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Molecular characterization of a novel mycovirus of the family *Tymoviridae* isolated from the plant pathogenic fungus *Fusarium graminearum*

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ABSTRACT

We isolated a novel mycovirus, *Fusarium graminearum* mycotymovirus 1 (FgMTV1/SX64), which is related to members of the family *Tymoviridae*, from the plant pathogenic fungus *F. graminearum* strain SX64. The complete 7863 nucleotide sequence of FgMTV1/SX64, excluding the poly (A) tail, was determined. The genome of FgMTV1/SX64 is predicted to contain four open reading frames (ORFs). The largest ORF1 is 6723 nucleotides (nt) in length and encodes a putative polyprotein of 2242 amino acids (aa), which contains four conserved domains, a methyltransferase (Mtr), tymovirus endopeptidase (Pro), viral RNA helicase (Hel), and RNA-dependent RNA polymerase (RdRp), of the replication-associated proteins (RPs) of the positive-strand RNA viruses. ORFs 2–4 putatively encode three putative small hypothetical proteins, but their functions are still unknown. Sequence alignments and phylogenetic analyses based on the putative RP protein and the three conserved domains (Mtr, Hel and RdRp) showed that FgMTV1/SX64 is most closely related to, but distinctly branched from, the viruses from the family *Tymoviridae*. Although FgMTV1/SX64 infection caused mild or no effect on conidia production, biomass and virulence of its host *F. graminearum* strain SX64, its infection had significant effects on the growth rate, colony diameter and deoxynivalenol (DON) production. This is the first molecular characterization of a tymo-like mycovirus isolated from a plant pathogenic fungus. It is proposed that the mycovirus FgMTV1/SX64 is a representative member of new proposed lineage *Mycotymovirus* in the family *Tymoviridae*.

Introduction

The plant pathogenic fungus *Fusarium graminearum* is the causal agent of Fusarium head blight (FHB), one of the most destructive crop diseases, and is distributed worldwide (Voigt et al., 2005). *F. graminearum*, with broad host range of pathogens, mostly infects crop plants, particularly wheat, maize, and barley, and results in a severe loss of grain yield as well as quality reduction. There have been seven reported mycoviruses (fungal viruses) isolated from the phytopathogenic fungus *F. graminearum*: FgV1 (*F. graminearum* virus1), FgV2, FgV3, FgV4, FgV-ch9, FgHV1/HN10 (*F. graminearum* hypovirus 1) and FgHV2/JS16, of which FgV1, FgV-ch9 and FgHV2/JS16 were associated with the hypovirulence of *F. graminearum* (Supplementary Table S1; Chu et al., 2002; Darissa et al., 2011; Li et al., 2015; Theisen et al., 2001; Wang et al., 2013; Yu et al., 2009).

The order *Tymovirales* consists of four families (*Alphaflexiviridae*, *Betaflexiviridae*, *Gammaflexiviridae* and *Tymoviridae*) (King et al., 2011). The largest protein encoded by members of the order *Tymovirales* is a replication-associated polyprotein of approximately 150–250 kDa that is close to the 5′ end of the genome and consists of a set of functional domains whose amino acid sequences and order are conserved in all viruses of the alphavirus-like superfamily of positive-stranded RNA viruses (King et al., 2011). Virions of the families *Alphaflexiviridae*, *Betaflexiviridae* and *Gammaflexiviridae* are flexuous filaments, while members of the family *Tymoviridae* have non-enveloped isometric particles (King et al., 2011).

The family *Tymoviridae*, a predominantly plant-infecting virus family, consists of three genera (*Tymovirus*, *Marafivirus* and *Maculavirus*), the members of which have the following common characteristics: non-enveloped isometric virions with a rounded contour and prominent surface structures; a monopartite positive-sense, single-stranded RNA genome (6.0–7.5 kb in length) with an unusually high cytosine content (32–50%); caps at the 5, terminus; a replication-associated polyprotein containing a set of functional domains; and cytopathic structures in infected cells (Martelli et al., 2002). There are 26, 4 and 1 confirmed virus species in the genera *Tymovirus*, *Marafivirus* and *Maculavirus*, respectively. Recently,

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many unconfirmed viral species have been reported, but no virus related to members of the family *Tymoviridae* has been isolated from plant pathogenic fungi.

In this study, we present the sequence and molecular characteristics of a novel mycovirus from the strain SX64 of *F. graminearum* and its effect on the phenotypic characteristics and virulence of SX64. We also describe the taxonomic status of the mycovirus FgMTV1/SX64.

Results

Detection and sequencing of dsRNA in F. graminearum strain SX64

The strain SX64 was identified as *F. graminearum* using polymerase chain reaction (PCR) amplification of the translation elongation factor (EF-1α) fragment (O'Donnell et al., 2000). *F. graminearum* isolate SX64 produced a distinct dsRNA band sized at about 8 kb (Fig. 1B). The band resisted digestion by DNase I and S1 nuclease, confirming it to be dsRNA. However, the virus-free strain SX64-F did not contain any dsRNA segments (Fig. 1B). The distinct dsRNA element of strain SX64 was gel purified and used as the template for cDNA cloning.

The complete genomic sequence was 7863 nts in length, excluding the poly (A) tail. A Blast X search of the complete nucleotide sequence showed a high amino acid (aa) sequence identity (29–42%) to viruses in the order *Tymovirales*, particularly to viruses of the family *Tymoviridae*. Thus, we tentatively assigned mycoviral dsRNA the name "*F. graminearum* mycotymovirus virus 1 (FgMTV1/SX64)". The sequence was deposited in GenBank under accession number KT360947.

Molecular characterization of FgMTV1/SX64

The genome organization of the coding strand of the dsRNA segment of FgMTV1/SX64 is shown in Fig. 2. Sequence analyses of the nucleotide sequence revealed the presence of four putative open reading frames (ORFs) (Fig. 2). Four untranslated regions (UTR) were present in the genome, comprising a region of 82 nucleotides preceding the initiation codon of ORF1, a 1 nucleotide between ORFs 1 and 3, a 4 nucleotides between ORFs 3 and 4 and a 3′-terminal region of 116 nucleotides followed by a poly(A) tract. Similar to members of family *Tymoviridae*, the genomic sequence of FgMTV1/SX64 contains a high percentage of cytosines (A, 17.8%; C, 35.9%; G, 23.6%; T, 22.7%) (Dreher, 2004; Martelli et al., 2002).

The largest ORF (ORF1; nt 83-6811) is 6729 nt in length and encodes a putative polyprotein of 2242 aa with a calculated molecular mass of 249 kDa (Fig. 2). ORF1 encodes a putative replication-associated polyprotein (RP) and contains four conserved domains, viral RNA methyltransferase (Mtr), tymovirus endopeptidase (Pro), viral RNA helicase (Hel) and RNA-dependent RNA polymerase (RdRp), which are contained in all of the members of the *Tymoviridae* family (Martelli et al., 2002). Although the presence of a cap structure in the genomic RNA has not been experimentally demonstrated, the existence of a methyltransferase region in the replicase gene suggests that FgMTV1/SX64 RNA is capped. Similar to members of the genus *Maculavirus*, ORF1 lacks the highly conserved 16-nt subgenomic RNA promoter, known as tymobox or marafibox, which has been identified near the end of the viral replicases of all of the sequenced tymoviruses and marafiviruses (Ding et al., 1990; Izadpanah et al., 2002).

ORF2 (nt 91–459) overlaps the ORF1 towards the 5′-terminus (Fig. 2). ORF2 is 369 nts in length and is predicted to encode a 122-aa protein with a molecular mass of 14.1 kDa. ORF3 (nt 6813–7196) is 384 nts long and encodes a putative 127-aa protein with a predicted molecular mass of 13.2 kDa (Fig. 2). Only one nucleotide (G) is situated between ORF1 and ORF 3. ORF4, in the same frame as ORF2, which encodes a putative protein with an expected molecular mass of 19.2 kDa, spans nucleotides 7201–7746 (Fig. 2). Database searches with the nucleotide and amino acid sequences of ORF 2–4 did not reveal any significant homology with other known sequences.

Sequence alignment and phylogenetic classification of FgMTV1/SX64

Alignments were obtained from the complete genomic sequence, the entire RP amino acid sequence and the four internal conserved replicase regions containing the Mtr, Pro, Hel and RdRp domains between FgMTV1/SX64 and typical viruses of the order *Tymovirales* (Table 2). GenBank accession numbers and acronyms for viruses used in analyses are listed in Table 1. The phylogenetic position of FgMTV1/SX64 within the *Tymovirales* was determined by aligning the RP, Mtr, Hel and RdRp amino acid sequences to those of members of the order (Fig. 3; Fig. S2A–C). The amino acid sequence alignments were obtained using the putative Mtr, Hel and RdRp domains of FgMTV1/SX64 and selected viruses in the family *Tymoviridae* (Fig. 4; Fig. S3A and B).

The overall nucleotide and RP amino acid sequences of FgMTV1/SX64 showed the highest identity with GFkV (family *Tymoviridae*, genus *Maculavirus*), of 33.84% and 21.26%, respectively (Table 2), compared to those of the representatives of the order *Tymovirales*. Phylogenetic analyses of the viral RP polyprotein of FgMTV1/SX64 and representatives of the order *Tymovirales* using the neighbor joining (NJ) method positioned FgMTV1/SX64 firmly within the *Tymoviridae*, but outside of the

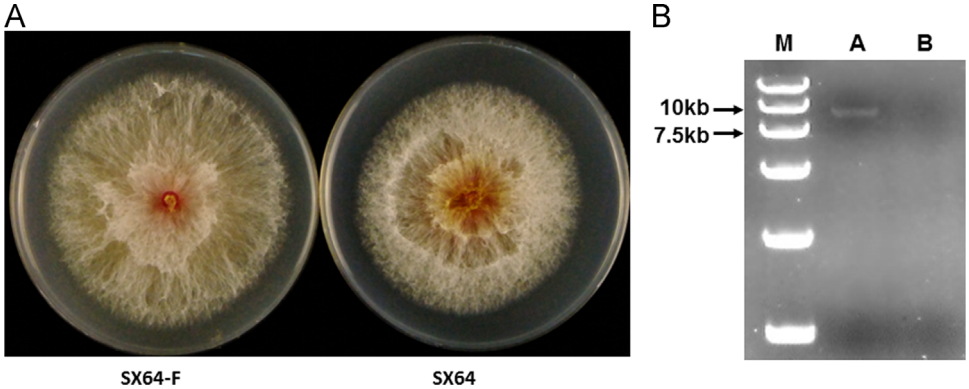

Fig. 1. (A) Colony morphology of strain SX64 (virus-carrying) and SX64-F (virus-free) after 4 days of culture on PDA in the dark. (B) DsRNA extraction of *F. graminearum* strains SX64 and SX64-F. The dsRNA fraction was electrophoresed in a 1% agarose gel and visualized under UV light after staining with ethidium bromide. Lane M, 15-kb DNA marker. Lane A, SX64. Lane B, SX64-F. All of the samples were treated with DNase I and S1 nuclease.