Nature Microbiology

A capsidless ssRNA virus hosted by an unrelated dsRNA virus

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MS information:

Title, 59 characters including space; Summary, 165 words; Main text, 2013 words excluding references; Figure legends, 384; Materials and Methods, 780; References, 33; Table, 0; Figures, 3; Supplemental table, 3; Supplemental figures, 4.

The complete nucleotide sequences reported in this article have been deposited with the EMBL/GenBank/DDBJ Data Library under Accession Nos. LC006253, LC006254, and LC006255.

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Summary (165 words)

Viruses typically encode their own capsid that encase their genome, while satellite viruses do not encode a replicase and depend on a helper virus for their replication[1]. Here, we report a virus/virus interplay between two RNA viruses termed yado-nushi virus 1 (YnV1) and yado-kari virus 1 (YkV1) in a

phytopathogenic fungus, *Rosellinia necatrix*[2]. YkV1 has a close phylogenetic affinity to positive-sense, single-stranded (+)ssRNA viruses such as animal caliciviruses[3], while YnV1 has an undivided double-stranded (ds) RNA genome with resemblance to fungal totiviruses[4]. Virion transfection and infectious full-length cDNA transformation showed that YkV1 depends on YnV1 for viability, although it probably encodes functional RNA-dependent RNA polymerase (RdRp). Immunological and molecular analyses revealed trans-encapsidation of not only YkV1 RNA but also RdRp by the capsid protein (CP) of the other virus (YnV1), and enhancement of YnV1 accumulation by YkV1. This study represents the virus/virus interplay where the capsidless (+)ssRNA virus, YkV1, highjacks CP of the dsRNA virus, YnV1, and replicates as if it were a dsRNA virus.

Lower eukaryotes including fungi, amoebae, and algae are important as virus hosts, from which a rapidly growing number of viruses have been reported in the past few decades[5-8]. These have contributed to further enhance our understanding of virus evolution and diversity. Simultaneously, some unusual viruses have shown their peculiarity and challenged the "common rules" of viruses in sizes and concepts. One such virus group includes the so called double-stranded (ds) DNA megaviruses (mimiviruses, pandoraviruses)[9]. Viruses were classically defined as nano-organisms (infectious virions with capsids encasing their genomes) that can pass through a bacteria-proof filter candle. However, the giant DNA viruses exceed those of bacterial parasites such as mycoplasmas and in coding capacity (>1.2 Mb) and particle size (>0.5 μm)[10]. Other unusual examples include "naked" or "capsidless" viruses, exemplified by fungal hypoviruses[11]. In addition, several groups of capsidless viruses such as umbraviruses, endornaviruses and narnaviruses are now recognised that infect either plants or fungi[3,12,13]. These capsidless viruses are considered to be positive-sense (+) single-stranded (ss) RNA viruses based on their phylogeny using hallmark proteins such as RNA-dependent RNA polymerase (RdRp). Capsidless narnaviruses with genomes of 2.3~3.6 kb represent a minimal virus form[14], encoding only RdRp, while others have relatively complex genome architectures.

Rosellinia necatrix is a filamentous ascomycete that causes white root rot in diverse perennial crops worldwide[2,15,16]. In this study, we show a mutualistic interplay between a capsidless, (+)ssRNA virus and a dsRNA virus tentatively termed yado-kari virus 1 (YkV1) and yado-nushi virus 1 (YnV1). "Yado-kari" literally means in Japanese "borrowing a room to stay" and biologically "hermit-crab," while "yado-nushi" means "landlord or room owner." In their interplay, YkV1 highjacks YnV1 CP to encapsidate its genomic RNA and RdRp, and probably replicates in the resultant particles, as if it were a dsRNA virus. YkV1 also trans-enhances YnV1 replication. This mutualism represents a unique virus "lifestyle."

The *R. necatrix* strain W1032 infected with several RNA viruses was isolated by Yaegashi et al.[17], which showed a debilitation phenotype when compared with an isogenic virus-free strain, W563 (Fig. 1a). A conventional virion purification method enabled us to purify one single type of spherical particles of approximately 40 nm in diameter (Fig. 1b). Particle fractions harbored at least three dsRNA elements designated as dsRNA-S5, dsRNA-S5a and dsRNA-S6 (Fig. 1c)[17] and one single protein of 120 kDa (Fig. 1d). The chemical nature of these RNAs was determined to be dsRNA, as described by Lin et al.[18]. Sequence analyses revealed that dsRNA-S5 represents the genome of a dsRNA mycovirus, YnV1, which shows low sequence similarity to other dsRNA mycoviruses (Fig. 1e), while dsRNA-S5a represents a defective (D)-RNA of YnV1 (Fig. 1e). The YnV1 genome of 9.0 kb encodes two ORFs, ORF1 and ORF2 situated in -1 frame relative to ORF1 (Fig. 1e, -1FS), as observed for some mycoviruses[19,20]. A blastp search showed no significant sequence similarity between YnV1 ORF1-encded CP precursor (see below) and known protein sequences. YnV1 ORF2-encoded RdRp shows moderate levels of sequence identity to counterparts of dsRNA viruses such as Sclerotinia sclerotiorum dsRNA mycovirus-L[21], and Glomus sp. RF1 medium virus (GRF1V-M)[22]. Phylogenetically, YnV1 is grouped with GRF1V-M (Table S1, Fig. 1), but distantly related to other members of the family *Totiviridae* (Fig. 1g). The YnV1 ORF2-coded protein possesses the Phytoreo-S7 domain, upstream of the RdRp motifs (Fig. 1e), which is widespread in fungal dsRNA viruses[21,23]. YnV1 sequence heterogeneity (Fig. 1e) will be reported elsewhere.

The dsRNA-S6 turned out to be the replicative form of the genome of a (+)ssRNA virus, YkV1. The YkV1 genome is 6.3 kb in size and possesses one single ORF encoding an RdRp domain close to the centre of the N-terminal region. The RdRp domain shows a distant relatedness to (+)ssRNA viruses such as caliciviruses including human-pathogenic sapoviruses. YkV1 shared the highest sequence identity (51%) with a little-explored RNA mycovirus, Aspergillus foetidus slow virus 2 (AfSV2) which has a similar genome organisation[24]. Modest levels of sequence identity (20–30%) were detected between the YkV1-encoded protein and RdRps from members of the family *Caliciviridae* (Table S2). A phylogenetic analysis revealed that YkV1 and AfSV2 cluster together, but confirms their large evolutionary distance from other known viruses (Fig. 1h).

We further analysed the 120-kDa protein detectable in W1032 virion fractions (Fig. 1d). In peptide mass fingerprinting (PMF), the m/z (mass-to-charge ratio) of 33 fragments matched the theoretical tryptic digests of YnV1 ORF1 (Table S3). Importantly, no peptide fragments were assigned to YkV1 ORF-encoded protein. The N-terminal sequences were determined to be ST/AEIKKMFDT (map positions 636 to 645, major peak) and GVYDLKKKEW (map positions 584 to 593, minor peak), which suggests the presence of the two co-carboxyl-terminal versions of CP (Fig. 1e). Protein and RNA analysis of virions raised an interesting question: how is YkV1 RNA packaged? To address this question, we

performed immunoprecipitation analysis of virions. Purified virion solution was immunoprecipitated by anti-YnV1-CP serum and a pre-immune serum (Fig. 1f). DsRNA was extracted from respective precipitates and analysed by gel electrophoresis. Interestingly, a dsRNA profile similar to the input virion fractions was obtained with the anti-YnV1-CP antibodies, while the pre-immune antibodies yielded no dsRNA. These results clearly indicated the trans-encapsidation of YkV1 RNA by YnV1 CP. The terminal sequences of the genomic RNA, which would serve as cis-elements involved in RdRp recognition, completely differ between the two viruses. Thus, it is highly unlikely that YkV1 RNA is replicated by YnV1 RdRp. We tested the possibility that YkV1 RdRp is also trans-packaged by YnV1 capsids. Western blotting with anti-YkV1 RdRp antibodies showed that polypeptides of approximately 100 kDa and 40 kDa are observed specifically in virion fractions obtained from mycelia co-infected with YkV1 and YnV1, but not from mycelia singly infected by YnV1 (Fig. S3). Fungal strains infected singly by YnV1 were obtained by virion transfection as stated below. This strongly suggests that YnV1 capsids encase not only YkV1 RNA but also its RdRp protein.

Given the trans-encapsidation of YkV1 RNA, we reasoned that YkV1 behaves like satellite viruses. In order to determine whether YkV1 requires YnV1 to survive, we utilised the transfection assay using purified virions and virus-free fungi (standard strain W97), which occasionally allows the isolation of co-infecting viruses[15]. Analysis of a number of resultant transfectants allowed for classification into four groups based on dsRNA gel profiles (Fig. 2a). The first group carried the same set of three dsRNAs as the parental strain W1032. The second group had YnV1 and YnV1-DI dsRNAs. The third group harbored YnV1 and YkV1. The last group had YnV1 dsRNA alone. Interestingly, a transfectant infected by YkV1 alone was not obtained (Fig. 2b). These results strongly suggest YkV1 relies for its survival on YnV1.

We explored the possibility that transcripts from chromosomally integrated full-length cDNA of YkV1 was infectious, in order to further confirm the dependence of YkV1 on YnV1. The full-length cDNA construct of the YkV1 was flanked by dual ribozymes (Fig. 2c) that allow synthesis of authentic viral transcripts in transformed fungal cells. Transformants derived from *R. necatrix* W97 are genetically barcoded by the benomyl resistance gene. To test whether YkV1 replication required YnV1 capsids, W97 transformants were fused with YnV1-transfected W97 (Fig. 2d). Expectedly, W97 transformed with the YkV1 cDNA launched infection upon receipt of YnV1 that then moved to YnV1-infected W97 strains. Importantly, YkV1 moved laterally to the benomyl-susceptible transfectant side (Fig. 2d). Although ssRNA transcripts of YnV1 cDNA were detectable by RT-PCR, no dsRNA was observed, when transformants were co-cultured with virus-free W97 (Fig. 2e). Establishment of YkV1 infection derived from the full-length cDNA was confirmed with different transformant strains. Moreover, we prepared spheroplasts from strain W97 infected by YnV1 and transformed with pCPXBnYkV1. Of 15, 9

independent transformants were found to support YkV1 replication (Fig. S4). These combined results compellingly show the infectiousness of the full-length cDNA of YkV1 only in the presence of YnV1, and convincingly show the dependency of YkV1 viability on YnV1.

Whether interplays between YkV1 and YnV1 are commensal or mutual is our next question. To address this, we compared the accumulation of YnV1 genomic dsRNA between mycelia infected singly and doubly that had been obtained by fusing YkV1 cDNA transformants and YnV1 transfectants. Consequently, YnV1 dsRNA accumulation was increased in fungal isolates infected by both viruses, relative to those infected by YnV1 alone when normalised to ribosomal RNA (Fig. 2f). Comparison of two different sets of fungal strains showed slightly different levels (two- to four-fold) of increase in co-infected fungal strains (Fig. 2f). These results suggest that YkV1 benefits YnV1 replication and enhances production of YnV1 CP. The mutualistic interaction between YnV1 and YkV1 appears quite distinct from any other reported virus/virus interactions and greatly expands our knowledge of diverse lifestyles of viruses.

One may refer to YkV1 as a dsRNA virus, but this is not appropriate because a viral "genome" is defined as the nucleic acids packaged by its own CP. Rather, our phylogenetic analysis with RdRps compellingly showed that while YnV1 is a genuine dsRNA virus, YkV1 is placed into a cluster comprising established animal (+)ssRNA viruses (*Caliciviridae* family) (Fig. 1h). Virions of dsRNA viruses in general have enzymatic activities for RNA synthesis. (+)ssRNA viruses induce membrane remodeling and form spherule encasing RNA-replication complexes[25,26]. For hypoviruses, representative capsidless (+)ssRNA fungal viruses, infection-specifically augmented and Golgi-derived vesicles are probably used as the site for replication[27]. Taken together, an emerging picture for the interplays is that YnV1 is a full-fledged dsRNA virus, while YkV1 is a capsidless (+)ssRNA virus that highjacks YnV1 capsids for its replication (Fig. 3). Trans-encapsidated dsRNA of YkV1 can be regarded as a replicative form as in the case of dsRNAs detectable in fungal hosts infected by other capsidless (+)ssRNA viruses.

YkV1 is decisively different from precedent viruses or virus-like molecules. There are several types of satellite viruses and molecules. Neither satellite RNAs nor satellite RNA viruses encode RdRps[1]. YkV1 encodes its own RdRp probably responsible for its genome replication (negative-strand RNA synthesis) and transcription (positive-strand RNA synthesis). Importantly, YkV1 RdRp is incorporated into YnV1 capsids, as clearly shown in (Fig. S3). This feature is distinct from all other RNA satellites that depend for their genome amplification on helper viruses and that may or may not encode their own CP. Importantly, the genome type of a helper virus and a satellite virus always belong to the same category classified by Baltimore[28]. This is not the case for the YkV1 and YnV1 relationship. Plant (+)ssRNA umbraviruses are unique in that they depend for encapsidation and aphid vector transmission on co-infecting (+)ssRNA luteoviruses[12]. Umbraviruses are similar in trans-encapsidation to YkV1;

nevertheless, they replicate independently of the co-infecting viruses. Furthermore, YkV1 is also distinguishable from capsidless (+)ssRNA viruses. Many capsidless viruses appear to use host-derived membranous vesicles, not capsid of another virus, as replication sites.

Among capsidless fungal viruses, narnaviruses[14,29] are similar to YkV1 in their one-ORF genome organisation. Recently, a few probably capsidless viruses[30] were reported to contain one ORF genome, with close relatedness to those of encapsidated, bi-segmented dsRNA partitiviruses. Capsidless mycoviruses may have evolved from encapsidated parental viruses by loss of capsid genes[3], although the opposite direction of evolution could not be completely eliminated. YkV1 is most closely related to caliciviruses, while YnV1 is phylogenetically related to dsRNA totiviruses. Note that a mycovirus AfSV2 closely related to YkV1 was isolated from an ascomycete co-infected with a totivirus[24]. Caliciviruses are animal viruses able to form virions but have not yet been reported from fungi or plants thus far. An interesting speculation is that YkV1 might have been derived from an extinct or an as-yet-unrecognised calici-like virus infecting fungi or plants. Given the fact that not many such viruses have been reported, YkV1 may evolve into an independent virus that uses host-derived compartments as replication sites or into a full-fledged dsRNA virus, in a similar way as encapsidatred plant ourmiaviruses likely evolved from a capsidless narnavirus[3,31]. In this regard, Koonin and collaborators[32] noted a phylogenetic affinity between the families *Caliciviridae* ((+)ssRNA virus) and *Totiviridae* (dsRNA viruses), as both are members of the expanded picorna-like superfamily, and proposed their common ancestor. This notion is supported by a paralleled replication strategy between them[25].

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgments

The authors are grateful to Yomogi Inc. (to NS) and the Science and Technology Research Promotion Program for Agriculture, Forestry, Fisheries and Food Industry (25032AB) (to SK and NS) for financial support during this study. We thank Drs. Ralf Dietzgen, Kiwamu Hyodo, and Ida Bagus Andika for fruitful discussion and critical reading of the manuscript. The authors are also grateful to Ms. Tsugumi Shiokawa and Dr. Hiroko Tada at Division of Instrumental Analysis, Okayama University for the amino acid sequence analyses.

Author Contributions

S.K. and N.S. designed research; R.Z., S.H., A.T., and H.K. performed the experiments; S.K. contributed fungal materials; H.K. A.T., and N.S. analysed data; N.S. wrote the paper. All authors discussed the results and commented on the manuscript.

Author Information

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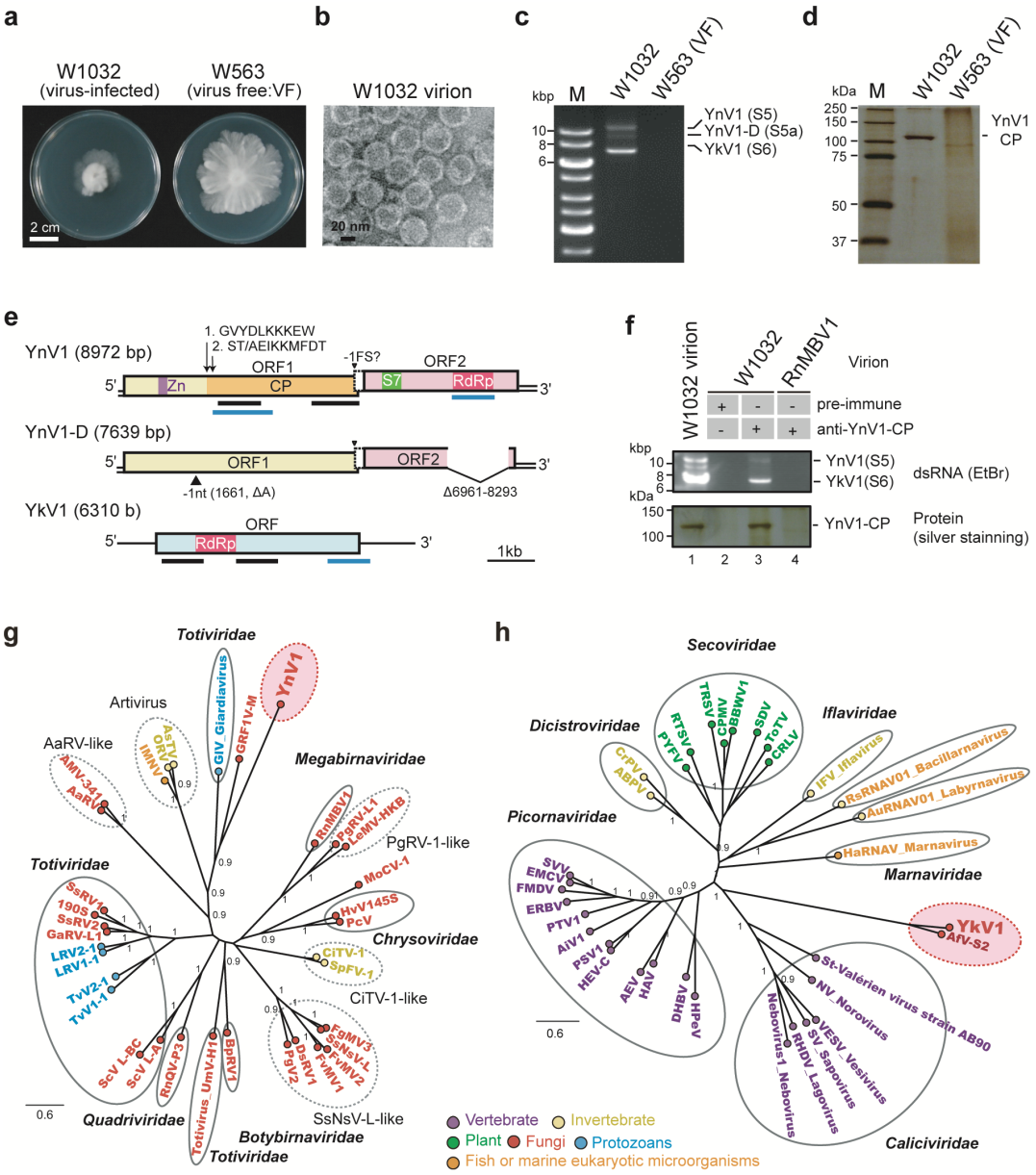

Figure 1. Characterization of yado-nushi virus 1 (YnV1) and yado-kari virus 1 (YkV1) infecting *R. necatrix* strain W1032 and composition of virus particles isolated from it. **(a)** Colony morphologies of virus-carrying field strain W1032 and virus-free isogenic strain W563 of *R. necatrix*. **(b)** Electron micrograph of virus particles negatively stained. **(c)** Double-stranded RNA agarose gel profile of virus particle fractions. **(d)** Protein components of W1032 particles analysed. **(e)** Diagrams of genetic organisation of YnV1 and YkV1. RdRp, zinc-finger (Zn), Phytoreo S7 (S7) sequence motifs are highlighted (Fig. S1). The arrows indicate positions of the amino-terminal of the 120-kDa CP. Black lines show portions expressed in *E. coli* for antigen preparation. Blue bars denote portions used as Northern probes (Fig. S2). **(f)** Immuno-precipitation of virus particles. Virion fractions from W1032 (YnV1 + YkV1) (lanes 1 to 3) or W779 (RnMBV1) (lane 4) were immune-precipitated with anti-YnV1 CP antibodies (lanes 3 and 4) or pre-immune serum (lane 2). **(g, h)** RdRp-based maximum likelihood phylogenetic trees of YnV1, YkV1 and their related viruses. Different host groups are differentiated by circles filled with different colours. The branch support values greater than 0.9 are shown. Representative pictures or gel images out of at least three biological replicates were shown in Fig. 1a–d, and f, and the subsequent figure Fig. 2a, b, e, and f.

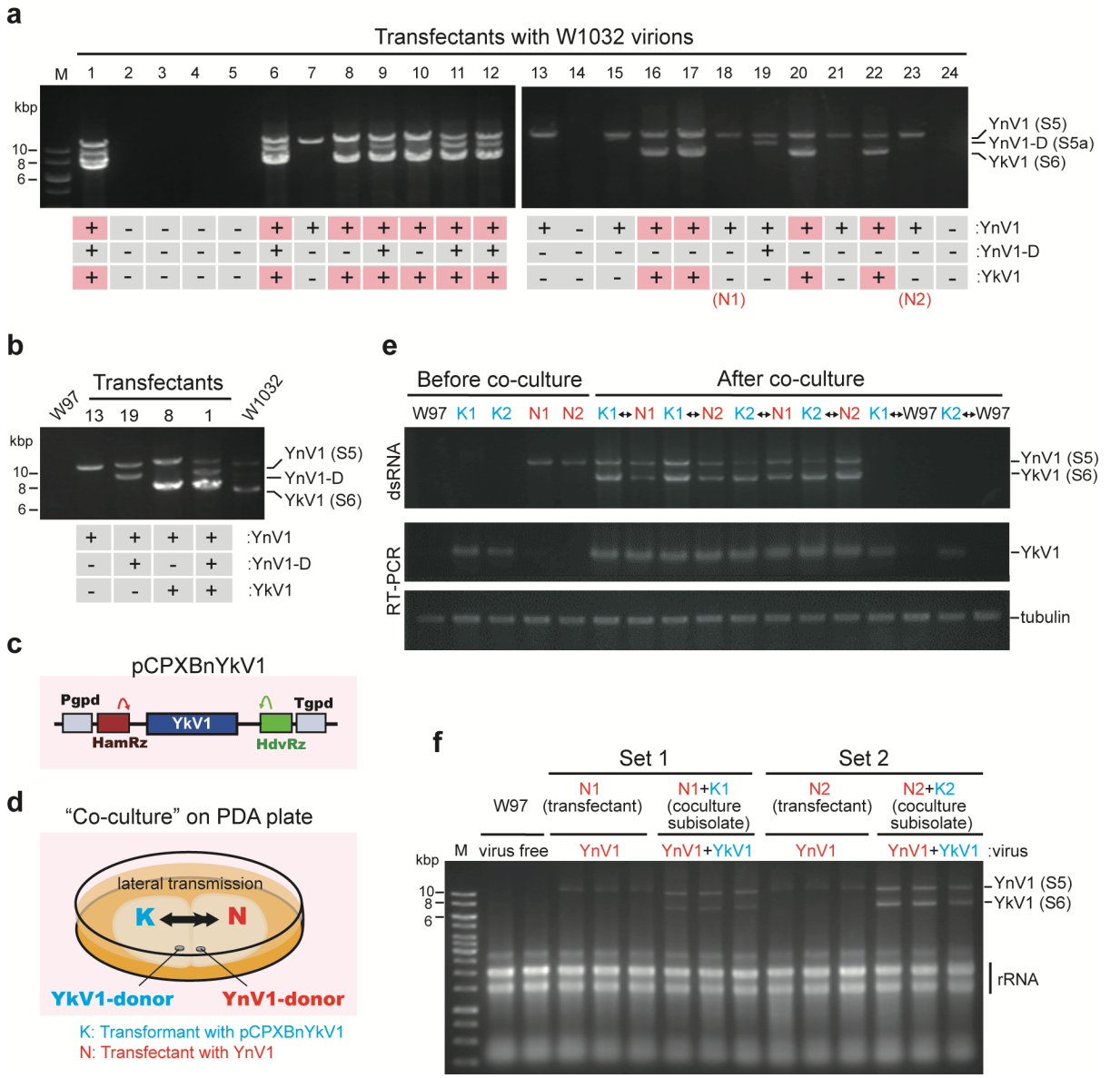

Figure 2. Interaction between yado-nushi virus 1 (YnV1) and yado-kari virus 1 (YkV1). **(a)** Agarose gel analysis of transfectants derived from the fungal strain W97. **(b)** DsRNA patterns of four representative transfectants. **(c)** Diagram of an infectious cDNA clone of YkV1 (pCPXBnYkV1). **(d)** Illustration of co-culture experiments. Transformants of W97 with pCPXBnYkV1 were co-cultured with YnV1-infected W97 strains to allow lateral virus transmission. **(e)** Agarose gel analysis of viral dsRNAs and RT-PCR fragments. Single-stranded and dsRNA fractions were prepared from original transformant and transfectant strains, and sub-isolates derived from co-cultures (positions marked with "K" and "N"). N1 and N2 refer to two independent YnV1-transfectants while K1 and K2 denote two independent transformants with pCPXBnYkV1. **(f)** Comparison of YnV1 RNA accumulation in the presence and absence of YkV1. Total RNA fractions were obtained from two sets of W97-derived strains infected singly (YnV1) and doubly (YnV1+YkV1).

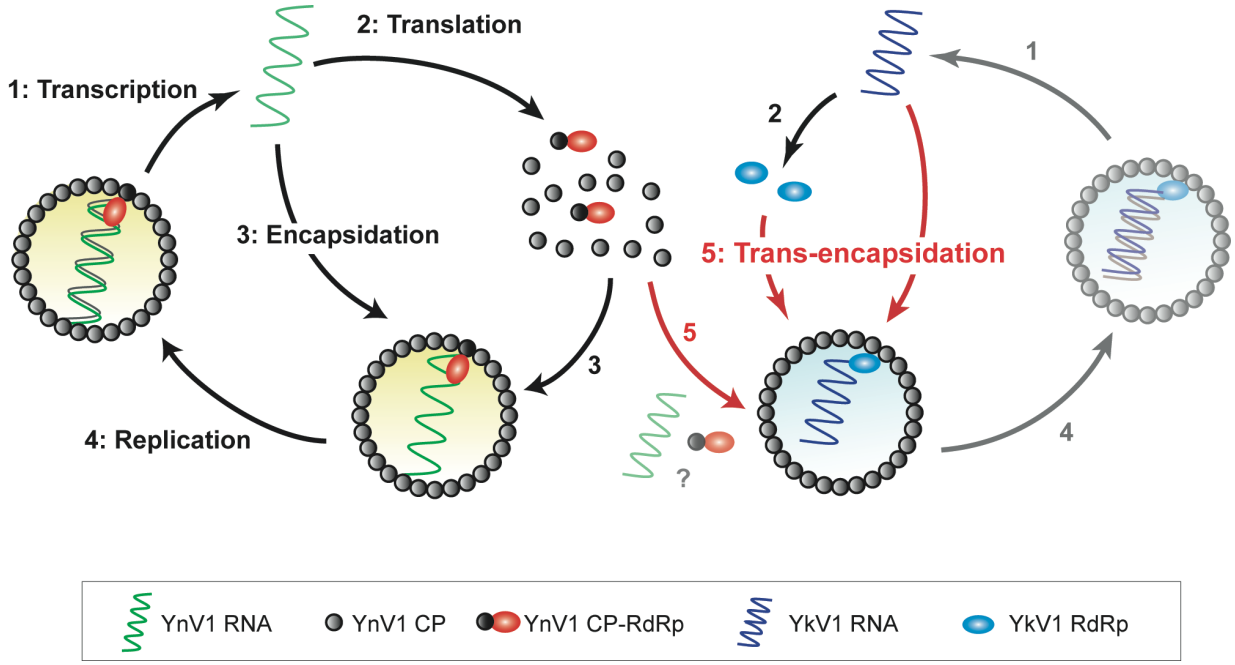

Figure 3. Proposed model for the interactions between yado-nushi virus 1 (YnV1) and yado-kari virus 1 (YkV1). YnV1 can complete its intracellular replication cycle without YkV1, as in the case for totiviruses[29]. YkV1 hijacks YnV1 CP for encapsidation and replicates as if it were a dsRNA virus.

Supplemental Information

A capsidless ssRNA virus hosted by an unrelated dsRNA virus

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METHODS

Fungal strains and culturing.

R. neactrix strain W1032 was described earlier by Yaegashi et al.[17], while *R. necatrix* standard strain W97 was reported by Kanematsu et al.[33]. Rosellinia necatri*x* megabirnavirus 1 (RnMBV1) with a bi-segmented dsRNA genome, which is harbored by W779, was used a reference virus [34]. These fungal strains are mycelially incompatible and incite incompatible programmed cell death upon when co-cultured. All *R. necatrix* strains were cultured on at 24°C on Difco potato dextrose agar (PDA; Becton Dickinson) or in Difco potato dextrose broth (PDB) in the dark.

All data were taken from more than three biological replicates.

Virion purification.

Virus particle fractions were obtained by the method of Chiba et al.[34]. that entailed homogenization of harvested mycelia, clarification with 20% (v/v) CCl_4, concentration with sodium chloride and polyethylene glycol (PEG) 6000, differential centrifugation, and sucrose gradient (10–40%) or cesium sulfate gradient (20–50%) centrifugation. A virus-containing zone located at a sucrose concentration of approximately 25–35% or cesium sulfate concentration of approximately 40% was further centrifuged to precipitate the particles.

Transformation and transfection.

Spheroplasts were prepared from virus-free *R. necatrix* W97 freshly grown in PDB as described by Kanematsu et al.[33]. For transfection, purified virions were introduced into spheroplasts in the presence PEG, as described by Hillman et al.[35] and Sasaki et al.[36]. For transformation, plasmid DNA was used to replace virions in the transfection procedure[33]. After the regeneration step, PDA media with 0.6 μg/ml benomyl was overlayed on regeneration media for selection.

RNA extraction and analyses.

The method of Eusebio-Cope and Suzuki[37] was applied for preparations of total RNA. DsRNA was further purified using CC41 cellulose. DsRNAs were analysed in agarose gel electrophoresis in a TAE buffer system. Northern blotting analysis of purified dsRNA was done using a digoxigenin detection kit as described by Lin et al.[38].

Sequencing of YnV1 and YkV1 RNAs and construction of a full-length cDNA clone of YkV1.

A cDNA library was prepared by a non-PCR-based method as described by Lin et al.[18] which entailed reverse transcription of a mixture of YnV1 and YkV1 RNAs. Approximately 70 plasmid clones carrying

cDNA inserts of 1.5 to 3.0 kb were sequenced from both directions. Terminal sequences were determined by RNA ligase mediated PCR (RLM-PCR) method[18].

The full-length cDNA of the YkV1 was synthesized by GENEWIZ, Inc. (South Plainfield, NJ 07080). The cDNA was flanked by two ribozymes, hammerhead (HamRz) and hepatitis delta virus ribozymes (HdvRz) to produce authentic viral transcript in transformed cells as shown in Fig. 2c. This cassette was cloned between the *Cryphonectria parasitica* glyceraldehyde-3-phosphate dehydrogenase promoter (Pgpd) and terminator of an expression vector, pCPXBn2[39] to make pCPXBnYkV1.

Amino acid sequence analyses of major capsid protein (CP).

Amino acid (aa) sequences of the major capsid protein of W1032 was analysed by two methods. The amino-terminal sequence of the 110-kDa CP blotted onto polyvinylidene difluoride (PVDF) membrane was determined on a gas-phase protein sequencer Shimadzu Model PPSQ-31A at the Department of Instrumental Analysis and Cryogenics, Advanced Science Research Centre, Okayama University.

Peptide mass fingerprinting (PMF) of the major CP of 110 kDa, was carried out by the method of Charoenpanich et al.[40]. Peptide masses were determined by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) and MALDI-TOF tandem mass spectrometry (MS/MS) on a UltraFLEX mass spectrometer (BRUKER Daltonics). Peptide masses were mapped to those deduced from sequences of YnV1- and YkV1 ORFs using the MASCOT program (Matrix Science).

Antisera preparation and immunoprecipitation.

YnV1 ORF1 truncated spanning aa 641-961 and carboxyl-termini 1361–1569 (Fig. 1e) were fused in frame maltose binding proteins (MBP), and expressed in *Escherichia coli* strain BL21 and BL21-CodonPlus using pMal-c2x (New England Biolabs) vector. YkV1 ORF truncated aa 61–329 and aa 584–863 were fused in frame MBP and glutathione S-transferase, and expressed in BL21 using pMal-c2x and pGEX-6P-1 (Promega). Purified recombinant fusion proteins, in native or denatured form, were used to immunize New Zealand white rabbits five times[41].

Purified virion fractions (OD_{260}=40, 200 □l), rProteinA-sepharose beads (GE Healthcare) were incubated with anti-YnV1-CP antibodies in H buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA) at 4°C for overnight. Immunocomplexes, precipitated by centrifugation at 500 g for 3 min, were washed in H buffer 4–5 times and used for dsRNA extraction by an SDS/phenol method.

Database search and phylogenetic analysis.

Blastp searches were conducted against sequence databases available from the National Centre for

Biotechnology Information using protein sequences deduced from two YnV1 ORFs and one YkV1 as queries. Similar sequences with E-values of <0.01 were compiled in Tables S1 and S2.

The two viruses were phylogenetically analysed based on their RdRp sequences using a standard maximum-likelihood method as described previously with minor modification[42].

34 Chiba, S. *et al.* A novel bipartite double-stranded RNA mycovirus from the white root rot fungus *Rosellinia necatrix*: molecular and biological characterization, taxonomic considerations, and potential for biological control. *J Virol* **83**, 12801-12812, doi:JVI.01830-09 [pii]10.1128/JVI.01830-09 (2009).

35 Hillman, B. I., Supyani, S., Kondo, H. & Suzuki, N. A reovirus of the fungus *Cryphonectria parasitica* that is infectious as particles and related to the *Coltivirus* genus of animal pathogens. *J Virol* **78**, 892-898 (2004).

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38 Lin, Y. H., Hisano, S., Yaegashi, H., Kanematsu, S. & Suzuki, N. A second quadrivirus strain from the phytopathogenic filamentous fungus *Rosellinia necatrix*. *Arch Virol* **158**, 1093-1098, doi:10.1007/s00705-012-1580-8 (2013).

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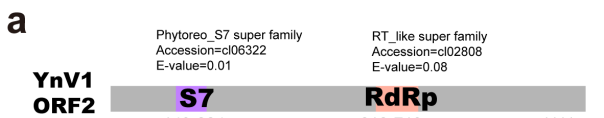
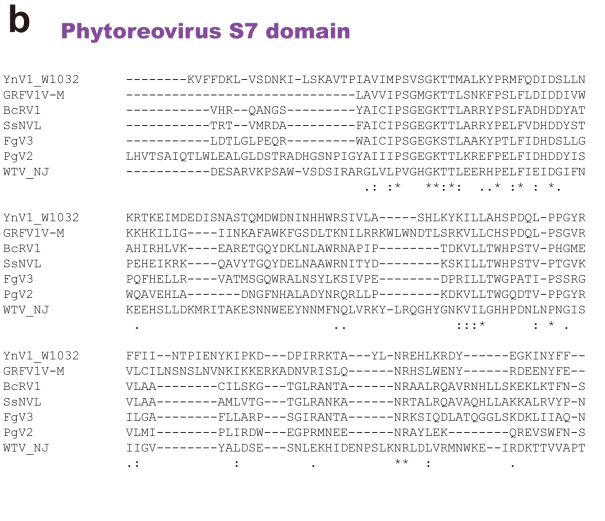
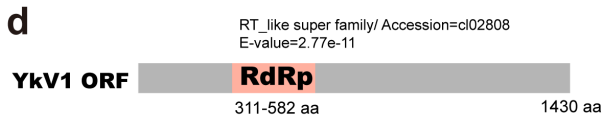
42 Kondo, H., Chiba, S., Sasaki, A., Kanematsu, S. & Suzuki, N. Evidence for negative-strand RNA Virus infection in fungi *Virology* **435**, 201-209 (2013).

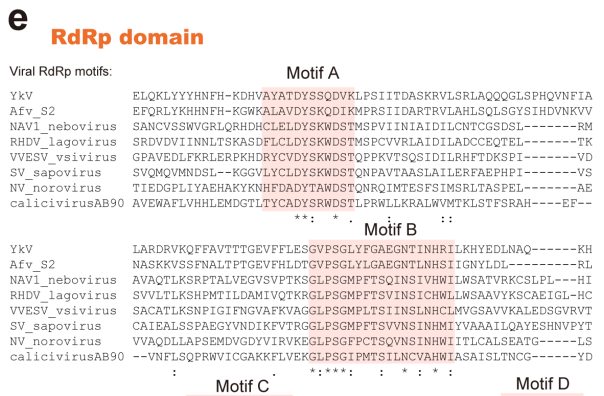
Supplementary Figures

Fig. S1









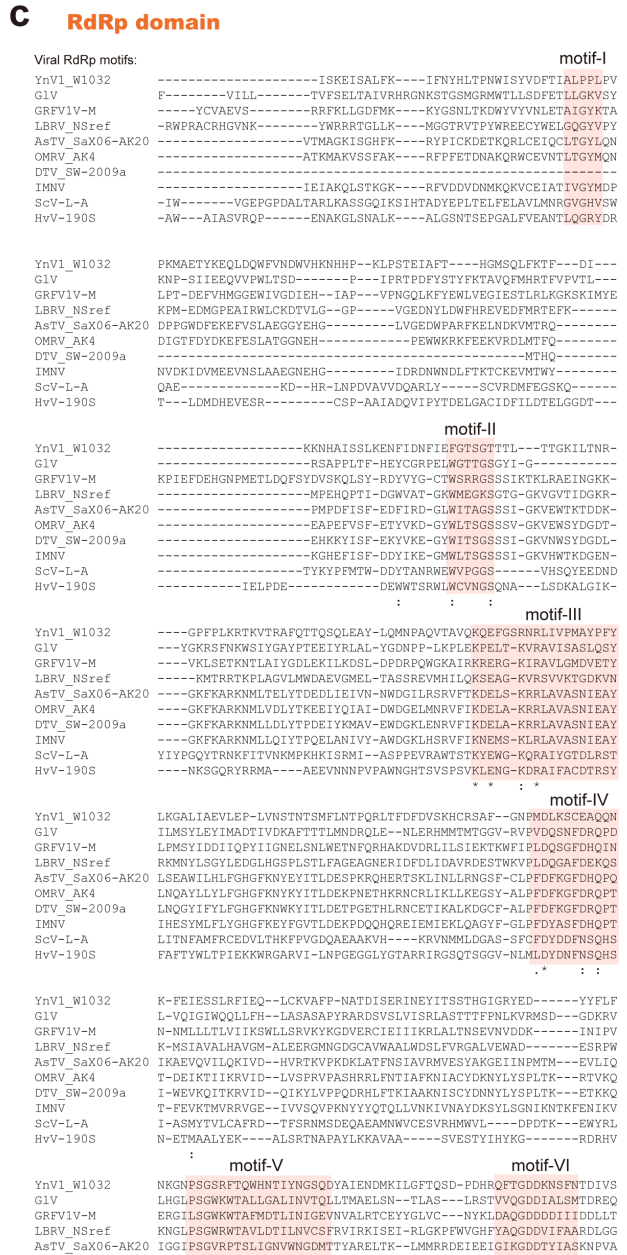


Fig. S1. Predicted conserved domains in RNA-dependent RNA polymerases (RdRp) of yado-nushi virus 1 (YnV1) (ORF2) and yado-kari virus 1 (YkV1) (ORF). **(a, d)** YnV1 ORF2 and YkV1 ORF proteins are predicted conserved core RdRp and Phytoreo_S7 domains with the CD-Search Tool at NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). **(b, c, e)** Multiple alignments of the amino acid sequences of the Phytoreo_S7 (b) or core RdRp (c and e) motifs of YnV1 and YkV1 with related viruses (selected). The alignment was made using MAFFT version 7 (http://mafft.cbrc.jp/alignment/server/). The asterisks indicate identical amino acid residues, the dots indicate low chemically similar amino acid residues. Conserved RdRp motifs are shaded in red.

Fig. S2

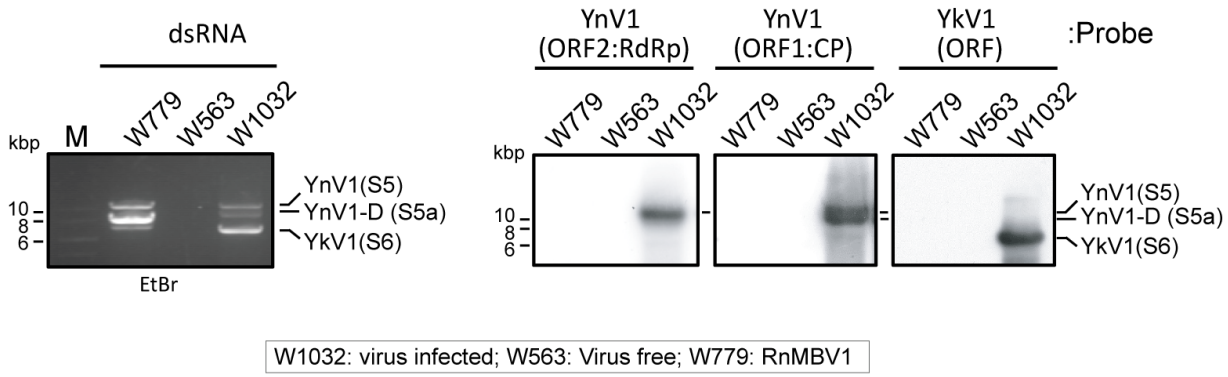


Fig. S2. Northern blotting of yado-nushi virus 1 (YnV1) and yado-kari virus 1 (YkV1) RNAs. DsRNA fraction (3 μg/lane), obtained from W1032 and virus-free W563, was electrophoresed under denaturing conditions and analysed in northern blotting. Rosellinia necatrix megabirnavirus 1 (RnMBV1, W779 strain) genomic dsRNAs were also analysed. Three digoxigenin-labeled DNA probes specific for YnV1 ORF1 and ORF2, and YkV1 ORF probes Yn1a, Yn1b, and YnK1 were used. The corresponding positions of these probes are shown diagrammatically in Fig. 1e. Representative gel or blot images out of at least three biological replicates are shown in this and the subsequent figure. M refer to 1kbp DNA size standards (Thermo Scientific).

Fig. S3

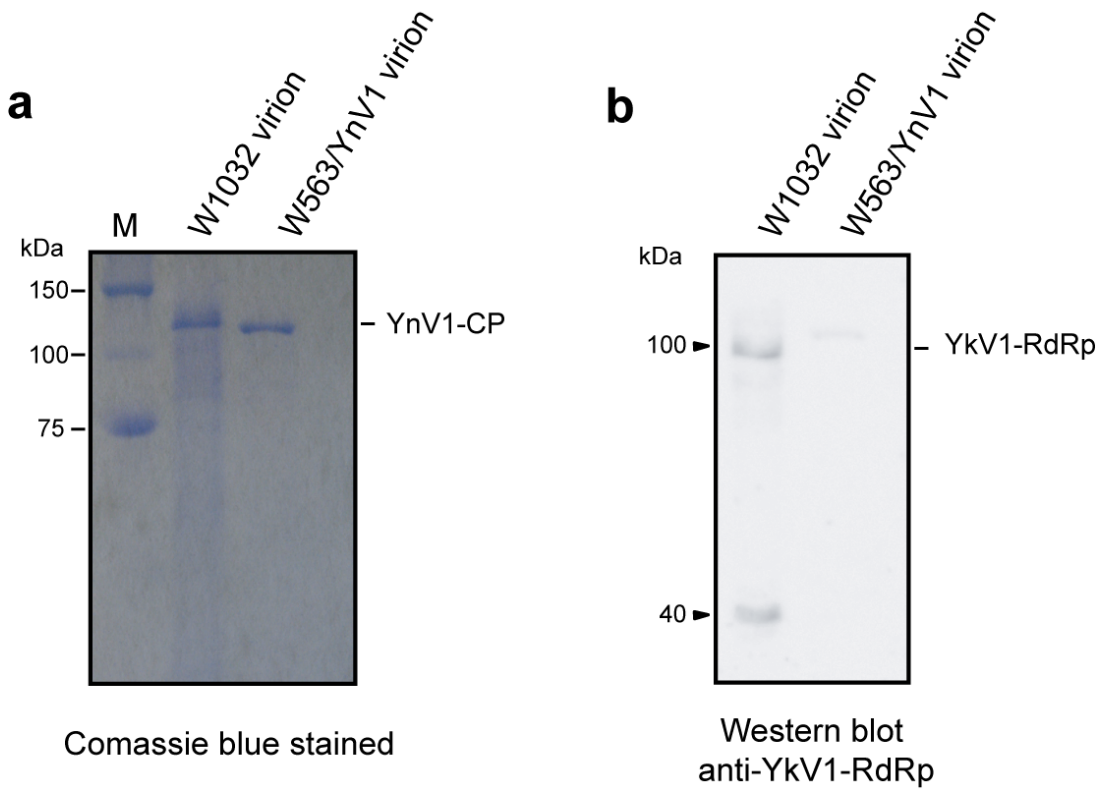


Fig. S3. Western blotting of yado-kari virus 1 (YkV1) RNA-dependent RNA polymerase (RdRp). An equal amount of purified virion fraction (OD_{260}=40, 10 µl/lane) from fungal strain W1032 and a W563 transfectant infected by **yado-nushi virus 1 (**YnV1) alone were analysed by SDS-PAGE **(a)** and western blotting **(b)**. An antiserum was used that was prepared against a mixture of the N- and C-terminal portions of YkV1 RdRp expressed in *Escherichia coli* (see Fig. 1e for expressed portions). M refer to prestained protein standards (Bio-Rad).

Fig. S4

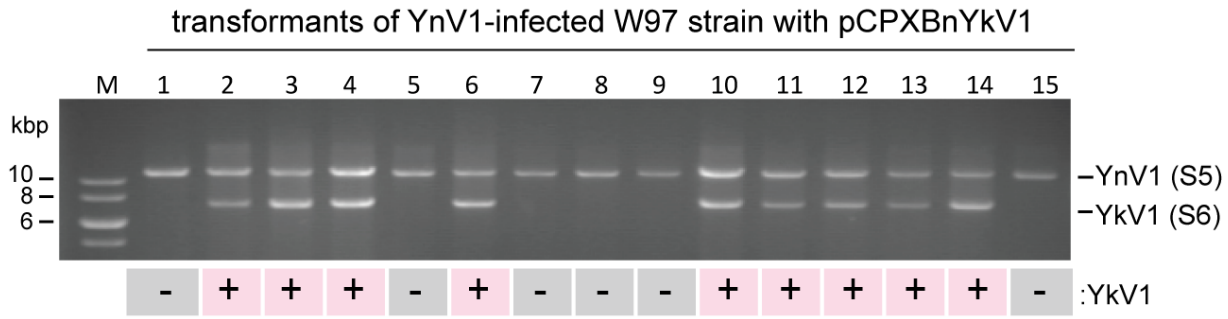


Fig. S4. DsRNA profiles of transformants of yado-kari virus 1 (YnV1)-infected fungal strain W97 with an infectious cDNA clone of yado-nushi virus 1 (YkV1) (pCPXBnYkV1). DsRNA fractions were obtained from a total of 15 independent transformants with pCPXBnYkV1 (see Fig. 2c), and electrophoresed in 1.2% agarose gel (lanes 1–15).

Table S1. Summary of protein blast search with YnV1-ORF2 (1111 aa)

Viruses	Identity	Overlap	Bit score	E value	Accession no.
RdRp domain					
Drosophila melanogaster totivirus SW-2009a	22%	68/305	49.3	0.020	YP_003289293
Leptopilina boulardi RNA virus	21%	103/484	47.4	0.084	YP_009072448
Penaeid shrimp infectious myonecrosis virus	22%	58/259	42.7	3.0	AIC34744
Armigeres subalbatus virus SaX06-AK20	23%	54/235	41.6	4.5	YP_003934934
Phytoreo_S7 domain					
Glomus sp. RF1 medium virus	33%	46/139	59.7	$1e^{-05}$	BAJ23142
Wound tumor virus (strain NJ)	27%	25/92	50.1	0.011	P31610
Botrytis cinerea RNA virus 1	27%	36/135	46.2	0.23	YP_009115498
Phlebiopsis gigantea mycovirus dsRNA 2	27%	37/135	43.9	1.2	CAJ34335
Sclerotinia sclerotiorum dsRNA mycovirus-L	34%	29/85	43.1	1.7	YP_006331065
Fusarium virguliforme dsRNA mycovirus 1	30%	27/89	40.8	8.7	AEZ54148

Table S2. Summary of protein blast search with YkV1-ORF (1430 aa)

Viruses	Identity	Overlap	Bit score	E value	Accession no.
Aspergillus foetidus slow virus 2	51%	337/659	616	0.0	CCD33025
Sapovirus SaKaeo-15/Thailand	26%	62/242	50.8	0.011	AAV69574
Sapovirus Hu/GII/Hokkaido/Nay1/2005/JPN	28%	51/181	50.1	0.012	ABO20832
Norovirus isolates	31%	40/130	47.4	0.013	AAB81329
California sea lion sapovirus 2	24%	72/295	47.4	0.11	AEM37580
Calicivirus pig/AB90/CAN	26%	55/214	47.8	0.11	YP_002905325

Table S3. Peptide mass fingerprinting analysis of the 120-kDa capsid protein of YnV1.

Amino acid position	Observed	Mr(expt)	Mr(calc)	Delta	Peptide
906-909	505.29	504.29	504.27	0.01	IMNK
349-353	561.31	560.31	560.29	0.02	LGESR
1478-1481	566.34	565.34	565.25	0.09	DFER
831-835	587.26	586.25	586.38	-0.13	VLSLR
430-434	603.34	602.33	602.36	-0.03	EILTK
323-328	706.38	705.38	705.32	0.05	NGLSCR
724-729	806.61	805.60	805.38	0.23	ASYFYR
1163-1170	962.82	961.81	961.56	0.25	YPITLLSR
1017-1024	980.75	979.74	979.48	0.26	SFFDSLHK
1025-1033	1015.77	1014.76	1014.48	0.28	LSSGYADFR
1557-1564	1029.79	1028.78	1028.54	0.24	YFISLCVK
1501-1508	1046.83	1045.82	1045.53	0.29	RPEDNFLR
1609-1616	1075.79	1074.78	1074.51	0.27	EEFWSHLK
108-117	1103.89	1102.88	1102.55	0.33	GESNLDDLIK
78-88	1202.95	1201.94	1201.72	0.22	VLAPQTPLVHK
1647-1657	1221.85	1220.84	1220.60	0.24	VKPSFSNLSEN
910-919	1260.00	1258.99	1258.67	0.32	LYPLFNHDIK
1388-1398	1264.93	1263.92	1263.59	0.33	DTFFPPGNDVR
963-973	1357.10	1356.10	1355.77	0.33	ENFLLQAIRPR
1060-1071	1361.05	1360.04	1359.70	0.34	ATVPLNENDFLK
1195-1206	1485.06	1484.05	1483.68	0.37	EHLTYSHDNVNR
642-654	1587.12	1586.11	1585.76	0.36	MFDTQTNLYNAIR
702-716	1651.29	1650.28	1649.87	0.41	QISSSISHLIPDTPR
1148-1162	1725.19	1724.19	1723.73	0.46	SNFTYDDGTVASEYR
1115-1129	1780.28	1779.28	1778.87	0.41	DTYMPVASLINQYHK
1565-1581	1840.41	1839.40	1838.94	0.47	LGTLQTFNDITGTTTTR
1131-1147	1906.50	1905.49	1904.99	0.50	NQLLCVDIVPHQGGSIR
1517-1534	2076.47	2075.47	2074.98	0.49	FDFSGFSTVNENNMVVLR
873-891	2132.45	2131.44	2130.96	0.48	HHAMTSSMSIGAFNQLEDR
1041-1059	2166.60	2165.59	2165.10	0.49	AINLPFTVVLSEDFDQTTR
740-758	2192.64	2191.63	2191.06	0.57	NQSVPYVPVYEHDPVASYK
1584-1603	2317.83	2316.82	2316.21	0.61	TPTYPTHLLAVNVITQTEYR
1617-1642	2918.23	2917.22	2916.47	0.75	SSIVITTPADVSIEQEQIFDDININR