Biological properties of Beet soil-borne mosaic virus and Beet necrotic yellow vein virus cDNA clones produced by isothermal in vitro recombination: Insights for reassortant appearance

Biological properties of *Beet soil-borne mosaic virus* and *Beet necrotic yellow vein virus* cDNA clones produced by isothermal *in vitro* recombination: Insights for reassortant appearance

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Abstract

Two members of the *Benyviridae* family and genus *Benyvirus*, *Beet soil-borne mosaic virus* (BSBMV) and *Beet necrotic yellow vein virus* (BNYVV), possess identical genome organization, host range and high sequence similarity; they infect *Beta vulgaris* with variable symptom expression. In the US, mixed infections are described with limited information about viral interactions. Vectors suitable for agroinoculation of all genome components of both viruses were constructed by isothermal *in vitro* recombination. All 35S promoter-driven cDNA clones allowed production of recombinant viruses competent for *Nicotiana benthamiana* and *Beta macrocarpa* systemic infection and *Polymyxa betae* transmission and were compared to available BNYVV B-type clone. BNYVV and BSBMV RNA1 + 2 reassortants were viable and spread long-distance in *N. benthamiana* with symptoms dependent on the BNYVV type. Small genomic RNAs were exchangeable and systemically infected *B. macrocarpa*. These infectious clones represent a powerful tool for the identification of specific molecular host-pathogen determinants.

Keywords: *Beet soil-borne mosaic virus*; *Beet necrotic yellow vein virus*; *Benyviridae*; Gibson assembly; ~~infectious~~Infectious cDNA full-length clones; *Polymyxa betae*; ~~reassortment~~Reassortment

1 Introduction

Beet necrotic yellow vein virus (BNYVV) and *Beet soil-borne mosaic virus* (BSBMV) are both members of the genus *Benyvirus* in the family *Benyviridae* with BNYVV representing the type species (Gilmer and Ratti, 2012, Gilmer and Rattiet al., 2017(Please modify as: "Gilmer et al., 2017" and link to the reference: "Gilmer, D., Ratti, C., Ictv, R.C., 2017. ICTV Virus Taxonomy Profile: *Benyviridae*. J. Gen. Virol. 98, 2017, 1571-1572")). Both virus species mainly infect plants of the family *Amaranthaceae* (Heidel et al., 1997). BNYVV is known as the causative agent of rhizomania with worldwide distribution in nearly all sugar beet-growing areas (Peltier et al., 2008). In contrast, BSBMV is currently restricted to the United States (Heidel et al., 1997; Lee et al., 2001). BNYVV and BSBMV are both vectored by the soil-borne Plasmodiophoromycete *Polymyxa betae* Keskin where viral particles persist in the protozoa resting spores and therefore in the soil for decades (Keskin, 1964; Tamada and Kondo, 2013). Although representing closely related species sharing a similar host range (Heidel et al., 1997) and vector species, the symptoms in the natural host sugar beet (*Beta vulgaris*) differ considerably. In the field BSBMV infected sugar beet roots appear symptomless, whereas leaves displays light yellow vein banding, mottling or mosaic patterns and growth disorders (Heidel and Rush, 1994; Rush and Heidel, 1995). In contrast BNYVV infections are mainly confined to the root system that displays extensive proliferation of necrotizing secondary rootlets, a stunted tap root and a brownish discolouration of the vascular system. The leaves in upright position only rarely show symptoms like vein yellowing and necrotic leaf tissue (reviewed in Peltier et al., 2008). The impact of BNYVV on root weight is higher for BNYVV than BSBMV after mechanical inoculation (Heidel et al., 1997) and corresponds to higher yield losses with BNYVV compared to BSBMV (Wisler et al., 2003). Remarkably, *Rz1* gene used for rhizomania control has no effect on BSBMV infection (Wisler et al., 2003). Among the three major BNYVV subgroups (namely A, B and P), B-type is so far limited to Central and Northern Europe, whereas A-type is present worldwide (Koenig and Lennefors, 2000). Specific P25 amino acid residue variations, required for *Rz1* resistance-breaking abilities in sugar beet have only been detected in A-type isolates (Bornemann et al., 2015; Koenig et al., 2008, 2009; Liu and Lewellen, 2007; Pferdmenges and Varrelmann, 2009). Geographic genetic variability in BSBMV has not been analysed yet.

Benyviruses represent multipartite single-stranded positive-sense RNA viruses and consist of four capped, polyadenylated RNA segments that are separately encapsidated in rod-shaped particles (reviewed in Peltier et al., 2008). Some isolates of BNYVV possess an additional fifth RNA species (Tamada et al., 1996). While BNYVV and BSBMV display a similar genome organization, sufficient sequences differences allow classification as distinct species (Lee et al., 2001; Gilmer and Ratti, 2012, Gilmer and Rattiet al., 2017(Please modify as: "Gilmer et al., 2017" and link to the reference: "Gilmer, D., Ratti, C., Ictv, R.C., 2017. ICTV Virus Taxonomy Profile: *Benyviridae*. J. Gen. Virol. 98, 2017, 1571-1572")). RNA1 and RNA2 carry genes required for replication, movement, silencing suppression, packaging and vector transmission (Lee et al., 2001; Peltier et al., 2008). BNYVV RNA1 + 2 segments alone are sufficient for systemic infection in the experimental host *Nicotiana benthamiana* (Rahim et al., 2007). The single 237 K open reading frame (ORF) on RNA1 produces a polypeptide possessing methyltransferase (MetT), helicase (Hel), papain-like protease (Prot) and RNA-dependent RNA polymerase (RdRp) signatures. The six ORFs of RNA2 encode the coat protein (CP) which leaky UAG stop codon allows the translation of the readthrough protein (RT) associated with vector transmission (Tamada and Kusume, 1991). The next three overlapping ORFs form a cluster named triple gene block (TGB1-3) essential for cell-to-cell movement (Gilmer et al., 1992; Verchot-Lubicz et al., 2010). The 3'-proximal ORF encodes for a 14 kDa cysteine-rich protein with viral suppressor of RNA silencing (VSR) activity (Chiba et al., 2013; Dunoyer et al., 2002). Nearly all the molecular biology of Benyvirus RNA1 and RNA2 has been investigated on BNYVV. However, the high sequence similarity of the different proteins encoded by the viruses suggests functional similarity of BSBMV (Lee et al., 2001). BNYVV RNA3 has been described to be involved in viral pathogenicity and required for long distance movement in *Beta macrocarpa* (Lauber et al., 1998; Peltier et al., 2012; Flobinus et al., 2016). It encodes the P25 protein responsible for virus pathogenicity and the rhizomania disease phenotype in sugar beets (Chiba et al., 2008; Koenig et al., 1991). BSBMV RNA3 is also involved in long-distance movement and encodes a P29 protein that shows 23% amino acid similarity compared to BNYVV P25 and a much higher (43%) similarity to BNYVV RNA5-encoded P26 (Ratti et al., 2009). Both RNA4-encoded BNYVV P31 and BSBMV P32 proteins are responsible for vector transmission (D'Alonzo et al., 2012; Tamada and Abe, 1989). Sequence similarity suggests that P32 might be involved in symptom expression and suppression of RNA silencing that has been evidenced for P31 only in *N. benthamiana* roots (Rahim et al., 2007). BSBMV smaller RNAs are replicated and encapsidated by the BNYVV housekeeping machinery and complement the corresponding cognate RNA functions in *trans* (D'Alonzo et al., 2012; Ratti et al., 2009). The opposite situation of BNYVV smaller RNA replication by BSBMV RNA1 + 2 has not been reported yet.

To understand functional differences in molecular biology, pathogenicity mechanisms, symptom expression as well as interaction with the host and between viral species, a reverse genetic system represents a prerequisite. For BNYVV B-type, infectious cDNA clones for agroinoculation for RNA1-4 are available (Delbianco et al., 2013); however, A-type and BSBMV cDNA clones infectious clones were lacking. Initial construction of BNYVV B-type infectious clone for generation of infectious *in vitro* transcripts of RNA2 (Ouillet et al., 1989), was associated with stability or toxicity problems in *Escherichia coli*. Therefore Delbianco et al. (2013) successfully transformed ligated plasmids from reamplified BNYVV B-type cDNA and binary expression vectors into *Agrobacterium tumefaciens*.

The aim of this study was to generate infectious BSBMV and BNYVV A-type cDNA clones. In order to avoid possible cloning problems, the standard restriction enzyme based cloning was replaced with a one-step isothermal *in vitro* recombination assembly named Gibson assembly (GA) (Gibson et al., 2009). Recently, this method was applied for the first time for the generation of an infectious full-length clone of tomato blistering mosaic virus (ToBMV) (Blawid and Nagata, 2015). The clones obtained were characterized for their ability to reproduce the entire viral cycle including systemic infection, symptom expression in different host plants and vector transmission to demonstrate major functionality of the virus encoded proteins. We applied this approach on BSBMV and A-type BNYVV to extend the availability of cDNA clones and study the biological properties of standardized isolates and artificial reassortants. Viral accumulation, symptom expression and long-distance movement were assayed in *N. benthamiana* and *B. macrocarpa* to demonstrate the exchangeability of genome components between species.

2 Materials and methods

2.1 Virus and plant material

A BSBMV isolate (BSBMV-CA) from California USA, originally isolated by H.-Y. Liu (United States Department of Agriculture, Salinas, CA) and a BNYVV A-type isolate BNYVV-Yu2 (Kruse et al., 1994), (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany PV-0649) originated from former Yugoslavia were provided by DSMZ. As BNYVV-Yu2 did not allow RNA4 amplification, roots from sugar beet plants grown in BNYVV A-type containing soil from Rovigo (Italy) were used as source for RNA4.

The benyviruses hosts *C. quinoa* (local lesion), *B. macrocarpa* Guss., *B. vulgaris* ssp. *vulgaris* (*B. vulgaris*) susceptible genotype (without *Rz1* or *Rz2* resistance) and *N. benthamiana* served as host plants for the experimental work under greenhouse conditions (24 °C/14 h 18 °C/10 h).

2.2 Virus detection

Plant total RNA extracts were prepared using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. For each genome fragment of BNYVV and BSBMV, respectively, specific primers were developed (Table S1, see Supporting information) to allow RT-PCR detection. The cDNA synthesis was performed using RevertAid H Minus Reverse transcriptase (Thermo Fisher Scientific) and specific antisense primers. The PCR reaction was conducted with Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific) according to the manufacturer´s instruction. PCR products were visualized following agarose gel electrophoresis.

BNYVV genomic RNA detection by Northern hybridization was performed as previously described (Link et al., 2005; Schmidlin et al., 2005) while BSBMV RNAs 1 and 2 were detected using ^{32}P labeled RNA probes corresponding to position 4747–6549 of RNA1 and 2311–3774 of RNA2.

Additionally, a specific enzyme-linked immunosorbent assay (ELISA) of infected *N. benthamiana* leaves was performed to determine the virus content as previously described (Pferdmenges and Varrelmann, 2009).

2.3 BNYVV B-type full-length clones

The BNYVV B-type full-length clones (RNA1-4) for agroinoculation have been described previously (Delbianco et al., 2013).

2.4 Generation of BSBMV and BNYVV A-type full-length clones

For generation of full-length cDNA clones of both benyvirus species, total RNA preparations (RNeasy Plant Mini Kit, Qiagen) and dsRNA preparations (Darissa et al., 2010) from *C. quinoa* virus-induced local lesions were produced. Gibson assembly was applied as *in vitro* recombination method for the cloning of full-length cDNA of BSBMV and BNYVV A-type RNA1-4 into a small binary vector. For the full-length clones construction the plasmid pDIVA was used (Acc. No. KX665539), which is based on the mini binary vector pCB (Xiang et al., 1999), supplemented with a cauliflower mosaic virus (CaMV) 35 S promoter followed by a hepatitis delta virus (HDV) ribozyme and the polyadenylation signal of CaMV. All fragments (viral cDNA inserts and vector fragments) were generated by PCR amplification using Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific) following the manufacturer´s instructions. The vector plasmid was linearized by means of PCR amplification with a sense primer annealing to the 5´-end of the HDV ribozyme and an antisense primer annealing to the exact end of 35S promoter sequence. For successful GA the following sequence overlaps were generated during PCR. The 5´-end of each viral genome fragment was supplied with 25 nucleotides overlap to the exact 35S end. If multiple viral cDNA fragments had to be produced, a *ca.* 30–50 nt overlap between fragments was generated. The 3´-viral cDNA end was supplied with 18 nt overlap to the 5´-end of the HDV ribozyme sequence. The following Genbank nucleotide sequences represented the basis for the primer design: BSBMV RNA1 (6 683 nt, NC_003506.1), RNA2 (4 615 nt, NC_003503.1), RNA3 (1 720 nt, NC_003507.1), RNA4 (1 730 nt, FJ424610.2), BNYVV RNA1 (6 746 nt, NC_003514.1), RNA2 (4 609 nt, NC_003515.1), RNA3 (1 774 nt, NC_003516.1), RNA4 (1 465 nt, NC_003517.1). Viral cDNA was generated with the appropriate 3´-end antisense primer for each genome fragment (including the overlap) with RevertAid H Minus Reverse Transcriptase. Primers for amplification of the different viral genome components are displayed in Table S1 (see Supporting information). To generate full-length cDNA clones BSBMV RNA1 (6 683 nt) was converted into cDNA and PCR amplified in three overlapping products with size ranging from 2 255–2 275 nt. Two overlapping PCR fragments (from 908 to 3 879 nt) were generated for BSBMV RNA2 (4 615 nt), A-type BNYVV RNA1 (6 746 nt), RNA2 (4 609 nt), RNA3 (1 774 nt), whereas the smaller BSBMV RNAs 3 (1 720 nt), 4 (1 730 nt) and BNYVV RNA4 (1 465 nt) were RT-PCR amplified in one fragment each. All PCR products were gel-purified with NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). The GA was performed as described by Gibson et al. (2009).

In vitro recombination products were transformed into chemical competent *E. coli* cells (strains DH5α or NM522) (Inoue et al., 1990). Viral inserts were sequenced (Eurofins MWG Operon, Ebersberg, Germany and Seqlab, Goettingen, Germany) with specific primers. Resulting sequences were assembled with the Molecular Evolutionary Genetics Analysis (Tamura et al., 2013) software. Clustal Omega of the European Molecular Biology Laboratory-European Bioinformatics Institute was used to create a multiple sequence alignment and to check for sequence similarity with NCBI published genome sequences (http://www.ebi.ac.uk/Tools/msa/clustalo/). Subsequently all plasmids

were electroporated into *Rhizobium radiobacter* (syn. Agrobacterium tumefaciens) strain GV2260 (pGV2260). Plant infection was performed by means of agroinoculation according to Voinnet et al. (1998) with an OD_{600} = 0.5 for *B. macrocarpa* infection at BBCH12 stage. Fourteen-day-old *N. benthamiana* were agroinfiltrated with an OD_{600} of 0.1 to avoid necrosis induction with more concentrated suspensions. *A. tumefaciens* cultures carrying the different clones were mixed in equal amounts.

Leaves of *C. quinoa* were mechanically rub-inoculated with plant sap (1/5 diluted in 0.05 M phosphate buffer) from systemically agroinfected *N. benthamiana* plants. Besides agroinoculation, vortex-inoculation was used to infect *B. vulgaris* seedlings (BBCH 10) as described by Bornemann and Varrelmann (2011).

2.5 Electron microscopy

To obtain evidence for particle formation, transmission electron microscopy (TEM) was performed. *N. benthamiana* leaf tissue, systemically infected with BSBMV and BNYVV RNA1-4 respectively, initiated from agroinoculation of lower leaves was used for preparation of plant sap that was applied for TEM specimen preparation and visualisation (Milne and Lesemann, 1984). Particle decoration with specific antisera was performed at Julius-Kühn-Institute, Institute for Epidemiology and Pathogen Diagnostics (Braunschweig, Germany).

2.6 Polymyxa betae transmission

The BSBMV and BNYVV full-length clones derived from RNA1-4, respectively, were used for agroinoculation of *N. benthamiana* and leaf tissue sap was used for mechanical inoculation of 42 sugar beet seedlings (BNYVV-susceptible sugar beet cultivar KWS03). As controls served *C. quinoa* local lesions of the Californian BSBMV isolate and of the BNYVV A-type isolate. Seven plants per pot (six pots in total) were planted into virus-free field soil from a sugar beet field in Reutershof, (Brandenburg, Germany) that contained *P. betae* according to Bornemann and Varrelmann (2011). After growth for five weeks, plants and roots were removed and new seedlings were planted into the virus loaded soil for another period of five weeks. Finally, lateral roots were harvested and virus infection was assayed by means of RT-PCR with specific primers for RNA3 and RNA4.

3 Results

3.1 Generation of full-length cDNA clones of BSBMV and BNYVV A-type for agroinoculation

Following GA *in vitro* recombination into pDIVA (Acc. No. KX665539), clone identification, sequencing and *in silico* assembly of the complete viral cDNA inserts, the following BSBMV genome sizes (excluding polyA-tail) were obtained: BSBMV RNA1 6 674 nt, RNA2 4 615 nt, RNA3 1 720 nt and RNA4 1 729 nt. For BNYVV A-type the different RNA components displayed the following lengths: RNA1 6 746 nt, RNA2 4 588 nt, RNA3 1 775 nt and RNA4 1 470 nt. Viral sequences were submitted to Genbank (Acc. No. KX352033, KX352170, KX352171, KX352034, KX665536, KX665537, KX665538 and MF476800).

Sequence comparisons with published sequences of BSBMV isolates MRM06 (originating from Texas, USA; D'Alonzo et al., unpublished; D´Alonzo et al., 2012; Ratti et al., 2009) and EA (originating from Colorado, USA; Lee et al., 2001) were performed (Tables S2 and S3, see Supporting information). Sequence similarity of isolate BSBMV-CA at nucleotide level over all RNA components was closer to isolate MRM06 than EA (Table S2). The amino acid sequence similarities between isolate BSBMV-CA and MRM06 was striking for all ORF except for the RNA3 encoded P29 and RNA4 encoded ~~32~~32 K protein, respectively (Table S3).

The BNYVV Yu2 sequence obtained here represents the second complete A-type genome sequence in addition to the Japanese A-type isolate (BNYVV-S) (Saito et al., 1996). We determined the relatedness of these two geographically distant A-type strains and compared them to European B-type and P-type strains, confirming the closer relationship between the two A-type isolates compared to the P-type isolate (Tables S4 and S5).

3.2 Proof of infectivity

To obtain evidence about infectivity comparable to wild-type isolates, each cDNA clone was transformed into *R. radiobacter* (GV2260) and agrobacterial clones harbouring RNA1-4 cDNA of each species were mixed and agroinfiltrated into leaf tissue of known host plants *N. benthamiana*, *Chenopodium quinoa*, *B. macrocarpa* and *B. vulgaris* (Young et al., 2001). In *N. benthamiana* BSBMV RNA1-4 cDNA clones produced systemic symptoms of chlorotic vein banding, yellow blotches and leaf crinkling 12–16 days post-infiltration (dpi) and necrosis at 22 dpi (Fig. 1a). BNYVV A-type RNA1-4 cDNA clones led to systemic infection with deviating symptoms consisting of light yellow chlorosis that appeared delayed at 20–22 dpi (Fig. 1b).

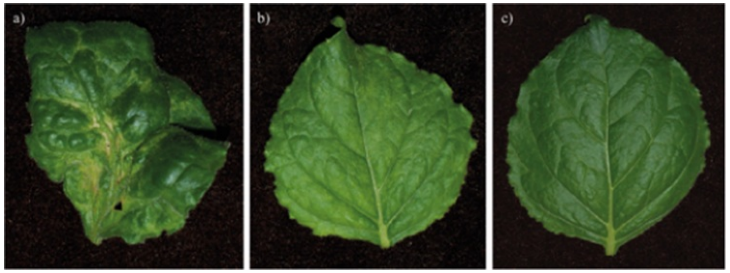

Fig. 1 Symptom expression on *Nicotiana benthamiana* leaves obtained after agroinoculation of (a) BSBMV cDNA clones RNA1-4 and (b) BNYVV cDNA clones RNA1-4 compared to (c) mock-inoculated healthy control at 22 dpi.

alt-text: Fig. 1

The local lesion host *C. quinoa* turned out to be resistant towards agroinoculation. Therefore, sap from systemically infected tissues of agroinoculated *N. benthamiana* was used for rub-inoculations. Both viruses induced typical specific local lesions at 7–10 dpi with BSBMV lesions developing to necrotic spots quite rapidly comparable to wild-type viruses. Agroinoculation of *B. macrocarpa* leaves with cDNA clones (RNA1-4) of both viruses initially resulted in local lesion formation at 6–9 dpi inside the infiltrated patch. Systemic spread and symptoms development occurred at 25–33 dpi (BSBMV) and 19–22 dpi (BNYVV), respectively (Fig. 6, c, h, see below). BSBMV or BNYVV agroinoculation of *B. vulgaris* leaf tissue resulted in local lesions formation inside the infiltrated patch at 13 and 18 dpi, respectively. Although lesions increased in size and slow spreading to leaf veins was observed, systemic spread associated with virus symptoms in newly emerging leaves was not observed with any of the two viruses. Additionally, variation of agroinoculation methods like vacuum-infiltration, root-dipping or vortex-inoculation of *B. vulgaris* roots with agrobacterium suspensions did not result in development of systemic infection. Finally, vortex-inoculation of 12 days old seedlings was applied according to Bornemann and Varrelmann (2011) using sap from *C. quinoa* local lesions. Following this approach, first systemic viral symptoms were observed at 35 dpi with cDNA derived BSBMV, 26 dpi with wild-type BSBMV, 30 dpi with cDNA derived BNYVV and 26 dpi with wild-type BNYVV (Fig. 2). Both cDNA clones induced wild-type like symptoms (Fig. 2a-d). ELISA based detection of viral accumulation in *B. vulgaris* lateral roots resulted in mean absorbance ($A_{405nm-405\,nm}$) values that were similar between cDNA derived and wild-type virus (0.40 vs 0.44 for BSBMV and 1.18 vs 1.46 for BNYVV). RT-PCR with specific primers allowed the detection and validation of the replication of all BSBMV or BNYVV RNA-components in total RNA extracts from systemically infected *N. benthamiana*, *B. macrocarpa* (agroinoculated) and *B. vulgaris* (vortex-inoculated) according to infiltrated or inoculated combinations.

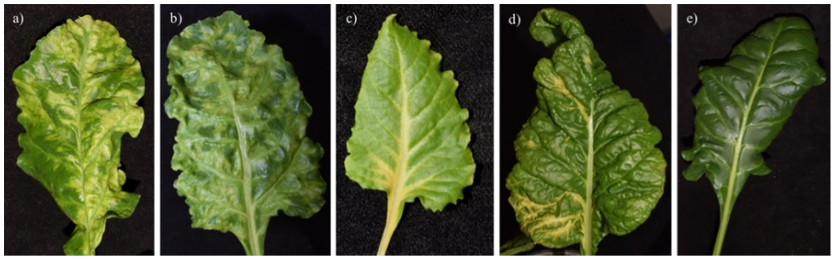

Fig. 2 Systemic symptom expression on *Beta vulgaris* leaves produced after mechanical root vortex-inoculation with plant sap from *C. quinoa* local lesions infected with (a) BSBMV cDNA clones RNA1-4, (b) wild-type BSBMV, (c) BNYVV cDNA clones RNA1-4 and (d) wild-type BNYVV compared to (e) healthy control at 48 dpi.

alt-text: Fig. 2

3.3 Electron microscopy

By means of TEM from BSBMV and BNYVV, respectively, cDNA clone infected *N. benthamiana*, rod shaped viral particles of varying lengths displaying a central core were observed from both samples (Fig. 3). Clear decoration with specific antisera was observed supporting virus identity.

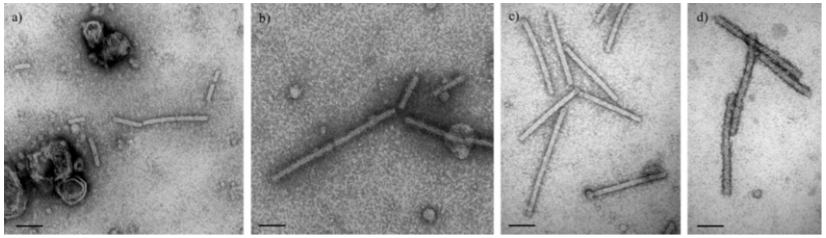

Fig. 3 (a) Transmission electron microscopy (TEM) of *Beet soil-borne mosaic virus* (BSBMV) and (c) *Beet necrotic yellow vein virus* (BNYVV) particles from systemically infected *N. benthamiana* leaf tissue and (b) BSBMV and (d) BNYVV particles, respectively, decorated with virus specific antisera. Bar represents 100 nm.

alt-text: Fig. 3

3.4 Polymyxa betae transmission of recombinant viruses

Furthermore, we verified the ability of BSBMV and BNYVV cDNA clones to be vectored by *P. betae.* After mechanical vortex inoculation of sugar beet and subsequent transplanting into the vector-containing soil, composite root samples from all source plants in each pot were ELISA-tested. BSBMV and BNYVV were detected in five out of six and six out of six pots, respectively (data not shown). Bait plants were subsequently grown in the loaded soil. RT–PCR confirmed the efficient transmission of BSBMV (4/6) and A-type BNYVV (4/7).

3.5 Viability of different BNYVV and BSBMV ~~RNA1+2~~RNA1 + 2 reassortants in N. benthamiana

We aimed to test for viability of RNA1 + 2 reassortants of the two viral species in *N. benthamiana* to assess the possibility for the production of reassortants during natural infection. As A- (BNa) and B-type (BNb) BNYVV differ substantially, we used both cDNA clones to produce BNYVV/BSBMV reassortants. To simplify the designation, we summarized the viral species by two capital followed if required by a lower case designating A- or B-type such as exemplified: BNa1BS2 corresponds to A-type BNYVV RNA1 inoculated in the presence of BSBMV RNA2, while BS1BN2b stands for BSBMV RNA1 inoculated in the presence of B-type BNYVV RNA2.

N. benthamiana symptoms produced by the inoculation of BS12 and BNa12 were undistinguishable from those produced after the inoculation of the all set of BSBMV and BNYVV RNAs suggesting that the smaller genomic RNA species did not significantly affect the systemic movement and symptom induction (compare Fig. 1a and b to Fig. 4a and b). BNa1BS2, BNb1BS2 and BS1BNb2 combinations were able to systemically infect *N. benthamiana* (9/9; 7/9 and 9/9 plants inoculated) (Fig. 4c, f, g and Table 1). However, symptoms appeared delayed and were less pronounced when compared to the natural RNA1 + 2 combination of each species. BNb1BS2 did not result in visible symptoms while BS1BNb2 displayed severe necrosis 21–23 dpi (Fig. 4 and Table 1). BNa12, BNb12 and BS12 infections appeared systemic 12–16 dpi while reassortants containing A-type BNYVV RNA species produced mild symptoms 16–23 dpi (BNa1BS2) and 23–29 dpi (BS1BNa2). Only one plant was systemically infected with BS1BNa2 and displayed faint systemic symptoms 23–29 dpi including rare occurrence of mild yellow veins. BNa1BS2 symptoms were similar but less pronounced to BS12. High ELISA values (29 dpi) were obtained for all plants displaying systemic symptoms as well as some symptomless plants inoculated by BNb12 and BN1bBS2 combinations (Table 1). Symptomless plants inoculated with BS1BNa2 produced ELISA values similar to the healthy control. BS12, BNa1BS2 and BNbBS2 were ELISA positive when assayed with BSBMV specific antiserum and BNa12, BNb12, BS1BNa2 and BS1BNb2 were detected with BNYVV specific antiserum.

Table 1 Occurrence of local and systemic symptoms in *Nicotiana benthamiana,* systemic infection rate and ELISA values after agroinoculation of BSBMV and BNYVV RNA1 + 2 (A or B-type) full-length clones compared to RNA1 + 2 BSBMV/BNYVV reassortants (see text for nomenclature).

alt-text: Table 1

Full-length clone	Symptom appearance (dpi)		Efficiency of systemic movement (%)	ELISA values (average)	Systemic symptoms
	Local	Systemic			
BS12	4–6	12–16	100 (9/9)	0.41	Chlorotic veins, leaf crinkling
BNa12	4–6	12–16	100 (9/9)	0.91	Light yellow chlorosis
BNb12	4–6	12–16	100 (9/9)	0.94	Mild yellowing/No symptoms
BNa1BS2	4–6	16–23	100 (9/9)	0.43	Chlorotic veins, leaf crinkling

BS1BNa2	4–6	23–29	11,1 (1/9)	0.83[a]	Faint leaf crinkling
BNb1BS2	6–7	26–28	77.7 (7/9)	0.40	Mild yellowing/No symptoms
BS1BNb2	6–7	21–23	100 (9/9)	0.86	Necrotic veins, leaf crinkling

[a] ~~only~~Only one plant was ~~infected~~infected.

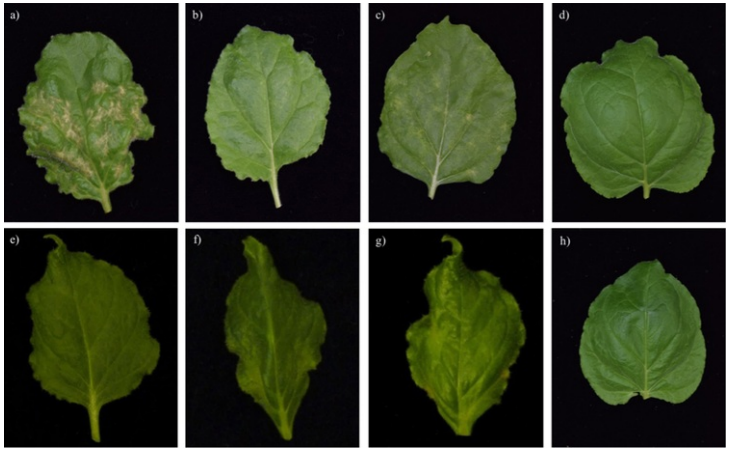

Fig. 4 Systemic symptom expression on *N. benthamiana* upper leaves at 17 days post agroinoculation of (a) BSBMV cDNA clones RNA1-2 (BS12), (b) BNYVV A-type cDNA clones RNA1-2 (BNa12) and ~~pseudorecombinants~~reassortants consisting of (c) BNYVV RNA1 + BSBMV RNA2 cDNA clones (BNa1BS2) and (d) BSBMV RNA1 + BNYVV RNA2 cDNA clones (BS1BNa2), (e) BNYVV B-type cDNA clones RNA1-2 (BNb12) and ~~pseudorecombinants~~reassortants consisting of (f) BNYVV B-type RNA1 + BSBMV RNA2 cDNA clones (BNb1BS2) and (g) BSBMV RNA1 + BNYVV B-type RNA2 cDNA clones (BS1BNb2) compared to (h) healthy control.

alt-text: Fig. 4

3.6 Influence of different RNA components on local lesion expression in C. quinoa

Sap from systemic leaves of *N. benthamiana* inoculated with BNa12, BNb12, BS12, BNa1BS2, BS1BNa2, BNb1BS2 and BS1BNb2 was applied for rub-inoculation of *C. quinoa* leaves and lesions appeared at 7 dpi (Fig. S1). BNa12 and BNb12 lesions were faint whereas BS12 lesions quickly developed necrosis. Lesions produced by reassortants BNa1BS2 (Fig. S1c) were comparable to BS12 whereas BNb1BS2 (Fig. S1f) displayed a more faint and BS1BNb2 (Fig. S1g) showed necrotic appearance. Interestingly, BS1BNa2 sap from infiltrated leaves applied for rub-inoculation of *C. quinoa* leaves did not produce local lesions (Fig. S1d). As already suspected under *3.5* and shown in Fig. 4 for systemic movement in *N. benthamiana* there was also a consistent specific RNA component effect on local lesion phenotype formation in *C. quinoa*.

3.7 Influence of different RNA components on viral RNA accumulation in C. quinoa

To evidence the effective replication of reassortants and find indications for an RNA species effect, lesions derived from BS12, BNb12 and corresponding reassortant infection were individually (5 each) collected for RNA extraction and northern blot analysis using RNA species specific probes (Flobinus et al., 2016). Signal processing using ImageJ software (Schneider et al., 2012) was used to estimate the accumulation of viral RNAs within each local lesion using RNA loading (ribosomal RNAs) normalization. We evidenced a higher accumulation of both genomic RNAs in BNb1BS2 reassortant when compared to BS1BNb2 and BNb12 or BS12 combinations (Fig. 5), confirming the specific RNA composition effect described above.

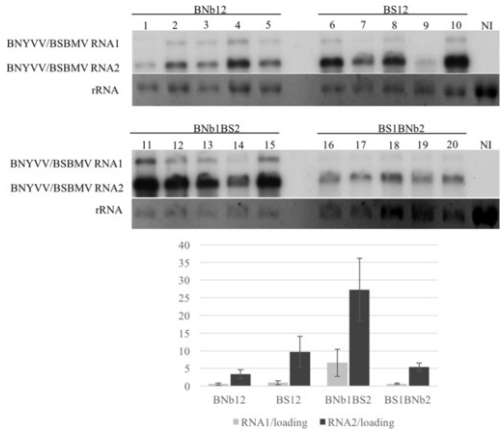

Fig. 5 Northern blot analysis of RNA extracted from local lesions of *C. quinoa* described in Fig. S1. BNYVV and BSBMV RNAs 1 and 2 were detected using specific ^{32}P labeled RNA probes while ribosomal RNAs (rRNA) have been used as loading control. Blotting image has been analysed by the ImageJ software to quantify the viral RNAs accumulation. Each bar in the presented graph indicates accumulation of BNYVV or BSBMV RNA1 and 2 normalized to the RNA loading (rRNAs).

alt-text: Fig. 5

3.8 Long distance movement function of BSBMV and BNYVV RNA3 in cis and in trans

In the USA, natural mixed infections occur between BNYVV A-type and BSBMV. As BSBMV RNA3 can substitute BNYVV RNA3 in BNYVV long-distance movement in *B. macrocarpa*, we focused on this combination for further experiments. As expected, agroinoculation of *B. macrocarpa* with BSBMV and BNYVV RNA1-2, respectively, did not lead to systemic infection (0/10 plants inoculated) (Fig. 6a, f, Table 2). Agroinoculation of primary leaves resulted in yellowing at 4–6 dpi and necrosis development at 12–18 dpi with no phenotypic differences between species and no such effect was observed when empty binary vector was used (data not shown). Systemic symptoms produced by wild-type BNYVV RNA1-3 (BNa1-3) were more pronounced and occurred rapidly (9/10 plants, 19–22 dpi) when compared to BSBMV RNA1-3 (BS1-3; 5/10 plants, 25–33 dpi). BSBMV infection induced yellow blotches and bands (Fig. 6b, c) while BNYVV mainly provoked vein yellowing (Fig. 6g, h). Systemic infection using BSBMV RNA1-2 + BNYVV RNA3 reassortants (BS12 + BNa3, Fig. 6d) was visible 19–25 dpi with symptoms comparable to those provoked by BS1-3. The systemic infection produced by BNYVV RNA1-2 + BSBMV RNA3 reassortants (BNa12 + BS3, Fig. 6i) appeared delayed (33–39 dpi) with symptoms similar to those formed by BS1-3 (Fig. 6b) and BS1-4 (Fig. 6c). When RNA4 was added to the three genomic species combinations, no differences were observed on the phenotype or infection kinetics (Fig. 6e, j and Table 2). In all treatments, presence or absence of individual viral RNAs in systemically infected *B. macrocarpa* tissue was assayed with specific primers by RT-PCR and corresponded to the input (data not shown).

Fig. 6 Systemic symptom expression in *Beta macrocarpa* obtained after agroinoculation of cDNA clones a) BSBMV RNA1 + 2, b) BSBMV RNA1-3, c) BSBMV RNA1-4, d) BSBMV RNA1 + 2 plus BNYVV RNA3, e) BSBMV RNA1-3 plus BNYVV RNA4, f) BNYVV RNA1 + 2, g) BNYVV RNA1-3, h) BNYVV RNA1-4, i) BNYVV RNA1 + 2 plus BSBMV RNA3, j) BNYVV RNA1-3 plus BSBMV RNA4, compared to k) healthy control at 43 dpi. BNYVV genome components are A-type derived.

alt-text: Fig. 6

Table 2 Development of local and systemic infection in *Beta macrocarpa* after agroinoculation of BSBMV and A-type BNYVV cDNA clones with different RNA composition compared to BSBMV and BNYVV reassortants.

alt-text: Table 2:

Full-length clone	Symptoms occurrence (dpi)		Efficiency of systemic movement (%)
	Local	Systemic	
BS12	6–9	–	0 (0/10)
BS1-3	6–9	25–33	50 (5/10)
BS1-4	6–9	25–33	30 (3/10)
BS12BNa3	6–9	19–25	80 (8/10)
BS1-3BNa4	6–9	22–26	70 (7/10)
BNa12	6–9	–	0 (0/10)
BNa1-3	6–9	19–22	90 (9/10)
BNa1-4	6–9	19–22	90 (9/10)
BNa12+BS3	6–9	33–39	80 (8/10)
BNa1-3BS4	6–9	19–22	100 (10/10)

4 Discussion

This study describes successful application of the GA for the generation of agroinfectious cDNA clones of two multipartite RNA viruses and to our knowledge it is the first example that describes successful assembly of three cDNA fragments in the case of BSBMV RNA1 (6 674 nt total cDNA) and two fragments for BNYVV RNA1 (6 746 nt) without any detectable functional errors. Identical properties were found when cDNA clones and natural isolates were compared.

Agroinoculation represents a quick and easy method to infect plants with cDNA clones of viruses (Nagyová and Subr, 2007). However, our study illustrated also some limitations of agroinoculation that failed in *C. quinoa* leaves and *B. vulgaris* roots. Solís et al. (2003) and Komari (1990) reported difficulties to transform *C. quinoa* by *A. tumefaciens*. Agroinoculation failure in sugar beet roots could be explained by a general lower efficiency of *A. tumefaciens* to transform root cells (Grevelding et al., 1993) and particularly by the sugar beets ability being recalcitrant to transformation (Krens et al., 1996; Wozniak, 1999), combined with a synergistic antiviral defence. Further work is required to bypass this issue, possibly by using *A. rhizogenes* described to efficiently transform sugar beet roots (Cai et al., 1997; Pavli et al., 2010). For such purpose, disarmed *A. rhizogenes* described by Mankin et al. (2007) will be required to alleviate undesired hairy roots phenotypic effect. Meanwhile, root inoculation with sap issued from agroinfected tissues provided an alternative inoculation method that successfully produced specific symptoms with viral accumulation assays similar to those observed in naturally infected plants (Heidel et al., 1997; Peltier et al., 2008).

This work also describes for the first time a direct comparison of BSBMV and BNYVV effects on several host plants including the natural host sugar beet. Specific symptoms observed on *N. benthamiana*, *B. macrocarpa* and *B. vulgaris* reflect the interspecies genetic variability observed and reproduced with the artificial clones. A-type BNYVV RNA1 + 2 and BSBMV RNA1 + 2 derived from cDNA clones are sufficient for long-distance movement in *N. benthamiana* confirming previous observations with BNYVV (Rahim et al., 2007). Such behaviour underlines the functional similarities of the two large genomic RNAs for both species. Our work also confirmed the involvement of both BSBMV and BNYVV RNA3 in virus long distance movement in *Beta* species (Lauber et al., 1998; Ratti et al., 2009; Peltier et al., 2012) as well as for the viral long distance movement of the reassortants produced (Table 2). The same observation applied as well for the RNA4 species for their involvement in vector transmission. While some RNA segment exchanges were performed in our study, all chimeric combinations were not assessed. However, taking advantage of infectious cDNA agroclones availability, there might be no limit for segment exchanges and recombinant production. Gene-exchange recombinants or mutants using A- and B-type BNYVV as well as BSBMV (not targeted by *Rz1*) will provide more precise information about the molecular basis of rhizomania resistance recognition by *Rz1* and/or *Rz2* as well as *Rz1* resistance breaking BNYVV strains (Bornemann and Varrelmann, 2013~~;and~~ Bornemann and Varrelmann, 2011(Please link to: "Bornemann K. and Varrelmann M., Analysis of the resistance-breaking ability of different Beet necrotic yellow vein virus isolates loaded into a single Polymyxa betae population in soil, Phytopathology 101, 2011, 718–724."); Koenig et al., 2009).

Benyvirus (BSBMV and BNYVV) RNA1 and 2 chimeric combinations analysed in *N. benthamiana* gave a first clear hint for the involvement of genome segments in symptom development mainly determined by the RNA2 species that encode the most divergent proteins. Interestingly, BS1BN2b reassortant was efficiently amplified and moved long distance in *N. benthamiana* whereas the same combination using A-type BNYVV did not led to reproducible and significant infection. Some of the combinations tested revealed the possible fitness penalty of reassortants compared to wild-type isolates. These combinations require further extensive analyses that were not the first objective of this study. An open question remains about the phenotypes observed in host plants where some reassortants symptoms appeared more severe than wild-type viruses or did not produced progeny in some hosts. One could expect a lethal effect of some combinations for infected cells or necrotic phenotype that would restrict the viability of the reassorted virus. So far, detection of reassorted BSBMV/BNYVV in sugar beet has never been described in the USA. To investigate, if the two species are able to co-infect the same cell, labeling of individual genome components including RNA2 is required. Preliminary experiments using replicons derived from BSBMV and BNYVV RNA3 species already evidenced the exclusion of the smaller RNA species (Ratti et al., 2009). Interestingly, under natural mixed infections (Rush and Heidel, 1995), BSBMV infection is lowered by BNYVV (Wisler et al., 2003) and cross protection has been described (Mahmood and Rush, 1999). If true recombinants occur in natural infections, leading to new virus genotypes with different properties and abilities to cause damage and disease needs to be investigated.

Uncited references

Bouzoubaa et al., 1985~~(~~; Bouzoubaa et al., 1987~~)~~; Bouzoubaa et al., 1986; Boyer and Haenni, 1994; Gilmer et al., 2017; Klein et al., 2007.

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Author ~~Contributions~~contributions

MV, EM, CR and DG conceived the study and experiments; EM and HM designed and constructed pDIVA and generated the BNYVV (A-type) cDNA clones including infectivity tests; ML generated the BSBMV cDNA clones, performed infectivity tests, vector transmission and reassortant analysis; KRP performed the TEM experiments; MDA performed the BNYVV (B-type)/BSBMV reassortant experiments; SL performed the BNYVV (A-type)/BSBMV reassortant experiments on *C. quinoa*; MDA and CR performed the Northern blot experiments; ML, MV, CR and DG wrote the manuscript.

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Database

NCBI KX352033KX352033,, KX352170KX352170,, KX352171KX352171,, KX352034KX352034,, KX665536KX665536,, KX665537KX665537, KX665538, KX665538 and MF476800

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2018.01.029.

References

Blawid R. and Nagata T., Construction of an infectious clone of a plant RNA virus in a binary vector using one-step Gibson Assembly, *J. Virol. Meth.Methods* **222**, 2015, 11–15.

Bornemann K., Hanse B., Varrelmann M. and Stevens M., Occurrence of resistance-breaking strains of *Beet necrotic yellow vein virus* in sugar beet in northwestern Europe and identification of a new variant of the viral pathogenicity factor P25, *Plant Pathol.* **64**, 2015, 25–34.

Bornemann K. and Varrelmann M., Analysis of the resistance-breaking ability of different *Beet necrotic yellow vein virus* isolates loaded into a single *Polymyxa betae* population in soil, *Phytopathology* **101**, 2011, 718–724.

Bornemann K. and Varrelmann M., Effect of sugar beet genotype on the *Beet necrotic yellow vein virus* P25 pathogenicity factor and evidence for a fitness penalty in resistance-breaking strains, *Mol. Plant Pathol.* **14**, 2013, 356–364.

Bouzoubaa S., Guilley H., Jonard G., Richards K. and Putz C., Nucleotide sequence analysis of RNA 3 and RNA 4 of *Beet necrotic yellow vein virus*, isolates F2 and G1, *J. Gen. Virol.* **66**, 1985, 1553–1564. (Please delete it)

Bouzoubaa S., Quillet L., Guilley H., Jonard Gt and Richards K., Nucleotide sequence of *Beet necrotic yellow vein virus* RNA 1, *J. Gen. Virol.* **68**, 1987, 615–626. (Please delete it)

Bouzoubaa S., Ziegler V., Beck D., Guilley H., Richards K. and Jonard G., Nucleotide sequence of *Beet necrotic yellow vein virus* RNA 2, *J. Gen. Virol.* **67**, 1986, 1689–1700. (Please delete it)

Boyer J. C. and Haenni A. L., Infectious transcripts and cDNA clones of RNA viruses, *Virology* **198**, 1994, 415–426. (Please delete it)

Cai D., Kleine M., Kifle S., Harloff H.-J., Sandal N.N., Marcker K.A., Klein-Lankhorst R.M., Salentijn E.M., Lange W. and Stiekema W.J., Positional cloning of a gene for nematode resistance in sugar beet, *Science* **275**, 1997, 832–834.

Chiba S., Hleibieh K., Delbianco A., Klein E., Ratti C., Ziegler-Graff V., Bouzoubaa S. and Gilmer D., The *Benyvirus* RNA silencing suppressor is essential for long-distance movement, requires both zinc-finger and NoLS basic residues but not a nucleolar localization for its silencing-suppression activity, *MPMI* **26**, 2013, 168–181.

Chiba S., Miyanishi M., Andika I.B., Kondo H. and Tamada T., Identification of amino acids of the *Beet necrotic yellow vein virus* p25 protein required for induction of the resistance response in leaves of *Beta vulgaris* plants, *J. Gen. Virol.* **89**, 2008, 1314–1323.

D'Alonzo M., Delbianco A., Lanzoni C., Autonell C.R., Gilmer D. and Ratti C., *Beet soil-borne mosaic virus* RNA-4 encodes a 32 kDa protein involved in symptom expression and in virus transmission through *Polymyxa betae*, *Virology* **423**, 2012, 187–194.

Darissa O., Willingmann P. and Adam G., Optimized approaches for the sequence determination of double-stranded RNA templates, *J. Virol. Methods.Methods* **169**, 2010, 397–403.

Delbianco A., Lanzoni C., Klein E., Rubies Autonell C., Gilmer D. and Ratti C., Agroinoculation of *Beet necrotic yellow vein virus* cDNA clones results in plant systemic infection and efficient *Polymyxa betae* transmission, *Mol. Plant Pathol.* **14**, 2013, 422–428.

Dunoyer P., Pfeffer S., Fritsch C., Hemmer O., Voinnet O. and Richards K.E., Identification, subcellular localization and some properties of a cysteine-rich suppressor of gene silencing encoded by *peanut clump virus*, *Plant J.* **29**, 2002, 555–567.

Flobinus A., Hleibieh K., Klein E., Ratti C., Bouzoubaa S. and Gilmer D., A ~~Viral Noncoding~~viral noncoding RNA ~~Complements~~complements a ~~Weakened Viral~~weakened viral RNA ~~Silencing Suppressor~~silencing suppressor and ~~Promote Efficient Systemic Host Infection~~promotes efficient systemic host infection, *Viruses* **8**, 2016, 272.

Gibson D.G., Young L., Chuang R.-Y., Venter J.C., Hutchison C.A. and Smith H.O., Enzymatic assembly of DNA molecules up to several hundred kilobases, ~~*Nature Meth.*~~*Nat. Methods* **6**, 2009, 343–345.

Gilmer D., Bouzoubaa S., Hehn A., Guilley H., Richards K. and Jonard G., Efficient cell-to-cell movement of *Beet necrotic yellow vein virus* requires 3' proximal genes located on RNA 2, *Virology* **189**, 1992, 40–47.

Gilmer, D., Ratti, C., 2012. Genus *Benyvirus*. Virus Taxonomy: Classification and Nomenclature. Ninth Report of the International Committee on Taxonomy of Viruses, 1163-1168.

Gilmer D., Ratti C. and Ictv R.C., ICTV Virus Taxonomy Profile: *Benyviridae*, ~~*J Gen*~~*J. Gen. Virol.* **98**, 2017, 1571–1572.

Grevelding C., Fantes V., Kemper E., Schell J. and Masterson R., Single-copy T-DNA insertions in *Arabidopsis* are the predominant form of integration in root-derived transgenics, whereas multiple insertions are found in leaf discs, *Plant Mol. Biol.* **23**, 1993, 847–860.

Heidel G. and Rush C., Distribution of *Beet necrotic yellow vein virus*, beet distortion mosaic virus, and an unnamed soilborne sugar beet virus in Texas and New Mexico, *Plant Dis.* **78**, 1994, 603–606.

Heidel G., Rush C., Kendall T., Lommel S. and French R.C., Characteristics of *Beet soilborne mosaic virus*, a furo-like virus infecting sugar beet, *Plant Dis.* **81**, 1997, 1070–1076.

Inoue H., Nojima H. and Okayama H., High efficiency transformation of *Escherichia coli* with plasmids, *Gene* **96**, 1990, 23–28.

Keskin B., *Polymyxa betae* n. sp., ein Parasit in den Wurzeln von *Beta vulgaris* Tournefort, besonders während der Jugendentwicklung der Zuckerrübe, *Arch. Mikrobiol.* **49**, 1964, 348–374.

~~Klein E., Link D., Schirmer A., Erhardt M. and Gilmer D., Sequence variation within *Beet necrotic yellow vein virus*p25 protein influences its oligomerization and isolate pathogenicity on *Tetragonia expansa*, *Virus Res.* **126**, 2007, 53–61.~~
(Please delete it)

Koenig R., Jarausch W., Li Y., Commandeur U., Burgermeister W., Gehrke M. and Luddecke P., Effect of recombinant *Beet necrotic yellow vein virus* with different RNA compositions on mechanically inoculated sugarbeets, *J. Gen Virol.* **72**, 1991, 2243–2246.

Koenig R., Kastirr U., Holtschulte B., Deml G. and Varrelmann M., Distribution of various types and P25 subtypes of *Beet necrotic yellow vein virus* in Germany and other European countries, *Arch. Virol.* **153**, 2008, 2139–2144

Koenig R. and Lennefors B.-L., Molecular analyses of European A, B and P type sources of *Beet necrotic yellow vein virus* and detection of the rare P type in Kazakhstan, *Arch. Virol.* **145**, 2000, 1561–1570.

Koenig R., Loss S., Specht J., Varrelmann M., Lüddecke P. and Deml G., A single U/C nucleotide substitution changing alanine to valine in the *Beet necrotic yellow vein virus*p25 protein promotes increased virus accumulation in roots of mechanically inoculated, partially resistant sugar beet seedlings, *J. Gen. Virol.* **90**, 2009, 759–763.

Komari T., Transformation of cultured cells of Chenopodium quinoa by binary vectors that carry a fragment of DNA from the virulence region of pTiBo542, *Plant Cell Rep.* **9**, 1990, 303–306.

Krens F.A., Trifonova A., Keizer L.P. and Hall R.D., The effect of exogenously-applied phytohormones on gene transfer efficiency in sugarbeet (*Beta vulgaris* L.), *Plant Sci.* **116**, 1996, 97–106.

Kruse M., Koenig R., Hoffmann A., Kaufmann A., Commandeur U., Solovyev A., Savenkov I. and Burgermeister W., Restriction fragment length polymorphism analysis of reverse transcription-PCR products reveals the existence of two major strain groups of *Beet necrotic yellow vein virus*, *J. Gen. Virol.* **75**, 1994, 1835–1842.

Lauber E., Guilley H., Tamada T., Richards K. and Jonard G., Vascular movement of *Beet necrotic yellow vein virus* in ~~*Beta*~~*beta macrocarpa* is probably dependent on an RNA 3 sequence domain rather than a gene product, *J. Gen. Virol.* **79**, 1998, 385–393.

Lee L., Telford E., Batten J., Scholthof K.-B. and Rush C., Complete nucleotide sequence and genome organization of *Beet soilborne mosaic virus*, a proposed member of the genus *Benyvirus*, *Arch. Virol.* **146**, 2001, 2443–2453.

Link D., Schmidlin L., Schirmer A., Klein E., Erhardt M., Geldreich A., Lemaire O. and Gilmer D., Functional characterization of the *Beet necrotic yellow vein virus* RNA-5-encoded p26 protein: evidence for structural pathogenicity determinants, *J. Gen. Virol.* **86**, 2005, 2115–2125.

Liu H.-Y. and Lewellen R., Distribution and molecular characterization of resistance-breaking isolates of *Beet necrotic yellow vein virus* in the United States, *Plant Dis.* **91**, 2007, 847–851.

Mahmood T. and Rush C.M., Evidence of Cross-Protection between Beet soilborne mosaic virus and *Beet necrotic yellow vein virus* in Sugar Beet, *Plant Dis.* **83**, 1999, 521–526.

Mankin S.L., Hill D.S., Olhoft P.M., Toren E., Wenck A.R., Nea L., Xing L., Brown J.A., Fu H. and Ireland L., Disarming and sequencing of *Agrobacterium rhizogenes* strain K599 (NCPPB2659) plasmid pRi2659, *In Vitro Cell. Dev. ~~Biol.-Plant.~~Biol. -Plant.* **43**, 2007, 521–535.

Milne R. and Lesemann D., Immunosorbent ~~electron microscopy~~Electron Microscopy in ~~plant virus studies~~Plant Virus Studies. Methods in Virology, ~~Methods in Virology~~1984, Academic Press; Orlando, 85–101.

Nagyová A. and Subr Z., Infectious full-length clones of plant viruses and their use for construction of viral vectors, *Acta Virol.* **51**, 2007, 223.

Ouillet L., Guilley H., Jonard G. and Richards K., In vitro synthesis of biologically active *Beet necrotic yellow vein virus* RNA, *Virology* **172**, 1989, 293–301.

Pavli O.I., Panopoulos N.J., Goldbach R. and Skaracis G.N., BNYVV-derived dsRNA confers resistance to rhizomania disease of sugar beet as evidenced by a novel transgenic hairy root approach, *Transgenic Res.* **19**, 2010, 915–922.

Peltier C., Hleibieh K., Thiel H., Klein E., Bragard C. and Gilmer D., Molecular biology of the *Beet necrotic yellow vein virus*, *Plant ~~Viruses~~Virus.* **2**, 2008, 14–24.

Peltier C., Klein E., Hleibieh K., D'Alonzo M., Hammann P., Bouzoubaa S., Ratti C. and Gilmer D., *Beet necrotic yellow vein virus* subgenomic RNA3 is a cleavage product leading to stable non-coding RNA required for long-distance movement, *J. Gen. Virol.* **93**, 2012, 1093–1102.

Pferdmenges F. and Varrelmann M., Breaking of *Beet necrotic yellow vein virus* resistance in sugar beet is independent of virus and vector inoculum densities, *Eur. J. Plant Pathol.* **124**, 2009, 231–245.

Rahim M.D., Andika I.B., Han C., Kondo H. and Tamada T., RNA4-encoded p31 of *Beet necrotic yellow vein virus* is involved in efficient vector transmission, symptom severity and silencing suppression in roots, *J. Gen. Virol.* **88**, 2007, 1611–1619.

Ratti C., Hleibieh K., Bianchi L., Schirmer A., Autonell C.R. and Gilmer D., *Beet soil-borne mosaic virus* RNA-3 is replicated and encapsidated in the presence of BNYVV RNA-1 and −2 and allows long distance movement in *Beta macrocarpa*, *Virology* **385**, 2009, 392–399.

Rush C. and Heidel G., Furovirus disease of sugar beets in the United States, *Plant Dis.* **79**, 1995, 868–875.

Saito M., Kiguchi T., Kusume T. and Tamada T., Complete nucleotide sequence of the Japanese isolate S of *Beet necrotic yellow vein virus* RNA and comparison with European isolates, *Arch. Virol.* **141**, 1996, 2163–2175.

Schmidlin L., Link D., Mutterer Jm, Guilley H. and Gilmer D., Use of a *Beet necrotic yellow vein virus* RNA-5-derived replicon as a new tool for gene expression, *J. Gen. Virol.* **86**, 2005, 463–467.

Schneider C.A., Rasband W.S. and Eliceiri K.W., NIH Image to ImageJ: 25 years of image analysis, ~~*Nature methods*~~*Nat. Methods* **9**, 2012, 671–675.

Solís J.F., Mlejnek P., Studená K. and Procházka S., Application of sonication-assisted Agrobacterium-mediated transformation in *Chenopodium rubrum*. L, *Plant Soil ~~Environ~~Environ.* **49**, 2003, 255–260.

Tamada T. and Abe H., Evidence that *Beet necrotic yellow vein virus* RNA-4 is essential for efficient transmission by the fungus *Polymyxa betae*, *J. Gen. Virol.* **70**, 1989, 3391–3398.

Tamada T. and Kondo H., Biological and genetic diversity of plasmodiophorid-transmitted viruses and their vectors, *J. Gen. Plant Pathol.* **79**, 2013, 307–320.

Tamada T. and Kusume T., Evidence that the 75K readthrough protein of *Beet necrotic yellow vein virus* RNA-2 is essential for transmission by the fungus *Polymyxa betae*, *J. Gen. Virol.* **72**, 1991, 1497–1504.

Tamada T., Schmitt C., Saito M., Guilley H., Richards K. and Jonard G., High resolution analysis of the readthrough domain of *Beet necrotic yellow vein virus* readthrough protein: a KTER motif is important for efficient transmission of the virus by *Polymyxa betae*, *J. Gen. Virol.* **77**, 1996, 1359–1367.

Tamura K., Stecher G., Peterson D., Filipski A. and Kumar S., MEGA6: molecular evolutionary genetics analysis version 6.0, *Mol. Biol. Evol.* **30**, 2013, 2725–2729.

Verchot-Lubicz J., Torrance L., Solovyev A.G., Morozov S.Y., Jackson A.O. and Gilmer D., Varied movement strategies employed by triple gene block–encoding viruses, *Mol. Plant Microbe Interact.* **23**, 2010, 1231–1247.

Voinnet O., Vain P., Angell S. and Baulcombe D.C., Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA, *Cell* **95**, 1998, 177–187.

Wisler G., Lewellen R., Sears J., Wasson J., Liu H.-Y. and Wintermantel W., Interactions between *Beet necrotic yellow vein virus* and *Beet soilborne mosaic virus* in sugar beet, *Plant Dis.* **87**, 2003, 1170–1175.

Wozniak C.A., Transgenic sugar beet: progress and development, ~~*Appl*~~*Appl. Plant Biotech.* **10**, 1999, 301–324.

Xiang C., Han P., Lutziger I., Wang K. and Oliver D.J., A mini binary vector series for plant transformation, *Plant Mol. Biol.* **40**, 1999, 711–717.

Young J.M., Kuykendall L.D., Martínez-Romero E., Kerr A. and Sawada H., A revision of *Rhizobium* Frank 1889, with an emended description of the genus, and the inclusion of all species of *Agrobacterium* Conn 1942 and *Allorhizobium undicola* de Lajudie *et al.* 1998 as new combinations: *Rhizobium radiobacter*, *R. rhizogenes*, *R. rubi*, *R. undicola* and *R. vitis*, *Int. J. Syst. Evol. Microbiol.* **51**, 2001, 89–103.

Appendix A. Supplementary material

Multimedia Component 1

Supplementary material

Highlights

- Generation of infectious clones of BNYVV and BSBMV by *in vitro* recombination.
- ~~•The~~The clone characteristics were comparable to the wild-type virus isolates.
- ~~•Small~~Small RNAs displayed interspecies exchangeability.
- ~~•Reassortant~~Reassortant viruses were able to systemically infect different host plants.

Queries and Answers

Query:

Please confirm that given names and surnames have been identified correctly and are presented in the desired order, and please carefully verify the spelling of all authors.

Answer: I verified. Please change the surname "Dall'Ara" using capital letter.

Query:

Your article is registered as a regular item and is being processed for inclusion in a regular issue of the journal. If this is NOT correct and your article belongs to a Special Issue/Collection please contact m.vs@elsevier.com immediately prior to returning your corrections.

Answer: Our article is a regular item

Query:

The reference given here is cited in the text but is missing from the reference list – please make the list complete or remove the reference from the text: Gilmer and Ratti, 2017, Bornemann and Varrelmann, 2011.

Answer: Problems fixed, please see instructions - replace and Ratti by et al.

Query:

Reference(s) given here were noted in the reference list but are missing from the text – please position each reference in the text or delete it from the list.

Answer: All references have been deleted from the list except Gilmer et al., 2017 (see query 3 and instructions). Gilmer et al., 2017 replaces Gilmer et Ratti, 2017 - Remove Uncited references part