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Sequence and phylogenetic analyses of novel totivirus-like double-stranded RNAs from field-collected powdery mildew fungi

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ABSTRACT

The identification of mycoviruses contributes greatly to understanding of the diversity and evolutionary aspects of viruses. Powdery mildew fungi are important and widely studied obligate phytopathogenic agents, but there has been no report on mycoviruses infecting these fungi. In this study, we used a deep sequencing approach to analyze the double-stranded RNA (dsRNA) segments isolated from field-collected samples of powdery mildew fungus-infected red clover plants in Japan. Database searches identified the presence of at least ten totivirus (genus *Totivirus*)-like sequences, termed red clover powdery mildew-associated totiviruses (RPaTVs). The majority of these sequences shared moderate amino acid sequence identity with each other (<44%) and with other known totiviruses (<59%). Nine of these identified sequences (RPaTV1a, 1b and 2–8) resembled the genome of the prototype totivirus, Saccharomyces cerevisiae virus-L-A (ScV-L-A) in that they contained two overlapping open reading frames (ORFs) encoding a putative coat protein (CP) and an RNA dependent RNA polymerase (RdRp), while one sequence (RPaTV9) showed similarity to another totivirus, Ustilago maydis virus H1 (UmV-H1) that encodes a single polyprotein (CP-RdRp fusion). Similar to yeast totiviruses, each ScV-L-A-like RPaTV contains a –1 ribosomal frameshift site downstream of a predicted pseudoknot structure in the overlapping region of these ORFs, suggesting that the RdRp is translated as a CP-RdRp fusion. Moreover, several ScV-L-A-like sequences were also found by searches of the transcriptome shotgun assembly (TSA) libraries from rust fungi, plants and insects. Phylogenetic analyses show that nine ScV-L-A-like RPaTVs along with ScV-L-A-like sequences derived from TSA libraries are clustered with most established members of the genus *Totivirus*, while one RPaTV forms a new distinct clade with UmV-H1, possibly establishing an additional genus in the family. Taken together, our results indicate the presence of diverse, novel totiviruses in the powdery mildew fungus populations infecting red clover plants in the field.

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1. Introduction

Mycoviruses are widespread in all major groups of fungi (Ghabrial et al., 2015; Ghabrial and Suzuki, 2009). Although most mycoviruses have no significant impact on their fungal hosts, some viruses are associated with hypovirulence in plant pathogenic fungi, and thus can be potential biological control agents against fungal diseases (Ghabrial and Suzuki, 2009; Pearson et al., 2009; Kondo et al., 2013b; Xie and Jiang, 2014). The largest number of mycoviruses have double-stranded RNA (dsRNA) genomes, which are encapsidated in rigid virus particles. This dsRNA mycovirus group currently consists of six families (*Totiviridae*, *Partitiviridae*, *Chrysoviridae*, *Reoviridae*, *Megabirnaviridae* and *Quadriviridae*) and a recently proposed family *Botybirnaviridae* (http://talk.ictvonline.org/files/proposals/m/mediagallery/default.aspx). In paticular, members of the families *Totiviridae* (genus *Victorivirus*) and *Partitiviridae* are most frequently found in filamentous fungi.

Powdery mildew fungi (family Erysiphaceae, order Erysiphales) cause disease on a wide range of plant species including crop (e.g., wheat, barley), vegetable (e.g., cucumbers, pea), fruit (e.g., grapes, strawberry) and ornamental (e.g., rose) plants. This fungal family consists of 873 species belonging to 16 genera (Braun

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and Cook, 2012). These filamentous ascomycetous fungi are obligate biotrophs that depend on living host tissue for their nutrition. *Erysiphe* is the largest genus in the Erysiphaceae and constitutes more than 50% of the members of this family (Braun and Cook, 2012; Takamatsu et al., 2015). *Erysiphe pisi* was once believed to be the only causal agent of powdery mildew disease in pea (*Pisum sativum* L.) that led to heavy yield losses. However, recent studies demonstrated that *Erysiphe trifoliorum* (syn. *E. trifolii*), a closely related species to *E. pisi*, is a second causal agent of pea powdery mildew and is able to overcome *er1* and *Er3* resistance genes against *E. pisi* in pea lines (Attanayake et al., 2010; Fondevilla et al., 2013). *E. trifoliorum* has also been reported to infect other legumes, such as clover (*Trifolium* spp.) and lentil plants (*Lens culinaris*) (Attanayake et al., 2009). The recent draft genome sequencing of some powdery mildew species, including *E. pisi* and *E. necator* (pathogenic on grapes), revealed that their genomes (~120 Mb) are more than four times larger than the normal ascomycetes (Douchkov et al., 2014; Wicker et al., 2013; Spanu et al., 2010). These genomes include a massive expansion of transposable elements, and lost a set of carbohydrate-active enzymes, probably reflecting its evolution towards an obligate biotrophic life style (Spanu et al., 2010). Until now, there have been no reports regarding mycoviruses or virus-like agents infecting powdery mildew fungi.

The family *Totiviridae* is currently divided into five genera, consisting of *Totivirus*, *Victorivirus*, *Giardiavirus*, *Trichomonasvirus* and *Leishmaniavirus*. Viruses that infect fungi have been classified to the first two genera, and those that infect protozoa to the remaining three genera (Goodman et al., 2011; Wickner et al., 2011). Some recently identified viruses infecting arthropods and fish are also proposed as members of a tentative genus "*Artivirus*" of the family *Totiviridae* (Dantas et al., 2016; Haugland et al., 2011; Zhai et al., 2010). Members of family *Totiviridae* are characterized by the non-enveloped isometric virions, 30 to 40 nm in diameter (Goodman et al., 2011; Wickner et al., 2011). Although many characterized dsRNA viruses have segmented genomes, members of family *Totiviridae* have a non-segmented dsRNA genome (4.6–6.7 kb) that commonly contains two overlapping open reading frames (ORFs) encoding a capsid protein (CP or gag) and an RNA-dependent RNA polymerase (RdRp or pol) (Wickner et al., 2011).

Members of genus *Totivirus* infect yeast strains (*Saccharomyces cerevisiae*, *Scheffersomyces segobiensis* and *Xanthophyllomyces dendrorhous*) (Baeza et al., 2012; Bruenn, 1993; Taylor et al., 2013), the corn smut fungus (*Ustilago maydis*) and the subterranean fungus (*Tuber aestivum*, the black summer truffle) (Kang et al., 2001; Stielow and Menzel, 2010). Saccharomyces cerevisiae virus-L-A (ScV-L-A), the so called "yeast LA virus" is the type strain of this genus and is one of the best studied mycoviruses at the molecular level (Wickner et al., 2013). The ScV-L-A replicase is expressed as a Gag-Pol-like fusion protein (analogous to retroviral Gag-Pol fusion proteins) through a minus one (−1) ribosomal frameshifting (Dinman et al., 1991), while the Ustilago maydis virus H1 (UmV-H1) encodes a large polyprotein and its RdRp is predicted to be released from the fusion by proteolysis (Kang et al., 2001). ScV-L-A and ScV-L-BC (another yeast totivirus closely related to L-A virus) possess a novel cap-snatching mechanism in which the viral Gag (CP) can remove a 7-methyl-guanosine "cap" from cellular mRNA and transfer it to the 5′ end of *de novo* viral transcripts (Fujimura and Esteban, 2011, 2013). Some strains of ScV-L-A and UmV-H1 are known to have additional small satellite dsRNAs that code for secreted "killer" protein toxins (Bostian et al., 1984; Koltin and Day, 1976).

In this study, we report new ten complete or near-complete totivirus-like genome sequences determined by deep sequencing on dsRNA isolated from field-collected powdery mildew fungus that infected red clover plants (the term "totivirus" refers to putative members of the genus, not the family). Interesting insights into the evolution of members of the genus *Totivirus* was provided by phylogenetic analyses of the newly identified genome sequences along with several other totivirus-related sequences detectable in the transcriptome shotgun assembly (TSA) libraries from some species of fungi, plants and insects. This study represents the first report of mycovirus infection of powdery mildew fungi.

2. Materials and method

2.1. Collection of fungal samples and species determinations

Samples of powdery mildew fungus were obtained from red clover (*Triticum aestivum* L. cv. Hokuseki) plants that grew in an experimental field (approximately 3 m × 3 m area) at the Institute of Plant Science and Resources (IPSR), Okayama University, Japan in the early spring of 2013. Conidia (and conidiophores) of powdery mildew fungus were collected from heavily infected leaves by washing the leaves with sterilized water and the samples were stored at −80 °C.

For species determination, internal transcribed spacer (ITS) sequences were analyzed. Total genomic DNA was extracted from conidia (and conidiophore)-enriched samples using DNeasy® Blood and Tissue Kit (Qiagen) following the manufacturer's instructions, to generate a PCR template. ITS sequences of ribosomal DNA were amplified by PCR using the *Erysiphe*-specific ITS primer pair, EryF and EryR (Attanayake et al., 2009) (Table S1). Amplified DNA fragments were sequenced using an ABI3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

2.2. Nucleic acid extraction and analysis

Total nucleic acid was extracted as described previously (Sun and Suzuki, 2008). After precipitation of total nucleic acids in ethanol, samples were treated with S1 nuclease (Fermentas) and subsequently with RQ1 RNase-free DNase I (Promega) to digest single-stranded RNAs (rRNAs and mRNAs) and DNAs (genomic DNA), respectively. The undigested nucleic acids were further purified by affinity column chromatography using CC41 cellulose powder (Whatman) to obtain dsRNA-enriched fractions as described previously (Eusebio-Cope and Suzuki, 2015).

Reverse transcription (RT)-PCR was performed as described previously (Lin et al., 2012). cDNAs were synthesized with SuperScript II RT (Invitrogen) using random hexamers following the manufacturer's instructions. The resultant cDNA was used as templates for PCR amplification. We took advantage of the viral genomic dsRNA to simultaneously determine both 5′- and 3′-terminal of totivirus-related sequences using 3′-RLM-RACE (Potgieter et al., 2009; Chiba et al., 2009). PCR products were then sequenced using the Sanger sequencing method.

2.3. RNA-seq and reads assembly

The mixture of dsRNA samples (total 3.7 μg) including three independent dsRNA samples derived from other phytopathogenic fungi (sequence data from these fungi will be reported elsewhere) was used for cDNA library construction using the TruSeq RNA Sample Preparation Kit (Illumina). In this method, ds-cDNA is end-repaired and adenylated prior to adaptor ligation, library construction and amplification. The sequenced-ready library was then subjected to paired-end sequencing of 100 nucleotide (nt) reads using Illumina HiSeq 2000 technology (Illumina). The cDNA library construction and deep sequencing analysis were carried out by Hokkaido System Science Co., Ltd.

After deep sequencing, adapter sequences attached to cDNA sequences were removed from sequence reads (61,387,280 raw reads) using cutadapt (https://code.google.com/p/cutadapt/). *De*

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