**Seed-borne viral dsRNA elements in three cultivated
Raphanus and Brassica plants suggest three cryptoviruses**

Journal:	<i>Canadian Journal of Microbiology</i>
Manuscript ID	cjm-2015-0788.R1
Manuscript Type:	Rapid communication
Date Submitted by the Author:	31-Jan-2016
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Keyword:	dsRNA virus, Raphanus, Brassica, Partitivirus, Plant virus

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1 Seed-borne viral dsRNA elements in three cultivated *Raphanus* and
2 *Brassica* plants suggest three cryptoviruses

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19 Footnotes: The GenBank accession numbers of the sequences reported in this paper
20 are KT285019 for *Raphanus sativus* partitivirus 1 ds1900, KT285020 and KT285021
21 for *Sinapis alba* cryptic virus 1 dsR1 and dsR2, respectively, and KT285022 for
22 *Brassica rapa* cryptic virus 1 dsR1.

23

Abstract

Since the 1970s, several dsRNA viruses, including *radish yellow edge virus* (RYEV), *Raphanus sativus* virus 1 (RsV1), *Raphanus sativus* virus 2 (RsV2) and *Raphanus sativus* virus 3 (RsV3) have been identified and reported as infecting radish. Here, in conjunction with a survey of seed-borne viruses in cultivated *Brassica* and *Raphanus* using the dsRNA diagnostic method, we discovered three novel cryptoviruses that infect *Brassica* and *Raphanus*: *Raphanus sativus* partitivirus 1 (RsPV1), which infects radish (*Raphanus sativus*); *Sinapis alba* cryptic virus 1 (SaCV1), which infects *Sinapis alba*; and *Brassica rapa* cryptic virus 1 (BrCV1), which infects *Brassica rapa*. The genomic organization of these cryptoviruses was analyzed and characterized. BrCV1 might represent the first plant partitivirus found in *Gammapartitivirus*. Additionally, the evolutionary relationships among all of the partitiviruses reported in *Raphanus* and *Brassica* were analyzed.

Keyword: dsRNA virus, *Raphanus*, *Brassica*, Partitivirus, Plant virus

Introduction

The genomes of the partitiviruses identified to date typically comprise two dsRNA segments, which range in size from 1.4 to 3.0 kbp. The larger dsRNA encodes RNA-dependent RNA polymerase (RdRp), and the smaller dsRNA encodes capsid protein (CP) (King et al. 2012). Though widely presenting in fungi, plants, some protozoa and possibly some higher animals (King et al. 2012; Liu et al. 2012), almost all reported partitiviruses cause asymptomatic infections in their hosts (Antoniw et al. 1986; Boccardo et al. 1985; Dodds et al. 1984; Lisa et al. 1981). Partitiviruses that

1 infect plants are usually designated as cryptoviruses. Since the 1970s, cryptoviruses
2 have been detected in the beet (Antoniw et al. 1986; Xie et al. 1993a; Xie et al.
3 1993b), broad bean (Abo-elnasr et al. 1985; Antoniow et al. 1986), white clover
4 (Boccardo and Candresse 2005a, b; Boccardo et al. 1985), carnation (Lisa et al. 1981),
5 radish (Chen et al. 2006a; Chen et al. 2006b; Natsuaki et al. 1983; Natsuaki et al.
6 1979), *Brassica* spp., hop trefoil, alfalfa, Italian ryegrass, meadow fescue, red clover,
7 spinach, carrot (Dodds et al. 1984), rose (Salem et al. 2008; Tzanetakis et al. 2008),
8 pear (Osaki et al. 1998), and pine (Veliceasa et al. 2006).

9 *Brassica* and *Raphanus*, members of the Brassicaceae family, are two closely
10 related plant genera. These two genera include various important agricultural plants
11 that are grown worldwide, including vegetables, ornamental plants, and plants used
12 for oil production and as fodder. As early as the 1970s, a cryptovirus named radish
13 yellow edge virus (RYEV) was reported to occur in Japanese radish (Natsuaki et al.
14 1979). Five dsRNA fragments were detected in RYEV-infected radish plants using
15 polyacrylamide gel electrophoresis (Natsuaki et al. 1983). In 2006, members of our
16 laboratory reported on two partitiviruses in a Chinese radish cultivar, *Raphanus*
17 *sativus* virus 1 (RsV1) and *Raphanus sativus* virus 2 (RsV2) (Chen et al. 2006a; Chen
18 et al. 2006b). Recently, we characterized another radish cryptovirus containing three
19 genomic dsRNA segments and found that various seed-borne dsRNA viruses are
20 present in many different radish cultivars (Li 2013; Li et al. 2013).

21 In this study, we identified three novel partitiviruses that infect three *Brassica* and
22 *Raphanus* species: *Raphanus sativus* partitivirus 1 (RsPV1), which infects radish (*R.*

1 *sativus*); *Sinapis alba* cryptic virus 1 (SaCV1), which infects *S. alba*; and *Brassica*
2 *rapa* cryptic virus 1 (BrCV1), which infects *B. rapa*. The complete genomic
3 sequences of SaCV1 and single dsRNA segments of BrCV1 and RsPV1 were
4 obtained using a modified single-primer amplification technique (M-SPAT). The
5 genomic organization of these partitiviruses was analyzed and characterized.
6 Furthermore, the evolutionary relationships among all of the partitiviruses reported in
7 *Raphanus* and *Brassica* were investigated.

8 **Materials and Methods**

9 **Plant materials**

10 *R. sativus* cv. Daqingtou (DQT) and *B. rapa* cv. Youdongercaitai (YDECT) seeds
11 were obtained from commercial seed sources. DQT was obtained from Kexing Seed
12 Company, Mianyang City, Sichuan Province, China, and YDECT was obtained from
13 Kefeng Seed Company, Hangzhou City, Zhejiang Province, China. DQT, a root
14 vegetable, and YDECT, a leaf vegetable, are widely grown in China. *S. alba* cv.
15 Lintaobaijie (LTBJ) seeds were kindly provided by Professor Wancang Sun of Gansu
16 Academy of Agricultural Sciences, China. The plants were grown in a greenhouse
17 environment to protect them from wild epidemic viruses.

18 **Extraction and fractionation of double-stranded RNA**

19 Total dsRNA was extracted from plant leaves as described previously (Chen et al.
20 2006a; Li et al. 2013) using a method modified from Morris and Dodds (1979).
21 Briefly, 3-5 g of fresh plant leaves was pulverized in liquid nitrogen. Phenol,
22 chloroform, SDS, and β -mercaptoethanol were then added to break down the plant

1 cells to release the nucleic acids by vortex-mixing for 20 min. After centrifugation,
2 the supernatant was collected and blended with pure ethanol, which was added to a
3 final concentration of 17%. The mixture was then passed through a CF-11 cellulose
4 chromatographic column (Sigma-Aldrich, St. Louis, USA), which had been soaked
5 with a solution of 17% ethanol-1× STE (50 mM Tris, 0.1 M NaCl, and 1 mM EDTA).
6 The column was then rinsed with 100 ml of 17% ethanol in 1× STE. The dsRNA
7 sample was finally eluted from the column using 1× STE buffer. The eluted solution
8 was precipitated with an equivalent volume of isopropyl alcohol, and the precipitate
9 was washed with 75% ethanol. The precipitate was then re-suspended in
10 DEPC-treated water for northern blot hybridization. Segments of dsRNA were
11 fractionated in a 1% agarose gel. For cDNA synthesis, dsRNAs were resolved and
12 individually purified using an AxyPrep™ DNA Gel Extraction Kit (Axygen,
13 Hangzhou, China).

14 **Reverse transcription, PCR amplification, cloning and sequencing**

15 Full-length cDNA clones corresponding to dsRNA segments were obtained using a
16 single-primer amplification technique (M-SPAT) modified from Lambden et al.
17 (M-SPAT) (Chen et al. 2006a; Lambden et al. 1992; Li et al. 2013). Briefly, a
18 single-stranded DNA adaptor (primer A:
19 5'-PO4-TCTTCGGGTGTCCTTCCTCG-NH₂-3') was ligated to the 3' ends of the
20 dsRNA segments using T4 RNA ligase (Fermentas, Canada) and T4 DNA ligase
21 (Fermentas, Canada). Then, the blunt 5' end was then elongated using Taq DNA
22 polymerase (TaKaRa, Dalian, China). cDNA was obtained through reverse

transcription with primer B (5'-CGAGGAAGGACACCCGAAGA-3'), which was complementary to primer A. The synthesized cDNAs were used as templates for PCR amplification using primer B. The PCR products were purified using an Axygen DNA gel recycle kit (Axygen, China) and ligated into the pGEM T-easy vector (Fermentas, USA). The products were then transformed into *Escherichia coli*. All clones were selected and sequenced, at least in triplicate, using a Sanger 3730 system to obtain consensus sequences.

Sequence analysis

Putative protein sequences were predicted by using BioEdit (Hall 1999). Searches for similar sequences were conducted using the BLAST program against the NCBI GenBank database (Altschul et al. 1990). Sequence alignments were performed using MUSCLE (Edgar 2004), and phylogenetic analyses were performed using MEGA version 5 (Tamura et al. 2011).

Viral RNA extraction and northern blot hybridization

Virus particles were isolated from young radish leaves (>50 g) using the method described in previous work (Li et al. 2013). Purified virus particles were observed under transmission electron microscopy after staining with 2% uranyl acetate. Viral RNA was extracted from the purified virions using TRIzol reagent (Invitrogen, USA) and phenol-chloroform mixtures. For northern blot hybridization, 50 ng of viral RNAs was denatured at 100 °C for 5 min and then resolved on a 1% TBE agarose gel. The gel was then soaked in DEPC water for 10 min, and the ssRNAs were transferred via capillary action to a Hybond-N+ nylon membrane (GE-healthcare) in 20× SSC buffer

overnight. The denatured viral nucleic acids were fixed on the membrane by heating at 80 °C for 2 hr.

Probes for detecting RsV1, RsV2 and RsPV1 were synthesized as follows: Templates for probe synthesis were obtained by PCR amplification from the corresponding plasmids using specific primer pairs (Supplemental Table 1). The obtained PCR products were then used to synthesize biotin-11-dUTP-labeled probes using 6-mer nucleotide random primers according to the protocol included in the Biotin DecaLabel DNA Labeling Kit (Fermentas, Canada). Pre-hybridization and hybridization were performed under high stringency conditions using a Biotin chromogenic detection kit (Fermentas, Canada).

Results

1. dsRNA analysis

Putative viral dsRNAs were found in the radish cultivar DQT, *S. alba* cv. LTBJ and *B. rapa* cv. YDECT with high frequency. No symptoms of viral infection were apparent in these dsRNA-containing plants. Three dsRNA bands (approximately 1.6 kbp, 1.8 kbp and 1.9 kbp in size) were resolved from DQT (indicated by the black arrows in Figure 1a; Figure 2a). The 1.9-kbp dsRNA fragment was detected with a frequency of 20.0% (in 4 of 20 examined DQT plants), and was found in association with the 1.8-kbp dsRNA. The 1.8-kbp dsRNA was found at a high frequency of 95% (in 19 of 20 examined DQT plants). The 1.6-kbp dsRNA was discovered in 2 of 20 (10%) examined DQT plants.

In LTBJ, one band (approximately 1.6 kbp) was present at a much higher frequency

1 of 87.5% (in 7 of 8 individuals) (indicated by the black arrow in Figure 1b). Two
2 smaller bands (approximately 1.5 kbp and 1.0 kbp, indicated by the gray arrows in
3 Figure 1b) were found in most of the investigated *S. alba* (in 6 of 8 individuals).
4 These two major smaller dsRNA bands always appeared simultaneously and were not
5 as stable as larger dsRNA bands (Figure 1b). Following M-SPAT cloning of the 1.5
6 kbp band in LTBJ more than 3 times, no viral-related genetic elements were obtained.
7 We suspected that these smaller bands represented plant genomic RNA with a dsRNA
8 structure that was occasionally extracted due to the instability of the dsRNA
9 extraction method (data not shown); thus, these bands were not studied further.

10 A clear 1.5-kbp dsRNA band was observed in all of the investigated *B. rapa* cv.
11 YDECT plants (9 individuals) (indicated by the white arrow in Figure 1c). The bands
12 of approximately 1.0 kbp that were present in all of the investigated YDECT plants
13 were also considered to be of plant genomic origin and were not studied further.

14 2. Nucleotide sequence analysis

15 The viral dsRNA bands that were described above were gel-purified, cloned using
16 M-SPAT, and sequenced using the Sanger method.

17 *Raphanus sativus* partitivirus 1

18 Clone, sequencing and northern blotting results showed that the 1.6- and 1.8-kb
19 dsRNA bands in DQT corresponded to RsV2 and RsV1 (Chen et al. 2006a; Chen et al.
20 2006b), respectively (Figure 2). From the 1.9-kbp dsRNA band, one dsRNA molecule
21 (ds1900) was obtained (GenBank accession number: KT285019). ds1900 is 1,915 bp
22 in length and contains 31.33% A, 18.54% G, 26.37% T and 23.76% C. The G+C

1 content (42.30%) is much lower than the A+T content (57.70%). ds1900 contains a
2 major ORF from nt 80 (ATG) to nt 1,837 (TAG), which is 1,758 nt in length and
3 encodes a 68.04 kDa (p68, GenBank accession number: ALT00589) protein
4 comprising 586 aa residues (Figure 2a,b). No other putative ORFs that encoded
5 function proteins larger than 100 aa were found in either strand. BLASTP searches
6 using p68 as the seed revealed that all of the sequences that were closely related to
7 p68 were partitiviral RdRps, including those of *Vicia faba* partitivirus 1 (VfPV1;
8 pairwise coverage 98%, identity 68%), Oyster mushroom isometric virus II (95%,
9 52%), *Chondrostereum purpureum* cryptic virus 1 (96%, 36%), *Flammulina velutipes*
10 isometric virus (92%, 37%), *Sclerotinia sclerotiorum* partitivirus S (85%, 36%), RsV1
11 (92%, 35%), *Heterobasidion* RNA virus 5 (94%, 33%), *White clover cryptic virus 1*
12 (95%, 32%), *Flammulina velutipes* browning virus (98%, 32%), *Beet cryptic virus 1*
13 (95%, 33%), *Vicia cryptic virus* (95%, 32%), *Heterobasidion* RNA virus 4 (85%,
14 34%), *Helicobasidium mompa* dsRNA mycovirus (91%, 33%) and Carrot cryptic
15 virus (95%, 32%) (e-value less than $6e^{-70}$). Multiple alignment analysis indicated that
16 p68 contains the motifs 3-8 that characterize the RdRp of a dsRNA virus
17 (Supplemental figure 1a) (Bruenn 2003).

18 These data indicate that p68 shows large evolutionary distances from its related
19 partitiviral RdRps, as it yielded sequencing identities that ranged from 32-68%.
20 Additional searches using ds1900 as the seed confirmed these differences. Neither the
21 BLASTN or TBLASTX searches using the default set found any nucleotide
22 sequences related to ds1900. We then assessed the identity between dsR1900 and

1 *Vicia faba* partitivirus 1 dsRNA3, which encodes the putative RdRp most closely
2 related to p68, and obtained an identity of 65.2%, which is lower than the threshold
3 used to define a novel virus species. Collectively, these results suggest that dsR1900
4 is the genomic segment of a novel partitivirus (see the analysis below). In this
5 manuscript, we name the virus corresponding to dsR1900 *Raphanus sativus*
6 partitivirus 1 (RsPV1).

7 The genomes of most cryptoviruses identified to date comprise two dsRNAs, one
8 encoding RdRp and the other encoding the CP. To obtain the dsRNA encoding the
9 viral CP of RsPV1, we repeated the M-SPAT more than three times using the dsRNA
10 samples that contained dsR1900 and other co-occurring dsRNA bands, and analyzed
11 hundreds of plasmid clones by sequencing or endonuclease digestion (data not shown).
12 Unfortunately, no further novel virus-related sequences that are potentially associated
13 with RsPV1 were found.

14 However, the northern blotting results demonstrated that dsR1900 was incorporated
15 into the virion particles. Isometric virus-like particles (VLPs), approximately 30 nm in
16 diameter, were purified from an individual DQT plant (Supplemental figure 2).
17 Unexpectedly, dsR1900, as well as RsV1 dsR1 and RsV2 dsR1 (both encoding RdRp)
18 were selectively detected in these VLPs as identified by a northern blot analysis using
19 specific probes (Figure 2).

20 It is widely believed that UTR sequences play an important role in viral protein
21 recognition, leading to virion formation and genome replication. Similarity between
22 the UTR sequences of different viral segments has often been used to assess viral

relationships. To test the possibility that RsPV1 is a satellite virus of coexisting viruses, we performed a multiple alignment of the UTR sequences of RsPV1, RsV1, and RsV2. The 5' and 3' UTRs of RsV1 and RsPV1 exhibited high similarity and identity (Supplemental figure 3a, b) with several conserved regions in the 5' UTR. Moreover, RsPV1 consistently appeared together with RsV1 in DQT individuals, according to electrophoresis of the dsRNA preparations. Additionally, the northern blotting analysis revealed that RsPV1 dsRNA1 was incorporated into virion particles. Therefore, we hypothesized that RsPV1 might be a satellite virus of RsV1 that regenerates using the structural proteins of its co-virus.

Phylogenetic analysis based on p68 and partitivirus RdRPs showed that ds1900 is clustered together with VfPV1 (Figure 3). The RdRP-encoding the dsRNA segment of VfPV1 is similar in length to ds1900, and the CP-encoding dsRNA segment of VfPV1 has not been reported until now. Together, these findings suggest that RsPV1 and VfPV1 potentially represent a novel taxon of partitiviruses that can use the CP of other partitiviruses to form their virion structure and replicate their genomes.

***Sinapis alba* cryptic virus 1**

The 1.6-kbp dsRNA found in LTBJ contained two different dsRNA molecules of similar lengths, comprising the genome of another novel *alphacryptovirus*. This virus was named *Sinapis alba* cryptic virus 1 (SaCV1) (see the following analysis). The two genomic dsRNAs of SaCV1 are 1,583 bp (dsR1, GenBank accession number: KT285020) and 1,536 bp (dsR2, GenBank accession number: KT285021) in length (Figure 2d). Similar to the previously reported alphacryptoviruses, G is present at

1 lower levels (20.32% and 19.14% for dsR1 and dsR2, respectively) than other
2 nucleotides (29.97% and 24.48% for A; 27.44% and 27.93% for T; and 22.27% and
3 28.45% for C (values for dsR1 and dsR2, respectively)). The 5' UTRs of dsR1 and
4 dsR2 are 105 nt and 90 nt, respectively, and are highly conserved, sharing many
5 identical regions, such as the terminal sequence 5'-AGAATTTT-3'. The 3' UTRs of
6 dsR1 and dsR2 are 42 nt and 261 nt, respectively, and share several identical regions
7 (Supplemental figure 4).

8 The major ORF in the positive strand of dsR1 (nt 106 to nt 1541) is 1,437 nt in
9 length and encodes a 54.06 kDa protein (p54, GenBank accession number: ALT00590)
10 comprising 479 aa residues (Figure 2d). No other large ORF was found in either
11 strand. BLASTN searches using found only one nucleotide sequence, segment 1 of
12 RsV3, which encodes viral RdRp, that was related to SaCV1 dsR1, with 62.4%
13 identity. BLASTP searches revealed that p54 has much higher sequence similarity
14 with RdRPs encoded by alphacryptoviruses, including Pepper cryptic virus 1 (PCV1,
15 pairwise coverage 99%, average identity 67%), RsV3 (100%, 67%), Persimmon
16 cryptic virus (PerCV, 98%, 59%), Pepper cryptic virus 2 (PCV2, 98%, 58%), Beet
17 cryptic virus 2 (BCV2, 98%, 57%), Pittosporum cryptic virus-1 (47%, 73%), and
18 RsV2 (91%, 37%). A multiple alignment of P54 and the related sequences revealed
19 that eight conserved motifs, which are characteristic of viral RdRp, also exist in p54
20 (Supplemental figure 1b) (Bruenn 2003). Together, these results indicate that p54 is a
21 viral RdRp; i.e., dsR1 encodes the RdRp of SaCV1.

22 Similarly, only one large ORF (1,185 nt in length) was found in dsR2; this ORF

1 encodes a 45.05 kDa protein (p45, GenBank accession number: ALT00591)
2 comprising 395 aa residues (Figure 2d). BLASTP searches of p45 demonstrated that
3 this protein has high sequence similarity to the CPs of alphacryptoviruses, including
4 PCV1 (pairwise coverage 99%, average identity 67%), RsV3 (86%, 36%), PCV2
5 (92%, 34%), PerCV (96%, 33%), Mulberry cryptic virus 1 (78%, 34%) and BCV2
6 (89%, 34%). The profiles of these viruses, corresponding to the sequences related to
7 p45, are similar to the profile of viral RdRp, p54. These results confirm that dsR2
8 encodes the structural protein CP of the novel cryptovirus.

9 The above analysis revealed that p54 displays large evolutionary distances to its
10 related partitiviral RdRps, with sequencing identities ranging from 37 to 67% at
11 amino acid sequence level. Similarly, the CP p45 showed identities ranging from
12 34-67% to its close related proteins, and dsR2 showed 49.9% similarity to its most
13 closely related nucleotide sequence, segment RNA2 of PCV1. These results confirm
14 that *Sinapis alba* cryptic virus 1, which has a genome composed of dsR1 and dsR2, is
15 a novel cryptovirus that shows a large evolutionary distance to other related
16 partitiviruses.

17 **Putative *Brassica rapa* cryptic virus 1**

18 To determine the nature of the 1.5-kbp dsRNA in *Brassica*, purified dsRNA
19 extracted from YDECT was subjected to M-SPAT cloning. One dsRNA molecule was
20 obtained that possibly belongs to a novel partitivirus, named *Brassica rapa* cryptic
21 virus 1 (BrCV1) for convenience (see the analysis below). The dsRNA was
22 designated BrCV1 dsR1 (GenBank accession number: KT285022).

1 BrCV1 dsR1 is 1,758 nt in length and contains one major ORF on its positive
2 strand (nt 762 to nt 1,568). This ORF encodes a 29.9 kDa protein (p29, GenBank
3 accession number: ALT00592) comprising 268 aa residues (Figure 2e). Another,
4 smaller, ORF exists from nt 555 to nt 1,004 (5' to 3') on the minus strand of BrCV1
5 dsR1 (nt 1,150 to nt 701 on the positive strand), putatively encoding a 16.6 kDa
6 protein comprising 149 aa residues (Figure 2e). As with the above two novel viruses,
7 neither the BLASTN nor TBLASTX searches using the default set found any
8 nucleotide sequences related to BrCV1 dsR1. No sequence that showed high
9 similarity to p29 with an e-value less than 1 was retrieved from the NCBI GenBank
10 database using BLASTP searches. Moreover, several small ORFs were found
11 upstream and downstream of this ORF. The small upstream ORF might be involved in
12 ribosome recognition and other functional ORF translation (Morris and Geballe
13 2000).

14 Only four sequences that are closely related to p29 were obtained in the BLASTP
15 searches and were of low similarity. These sequences encoded two proteins of
16 unknown function that were isolated from the protozoan *Toxoplasma gondii*,
17 TGME49_111730 (GenBank accession number: XP_002364405, *Toxoplasma gondii*
18 ME49, e-value 0.34, 1169 aa) and a conserved hypothetical protein (EEE25710,
19 *Toxoplasma gondii* GT1, e-value 0.34, 1172 aa), and two viral proteins from two
20 different partitiviruses infecting the same plant pathogenic fungi, *Gremmeniella*
21 *abietina*: GaRVMS2s3gp1 (YP_138542, e-value 1.2; pairwise coverage 39%, identity
22 31%) of *Gremmeniella abietina* RNA virus MS2 (GaRVMS2) (Tuomivirta and

1 Hantula 2005) and GaRvM1s3gp1 (NP_659029.1, e-value 1.8; 39%, 31%) of
2 *Gremmeniella abietina* RNA virus MS1 (GaRVMS1) (Tuomivirta and Hantula 2003).
3 Both GaRVMS1 and GaRVMS2 are tri-segmented partitiviruses and typical species
4 of the novel genus *Gammapartitivirus*, as designated by Nibert et al. (2014).
5 GaRVMS2s3gp1 and GaRvM1s3gp1 are respectively encoded by the third dsRNA
6 segments of GaRVMS1 and GaRVMS2, and are of unknown function. Like the third
7 dsRNAs of GaRVMS1 and GaRVMS2, BrCV1 dsR1 also has a long 5' UTR (761 nt),
8 which accounts for nearly half of its entire length and is longer than that of the normal
9 genomic dsRNAs of partitiviruses. Thus, BrCV1 dsR1, which putatively encodes p29,
10 possibly belongs to a novel cryptovirus in *B. rapa* cv. YDECT. This virus was named
11 *Brassica rapa* cryptic virus 1 in this study.

12 Until now, no cryptovirus has yet been reported among *Gammapartitivirus*.
13 Pairwise alignment indicated that BrCV1 dsR1 showed 41.7% and 42.4% identity
14 with the third dsRNAs (NC_004020 and NC_006446) of GaRVMS1 and GaRVMS2,
15 respectively. Phylogenetic analysis, based on the alignment of p29, GaRVMS2s3gp1,
16 GaRvM1s3gp1 and related viral protein from other gammapartitivirus, further
17 confirmed that BrCV1 is closely related to GaRVMS1 and GaRVMS2 (Supplemental
18 figure 5). Taken together, these data indicate that BrCV1 might be the first cryptovirus
19 to be found in *Gammapartitivirus* and that there is a large evolutionary distance to the
20 other reported *Gammapartitivirus* taxa.

21 3. Phylogenetic analysis of partitivirus in *Raphanus* and *Brassica*

22 To date, six partitiviruses, RsV1, 2, 3 and the three uncovered in this study, have

1 been found in *Raphanus* and *Brassica*. To investigate the evolutionary relationships
2 among these partitiviruses, a phylogenetic analysis was performed based on the
3 multiple alignment of their RdRps. The phylogenetic analysis indicated that these six
4 viruses are all clustered into plant virus groups. SaCV1 is closely related to pepper
5 cryptic virus 1 and RsV3; RsPV1 is closely related to VfPV1 (Figure 3). RsPV1 and
6 RsV1 are clustered into the clade *Alphacryptovirus*, whereas RsV2, SaCV1 and RsV3
7 are clustered into another clade, *Deltapartitivirus*, as supported by high bootstrap
8 values of greater than 90 (Figure 3). The genomic segment lengths of RsPV1 (1,915
9 bp, 586 aa) and RsV1 (RdRp, 1,866 bp, 573 aa and CP 1,791 bp, 505 aa) are in the
10 typical range of *Alphacryptovirus* (1,866–2,027 bp for RdRp and 1,708–1,866 bp for
11 CP). Similarly, the genomic segment lengths of RsV2, SaCV1 and RsV3 are in the
12 typical range of *Deltapartitivirus* (1,563–1,762 bp for RdRp and 1,415–1,598 bp for
13 CP).

14 Similar to our previous report regarding a novel plant chrysovirus (Li et al. 2013),
15 no endogenous or symbiotic fungi were observed in the *Raphanus* and *Brassica*
16 leaves containing RsPV1, SaCV1 or BrCV1 on examination with a light microscope
17 after staining with Trypan blue (data not shown). Considering the seed-borne features,
18 these findings supported the notion that RsPV1, SaCV1 and BrCV1 infect *Raphanus*
19 and *Brassica* plants rather than endogenous or symbiotic fungi of the plants. We
20 suggest that RsPV1 should be classified as a novel member of *Alphacryptovirus*, and
21 that SaCV1 should be classified as a novel member of *Deltapartitivirus*.

22 Discussion

1 Cultivated *Brassicaceae* plants are among the most widely consumed vegetables;
2 the plants are also used as fodder and as sources of oil. Several seed-borne
3 partitiviruses have previously been found in cultivated radish (Chen et al. 2006a;
4 Chen et al. 2006b; Li et al. 2013; Natsuaki et al. 1983; Natsuaki et al. 1979). Based on
5 a comparison of our data with the partial genetic information of RYEV published in
6 other papers (Liu et al. 2012; Nogawa et al. 1996), we found that RsV1 and RsV2
7 might be a Chinese counterpart of RYEV. Namely, RYEV is likely to represent a
8 complex of partitiviruses rather than a single virus. In this study, along with the
9 survey of seed-borne viruses in cultivated *Brassica* and *Raphanus* (Li 2013), we
10 identified three novel partitiviruses: RsPV1, SaCV1 and BrCV1; these viruses infect
11 radish, *S. alba* and *B. rapa*, respectively.

12 We found that seed-borne cryptoviruses, including putative cryptoviruses and
13 chrysovirus, are widely distributed in *Brassica* and *Raphanus* with high frequency
14 (Li 2013; Li et al. 2013). We speculate that these seed-and-pollen-transmitted cryptic
15 viruses are widely distributed across various *Brassica* and *Raphanus* and even their
16 ancestor species during the hybridization and breeding of these two vegetable genera,
17 reflecting what is likely a very long history. Although isolated from similar hosts,
18 RsV1, RsV2, RsV3, RsPV1, SaCV1 and BrCV1 are distantly related to each other.
19 Although most reported partitiviruses are asymptomatic, some evidence exists that
20 partitiviruses might play a positive role and synergize with their hosts (Ahn and Lee
21 2001; Marquez et al. 2007; Nakatsukasa-Akune et al. 2005). It is conceivable that
22 further details regarding these five viruses in *Brassicaceae* plants will shed more light

1 on the transmission and evolution of cryptoviruses at the microbial level. Studies of
2 this nature are therefore of economic importance due to the agricultural features of the
3 host plants.

4 **Acknowledgments**

5 We thank Professor Wancang Sun (Gansu Academy of Agricultural Sciences, China)
6 for graciously providing the *S. alba* cv. Lintaobaijie (LTBJ) seeds. We thank
7 Professor Karel Petrzik for his constructive advice. This research was supported by
8 the National Natural Science Fund of China (No. 30971898).

9

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9

10 **Figure and table legends**

11 **Figure 1.** dsRNAs of *R. sativus* cv. Daqingtou (DQT) (a), *S. alba* cv. Lintaobaijie
12 (LTBJ) (b) and *Brassica rapa* cv. Youdongercaitai (YDECT) (c). The largest bands in
13 these figures are plant genomic DNAs. Different lanes represent different individuals,
14 and lane M contains a DNA molecular ladder.

15 **Figure 2.** The genetic organization of dsRNA genomic segments of *Raphanus sativus*
16 partitivirus 1 (RsPV1), *Sinapis alba* cryptic virus 1 (SaCV1) and *Brassica rapa*
17 cryptic virus 1 (BrCV1). (a, b) The genomic organization of RsPV1, and (c) northern
18 blot analyses of RsPV1, RsV1 and RsV2 for the detection of their RdRp sequences. (d)
19 The genetic structure of SaCV1. (e) The genetic structure of BrCV1 dsR1.

20 **Figure 3.** The phylogenetic relationships among RsPV1, SaCV1, RsV1, 2, and 3 and
21 other plant partitiviruses. All sequences were retrieved from the NCBI GenBank
22 database; viruses and their accession numbers are displayed in the branches.

1 Sequences were aligned using MUSCLE with the UPGMB method, and a
2 phylogenetic tree was constructed using the maximum parsimony (MP) method and
3 default set. A bootstrap test was performed with 1,000 re-samplings of the MP trees.
4 Plant partitivirus branches are shown in green; the branches shown in red represent
5 fungi partitiviruses.

6
7 **Supplemental figure 1.** The conserved RdRp motifs in p68 of RsPV1 and p54 of
8 SaCV1. Black, dark gray and light gray shading indicates that the residues at the
9 specified position are 100%, 80% and 60% identical respectively.

10 **Supplemental figure 2.** Virus-like particle purification from *R. sativus* cv. Daqingtou
11 infected by RsPV1, RsV1 and RsV2. The virions were purified from radish plants by
12 CsCl gradient ultracentrifugation and negatively stained with 2% uranyl acetate. Bar,
13 50 nm.

14 **Supplemental figure 3.** Comparison of the 5' and 3' UTRs of dsR1 and dsR2 (a, b).
15 Multiple sequence alignments were obtained using a MUSCLE algorithm
16 implemented in MEGA5.0. Black shading signifies that the residues at the indicated
17 position are 100% identical.

18 **Supplemental figure 4.** Comparison of the 5' and 3' UTRs of RsPV1 and RsV1, 2, 3
19 (a, b). Multiple sequence alignments were obtained using a MUSCLE algorithm
20 implemented in MEGA5.0. Black, dark gray and light gray shading indicate that the
21 residues at the specified position are 100%, 80% and 60% identical respectively.

22 **Supplemental figure 5.** The phylogenetic relationships among BrCV1, GaRVMS1,

1 GaRVMS2 and other gammapartitivirus. All sequences were retrieved from the NCBI
2 GenBank database; viruses and their accession numbers are displayed in the branches.
3 Sequences were aligned using MUSCLE with the UPGMB method, and a
4 phylogenetic tree was constructed using the maximum parsimony (MP) method and
5 default set. A bootstrap test was performed with 1,000 re-samplings of the MP trees.
6
7 **Supplemental Table 1.** The primers used to synthesize the northern blotting probes.

Draft

Figure 1a

b

S. alba cv. LTBJ

c

Figure 2a,b,c

Figure 2d,e

d **e**

BrCV1 dsR1

Alphacryptovirus