

An integrated badnavirus is prevalent in fig germplasm

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ABSTRACT

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Fig mosaic occurs worldwide and is the most common and important viral disease of fig. In the quest to identify the causal agent of the disease, several new viruses have been identified, including a new DNA virus, the subject of this communication. Phylogenetic analysis placed the virus, provisionally named Fig badnavirus 1 (FBV-1) in the genus *Badnavirus*, family *Caulimoviridae*. The experimental host range of FBV-1 was evaluated and the virus was mechanically transmitted to several herbaceous hosts. FBV-1 was detected in the National Clonal Germplasm Repository fig collection and additional samples from Arkansas, California, Florida, Michigan, Ohio, Oregon and South Carolina, suggesting its wide distribution in the United States. Further tests revealed the presence of FBV-1 in seedlings and meristem tissue culture plants. Forty-four isolates were used in a study evaluating the population structure of the virus in the United States. Evidence that FBV-1 is integrated in the fig genome is presented and discussed.

INTRODUCTION

Fig (*Ficus carica*, L.; family Moraceae) is a deciduous fruit tree, native of Eurasia. It has been cultivated for thousands of years for its edible fruit and is usually propagated from hardwood cuttings. Fig is susceptible to only a few pathogens and diseases with *Armillaria* and *Rosellinia* root rots (caused by *Armillaria mellea* and *Rosellinia necatrix*, respectively) and Mosaic being of major concern (26, 29).

Fig mosaic (FM) was first described in California in 1933 (5) and has since been found worldwide (11, 12). The putative causal agent of FM, Fig mosaic virus (FMV), was only recently discovered and characterized (11, 12, 44). In addition to FMV, several new viruses were found in FM trees including at least five species in the family *Closteroviridae* (9, 10, 13, 15, 32, 43) and one member in each of the genera *Trichovirus*, *Umbravirus*, *Luteovirus*, *Carlavirus*, *Potyvirus* and *Alphacryptovirus* (14, 16, 44). A survey of almost 190 trees in California found FMV in the vast majority but not all FM trees (44) and Koch postulates have yet to be completed for FMV/FM. Also, FM symptomology varies greatly between trees of the same or different cultivars ranging from mild mottling to severe mosaic to leaf distortion and ringspots (1, 14; Fig. 1). The inability to complete Koch postulates, lack of absolute association between FMV and FM, discovery of the myriad of new viruses in FM trees and extreme symptom variability imply that the disease is more complex than originally thought and symptoms may not only be caused by FMV, but also by mixed virus infections as is the case with diseases of several other perennial crops (27, 31, 37).

In 2008, and in the process of characterizing the fig mosaic agent, several new viruses were discovered in a FM tree (43). One of the viruses had sequence similarities to *Cacao swollen shoot virus* (CSSV) and *Citrus yellow mosaic virus* (CiYMV), members of the genus *Badnavirus*. Badnaviruses are dsDNA pararetroviruses that can integrate in the host genome (20), but also actively replicate as episomal, infectious viruses. Badnaviruses have circular genomes of 7-8 kilobases and are encapsidated in non-enveloped bacilliform virions. They are usually transmitted by mealybugs, although one of them, *Rubus yellow net virus*, is transmitted by aphids (23). Badnavirus symptoms vary greatly from latent infections, as is the case of *Sugarcane bacilliform virus* (SCBV) in sugarcane (3, 4), to severe, such as the disease caused by CSSV (35) or the Banana streak virus complexes (6, 21, 30) in cacao and banana, respectively.

The new virus, provisionally named Fig badnavirus 1 (FBV-1), was fully characterized at the molecular level and several experimental hosts were identified. Detection tests were developed and used to determine the presence of the virus in both FM and asymptomatic trees. FBV-1 was found in a high percentage of fig accessions from the National Clonal Germplasm Repository (NCGR) in Davis, California and a population structure study was initiated to determine whether there are distinct virus populations in the United States or whether isolates were imported with one or more accessions from their countries of origin. The possibility that FBV-1 integrates into the fig genome was explored using several approaches.

MATERIALS AND METHODS

Virus characterization. Nucleic acids were extracted from fig tissue using modified forms of the Yoshikawa and Converse (47) and Tzanetakis and Martin (42) protocols. Nucleic acids were subjected to RNase digestion at 37°C overnight for both methods. After digestion, the nucleic acids were bound to silica milk using STE (10 mM Tris-HCl, 1 mM EDTA pH 8.0) / 50% ethanol (39), eluted in 100-µl TE, reverse transcribed, amplified and cloned as described (42). The obtained sequences were subjected to BLAST (2) to identify viral sequences and compiled into contigs using CAP3 (20). Several virus sequences were acquired including those of an apparently new badnavirus. Sequence gaps between badnavirus sequences were obtained using PCR with virus-specific primers or combination of rolling circle amplification (RCA; 7, 22) and PCR (Table 1). All amplicons were cloned, sequenced and assembled using CAP3 (20) to obtain the virus genome with an at least 3x genome coverage (GenBank accession number JF411989).

Protein conserved domains were identified using the Conserved Domain Database (CDD; 25) and proteins were subjected to BindN (45) to determine DNA-binding domains (at 85% specificity). Phylogenetic analysis was performed with ClustalW (38) using the neighbor-joining algorithm, Kimura's correction and bootstrap consisting of 1000 pseudoreplicates. Trees were visualized on TreeView (33).

Detection. Total nucleic acids were purified as described before (40) and used as template for PCR amplification using primers 1094F/1567R (400nM final concentration; Table 1). The PCR was performed using Taq polymerase (GenScript) and a program consisting of initial denaturation at 94°C for 2 min followed by 94°C for 30 sec, 55°C for 20 sec, and 72°C for 30 sec repeated for 40 cycles. The protocol terminated with a final

extension step at 72°C for 10 min. Thirty-five amplicons were sequenced and all were FBV-1, verifying virus-specific detection.

Southern blot hybridization was employed to determine whether the virus is integrated in the fig genome or is actively replicating. Fig genomic DNA was extracted as previously described (8) followed by overnight digestion with T1 RNase (10u, Sigma) at 37°C and phenol-chloroform extraction. Equal amount of DNA extracts (15-20µg each) were digested overnight with *Sspl* and *EcoRI* at 37°C. After gel electrophoresis, the DNA was transferred overnight onto Hybond N+ membrane (GE Healthcare) and UV cross-linked. The probe was prepared using a 1094F/1567R purified amplicon (20ng) and labeled using the DIG-High Prime Kit (Roche Applied Science) according to the manufacturer's recommendations. After hybridization, membranes were washed in 0.5× SSC/0.1% SDS at 65°C and exposed to X-ray film.

For RCA, total nucleic acids were extracted from symptomatic tissue (40) and amplified using the Illustra TempliPhi 100 amplification kit (GE Healthcare) and primer 5900F (800nM final concentration; Table 1) according to manufacturer's recommendations. Amplified DNA was digested with *Sspl* or *MluI*, both of which cut the genome at a single site. Product were blotted for Southern blot as described above or purified using an Illustra column (GE Healthcare) for use as template at a 1/100 dilution for PCR amplification as described above. A total of 16 direct PCR FBV-1-positive samples were analyzed using this approach.

For RT-PCR, total nucleic acids from the same 16 samples were digested with 1u DNase I in the corresponding buffer (Fermentas) and 40 units RiboLock® RNase inhibitor for 1 h at 37°C. The digested material was adjusted to 50% ethanol and bound

to silica milk to sequester the RNA and remove proteins (40). For the reverse transcription, 100u Maxima® Reverse Transcriptase (Fermentas), 5x RT buffer, 20u RiboLock® RNase inhibitor, 5µl DNase I-digested template, and water to 50µl was incubated at 50°C for 1 h , 85°C for 5 min for enzyme inactivation followed by PCR as described above.

Geographic range and population structure. To determine the FBV-1 distribution, 39 trees from the NCGR in Davis, California, 60 seedlings generated through meristem tip-cultured tissue, and 18 additional samples from Arkansas, California, Florida, Michigan, Ohio, Oregon and South Carolina were tested using the detection protocol mentioned above (Table 2). The diversity for 44 isolates, 27 from the NCGR, six from Arkansas, including the original isolate, three from Oregon, two meristem plantlets, two seedlings, and one each from Florida, Michigan, Ohio and South Carolina were investigated by RCA to confirm the episomal form of the studied isolates. A 1,091-nt region of the virus genome was amplified with primers 580F/1650R (Table 1). Sequences were aligned and compared using ClustalW (38) at the nucleotide and amino acid level (GenBank accession numbers JN050858-84, JN112365-69, and JQ282668-78).

Host range. At least six plants from 19 herbaceous host indicator species (Table 3) were inoculated with leaf tissue from the fig tree used in the virus characterization. Tissue was ground in cold 0.05M phosphate buffer (pH 7.0) at approximately a 1/10 (w/v) ratio. The plants were kept in an insect-free greenhouse with 14-h light/10-h dark photoperiod and tested by PCR one month post-inoculation. All positive PCR amplicons were verified by sequencing.

RESULTS

Virus acquisition and characterization. The viral genome comprises 7,140 nucleotides (nt) and contains a negative strand tRNA^{MET} primer-binding site (TGGTATCAGAGC₁₋₁₂), which was chosen as the starting location for the genome (3). A TATA-box was found upstream of the primer site (TATAAG₆₉₅₀₋₆₉₅₅), and is part of the promoter region for badnavirus replication (28), though the exact promoter location requires experimental confirmation. The genome encodes four proteins (P1-P4) of 143, 135, 1837, and 142 amino acids (aa) with molecular masses of 15.3, 16.5, 212.3 and 17.0 kDa respectively (Fig. 2). FBV-1 is closely related to CSSV, CiYMV, and *Dioscorea bacilliform* AL virus all of which are dicot-infecting badnaviruses (Fig. 3). FBV-1 shares 61% and 64% nt identities with CSSV and CiYMV over the length of the genome. P1 shares 59% and 55% identities to the CSSV and CiYMV orthologs respectively. The protein function is yet to be elucidated. P2, a putative DNA binding protein (21), shares over 40% identities to both CSSV and CiYMV orthologs. As in the case of CSSV (21), the terminal residues of FBV-1 p2 are predicted to strongly bind DNA (45). The key *Badnavirus* motifs were found in the polyprotein; however, no protease cleavage sites could be identified *in silico* similar to other badnaviruses. The signature motifs (3) include: movement protein (Arg₁₃₅-Pro₁₉₆), the coat protein zinc-finger domain (Lys₈₁₃-Pro₈₂₉), pepsin-like aspartate protease (Val₁₀₈₅-Gly₁₁₆₃), reverse transcriptase (Leu₁₂₇₉-Gly₁₄₈₁), and RNase H (Thr₁₅₈₃-Leu₁₇₀₄). The protein shares 54% and 57% identities to CSSV and CiYMV orthologs, respectively. The reverse transcriptase and RNase H regions share less than 74% identities with any sequenced

badnavirus. FBV-1 has four ORFs, similar to CSSV but unlike most other badnaviruses. ORF4 codes for a putative protein of unknown function. No significant similarity, except for the putative CSSV P4 ortholog (28% aa identities), was identified and no function was elucidated using CDD, Psi- and Phi-Blast. According to the guidelines of the Ninth ICTV report (24), FBV-1 is a new member of the genus *Badnavirus* as the RT/RNaseH region shares less than 80% identity to other members of the genus.

Integration and replication. The taxonomic placement of FBV-1 as a new badnavirus led to the notion that the virus may, in addition to actively replicate, also integrate into the fig genome. To explore this hypothesis, genomic fig DNA was used in Southern blot hybridization. Digestion with *SspI*, an endonuclease with a single recognition site in the FBV-1 genome, produced signal at the approximate genomic size of 7 kb; additional signals were detected at 3, 4.5, and 6 kb (Fig.4). *EcoRI* has three recognition sites in the virus genome, with the probe hybridizing to a 5.4-kb fragment. In addition to the 5.4-kb signal, additional bands were detected at 3.5, 4, 4.5, 7 and 7.5 kb. The hybridization signal from undigested DNA occurred much above 10 kb (Fig. 4). The additional bands detected in the digested material, in addition to the signal at high molecular weight of the undigested samples, provide evidence that FBV-1 integrates in the fig genome. RCA and RT-PCR were also employed to differentiate between integrated and episomal virus in different fig accessions as described previously (22). Genome amplification using RCA followed by Southern blot verified that FBV-1 is episomal in the material used for the virus characterization (Fig. 4). However we considered that not all fig samples might contain episomal forms of the virus. For this reason, 16 direct PCR-positive samples were also assayed by RCA and RT-PCR. Total

nucleic acids were subjected to RCA followed by digestion using *SspI*. Fragments of nearly 7 Kb in size were only observed in seven out of sixteen fig samples whereas RT-PCR assays were in agreement with the RCA results (Fig 5). These results provide evidence that FBV-1 exists in both episomal and/or integrated forms.

Geographic range and diversity. A diverse array of different fig accessions were tested using direct PCR for FBV-1 including: 39 FM accessions from the NCGR in Davis, California; 60 plantlets generated by meristem tissue culture; six asymptomatic trees from Arkansas; three fig seedlings, three samples from Oregon, and one each from nurseries in California, Florida, Michigan, Ohio and South Carolina. FBV-1 was found in both FM-symptomatic and asymptomatic trees (Fig. 6). A total of 114 samples out of the 117 tested were found infected with FBV-1, including all the meristem tip culture plants and three seedlings; only two samples from the NCGR and one from Arkansas tested negative for FBV-1. Those samples were tested three times starting from tissue extraction and using all the detection techniques described above other than Southern blot hybridization. In order to verify the extraction of good quality material, an internal control was used as described (41).

The virus population, as studied by sequencing two genes of 44 isolates, proved to be homogeneous with nt identities ranging from 99 to 100%. ORF2 aa identities ranged from 98 to 100%. Similarly, sequence analysis of the N' terminus of ORF3 (196 aa) revealed aa identities ranging from 97 to 100%.

Host range. FBV-1 was detected in systemic leaves of *Nicotiana occidentalis*, *N. tabacum*, *N. sylvestris*, *N. rustica*, *Pisum sativum*, *Cucurbita pepo*, and *Glycine max* with the latter two hosts developing mosaic symptoms. However, inoculations were

performed and tissue tested before the identification of the several new viruses discovered in fig and thus symptoms cannot be attributed solely to FBV-1 as the herbaceous hosts were not tested for some of the ten or more new viruses that may have been present in the source tree.

DISCUSSION

Badnaviruses are some of the most important viruses for world agriculture, not only due to the severe diseases they cause (6, 21, 23, 35), but also because of their ability to integrate in the host genome (6, 19, 30). Genome integration can also be a research calamity as presence of an integrated virus does not always translate to an active virus. One of the most important questions after the discovery of badnavirus sequences, known to bear similarities to retrotransposons, is whether the virus-like findings are integrated in the host genome and/or are found in episomal form and therefore actively replicating. Using RCA, Southern blotting, RT-PCR, and mechanical transmission to herbaceous hosts, we determined that FBV-1 can be episomal and not an inactive, genetic fossil integrated in the host genome.

After confirmation of active virus replication, the obvious question was whether the pathogen causes disease. FBV-1 was detected in several asymptomatic samples, including some from Arkansas that were monitored for more than two years, evidence that FBV-1 is asymptomatic in single infections, at least in some cultivars. However, given the great number of cultivars available, this may not be true for others. There is also the distinct possibility that FBV-1 infection alters the internal physiology of the host as has been recently shown with *Fig leaf mottle-associated virus-1* (15). Badnaviruses

are also known to play an important role in disease complexes, as is the case of *Rubus yellow net virus*, an integral part of the Raspberry mosaic disease (23), and there are numerous reports of mixed infections causing severe symptoms in clonally propagated crops (23, 37, 39, 40, 46). The implication of the potential mayhem caused directly or indirectly by FBV-1 warrants further study so as to understand the virus epidemiology and population structure and set the basis for virus control in the nursery propagation stage.

There are several fig nurseries in the United States from California to Florida, propagating a wide assortment of fig germplasm. In the case of a new, apparently symptomless virus such as FBV-1, this practice can lead to dissemination of highly variable isolates which may have different effects on the onset of disease in the presence of other viruses, as noted above, or to possible recombination events and the emergence of more virulent isolates (17, 18, 36). For this reason, and given the high number of infected trees, we studied the population diversity of the virus in trees that are separated by thousands of kilometers. The results show that virus diversity is minimal. It is noticeable that isolates from the Near and Middle East are very similar to the U.S. isolates (Barutcu, personal communication). This can be indicative of FBV-1 being under purification selection or one or few isolates that were imported to the United States still circulate in the germplasm without much change.

As FBV-1 was found in a large number of trees of different origins, it is also possible that the virus is being actively transmitted in the United States by a yet undetermined vector, although mechanical transmission through pruning cannot be excluded given that FBV-1 appears to be easily transmissible by mechanical means.

252 However, no commercially available figs or meristem tip culture plants and fig seedlings
253 were found free of FBV-1. Two FBV-1-free accessions from NCGR are infected with at
254 least FMV, and an asymptomatic accession from Arkansas was infected with at least
255 two other viruses (unpublished data) making any trials for FBV-1 mechanical
256 transmission unreliable.

257 Another more plausible explanation for the widespread nature of FBV-1 is its
258 ability to integrate into the fig genome, given that 113 out of 116 figs tested were FBV-1-
259 positive when using direct PCR. However, the picture is not as clear as a 98% detection
260 rate. Southern blotting verified virus integration but RCA and RT-PCR assays detected
261 the episomal virus only in a subset of the samples tested, making virus epidemiology
262 rather complex.

263 The high incidence of FBV-1 integration makes its elimination nearly impossible.
264 Even if the episomal form is eliminated by thermo- and chemotherapy (34, 46), there is
265 always the possibility of virus reactivation and disease as found in other crops (30). For
266 this reason, it is important to be aware of virus presence and eliminate infected material
267 from the propagation pipeline. As such, we developed sensitive detection tests for FBV-
268 1 that target the integrated and/or the episomal form of the virus. To minimize the
269 possibility of false negatives because of sequence variations, the detection primers
270 were developed to be 100% identical to all 44 isolates sequenced.

271 FBV-1 is not the only badnavirus found in fig as there are unpublished reports of
272 additional badnaviruses from the United States and the Mediterranean (unpublished
273 data; Elbeaino, personal communication). Given the association of badnaviruses with
274 severe diseases and their significant role in virus complexes, it is possible that they

present a new, unrecognized problem for the fig industry, not only in the United States but also around the world.

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496 **Table 1:** Primers used for acquisition of Fig badnavirus-1 genome and detection.

Primer Type	Primer Name	Primer Sequence
Sequencing	344R	ACTGCTAGATTATGGTAAAGGT
	1400R	TACACAAAGCATCGCTTCCTCGGATCT
	1800F	AATGTTGGATTTGCTTACAGAATA
	2202F	ACCCTGGGAGAACCAAGCGG
	2724R	CTACCATCAGCCCAACA
	3260R	GTACCACCCCTGATACGACTCCTG
	3900F	ATTGTCCTTTGTGTCTCCTAACA
	4955R	TTGCCCTGATGACTCCTAAATCCA
	5900F ^a	CCTATGCTAGTGGGAAGTTTA
	6409R	CTTCTTCAACCTCCCTGAGT
	6456F	TGGAACAATACAGGCAGATT
	1094F	ACCAGACGGAGGGAAGAAAT
	1567R	TCCTTGCCATCGGTTATCTC
Diversity	580F	AGGCTCTAAGGTAACTGAAG
	1650R	ATCATCATCGTGTGTCAGGTATC

497 a: Primer used in the rolling circle amplification reaction to amplify Fig badnavirus 1-
 498 specific concatemers. F: Forward; R: Reverse
 499

Table 2: Cultivars from Arkansas, California, Florida, Michigan, Ohio, Oregon, South Carolina and trees grown from seed tested for Fig badnavirus-1.

Lab Sample	DFIC #	Cultivar	FBV-1 ^a presence	Symptoms	GenBank Accession Number
AR1 ^b	N/A	Unknown	+	FM ^c	JF411989 ^d
AR2	N/A	Unknown	+	None	JN112365
AR3	N/A	Brown Turkey	+	None	JN112366
AR4	N/A	Black Mission	+	None	JN112367
AR5	N/A	Kadota	+	None	JN112368
AR6	N/A	Kadota	+	None	JN112369
AR7	N/A	Unknown	-	None	N/A
CA1	1	Vernino	-	FM	N/A
CA2	6	UCR 347-1	-	FM	N/A
CA3	9	Flanders	+	FM	N/A
CA4	15	DiRedo	+	FM	N/A
CA5	17	Brown Turkey	+	FM	N/A
CA6	36	Zidi	+	FM	N/A
CA7	53	St Jean	+	FM	N/A
CA8	57	Calimyrna	+	FM	N/A
CA9	63	Violette De	+	FM	N/A
CA10	63	Violette De	+	FM	JN050858
CA11	65	UCR 143-28	+	FM	JN050859
CA12	67	Monstrueuse	+	FM	JN050860
CA13	69	Barnissotte	+	FM	JN050861
CA14	77	Calvert	+	FM	JN050862
CA15	80	Celeste	+	FM	JN050863
CA16	84	Dauphine	+	FM	JN050864
CA17	87	Snowden	+	FM	JN050865
CA18	88	Pied De Boef	+	FM	JN050866
CA19	90	Ischis Black	+	FM	JN050867
CA20	101	UCR 326-1	+	FM	N/A
CA21	102	UCR 284-11	+	FM	JN050868
CA22	102B	UCR 284-11	+	FM	JN050869
CA23	105	UCR K-7-11	+	FM	JN050870
CA24	105	UCR K-7-11	+	FM	N/A
CA25	105	UCR K-7-11	+	FM	JN050871
CA26	110	hybrid	+	FM	JN050872
CA27	111	Santa Cruz White	+	FM	JN050873
CA28	112	Rattlesnake	+	FM	JN050874
CA29	112	Rattlesnake	+	FM	N/A
CA30	113	Capitola Long	+	FM	JN050875
CA31	124	Capri P	+	FM	JN050876
CA32	147	Black Fig 1	+	FM	JN050877

CA33	147	Black Fig 1	+	FM	JN050878
CA34	153	Castle Kennedy	+	FM	JN050879
CA35	155	Californina Grown	+	FM	JN050880
CA36	158	Pseudocarica	+	FM	JN050881
CA37	158	Pseudocarica	+	FM	JN050882
CA38	185	KAC 11-7W	+	FM	JN050883
CA39	187	KAC 16-32W	+	FM	JN050884
CA40	N/A	White Genoa	+	FM	N/A
CA41	N/A	Sierra	+	None	JQ282675
CA42	N/A	Sequoia	+	None	JQ282676
FL1	N/A	Ischia	+	None	JQ282674
FS1	N/A	Unknown	+	None	JQ282677
FS2	N/A	Unknown	+	None	JQ282678
MI1	N/A	Green Ischia	+	None	JQ282673
OH1	N/A	Hardy Chicago	+	None	JQ282668
OR1	N/A	Brown Turkey	+	FM	JQ282669
OR2	N/A	Desert King	+	None	JQ282670
OR3	N/A	Black Mission	+	FM	JQ282671
SC1	N/A	Texas Everbearing	+	None	JQ282672

a: FBV-1: Fig badnavirus 1

b: Abbreviations: AR- Arkansas; CA- California; FL- Florida; FS- fig seedling grown from seed; MI- Michigan; OH- Ohio; OR- Oregon; SC- South Carolina.

c: FM: Fig mosaic

d: Complete genome sequence

Table 3: Herbaceous hosts mechanically inoculated with accession AR1, source to the type isolate of Fig badnavirus-1.

Indicator species	Symptoms	FBV-1 PCR Positives ^{a,b}
<i>Cucurbita pepo</i> cv. Conneticut Field Pumpkin	Mosaic	8/12
<i>Glycine max</i> cv. Lee	Mild mosaic	2/36
<i>Pisum sativum</i> cv. Wando	None	11/20
<i>Nicotiana occidentalis</i>	None	6/8
<i>Nicotiana tabacum</i>	None	4/8
<i>Nicotiana sylvestris</i>	None	4/8
<i>Nicotiana rustica</i>	None	4/8
<i>Nicotiana benthamiana</i>	None	0/14
<i>Cucumis sativus</i> cv. National Pickling	None	0/20
<i>Cucumis melo</i> cv. Edisto 47 cantaloupe	None	0/6
<i>Vigna unguiculata</i> cv. Monarch	None	0/18
<i>Phaseolus vulgaris</i> var. Black Valentine	None	0/8
<i>Gomphrena globosa</i>	None	0/12
<i>Spinacia oleracea</i> cv. Bloomsdale	None	0/16
<i>Chenopodium amaranticolor</i>	None	0/18
<i>Chenopodium quinoa</i>	None	0/22
<i>Beta vulgaris</i> cv. Ruby Queen	None	0/12
<i>Solanum lycopersicum</i> cv. Beefsteak	None	0/12
<i>Brassica rapa</i> subspecies <i>pekinensis</i>	None	0/8

a: FBV-1: Fig badnavirus 1

b: All positive amplicons were verified by sequencing the product.

Figure 1: Variation of Fig mosaic disease symptoms.

Figure 2: Linear representation of Fig badnavirus-1 genome (A) showing tRNA^{MET} primer-binding site (denoted by an arrow); TATA-box (denoted by a box); ORF 1; ORF 2; ORF 3 with movement protein (MP), capsid protein zinc-finger domain (CP), pepsin-like aspartate protease (Pro), reverse transcriptase (RT) and RNase H (RNase H) motifs; and ORF 4. Cloning strategy for acquiring complete FBV-1 genome (B) with forward (F) and reverse (R) primers labeled according to nucleotide position, primers used to amplify the region for the diversity study, detection PCR (amplicon also serves as Southern blot probe) and sequences obtained by degenerate oligonucleotide-primed PCR (DOP-PCR).

Figure 3: Phylogram using the genome nucleotide sequence for members of the genus *Badnavirus*. Virus sequences used: Fig badnavirus-1 (FBV-1; JF411989); *Taro bacilliform virus* (TaBV; NC_004450); *Bougainvillea chlorotic vein-banding virus* (BCVBV; NC_011592); *Dioscorea bacilliform AL virus* (DBALV; NC_009010); *Cacao swollen shoot virus* (CSSV; NC_001574); *Citrus yellow mosaic virus* (CiYMV; NC_003382); *Commelina yellow mottle virus* (CoYMV; NC_001343); *Banana streak GF virus* (BSGFV; NC_007002); *Sugarcane bacilliform IM virus* (SCBIMV; NC_003031); *Sugarcane bacilliform MO virus* (SCBMOV; NC_008017); *Banana streak MY virus* (BSMYV; NC_006955); *Banana streak AY virus* (BSAYV; DQ092436); *Banana streak VN virus* (BSVNV; NC_007003); *Banana streak OL virus* (BSOLV; NC_003381); *Banana streak CA virus* (BSCAV; NC_015506); *Banana streak UM virus* (BSUMV; NC_015505); *Banana streak UL virus* (BSULV; NC_015504); *Banana streak UI virus* (BSUIV; NC_015503); *Banana streak UA virus* (BSUAV; NC_015502); *Gooseberry vein banding associated virus isolate BC* (GVBaV; HQ852250); *Pineapple bacilliform CO virus* (PBCOV; NC_014648); and *Kalanchoe top-spotting virus* (KTSV; NC_004540). *Cauliflower mosaic virus* (CaMV; NC_001497) was used as an outgroup. Bootstrap values less than 70% is not shown as they are considered unreliable. Bar represents 0.1 changes per site.

Figure 4: A. Southern blot hybridization for Fig badnavirus 1 (FBV-1) using fig DNA digested with *Sspl* (Lane 1), *EcoRI* (Lane 2), FBV-1 amplicon used to generate the hybridization probe (Lane 3) and undigested control (Lane 4). Expected signal size for episomal FBV-1 is 7.1 kb for *Sspl* (Lane 1) and 5.4 kb for *EcoRI* (Lane 2), marked with a (*). B. Left: Agarose gel electrophoresis of rolling circle amplification (RCA) products. Lane 1: undigested RCA products from FBV-1 infected fig; lane 2: *Sspl*-digested RCA products from FBV-1-free plant, lanes 3-4: RCA products derived from FBV-1 infected fig digested with *Sspl* and *MluI*, respectively; lane 5: FBV-1 amplicon used to generate the hybridization probe. Expected signal size for episomal FBV-1 is 7.1 kb (Lane 1) marked with a (*). Right: Southern blot verifying (*) that the RCA products belong to FBV-1.

Figure 5: Results of eight of the 16 samples used in amplification of fig samples using PCR, RT-PCR and RCA for discrimination between episomal and integrated forms of Fig badnavirus 1 (FBV-1). All samples were positive for FMV-1 when total nucleic acids were assayed using primer set 1094F/1567R (A). By contrast only three of the eight samples (lanes 1, 6 and 7) were positive when subjected to RCA (B; *SspI*-digested product indicated by arrowhead) or RT-PCR (C).

Figure 6: Detection of Fig badnavirus 1(FBV-1) in fig. Lane 1, isolate AR1 (Fig mosaic-type isolate); Lane 2, isolate AR2 (asymptomatic); Lane 3, isolate AR3 (asymptomatic); Lane 4, isolate CA15 (Fig mosaic); Lane 5, isolate CA30 (Fig mosaic); Lane 6, FBV-1-negative Arkansas fig AR7 (asymptomatic); Lane 7, FBV-1-negative California fig CA1 (Fig mosaic); Lane 8, negative control using water in place of a template.

Figure 1



Figure 2

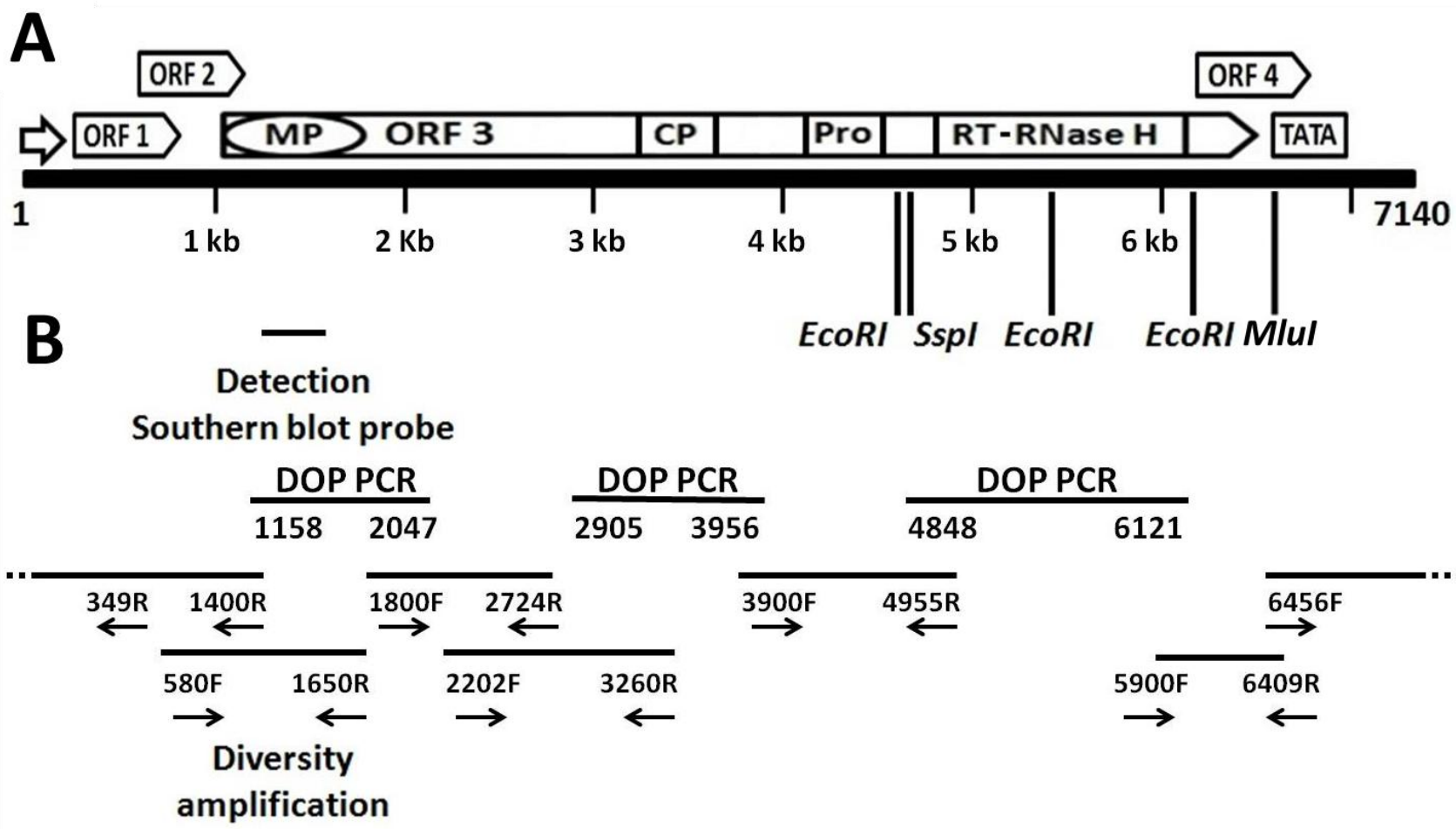


Figure 3

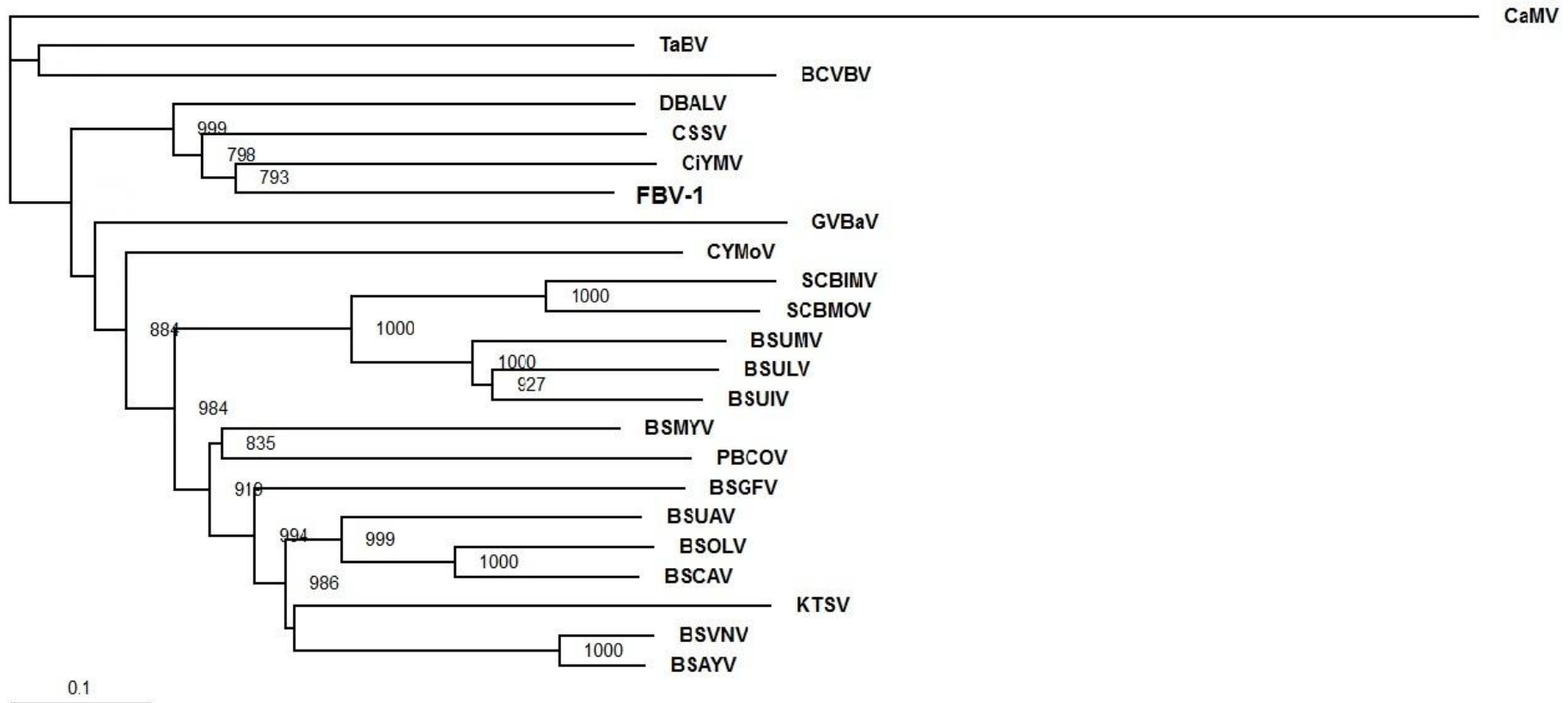


Figure 4

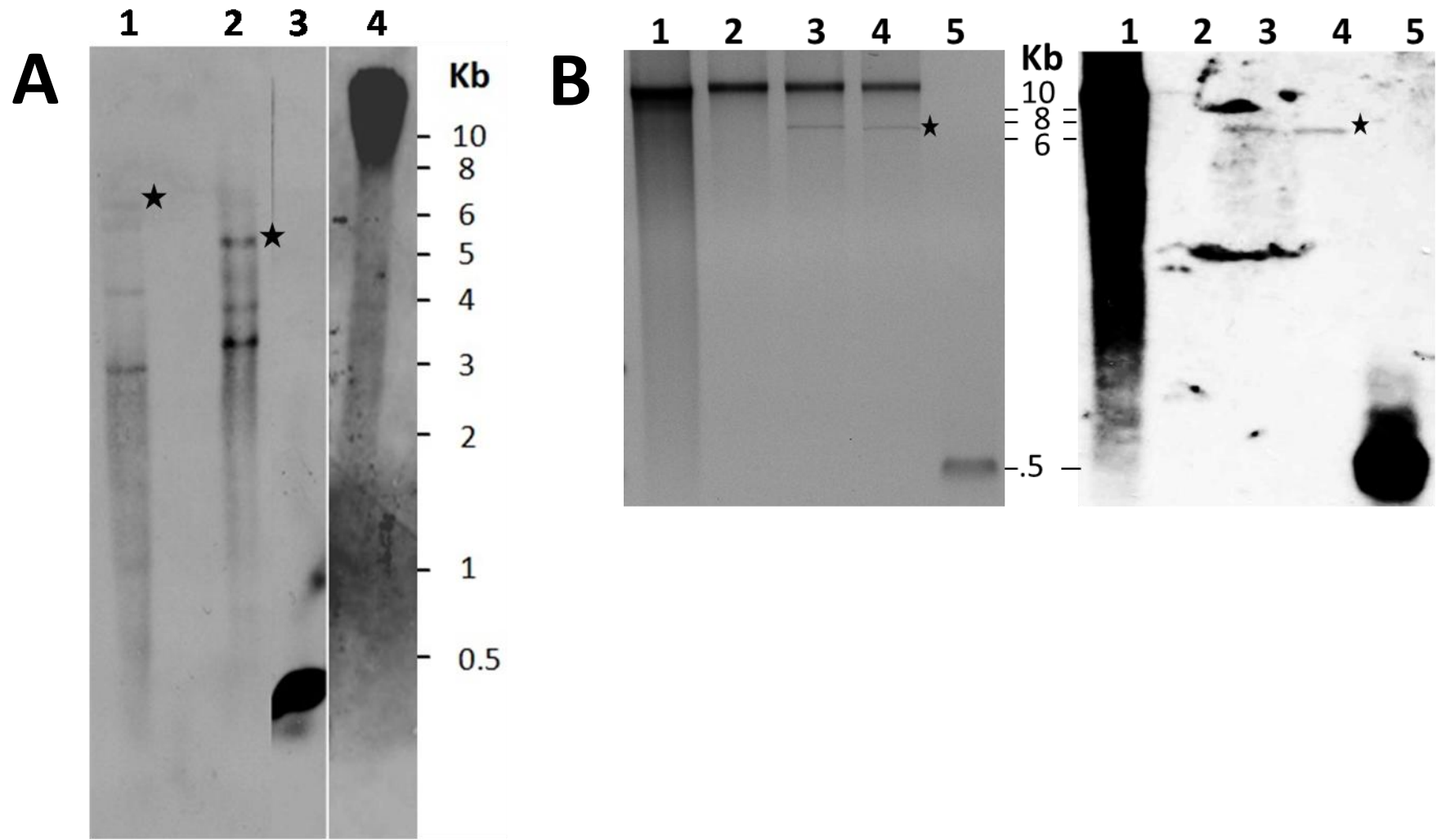


Figure 5

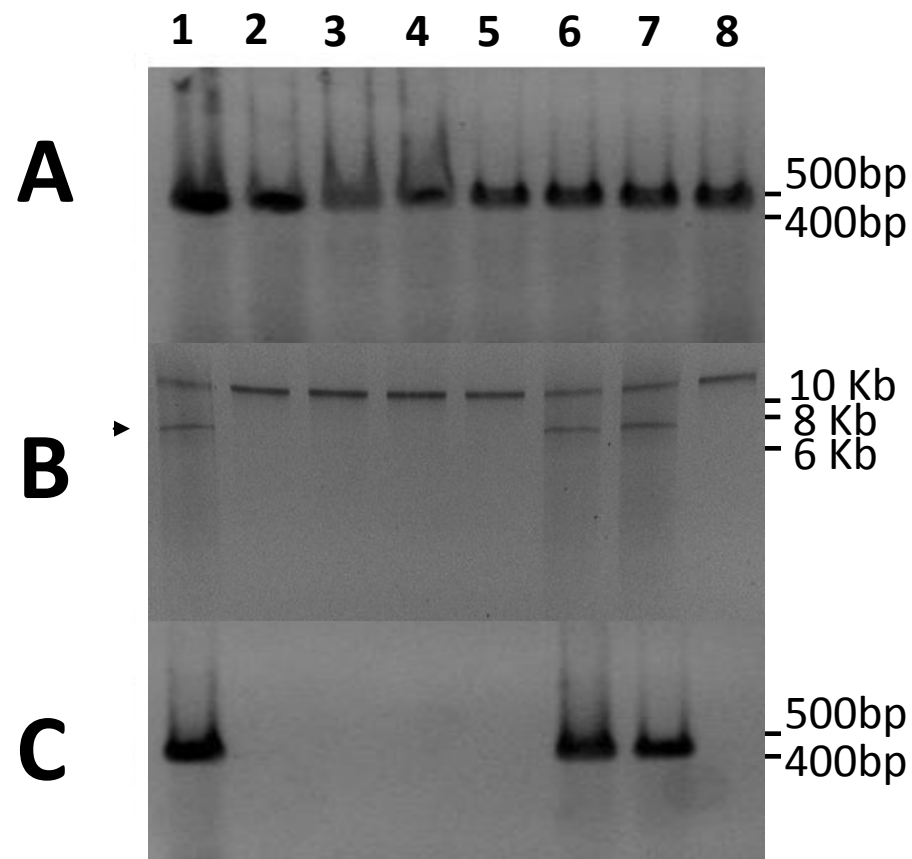


Figure 6

