Characterisation of mixed virus infections in *Ribes* species in Switzerland

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Abstract

Various virus disease-like symptoms are frequently observed in *Ribes* sp. in Switzerland but the aetiology remains poorly documented, although a number of viruses infecting *Ribes* sp. were described elsewhere. Therefore, symptomatic and apparently healthy plants from diverse origins were analysed by electron microscopy (EM), immuno-precipitation electron microscopy (IPEM), Western blot and (RT-)PCR. By EM, at least four different particle types, often in combination, were observed. (1) Bacilliform particles were typical for the *Badnavirus* genus with dimensions of 145 x 28 nm. This virus was identified by PCR as the *Gooseberry vein banding associated virus* (GVBaV). (2) Filamentous particles were mainly observed on black currants with downward rolling of leaves with interveinal reddening during summer and fall. We tentatively named this unknown virus Blackcurrant leafroll-associated virus 1 (BCLRaV-1). In phylogenetic analysis of HSP70h nucleotide sequences, BCLRaV-1 felt in the *Closterovirus* genus. In Western blot analysis, one dominant protein with an estimated molecular weight of about 28 kDa was detectable.

The virus was shown to be different from the *Raspberry mottle closterovirus* (RMoV) by IPEM and RT-PCR. (3) RT-PCR and sequencing of products also clearly demonstrated the presence in our *Ribes* samples of *Rubus chlorotic mottle virus* (RuCMV), a *Sobemovirus* recently described in Scotland. This finding correlates with the presence of the 30 nm diameter particles observed by EM. (4) A further structure with isometrical particles of 60 nm could not yet be attributed to a particular genus. Altogether, our data suggest the presence of multiple virus infections in *Ribes* sp. in Switzerland and emphasize the need for an efficient sanitary selection process.

Keywords: *Ribes* sp., *Gooseberry vein banding associated virus* (GVBaV), *Blackcurrant leafroll-associated virus* 1 (BCLRaV-1), *Rubus chlorotic mottle virus* (RuCMV)

Introduction

Over the past few years, symptoms of reversion, leafroll, vein clearing, defoliation, fruit failure, reduction of fruit calibre have been observed in *Ribes* sp. in Switzerland. So far, the sanitary status of commercial *Ribes* sp. in Switzerland has never been assessed. In this preliminary work, we focused on some accessions of *Ribes* sp. with symptoms of leafroll and early defoliation (Fig. 1). To our knowledge, such disorders have not yet been reported in *Ribes* species. In an attempt to determine if these disorders were associated with a viral agent, we analysed a few accessions by electron microscopy (EM), immuno-precipitation electron microscopy (IPEM), Western blot and RT-PCR.

Fig. 1 Left: leafroll symptoms characterised by the downward rolling of leaves with interveinal reddening in summer and fall. Right: early defoliation of a black currant bush.

Material and methods

All *Ribes* and *Rubus* sp. used in this study were from the reference collection of Agroscope ACW. Viral nucleoproteins were purified from leaves as previously published (Gugerli, Brugger et al. 1984). Purified extracts were observed by electron microscopy (EM) in negative contrast with phosphotungstic acid according to Bovey (1971). Mechanical inoculation to herbaceous hosts was performed in phosphate buffer 0.02 M + 0.01 M sodium diethyldithiocarbamate pH 7.6. The production of antiserum, purification of immunoglobulins, IPEM, electrophoresis and Western blot analysis were essentially done as described elsewhere (Gugerli 1986).

Leaf samples were tested by double-antibody-sandwich ELISA (DAS-ELISA) according to Gugerli (1986) with commercial kits from BIOREBA AG (Reinach, Switzerland) as follows: *Arabis mosaic virus* (ArMV), *Tomato black ring virus* (TBRV), *Strawberry latent ringspot virus* (SLRSV) and *Raspberry ringspot virus*-cherry strain (RpRSV-ch). Extraction of viral RNA or DNA was performed according to Rütsche (2008), whereby the DNeasy® Plant Mini Kit (QIAGEN, Switzerland) was used for DNA purification. RT-PCR protocols and primers' sequences for the detection of *Gooseberry vein banding associated virus* (GVBaV) (Jones, Mcgavin et al. 2001), *Raspberry mottle virus* (RMoV) (Tzanetakis, Halgren et al. 2007), *Rubus chlorotic mottle virus* (RuCMV) (Mcgavin and Macfarlane 2009) and HSP70h gene of the members of the *Closteroviridae* family (Dovas and Katis 2003) are described in the corresponding publication.

Detection of *Blackcurrant reversion virus* (BRV) was performed according to Lemmetty et al. (1998) with the primers P1/ P2 and P5/ P6, however without the immunocapture step. Amplification products were purified with the QIAquick PCR Purification Kit (QIAGEN, Switzerland) according to the manufacturer's instructions. DNA sequencing was performed by FASTERIS SA (Geneva, Switzerland). Oligonucleotide primers for the specific detection of the filamentous virus observed in currants were: Cass_Fw 5'-TCCTACCAGACGCTTC-3' and Cass_Rv2 5'-AGTGCGCTGTATTGTG-3'; Clost1Fw 5'-CTCATCTCGGGACA-3' and Clost2Rv 5'-ACAGAGCATACGAC-3'. RT-PCR setup and thermal conditions were described by Rütsche (2008) with an annealing temperature of 55°C. Alignments were performed in MacClade (Maddison and Maddison 2003) and ambiguously aligned regions were excluded from phylogenetic analyses. Searches for the most parsimonious tree(s) were conducted in PAUPv.4* (Swofford 2003) and used 500 RAS searches, with MAXTREE=unlimited and TBR branch swapping. Branch support was estimated based on 500 boostrap (BS) replicates, with the same settings as for the best tree(s) searches.

Results

Electron microscopy analysis: During the last years, several small fruits accessions (mainly *Ribes* sp. and *Rubus* sp.) were collected from various sources and maintained as references in the field or in greenhouse at Agroscope, Nyon. EM of purified extracts from selected symptomatic and apparently healthy *Ribes* sp. accessions (Table 1) disclosed the presence of at least four different particle types Fig. 2), often observed in combination. Bacilliform particles measured about 145 x 28 nm and were mainly observed in red currants samples.

Filamentous particles had an approximate dominant maximum length of 1500 nm based on 129 measures (BC No3SB 28074) and were observed in leafroll-affected *Ribes* sp. (downward rolling of leaves with interveinal reddening in summer and fall with symptoms on black currants being more pronounced than on red currants). At least two types of isometrical particles were observed: ~30 nm particles that were observed consistently in three accessions (Table 1) and ~60 nm particles that appeared more common. A significant variability was noticed with bigger spherical particles size, whereby diameters ranged from 40 to 80 nm, disclosing the possible presence of other particle types.

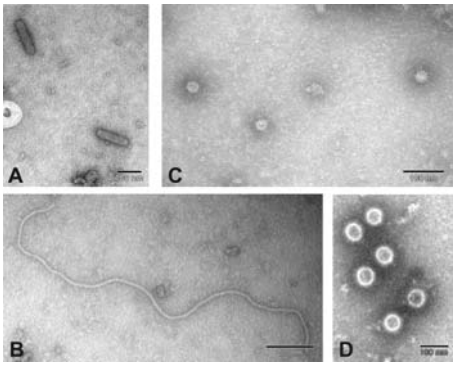

Fig. 2 Virus particles observed in EM from various *Ribes* sp. accessions. (A) Bacilliform particles. (B) Filamentous particles. (C) ~30 nm isometrical particles. (D) ~60 nm isometrical particles.

Tab. 1 EM analysis of symptomatic and apparently healthy *Ribes* accessions. +: virus particles observed in every extracts analysed; (+): virus particles observed in some extracts analysed.

		Particle types			
Accession	**Symptoms**	**Bacilliform**	**Filamentous**	**Spherical (~60 nm)**	**Spherical (~30 nm)**
Red currant No2SB 27986	Vein clearing and leafroll	+	+		
Red currant No3SB 28005	Vein clearing and leafroll	+	+	+	(+)
Red currant TE 27966	Vein clearing and leafroll	+	+		(+)
Red currant 0g 28650	No symptom			+	+
Black currant Titania 8/6 28636	Defoliation and leafroll		+		+
Black currant Titania 8/8 28640	Defoliation and leafroll		+	+	+
Black currant TE 27940	No symptom	(+)		+	
Black currant Titania 7/13 28119	Strong defoliation	(+)		+	
Black currant No3SB 28074	Strong leafroll	(+)	+	+	

Serological and molecular characterization of filamentous particles: Two antisera were produced, the first against the filamentous virus observed in currants (As 83) and the second against RMoV (As 160). Immunoglobulins were purified by DEAE chromatography and subsequently used in IPEM and Western blot. In IPEM, As 83 aggregated filamentous particles of all *Ribes* accessions tested so far (Fig. 3), whereas it did not react with RMoV virions. Furthermore, as 160 did not cross-react with filamentous virions observed in currants. Hence, these results indicated that these two viruses were not serologically related. In Western blot analysis, the estimated capsid protein molecular weight of the filamentous virus observed in currants was about 28 kDa (Fig. 3).

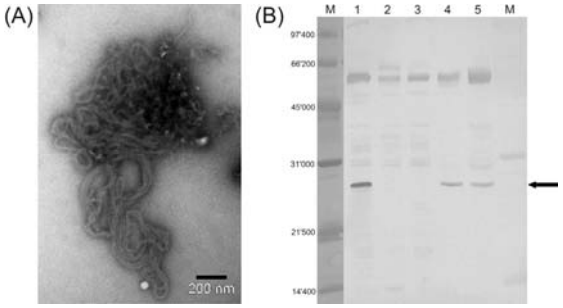

Fig. 3 Serological detection with As 83 of the filamentous virus observed in currants. (A) Decoration and aggregation of filamentous virions (BC No3SB 28074) by IPEM. (B) Staining of the ~28 kDa capsid protein by Western blot analysis (black arrow). M: marker (Da); 1) BC No3SB 28074; 2) BC Titania 7/13 28119; 3) RC 0g 28650; 4) RC TE 27966; 5) RC No2SB 27986.

Partial HSP70h genes of the filamentous virus identified in the accessions BC No3SB 28074 and RC TE 27966 were sequenced using the method developed by Dovas and Katis (2003). The generated nucleotide fragments diverged from 26% (sequence similarity of 74%), suggesting the existence of molecular variants (hereafter referred to as the BC and RC variants). In the maximum parsimony (MP) phylogenetic analysis of HSP70h partial nucleotide sequences, the two variants clustered together (BS = 91%) within the genus *Closterovirus* (BS = 90%). Based on nucleotide sequence information, an RT-PCR procedure for the variant-specific detection of the filamentous virus observed in currants was developed (Fig. 4).

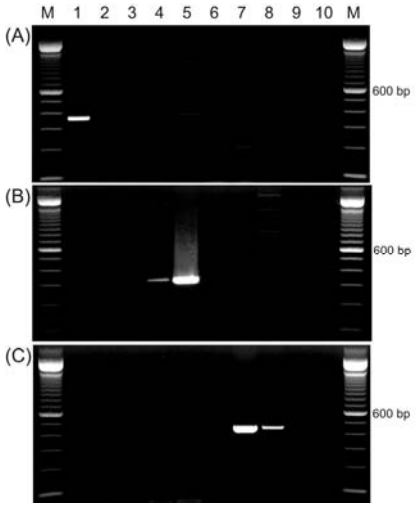

Fig. 4 RT-PCR detection of the filamentous virus observed in currants (A and B) and RMoV (C). (A) Detection of the BC variant (Cass_Fw/ Rv2; 365 bp). (B) Detection of the RC variant (Clost1Fw/ 2Rv; 324 bp). (C) Detection of RMoV according to Tzanetakis *et al.* (2007) (primers CPhF/ CPhR; 452 bp). M : 100 bp marker (bp) ; 1) BC No3SB 28074; 2) BC Titania 7/13 28119; 3) BC TE 27940; 4) RC No2SB 27986; 5) RC TE 27966; 6) RC 0g 28650; 7) Raspberry 5eL RMoV reference; 8) Raspberry 25081 RMoV reference; 9) Raspberry 12205; 10) H_2O.

Despite several attempts, the filamentous virus observed in currants could not be transmitted by mechanical sap inoculation to *Nicotiana benthamiana*.

Evidence of infection by the *Gooseberry vein banding associated virus* and the *Rubus chlorotic mottle virus*: Bacilliform particles were typical for the *Badnavirus* genus. Following the PCR protocol of Jones et al. (2001), this virus was identified as *Gooseberry vein banding associated virus* (GVBaV). Sequencing of the amplicon generated by the primers GVB1 for/ rev showed a similarity of 97% between the original sequence (AF298883) and those obtained from our red currant isolates (RC TE 27966; RC No3SB 28005). GVBaV-infection in some of the black currant accessions was also confirmed by PCR and sequencing (BC No3SB 28074; BC Titania 7/13 28119).

Following the RT-PCR procedure of McGavin and MacFarlane (2009), we clearly demonstrated the presence in our *Ribes* samples of *Rubus chlorotic mottle virus* (RuCMV), a *Sobemovirus* recently described in Scotland. A similarity of 91% was obtained between the sequenced RT-PCR product (primer 1082/ 1083) generated from the accession RC 0g 28650 and the reference sequence AM940437. This finding correlates with the presence of the 30 nm diameter particles observed by EM. Selected accessions analysed by EM (Table 1) tested negative for ArMV, TBRV, RpRSV, SLRSV in DAS-ELISA and BRV by RT-PCR.

Discussion

Closterovirus-like particles have been observed 12 years ago in *Ribes* samples (Roberts and Jones 1997). However, these particles were neither characterised nor associated with particular symptoms. In this work, EM analysis of nine reference accessions suggested an association between leafroll symptoms and the presence of a filamentous virus.

Therefore, we tentatively propose the name of Blackcurrant leafroll-associated virus 1 (BCLRaV-1) for this new virus. BCLRaV-1 was shown to be different from the RMoV by IPEM and RT-PCR. BCLRaV-1 virions have an approximate maximum length of 1500 nm and a capsid protein molecular weight of about 28 kDa. In phylogenetic analysis of partial HSP70h nucleotide sequences, BCLRaV-1 fell in the *Closterovirus* genus. Interestingly, we were able to distinguish two molecular BCLRaV-1 variants. Closteroviruses are known to display a high genetic variability (Maliogka, Dovas et al. 2008) with populations composed of variants or isolates with different biological properties (Meng, Li et al. 2005;Kong, Rubio et al. 2000;Beuve, Sempe et al. 2007). The question of considering the molecular variants of BCLRaV-1 described here as two variants of the same species or different virus species remains open, until their biological and molecular properties have been further analysed.

Symptoms of defoliation seems not to be associated with a particular virus or a complex of viruses and are possibly caused by physiological problems rather than having a viral aetiology. GVBaV was shown to be abundant in extracts of red currant and may be responsible for the vein clearing symptoms observed on three red currant accessions (Table 1) (Jones, Mcgavin, Geering, and Lockhart 2001). Furthermore, we report RuCMV infection of *Ribes* samples. RuCMV was isolated from a bramble with chlorotic mottle leaf symptoms and was shown to infect raspberry (Mcgavin and Macfarlane 2009). Its symptomatology on *Ribes* sp. needs however to be determined. Finally, we observed by EM in most of our samples spherical particles with a diameter of about 60 nm of an other uncharacterized entity.

This preliminary work pointed out the presence of multiple virus infections in *Ribes* sp. in Switzerland and emphasizes the need for an efficient sanitary selection process. A prerequisite is however the development of reliable and affordable diagnostic reagents such as monoclonal antibodies. The development of such diagnostic tools will not only be of interest for sanitary selection but they will also help us to pursue the study of *Ribes* viruses that frequently occur in mixed infection.

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