is a distinct potyvirus and reidentification of Welsh onion yellow stripe virus as a SYSV strain. Phytopathology 89:148-155.
Van Koot, Y., van Slogteren, D.H.M., Cremer, M.C. and Camfferman, J. 1954. Tijdschr. Plziekt. 60:157.
Van Slogteren, D.H.M. 1958. Versl. Werkzaamh. Bloemboll. Lisse, 1957.

Tables

Table 1. List of viruses and accompanying Genbank accession numbers identified in the investigated bulbous crops. Both viruses reported by the ICTV and viruses not reported by the ICTV were identified. Most crops were infected by more than one virus species.

Crop	Viruses (abbreviation) accepted by ICTV	Viruses (abbreviation) not accepted by ICTV
Anemone	*Ranunculus mild mosaic virus* (RanMMV) EU042756	Anemone mosaic virus (AneMV) EU042755
Muscari	*Hyacinth mosaic virus* (HyaMV) EU042754	Muscari mosaic virus (MMV) EU042752
Stenomesson	*Nerine yellow stripe virus* (NeYSV) EU042758	Stenomesson mosaic virus (SteMV) EU042757
Veltheimia	*Hyacinth mosaic virus* (HyaMV) EF203680	Veltheimia mosaic virus (VelMV) EF203686
Allium	*Leek yellow stripe virus* (LYSV)	Ornamental onion stripe mosaic virus (OOSMV) EU042750
Ornithogalum		Ornithogalum virus 4 (OrV4) EU042753
Galtonia		Galtonia mosaic virus EU042751