Complete genome sequence of a novel toti-like virus infecting the phytopathogenic fungus Rhizopus stolonifer

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Abstract

In this study, we report the complete genome sequence of a novel toti-like virus, tentatively named Rhizopus stolonifer toti-like virus 1 (RsTV1), identified from a phytopathogenic fungal agent of apple fruit rot disease, *Rhizopus stolonifer* strain A2-1. RsTV1 had a double-stranded RNA genome. The complete RsTV1 genome was 5178 nucleotides (nt) in length and contained two open reading frames (ORFs) encoding a putative coat protein (CP) and RNA-dependent RNA polymerase (RdRp). Phylogenetic analyses based on the RdRp and CP amino acid sequences revealed that RsTV1 was closely related to viruses of the unclassified totiviruses. In stress-inducing Vogel's minimal and sodium dodecyl sulfate-containing media, the hyphal growth of A2-1 was suppressed, but the accumulation of RsTV1 RNA was increased, indicating that stresses promote RsTV1 replication. To our knowledge, this is the first report of a mycovirus found in *R. stolonifer*.

Full Text

Fungal viruses, also known as mycoviruses, infect nearly all major fungal groups, including phytopathogenic fungi, yeasts, and mushrooms [1]. The field of mycovirology was established in 1962 when Hollings observed three spherical or elongated particles in a cultivated mushroom (*Agaricus bisporus*) under the electron microscope [2]. In recent years, the application of next-generation sequencing technology in metatranscriptomics is rapidly accelerating efforts to identify mycoviruses [3]. Most mycoviruses have double-stranded (ds) or single-stranded (ss) RNA genomes, while identified DNA mycoviruses are few in number [4]. According to the latest report from the International Committee on Taxonomy of Viruses (https://ictv.global/), dsRNA mycoviruses are currently divided into 10 families and one unclassified genus, namely *Alternaviridae*, *Amalgaviridae*, *Chrysoviridae*, *Curvulaviridae*, *Megabirnaviridae*, *Partitiviridae*, *Polymycovirus*, *Quadriviridae*, *Spinareoviridae*, *Totiviridae*, and *Botybirnavirus*.

Although the majority of fungal viruses have no obvious effect on their host fungi, increasing numbers of fungal viruses have been found to significantly alter some biological characteristics of their fungal hosts, such as mycelium growth, pigment accumulation, and virulence [3]. Some mycoviruses are beneficial to their fungal hosts, conferring advantages such as increased growth, enhanced stress tolerance, or hypervirulence [5, 6]. On the other hand, a number of mycoviruses inhibit fungal growth and pathogenicity [7, 8]. Several hypovirulence-inducing mycoviruses identified from phytopathogenic fungi have been developed as biocontrol agents for plant fungal diseases [9, 10].

Rhizopus stolonifer belongs to phylum Zygomycota and is an important fungal pathogen causing post-harvest diseases of many fruits and vegetables [11]. Its spores need to pass through a wound to invade host plants, while its mycelia rapidly colonialize plant tissue [12]. Following *R. stolonifer* infection, fruits usually become soft and watery, rot, and produce a black layer of mildew composed of multiple sporangiospores within a few days [13]. *R. stolonifer*-induced disease is known as black mold, Rhizopus rot, or soft rot and is considered to be one of the most devastating diseases of many horticultural

products during storage [11]. At present, the application of chemical fungicides is the primary method of controlling diseases caused by post-harvest pathogens [14]. However, the use of fungicides during the storage period has led to public concerns about chemical residues in agricultural products, along with environmental pollution and increasing resistance among pathogens [15]. The use of hypovirulence-inducing mycoviruses for plant fungal disease control has attracted many researchers. However, no mycovirus of *R. stolonifer* has been reported thus far.

A total of 18 fungal strains were isolated from the rotten skin tissues of apple fruits obtained from Yangling in Shaanxi Province, China, in 2022. The fungal isolates were cultured on potato dextrose agar (PDA) at 25 ℃. The fungal mycelium was cultured on PDA covered with cellophane at 25 ℃ for 3 days on a benchtop to extract fungal DNA, dsRNA, and total RNA as described previously [16-18]. The purified dsRNA was separated via 1% agarose gel electrophoresis in 1x Tris-Acetate-EDTA (TAE) buffer. The results showed that a specific dsRNA band was present in the dsRNA samples extracted from the above fungal isolates (Fig. 1a and Supplementary Fig. S1). The phenotype of these fungal isolates growing on PDA was identical (Supplementary Fig. S1), and isolate A2-1 was selected for fungal species identification via sequencing of the internal transcribed spacer (ITS) region. The DNA of A2-1 was used as a template, and the ITS fragment was amplified using ITS1 and ITS4 primers (Supplementary Table S1) [19]. Sequence analysis revealed that the fungal isolates were *R. stolonifera.* Thereafter, the *R. stolonifera* strain A2-1 was selected for further study.

To obtain the sequence of the dsRNA, a cDNA library was constructed using the dsRNA of strain A2-1 for next-generation sequencing, performed as described previously [20]. The terminal sequences were obtained by RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) as described by Lian and colleagues [20]. All primers used for obtaining the viral cDNA sequences are listed in Supplementary Table S1. The partial sequence and terminal sequences of the virus were assembled using DNAMAN version 5.0 software to obtain the full-length cDNA sequence of the virus (GenBank Accession No. OR089090).

The complete RNA sequence was 5178 nucleotides (nt) in length, with a GC content of 53.86%, and contained two open reading frames (ORFs), ORF A and ORF B (Fig. 1b). ORF A was 2319 nt long, encoding a putative protein with an estimated molecular weight of 80.51 kDa. ORF B was 2484 nt long, encoding a putative protein with an estimated molecular weight of 91.06 kDa. A BlastP search indicated that the virus was related to viruses belonging to family *Totiviridae*; hence, it was tentatively named Rhizopus stolonifera toti-like virus 1 (RsTV1). The viruses with the highest identity scores and other members of family *Totiviridae* (Supplementary Tables S2 and S3) in the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/) were selected for subsequent analyses.

The members of family *Totiviridae* consist of fungal and protozoan viruses and are classified into five genera, namely *Totivirus*, *Victorivirus*, *Giardiavirus*, *Leishmaniavirus*, and *Trichomonasvirus* [21, 22]. The members of family *Totiviridae* have a dsRNA genome about 4.6–7.0 kb in length, containing two ORFs

(ORF A and ORF B) that encode a capsid/coat protein (CP) and RNA-dependent RNA polymerase (RdRp), respectively [23]. The BlastP analysis showed that the RdRp (ORF B) of RsTV1 was most similar to that of Umbelopsis ramanniana virus 2 (UrV2, Accession No. VFI65724.1), which belongs to unclassified totiviruses, with a query cover of 92%, E-value of 0.0, and identity of 59.56%. The CP (ORF A) of RsTV1 was most similar to that of Mucor hiemalis virus 1 (MhV1, Accession No. CAE9672661.1), a member of unclassified *Totiviridae,* with a query cover of 96%, E-value of 0.0, and identity of 50.72%. These results suggested that RsTV1 is a novel mycovirus related to the members of unclassified totiviruses. It is worth noting that the CP amino acid sequence of UrV2 and the RdRp amino acid sequence of MhV1 have not been reported yet. Therefore, more information about the genome of these viruses was required for further analyses.

Phylogenetic trees were constructed based on the RdRp and CP amino acid sequences using the neighbor-joining (NJ) tree method provided by MEGA version 11 software. 2D graphs of pairwise sequence alignments were constructed based on the RdRp and CP amino acid sequences using SDT version 1.2 software. The RdRp-based phylogenetic tree showed that RsTV1 clustered (with 100% bootstrap support) with UrV2, Thelebolus microspores totivirus 1 (TmTV1), Tolypocladium ophioglossoides totivirus 1 (ToTV1), and Xiang Yun toti-like virus 1 (XtLV1) to form a branch separated from genus *Victorivirus.* Similarly, the CP-based phylogenetic tree indicated that RsTV1 was grouped (with 100% bootstrap support) with MhV1, TmTV1, and ToTV1 to form a branch separated from genus *Victorivirus* (Fig. 1c, 1d). Thus, these analyses suggested that RsTV1 and the members of unclassified totiviruses selected for the phylogenetic analysis might be considered the members of a new genus of family *Totiviridae*. However, the pairwise alignment results of the RdRp and CP amino acid sequences showed that RsTV1 and the members of unclassified totiviruses and members of the genus *Victorivirus* were relatively similar to each other but significantly different from members of other genera (Fig. 2a, 2b). Hence, we suggest that RsTV1 and these unclassified totiviruses are more related to members of the genus *Victorivirus*.

To examine the effects of RsTV1 on *R. stolonifer*, we attempted to obtain a homokaryotic virus-free strain from *R. stolonifer* strain A2-1 via single conidial isolation. The colony of *R. stolonifer* strain A2-1 was cultured on PDA for 7 days on a benchtop at 25 ℃ for sporulation. The conidial spores were liberated in distilled water at appropriate dilutions, and after filtering out hyphae, laid on 10 cm PDA plates for 24 h at 25 ℃. The regenerated fungal progenies from a single spore were transferred to a new PDA plate as single conidial cultures. The presence of RsTV1 in the single conidial cultures was detected by reverse transcription-polymerase chain reaction (RT-PCR) (Supplementary Fig. S3). The results showed that all 30 fungal isolates tested carried RsTV1, suggesting that RsTV1 has a high vertical transmission rate and is relatively stable in the host fungus. Since we failed to obtain a virus-free strain from *R. stolonifer* strain A2-1, the effects of RsTV1 infection on *R. stolonifer* need to be to further investigated in future studies.

Fungi are highly adaptable organisms that can grow under a wide range of environmental conditions. Stresses induced by nutrient deficiencies or osmotic, oxidative, and cell wall stressor agents can significantly suppress fungal growth. Moreover, stress conditions can influence the interactions between

mycoviruses and fungi [24]. Under normal conditions, certain mycoviruses may have minimal effects on fungal growth. However, stress conditions can trigger changes in viral replication, gene expression, or virus-host interactions.

To examine whether culturing the fungal host under conditions of stress would affect RsTV1 replication, *R. stolonifer* strain A2-1 was grown on four different conditions/stressor agents, including minimal medium (Vogel's Medium N) and PDA plates with sorbitol (1 M), sodium dodecyl sulfate (SDS, 0.005% w/v) or CongO Red (0.07% w/v). Compared to the growth on PDA, the minimal medium (Vogel's Medium N) and SDS-containing media largely suppressed hyphal growth, while sorbitol- and CongO Red-containing media slightly affected hyphal growth (Fig. 3a, 3b). The total RNA was extracted from fungal mycelia grown under stress conditions and used as a template for the semi-quantitative RT-PCR detection of RsTV1 (Fig. 3c). The results showed that RsTV1 replication was markedly increased in the fungal hosts grown on Vogel and SDS-containing media compared to those grown on PDA and the other stress-inducing media (Fig. 3c). These results indicate that N-nutrient deficiency (Vogel's) and oxidative membrane stress induced by SDS [25] alter the interactions between RsTV1 and *R. stolonifer* and promote virus replication.

In this study, we characterized RsTV1, a novel toti-like virus related to members of unclassified totiviruses. This is the first report of a mycovirus discovered in *R. stolonifer*. As a common fungus that spreads globally, *R. stolonifer* is highly adaptable and grows rapidly. We showed that stress conditions influence *R. stolonifer* growth and alter the dynamics of RsTV1-fungal host interactions. Nutrient limitations and membrane stress can enhance virus replication in infected fungal cells. Thus, stress conditions may influence mycovirus prevalence in fungal populations. Likewise, RsTV1 might modulate the physiology of the fungal host in response to environmental stresses. Further research is necessary to fully understand the intricacies of these relationships and their ecological implications.

Declarations

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Compliance with ethical standards:

Conflicts of interest: All authors declare that they have no conflicts of interest.

Ethical approval: This article does not contain any studies on human participants or animals performed by any of the authors.

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Figures

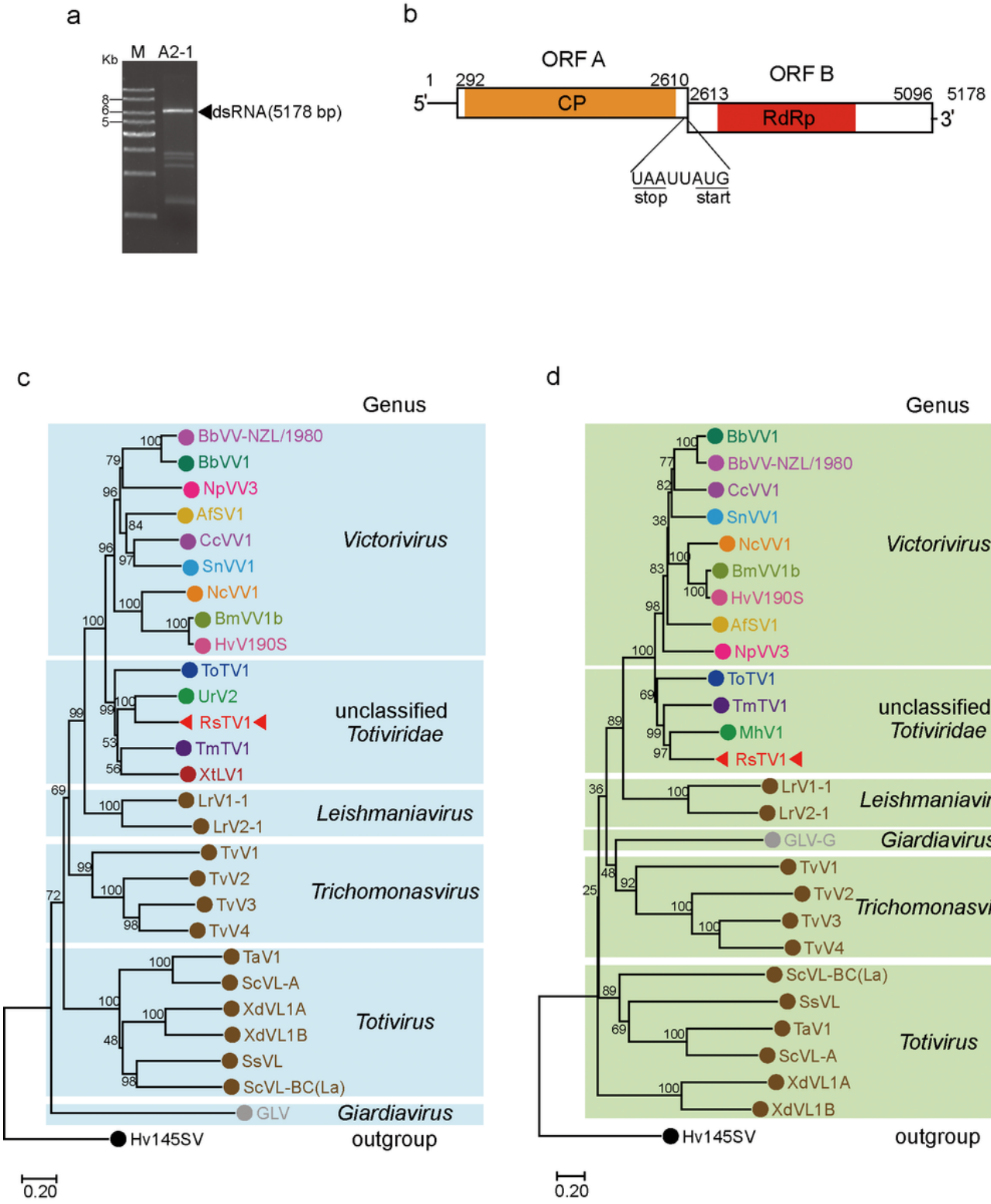

Figure 1

Genome structure and phylogenetic analysis of Rhizopus stolonifertoti-like virus 1 (RsTV1)

(a) Isolation of double-stranded RNA (dsRNA) from *R. stolonifer* strain A2-1. The extracted dsRNA sample was separated on a 1% agarose gel and stained with ethidium bromide. The black arrowhead indicates the dsRNA band corresponding to RsTV1. M, DNA marker; A2-1, the dsRNA sample of *R. stolonifer* strain

A2-1. (b) Genome organization of RsTV1. The positions of two open reading frames (ORFs) are depicted with two black frames. The sequence "UAAUUAUG" between the frames indicates the region from the stop codon of ORF A to the start codon of ORF B. The yellow and red squares in the two frames show the positions of conserved domains of the coat protein (CP) and RNA-dependent RNA polymerase (RdRp), respectively. (c-d) Phylogenetic tree of RdRp and CP using the neighbor-joining tree (NJ tree) method with a Poisson model. The phylogenetic trees were structured using RsTV1 and the members of family *Totiviridae*. Helminthosporium victoriae virus 145S (Hv145SV), a member of family *Chrysoviridae*, was used as an outgroup. The numbers of all branches are bootstrap values (1000 replicates). The positions of RsTV1 are highlighted with red arrowheads. The complete virus names and GenBank Protein_ID are presented in Supplementary Tables S2 and S3.

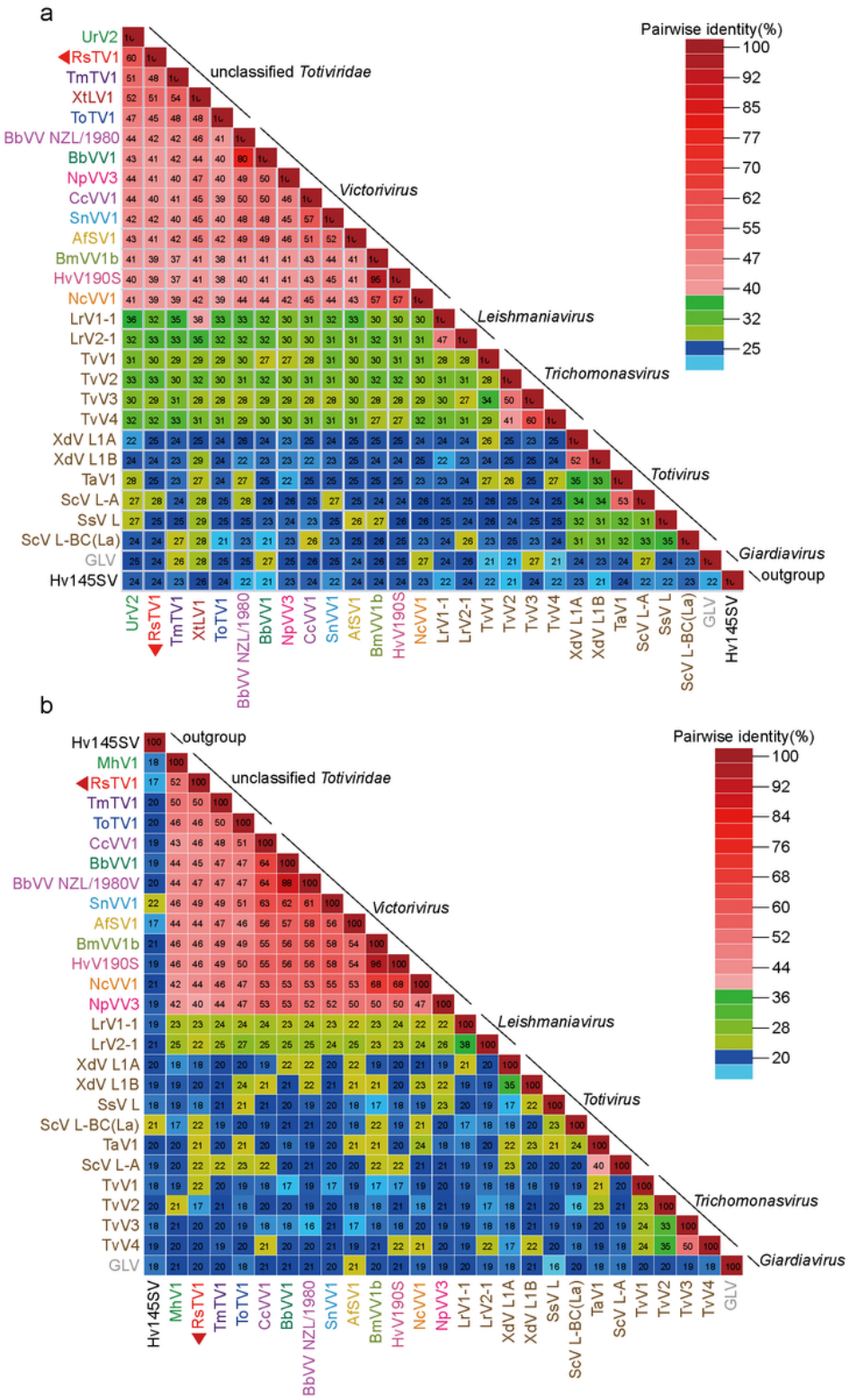

Figure 2

2D graphs of pairwise sequence alignment

(a) Pairwise sequence alignment of RdRp amino acids using SDTv1.2. (b) Pairwise sequence alignment of CP amino acids using SDTv1.2. The 2D graphs were structured using RsTV1 and the members of family *Totiviridae*. Helminthosporium victoriae virus 145S (Hv145SV), a member of family *Chrysoviridae*,

was used as an outgroup. The numbers inside the squares indicate the identity values between pairwise sequences. The positions of RsTV1 are highlighted with red arrowheads. The complete virus names and GenBank Protein_ID are presented in Supplementary Tables S2 and S3.

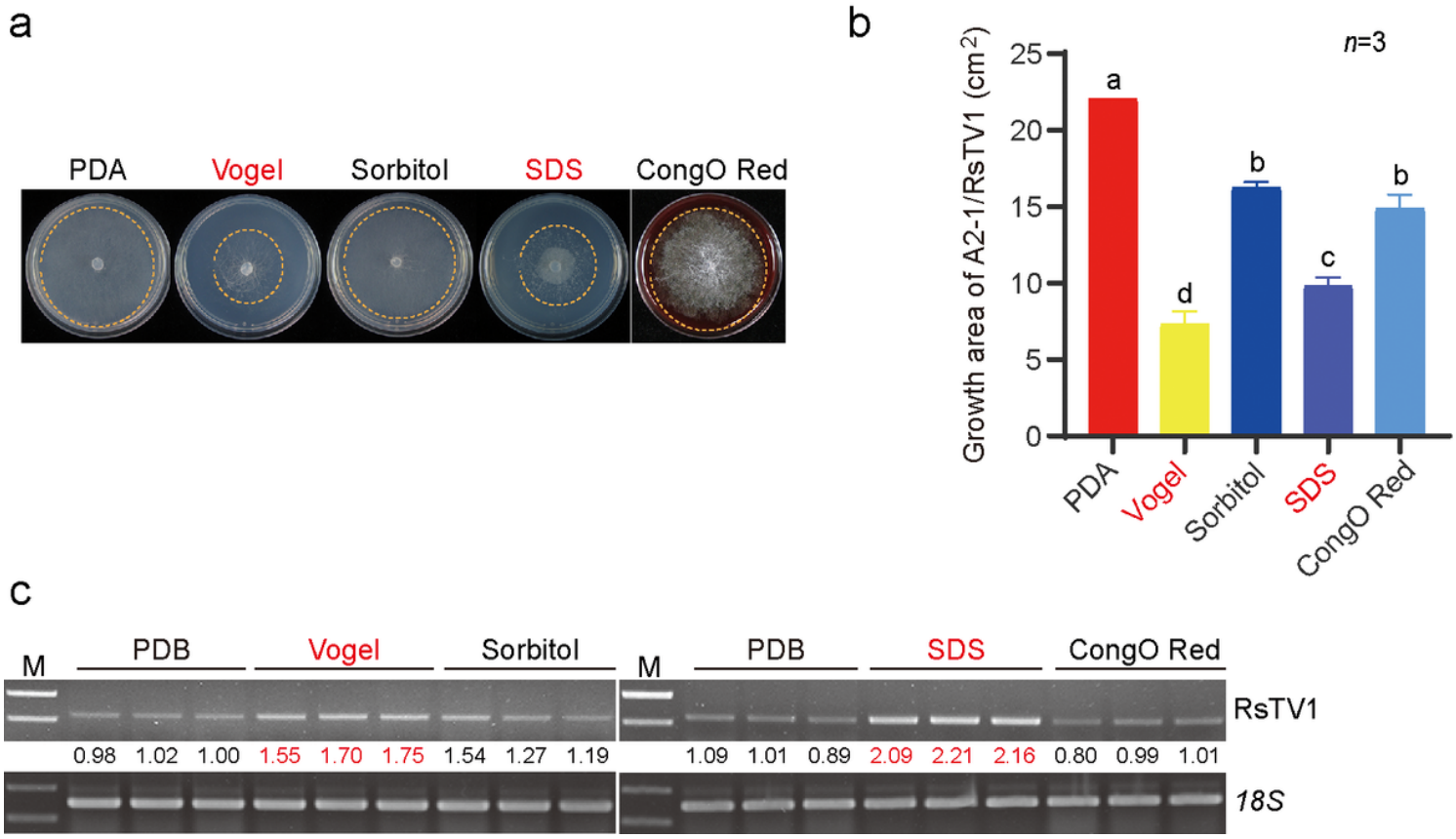

Figure 3

Effects of stresses on RsTV1 accumulation in *R. stolonifer*

(a) *R. stolonifer*A2-1 grown on PDA and stress-inducing media, including Vogel's and sorbitol-, SDS-, or CongO Red-containing media. Colonies were grown for 24 h on a benchtop at 25 ℃ and photographed. (b) The growth area of fungal colonies. Data are mean ± SD (n = 3). Different letters indicate a significant difference at $p < 0.01$ (one-way ANOVA). (c) The accumulation of RsTV1 RNA was examined by semi-quantitative RT-PCR. Total RNA was extracted from mycelia grown on PDA and stress-inducing media for 36 h on a benchtop at 25 ℃, with three independent cultures for each treatment. The RT-PCR detection of RsTV1 was performed using viral genome-specific primer sets (Supplementary Table S1). The *R. stolonifer 18S* gene was used as a reference gene. PCR samples were run on a 1.5% agarose gel and stained with ethidium bromide. M, DNA marker. The relative accumulation of RsTV1 was quantified using ImageJ software. The intensity of the viral PCR band was calculated by normalization using the *R. stolonifer 18S* band.

Supplementary Files

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