Supporting Information

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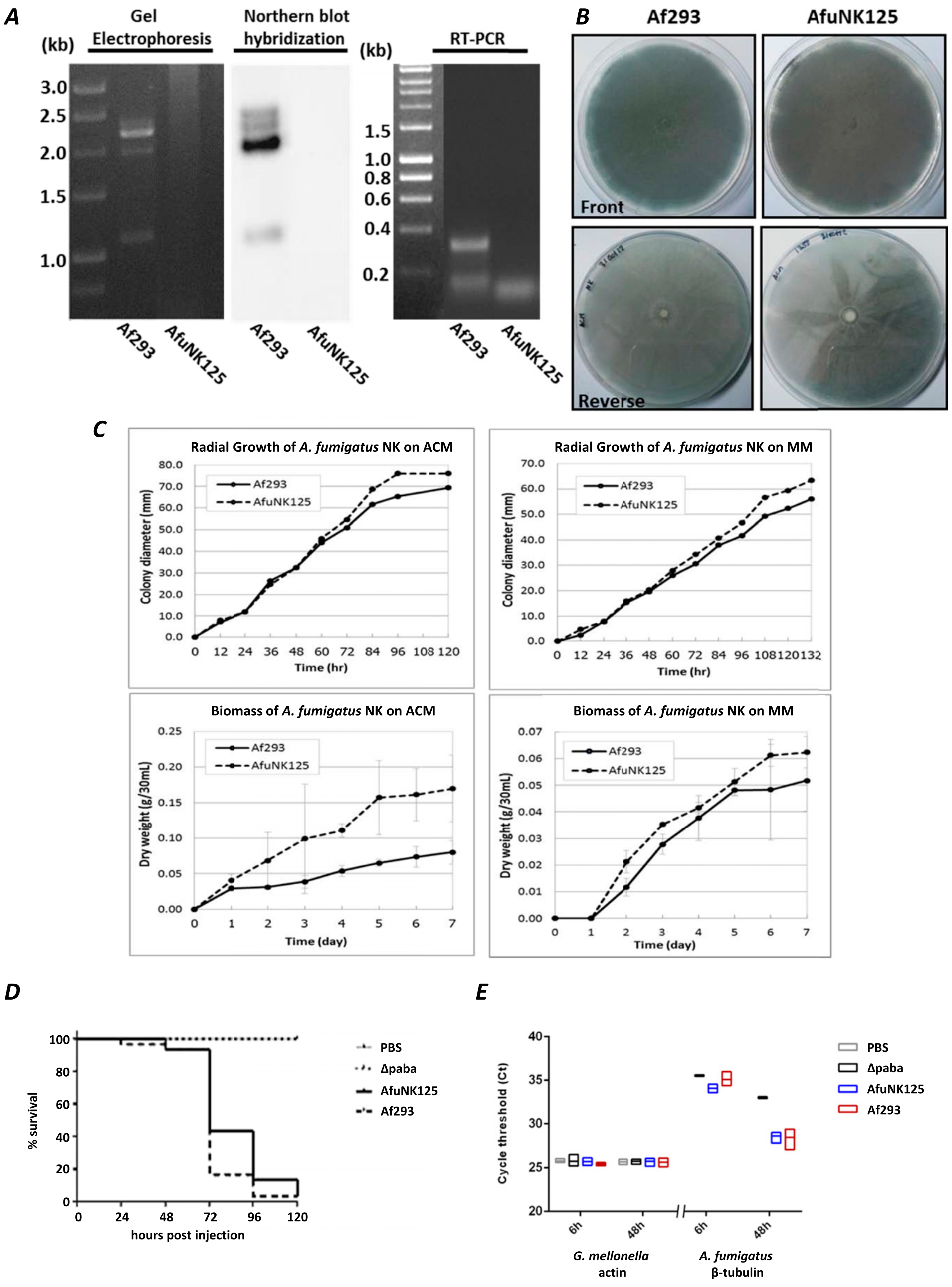

Fig. S1. (*A*) Agarose gel electrophoresis, Northern blot hybridization, and attempted RT-PCR amplification of a AfuTmV-1 dsRNA 2 337-bp fragment from the AfuTmV-1–infected *A. fumigatus* Af293 isolate and cycloheximide-treated and virus-free, isogenic isolate *A. fumigatus* AfuNK125 plus analysis of pathogenicity of both strains. Hyperladder 1 (Bioline) was used as marker. (*B*) Colonies of the Af293 and AfuNK125 grown on ACM at 37 °C for 6 d. (*C*) Comparison of the fungal growth under different cultivation conditions. Radial growth of Af293 and AfuNK125 on ACM plates (*Top Left)* and on MM plates (*Top Right*); biomass production of Af293 and AfuNK125 in ACM broth (*Bottom Left*) and in MM broth (*Bottom Right*). (*D*) Mean survival curves of *G. mellonella* larvae infected with Af293 and AfuNK125 (1×10^5 spores per larva). The *A. fumigatus* Δ*paba* strain was included as an avirulent control. (*E*) Fungal burden in *G. mellonella* larvae infected with Af293 and AfuNK125, as shown by quantitative PCR. Cycle of threshold (Ct) values indicate the PCR cycle where the amplified product was first detectable.

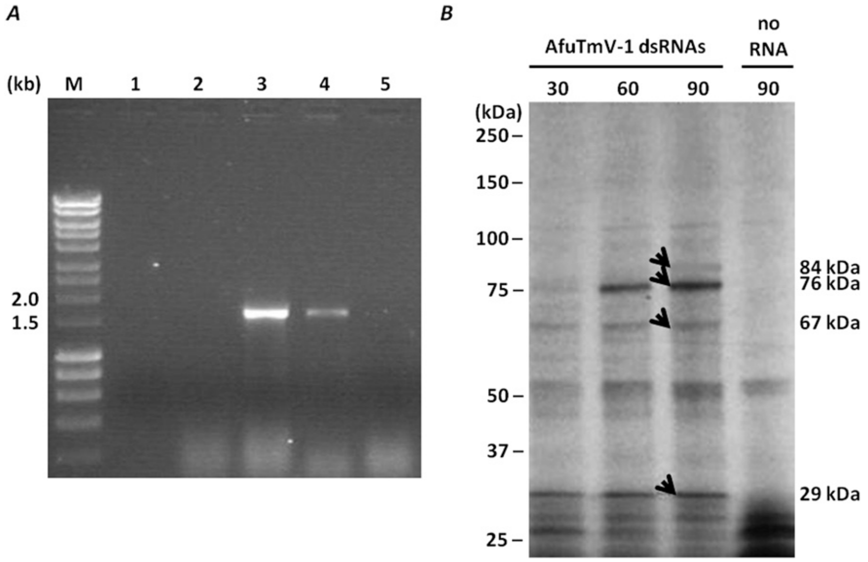

Fig. S2. (*A*) Transfection of *A. fumigatus* protoplasts with AfuPV-1 virions and AfuPV-1 dsRNA. Total RNA extracts were used for RT-PCR amplification using oligonucleotide primers designed to produce an amplicon corresponding to the complete AfuPV-1 dsRNA 1 RdRP gene 1,626 bp in size. Lane M, Hyperladder 1 marker DNAs (Bioline); RNA extracts of the following: lane 1, no RNA (negative control); lane 2, *A. fumigatus* isolate Af-237y (negative control); lane 3, purified AfuPV-1 (positive control); lane 4, *A. fumigatus* isolate Af-237y mycelia transfected with AfuPV-1 (positive control for transfection); lane 5, *A. fumigatus* isolate Af-237y mycelia transfected with AfuPV-1 dsRNA. (*B*) Analysis of in vitro translation products of AfuTmV-1 dsRNAs. In vitro translation products from unfractionated dsRNA were separated on a NuPAGE Novex 4–12% Bis-Tris protein gel (Invitrogen) and autoradiographed. The results shown represent samples taken after 30-, 60-, and 90-min incubation. A negative control (no RNA) incubated for 90 min facilitates identification of the major translation products—indicated by arrows and with their expected molecular weight to the *Right* of the gel—in the reactions that contain denatured dsRNA. The positions of marker proteins are indicated to the *Left* of the gel.

A **5’-terminal sequences (dsRNA1-4)**

```
dsRNA1   5’-CGAAUAAAAGGAAACUUCUCCGCUUGUGUGCUCCA------------------------------------
dsRNA3   5’-CGAAUAAAAGGAAACUAGAACGCUUGCAUGCUCCAACGCCCCGCAACCACC--------------------
dsRNA2   5’-UGAAUAAAAGAUUUGUUCUGGGCAAGCACGCUGCAACGUCGAGCAACUCCCAUAUAGGGACGA-UGAGAC-
dsRNA4    U/CGAAUAAAAGAUUUGAUCUGAGCUUGCAUGCUUCUUCGUCGACCUACUACCGUAUAAGCGCCGAGUGAGUCC
             *********.          **  * . *** *

dsRNA1      -------------- (35 nt)
dsRNA3      -------------- (51 nt)
dsRNA2      -------------- (70 nt)
dsRNA4      CCGAGAAGCCCACC (86 nt)
```

B **3’-terminal sequences (dsRNA1-4)**

```
dsRNA4      GCGUGCCCUUAGCACGCCCUUACCGUUCCCUGAACGGAUGCGCCCGGGAGAUGCCGCAGUACUCUUUCUCCCGG
dsRNA3      --------------------------------------------------------------------------
dsRNA1      --------------------------------------------------------------------------
dsRNA2      --------------------------------------------------------------------------


dsRNA4      GGAGGGACAUAGUGGGCUGCCUCAAGUGGUGAACUACACCUU-GUCUGUGGGAGUAGCGCCGAGGUUGCAUGUA
dsRNA3      ----------------GUGGAAC--GU--UGGAGAUGCUCGCGUCCCGACG--------C-GGCGGGGGGGCC
dsRNA1      ------------------UCACC--GCAC---ACAAGC---CGGCCCGGCG--------G-GAGGGGCGCGCG
dsRNA2      ----------ACUGCGCUGCAUC--GUGCUUGACCAGC-----GUCCGACG--------G-GGAGGGG-----C
                                  *  *      *            * *  *          *. *  *

dsRNA4      CUGUCU---CACGUACCCCCCCAAAAGGAUGGCUGACCCCUGUCCGGGGGGGACGGGGG- -3’ (205 nt)
dsRNA3      CUG-------GCCCCCCCCCGCG--------------------CCGGG----GCGGGGG- -3’  (74 nt)
dsRNA1      UUGUCGAUCCGCGUGCCCCCCCG--------------------CCGGG----GCGGGGGG -3’  (76 nt)
dsRNA2      UU--------AAUUGCCCCCCCG--------------------CCGGG----CCUGGGGG -3’  (72 nt)
             *         .    ****** *.                   *****     * ****
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C **3’-terminal sequences (dsRNA1-3)**

```
dsRNA3      ------GUGGAACGU--UGGAGAUGCUCGCGUCCCGACGCGGCGGGGGGGCCCUG-------GCCCCCCCCC
dsRNA1      --------UCACCGCAC---ACAAGC---CGGCCCGGCGGGAGGGGCGCGCGUUGUCGAUCCGCGUGCCCCC
dsRNA2      ACUGCGCUGCAUCGUGCUUGACCAGC-----GUCCGACGGGAGGGG-----CUU--------AAUUGCCCCC
                      * **      *   **      ***.** *. ****     *       .     *****

dsRNA3      CGCGCCGGGGCGGGGG- -3’ (74 nt)
dsRNA1      CCCGCCGGGGCGGGGGG -3’ (76 nt)
dsRNA2      CCCGCCGGGCCUGGGGG -3’ (72 nt)
            * ******* * ****
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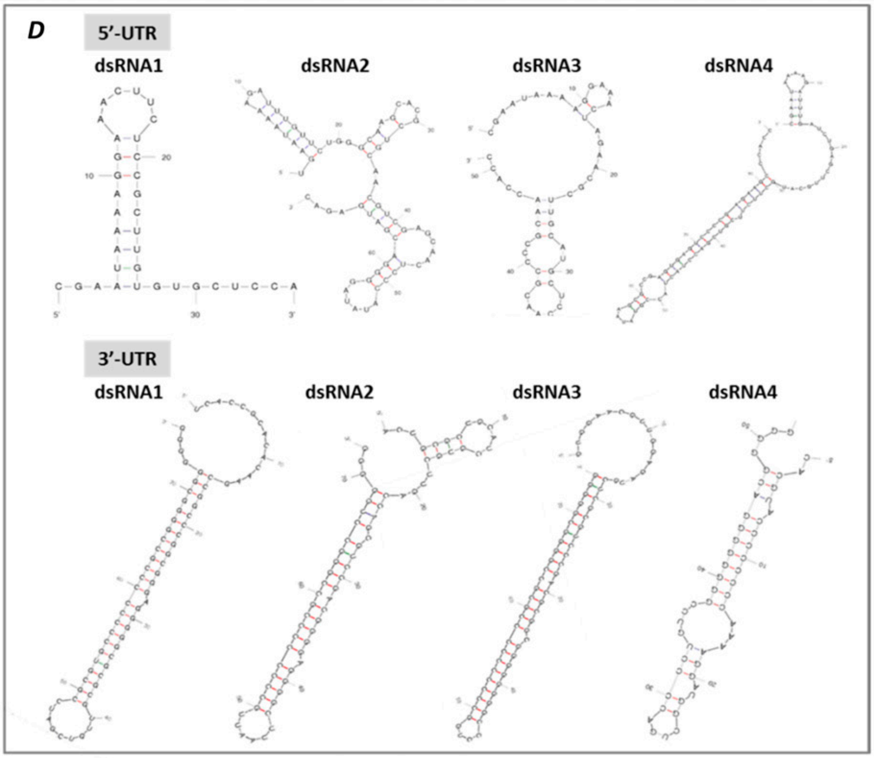

Fig. S3. Comparative alignment of the 5′-terminal sequences of dsRNAs 1–4 (*A*) and 3′-terminal sequences of AfuTmV-1 dsRNAs 1–3 (*B*) and dsRNAs 1–4 (*C*) using CLUSTALW. Asterisks signify identical nucleotides at the indicated positions. (*D*) Predicted secondary structure of the 5′-UTRs and 3′-UTRs of the AfuTmV-1 dsRNAs using mfold.

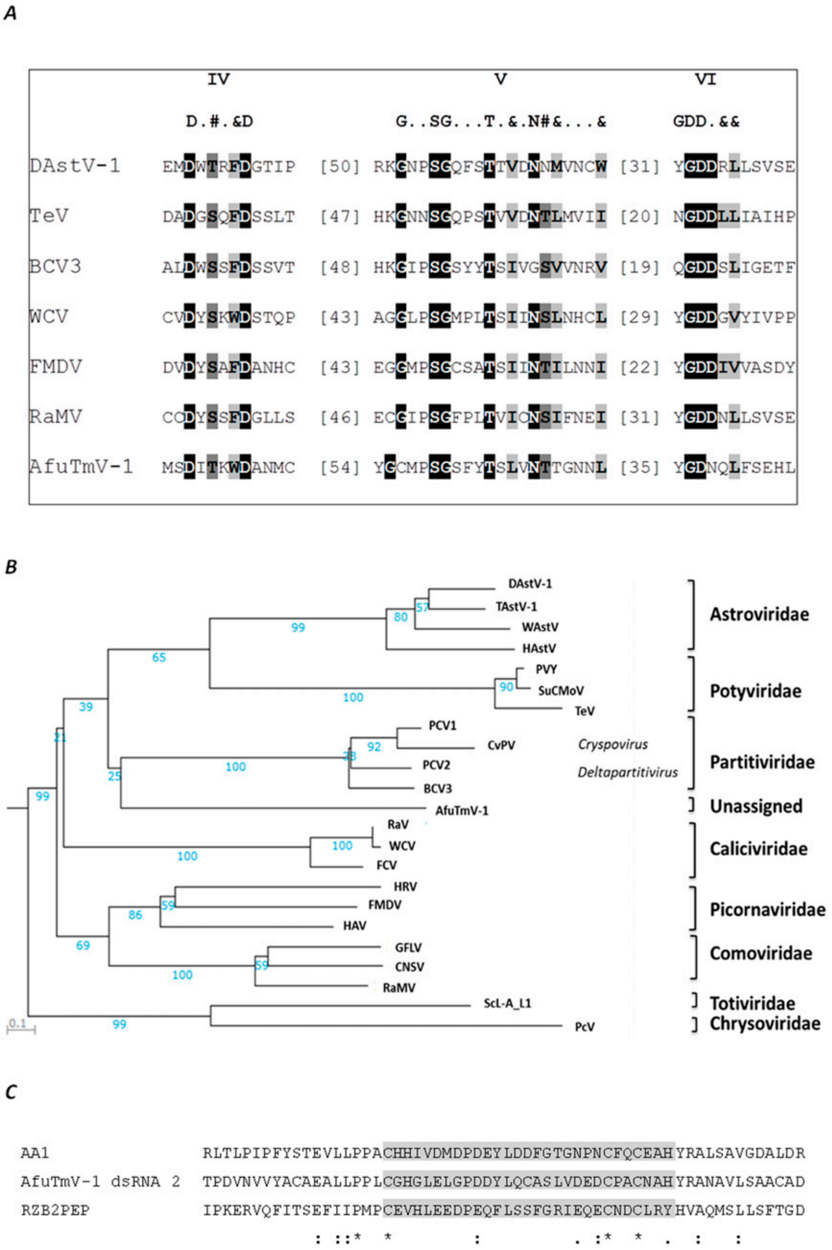

Fig. S4. (*A*) Amino acid sequence alignment of RdRP regions of AfuTmV-1 dsRNA 1 and selected RNA viruses. The alignment was generated using the PSI-Coffee program (version 10.0). Three conserved RdRP motif corresponding to motifs IV, V, and VI, respectively, according to Bruenn (1), are shown, and consensus sequences are highlighted either with black or gray shading. Black shading indicates identical amino acid residues, and gray shading indicates those that belong to the same group of consensus sequences. The "#" symbol refers to polar uncharged residues S or T, and "&" denotes any bulky hydrophobic residue (L, I, V, M, F, Y, W). Numbers within the brackets indicate the number of amino acids not shown. Virus names, acronyms, and GenBank accession numbers are listed in Table S2. (*B*) Phylogenetic analysis of AfuTmV-1 RdRP and related RdRPs. A phylogenetic tree was constructed using the fast Fourier transform MAFFT program L9INS-1 (2). A bootstrap test was conducted with 1,000 resamplings for the neighbor-joining trees. Virus genera and families are indicated by normal and embolded type respectively. Numbers on the nodes indicate percentage of bootstrap support from 1,000 replicates with branch lengths indicated. GenBank accession numbers and abbreviations for each sequence are shown in Table S2. (*C*) Amino acid sequence alignment of zinc finger-like motifs of regions on proteins of unknown function putatively encoded by AfuTmV-1 dsRNA 2, a 2,233-bp dsRNA isolated from *Alternaria* sp. FA0703 (AA1; accession no. ACL80752, and a ~2.3-kbp mycovirus dsRNA, nominated B2, isolated from the flax rust, *Melampsora lini* (RZB2). The motifs are shaded, and asterisks signify identical amino acid residues, colons signify highly conserved residues, and single dots signify less conserved but related residues.

1. Bruenn JA (1993) A closely related group of RNA-dependent RNA polymerases from double-stranded RNA viruses. *Nucleic Acids Res* 21(24):5667–5669.
2. Altschul SF, et al. (1997) Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res* 25(17):3389–3402.

A

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Vaccinia                        AVTKTDGIPITIRV
African swine fever virus       VTDKADGIRGIAVI
AfuTmV-1 ds RNA 3               ALVKYDGEHGVLVL
M. lini mycovirus dsRNA B3      VFPKFDGTPGILLM
                                .  * **      :
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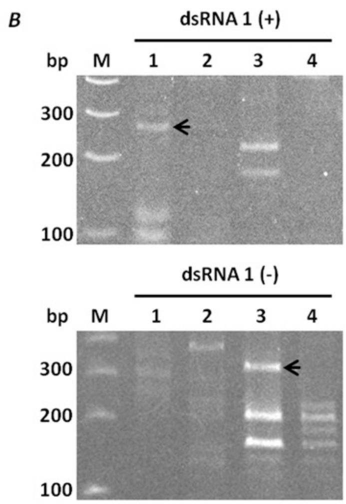

C

treatment	Lane 1	Lane 2	Lane 3	Lane 4
CIP	+	+	-	-
TAP	+	-	-	-
adaptor	+	+	+	-

Fig. S5. AfuTmV-1 dsRNA 3 encodes for a putative methyltransferase. (*A*) Amino acid sequence alignment of regions on proteins putatively encoded by AfuTmV-1 dsRNA 3, *Melampsora lini* dsRNA B3 (accession no. X64371), and vaccinia virus and African swine fever virus involved in 5′-capping of RNA. The principal lysine residue (K) is shown as part of a characteristic catalytic motif for viral GTPs. Asterisks signify identical amino acid residues, and colons signify highly conserved residues and single dots signify less conserved but related residues. (*B* and *C*) 5′-RLM RACE oligo-cap analysis of AfuTmV-1 dsRNA 1. Viral dsRNA was (*i*) treated with CIP and TAP and ligated to an oligoribonucleotide adaptor at the 5′ terminus, (*ii*) treated with CIP only and ligated, (*iii*) left untreated and ligated dsRNA, and (*iv*) left untreated and unligated. Subsequently, RT-PCR was perfomed with a primer homologous to the oligonucleotide adaptor and a sequence-specific primer, and the products were electrophoresed on a 4.5% (wt/vol) native polyacrylamide gel. Expected product sizes are 277 bp for the dsRNA 1 positive strand and 311 bp for the negative strand, and are indicated by arrows. The positive reaction in lane 1 for the positive strand indicates that it is capped, whereas the positive reaction in lane 3 for the negative strand indicates that it is phosphorylated but not capped.

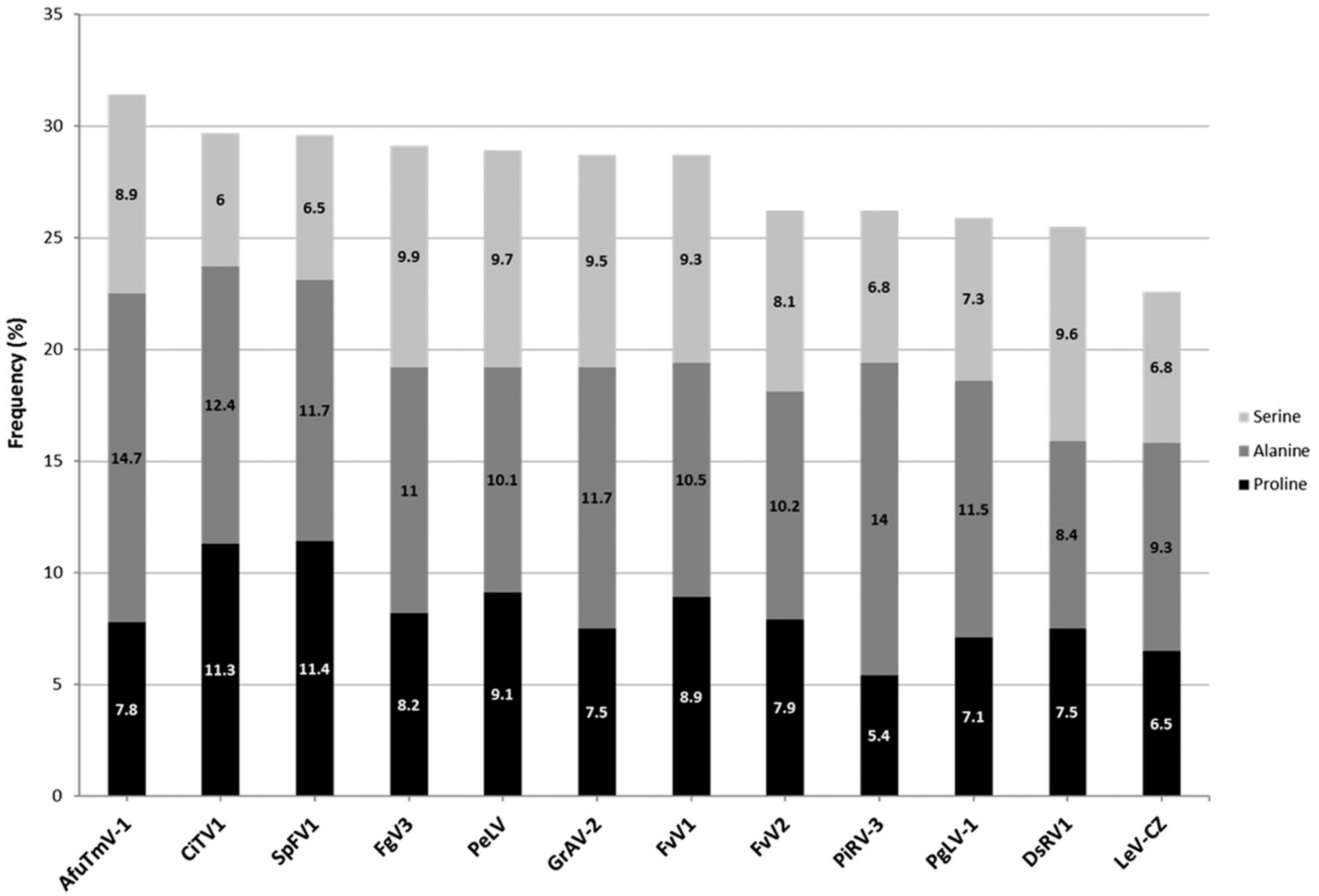

Fig. S6. Proline (P), alanine (A), and serine (S) ratios of PAS-rich proteins (PASrps) of AfuTmV-1 dsRNA4 and related unclassified viruses. Virus names, acronyms, and GenBank accession numbers are listed in Table S2. Amino acid lengths of complete ORFs and proline–alanine–serine (P–A–S) percent ratios of selected, unclassified insect, plant, and fungal viruses are summarized in Table S3.

Table S1. Properties of AfuTmV-1 dsRNAs

Segment	Accession no.	ORF size (aa, kDa)	Putative function	UTR length, nt	
				5′-UTR	3′-UTR
dsRNA 1 (2,403 bp)	HG975302	2,292 (763, 84.2)	RdRP	35	76
dsRNA 2 (2,233 bp)	HG975303	2,091 (696, 75.8)	Unknown*	70	72
dsRNA 3 (1,970 bp)	HG975304	1,845 (614, 66.7)	Methyltransferase	51	74
dsRNA 4 (1,131 bp)	HG975305	840 (279, 29.1)	PAS-rich protein (rp)	86	205

*Similar to proteins of unknown function encoded by respectively dsRNA isolated from *Alternaria* sp. FA0703 (ACL80752) and mycovirus dsRNA, B2, isolated from the flax rust, *Melampsora lini* (RZ1B2PEP).

Table S2. Viruses selected for phylogenetic analysis of RdRP and PASrp

Virus	Abbreviation	GenBank accession no. RdRP	GenBank accession no. PASrp	Ref.
Duck astrovirus C-NGB	DAstV-1	YP_002728002		1
Turkey astrovirus 1	TAstV-1	CAB95006		2
Wood pigeon astrovirus	WAstV	CBY02491.1		Unpublished
Human astrovirus 1	HAstV	NP_751905		3
Potato virus Y	PVY	EU563512		4
Tobacco etch virus	TeV	AEU12509.1		Unpublished
Sunflower chlorotic mottle virus	SuCMoV	YP_003580192.1		5
Pepper cryptic virus 1	PCV1	AEJ07890		6
Chlorella vulgaris partitivirus 2	CvPV	JX392808		Unpublished
Pepper cryptic virus 2	PCV2	AEJ07892		6
Beet cryptic virus 3	BCV3	AAB27624.1		7
Rabbit vesivirus	RaV	NC008580.1		8
Walrus calicivirus	WCV	NP_786919.1		9
Feline calicivirus	FCV	NP_783308		10
Human rhinovirus A	HRV	NP_740401.1		Unpublished
Foot and mouth disease virus	FMDV	AHW45732		11
Human hepatitis A virus	HAV	GNNYHB		12
Grapevine fan leaf virus	GFLV	BAA00761		13
Cycas necrotic stunt virus	CNSV	NP_734017		14
Radish mosaic virus	RaMV	AAY32935.1		15
Saccharomyces cerevisiae virus L-A	ScL-A (L1)	NP_620495		16
Penicillium chrysogenum virus	PcV	YP_392482		17
Persimmon latent virus	PeLV		BAN29037.1	18
Circulifer tenellus virus 1	CiTV1		YP_003800002.1	19
Spissistilus festinus virus 1	SpFV1		YP_003800000.1	19
Phlebiopsis gigantea large virus-1	PgLV-1		CAJ34331.3	20
Fusarium graminearum virus 3	FgV3		YP_003288788	21
Diplodia scrobiculata RNA virus 1	DsRV1		ACD91657	22
Lentinula edodes virus-CZ	LeV-CZ		AEB96149	23
Fusarium virguliforme dsRNA mycovirus 1	FvV1		AEZ54147.1	24
Fusarium virguliforme dsRNA mycovirus 2	FvV2		AEZ54148	24
Grapevine-associated totivirus-2	GrAV-2		ADO60932.1	25
Phytophthora infestans RNA virus-3	PiRV-3		AEX87901.1	26

1. Fu Y, et al. (2009) Complete sequence of a duck astrovirus associated with fatal hepatitis in ducklings. *J Gen Virol* 90(Pt 5):1104–1108.
2. Jonassen CM, Jonassen TO, Grinde B (1998) A common RNA motif in the 3′ end of the genomes of astroviruses, avian infectious bronchitis virus and an equine rhinovirus. *J Gen Virol* 79(Pt 4):715–718.
3. Geigenmüller U, Chew T, Ginzton N, Matsui SM (2002) Processing of nonstructural protein 1a of human astrovirus. *J Virol* 76(4):2003–2008.
4. Dullemans AM, Cuperus C, Verbeek M, van der Vlugt RA (2011) Complete nucleotide sequence of a potato isolate of strain group C of *Potato virus Y* from 1938. *Arch Virol* 156(3):473–477.
5. Bejerman N, Giolitti F, de Breuil S, Lenardon S (2010) Molecular characterization of *Sunflower chlorotic mottle virus*: A member of a distinct species in the genus *Potyvirus*. *Arch Virol* 155(8):1331–1335.
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14. Han SS, Karasev AV, Ieki H, Iwanami T (2002) Nucleotide sequence and taxonomy of Cycas necrotic stunt virus. Brief report. *Arch Virol* 147(11):2207–2214.
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16. Icho T, Wickner RB (1989) The double-stranded RNA genome of yeast virus L-A encodes its own putative RNA polymerase by fusing two open reading frames. *J Biol Chem* 264(12): 6716–6723.
17. Castón JR, et al. (2003) Three-dimensional structure of penicillium chrysogenum virus: A double-stranded RNA virus with a genuine T=1 capsid. *J Mol Biol* 331(2):417–431.
18. Ito T, Suzaki K, Nakano M (2013) Genetic characterization of novel putative rhabdovirus and dsRNA virus from Japanese persimmon. *J Gen Virol* 94(Pt 8):1917–1921.
19. Spear A, Sisterson MS, Yokomi R, Stenger DC (2010) Plant-feeding insects harbor double-stranded RNA viruses encoding a novel proline-alanine rich protein and a polymerase distantly related to that of fungal viruses. *Virology* 404(2):304–311.
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Table S3. Amino acid lengths of complete ORFs and proline–alanine–serine (P–A–S) percent ratios of selected unclassified insect, plant, and fungal viruses

		Proline–alanine–serine-rich protein (PArp)					
Virus	Abbreviation	Proline	Alanine	Serine	P–A	P–A–S	Length, aa
Aspergillus fumigatus tetramycovirus-1	AfuTmV-1	24 (8.6%)	40 (14.3%)	25 (8.9%)	64 (22.9%)	89 (31.9%)	279
Circulifer tenellus virus 1	CiTV1	128 (11.3%)	141 (12.4%)	68 (6.0%)	269 (23.7%)	337 (29.7%)	1,133
Spissistilus festinus virus 1	SpFV1	125 (11.4%)	129 (11.7%)	72 (6.5%)	254 (23.1%)	326 (29.6%)	1,101
Fusarium graminearum virus 3	FgV3	115 (8.2%)	151 (11.0%)	135 (9.9%)	266 (19.4%)	401 (29.3%)	1,369
Persimmon latent virus	PeLV	98 (9.1%)	109 (10.1%)	104 (9.7%)	207 (19.3%)	311 (28.9%)	1,075
Grapevine-associated totivirus-2	GrAV-2	99 (7.5%)	154 (11.7%)	125 (9.5%)	253 (19.3%)	378 (28.8%)	1,313
Fusarium virguliforme dsRNA mycovirus 1	FvV1	117 (8.9%)	137 (10.5%)	122 (9.3%)	254 (19.4%)	376 (28.7%)	1,311
Fusarium virguliforme dsRNA mycovirus 2	FvV2	107 (7.9%)	138 (10.2%)	109 (8.1%)	245 (18.2%)	354 (26.3%)	1,347
Phytophthora infestans RNA virus-3	PiRV-3	70 (5.4%)	182 (14%)	88 (6.8%)	252 (19.4%)	340 (26.2%)	1,296
Phlebiopsis gigantea large virus-1	PgLV-1	142 (7.1%)	232 (11.5%)	147 (7.3%)	374 (18.6%)	521 (25.9%)	2,012
Diplodia scrobiculata RNA virus 1	DsRV1	31 (7.5%)	35 (8.4%)	40 (9.6%)	66 (15.9%)	106 (25.5%)	416
Lentinula edodes virus-CZ	LeV-CZ	129 (6.5%)	184 (9.3%)	135 (6.8%)	313 (15.8%)	448 (22.7%)	1,975