

Double-stranded RNA viruses infecting *Epichloë festucae*

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Abstract

Some isolates of *Epichloë festucae* are asymptotically infected by *Epichloë festucae* virus 1 (EfV1), a member of the family *Totiviridae*. This virus has a genome composed by a 5109 bp molecule of double stranded RNA (dsRNA). In addition, a 3 kbp dsRNA molecule which could be the genome of another virus (EfV2) was frequently found in isolates of the fungal endophyte. In a survey of two populations of *E. festucae* it was found that 73% of the isolates were infected by one or both viruses. Although both viruses are very efficiently transmitted to conidia, a barrier to virus transmission occurs during ascospore formation.

Keywords: *Epichloë festucae*, virus, dsRNA, *Totivirus*

Introduction

In some ways, fungal viruses resemble fungal endophytes because it is common for their fungal hosts to remain symptomless. In fact, only a few mycoviruses are known to induce symptoms in their hosts; for most known fungal viruses no differences have been observed between virus infected and virus-free strains. Other characteristics of most mycoviruses are that their genomes are composed of double-stranded RNA (dsRNA), and their transmission to other individuals depends on hyphal fusion, no animal vectors are known for these viruses (Ghabrial 1998).

A 5 kbp dsRNA molecule encapsidated in spherical virus-like particles of about 50 nm in diameter, and a smaller 3 kbp unencapsidated dsRNA, were identified in some *E. festucae* isolates. Hybridisation experiments revealed that these two dsRNA molecules did not have similar nucleotide sequences. The dsRNA-infected isolates did not show any obvious symptoms when compared to uninfected isolates (Zabalgoeazcoa *et al.* 1998). The two dsRNA molecules seemed to represent the genomes of two different viruses, which we will refer as EfV1 (*Epichloë festucae* virus 1, 5 kbp dsRNA), and EfV2 (3kbp dsRNA genome). In this paper we identify EfV1 as a new member of the family *Totiviridae* (Romo *et al.* 2007; Wickner *et al.* 2005.), and report on the incidence and transmission of both viruses.

Methods

Fungal isolates, dsRNA purification, and viral genome characterisation

The incidence of dsRNA infections was estimated from 28 and 27 isolates of *Epichloë festucae* obtained in Palancar and Servandez, two savanna-like oak (*Quercus ilex*) grasslands located 40 km apart in the province of Salamanca, in western Spain. In addition, a few isolates from five other locations were analysed for the presence of dsRNA.

The presence of dsRNA was determined by CF-11 cellulose chromatography of nucleic acid extracts obtained from mycelium of each isolate (Morris & Dodds 1979).

DsRNA from strain 23-11, infected by EfV1, was used for cDNA synthesis. To synthesise cDNA, 2 µg of dsRNA dissolved in water were denatured in the presence of 1 µg of random nonanucleotides by heating at 95°C for 15 minutes, or alternatively, by heating for 10 minutes at 70° with 20% dimethylsulfoxide. The cDNA was synthesised using a Superscript Double Stranded

cDNA Synthesis kit (Invitrogen, Carlsbad, USA). The ends of the molecule were cloned using the method described by Zhang & Frohman (1997).

Phylogenetic analyses of amino acid sequences of the coat protein and RNA dependent RNA polymerase (RdRp) of EfV1 and other dsRNA viruses were done with MEGA3 software (Kumar *et al.* 2004), using the neighbour-joining method with Poisson corrected distances. Analysis of the RNA secondary structure was done with MFOLD (Zuker *et al.* 1999).

Transmission of virus to asexual and sexual spores

To find out if the viruses were transmitted to conidia, two sets of monospore isolates were made. A set of 22 isolates was obtained from strain V5, infected by both EfV1 and EfV2; another set of 42 monospore isolates was made from strain P23, infected by EfV1.

To test whether the virus was transmitted to ascospores, several crosses between virus-free and virus-infected strains were made. Strains 2210 and 2211 were virus-free, strain S33 was infected by EfV1 and EfV2, strain P23 by EfV1, and strain SE10 by EfV2. Plants infected by strain 2210 or 2211 developed external fungal stromata, which were fertilised with mycelium of the virus-infected strains (Leuchtmann *et al.* 1994). The ascospores ejected from mature perithecia were cultured and tested for the presence of dsRNA.

Results and Discussion

Characteristics of the EfV1 genome

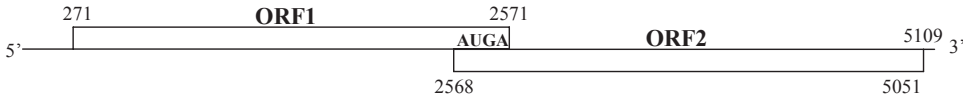
The complete genome of EfV1 has a length of 5109 nucleotides (Genbank accession number AM261427), and its coding strand contains two open reading frames (Fig. 1): ORF1 has 2301 bp and codes for a 765 amino acid protein. The sequence of this protein resembled those of coat proteins of several totiviruses. ORF2 has 2484 nt and codes an 826 amino acid protein whose sequence is similar to those of RdRps of viruses of the *Totiviridae* family. In addition, the eight conserved motifs of the amino acid sequences of RdRps of dsRNA viruses of fungi and protozoans (Bruenn 1993) were present in the ORF2 sequence.

Both ORFs are overlapped by four nucleotides at the sequence AUGA (Fig. 1). This sequence may function as a stop codon (UGA) for ORF1, and in a -1 frameshift as a start codon (AUG) for ORF2. This four nucleotide overlap is a characteristic of several members of the family *Totiviridae* (Huang & Ghabrial 1996; Cheng *et al.* 2003; Tuomivirta & Hantula 2003; Nomura *et al.* 2003).

The 5' untranslated region (UTR) has 270 nt and a GC content of 56.7%. The 3' UTR has 58 nt, and two GC-rich potential stem-loop structures occur in this region. This structure is very similar to the 3'UTR of yeast totivirus ScV-L-A, which has a stem essential for virus replication followed by the terminal sequence AUGCA (Wickner *et al.* 2002); the terminal sequence of EfV1 is AAGCA. The GC content of this region is 55.2%.

A comparative analysis of the amino acid sequence of the EfV1 coat protein and those of other members of the family *Totiviridae* showed that it resembled most those of a clade within the genus *Totivirus* (Fig. 2A). This clade is composed of nine viruses which infect filamentous ascomycetes and a basidiomycete

Figure 1 Organisation of the dsRNA genome of *Epichloë festucae* virus 1 (EfV1). ORF1 codes a putative coat protein, and ORF2 an RNA-directed RNA polymerase. Both reading frames are overlapped by four nucleotides at the sequence AUGA.



(*Helicobasidium mompa*). A phylogenetic tree with similar topology to that of the coat protein tree was obtained with RdRp amino acid sequences (Fig. 2B), and also with the sequences of the eight conserved RdRp motifs.

In conclusion, the genome organisation and phylogenetic analysis of EfV1 indicate that this virus is a member of the family *Totiviridae* (Wickner *et al.* 2005). With respect to EfV2, the other virus, its taxonomic identity can not be determined with the data available; however, it has some characteristics of viruses of the family *Narnaviridae* (Buck *et al.* 2005).

Incidence and transmission of viral dsRNA in populations of *Epichloë festucae*

In Palancar, out of 26 isolates, 8 contained dsRNAs indicative of the presence of EfV1 and EfV2, 1 isolate contained EfV1 dsRNA, and 13 isolates contained the EfV2 dsRNA. In Servandez, where 27 isolates were analysed, 11 were infected by both viruses, and 7 by EfV2. On average, in both populations 73% of the isolates were infected by one or both viruses. The only isolate infected only by EfV1 was the one we used to clone the EfV1 genome. Hybridisation experiments showed that the 5kbp dsRNA found in doubly infected strains has a nucleotide sequence similar to EfV1. EfV1 and EfV2 dsRNAs were also detected in isolates from five other populations from semiarid grasslands.

These results indicate that in the grassland ecosystems we studied, both viruses have a high incidence, and are geographically widespread in *Epichloë festucae* populations. The infection by both viruses seems to be persistent because infected strains have been maintained in our laboratory for periods of several years, and several attempts to cure infected strains have failed. Mixed virus infections like the ones we found with both

EfV1 and EfV2, have been described for other mycoviruses (Preisig *et al.* 1998; Park *et al.* 2005; Tuomivirta & Hantula 2003).

Regarding the mechanisms of transmission of the viruses, all 64 monospore isolates derived from strains P23 and V5, contained the dsRNAs present in the parental strain. This result shows that both dsRNA viruses are efficiently transmitted to mitotic spores.

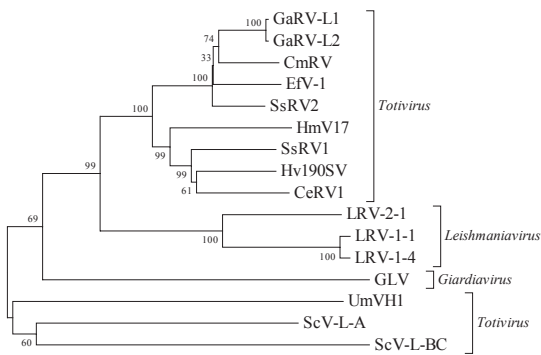
In contrast, when the ascospore progenies of crosses between virus-free and virus-infected strains were analysed, no dsRNA was found in any of the 19 ascospore progenies. This implies that the *Epichloë* viruses are not transmitted from infected gametes to ascospores. Therefore, progeny from sexual reproduction is always clean of viral parasites, if stromatal strains are virus-free. Maternal transmission from infected stromata to ascospores has not been tested.

The high frequency of virus infection that is observed in natural populations of *Epichloë festucae* may be explained by asexual reproduction which is the predominant mode of reproduction of the fungus, and indeed, was the only reproduction mode observed in the fungal populations of Palancar and Servandez. Clonal lineages and linkage disequilibrium detected in these natural populations suggest that asexual reproduction of the fungus is significant (Arroyo *et al.* 2002).

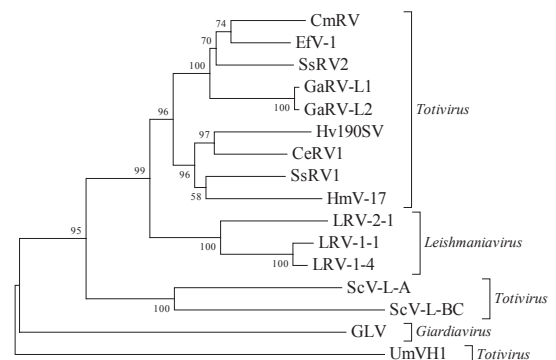
In semiarid grasslands of western Spain, *Festuca rubra* is a common species, and about 70% of the plants of this grass are asymptotically infected by the endophyte *Epichloë festucae* (Zabalgoatzea *et al.* 1999). It is remarkable how this plant-fungus association resembles the fungus-virus associations described in this study, where 73% of the fungal isolates are also asymptotically infected by viruses. Most fungal viruses appear to have no effect on the phenotype of their hosts, but perhaps the fungus may obtain selective advantages from the viruses, much like *Festuca rubra* plants from their association with the endophytic fungus.

Figure 2 Neighbour-joining phylogenograms of viruses of the *Totiviridae* family based on coat protein (a) and RdRp (b) amino acid sequences. Brackets enclose members of the same genus. Bootstrap values are based on 1000 replications. GaRV: *Gremmeniella abietina* viruses L1 and L2; CmRV: *Conyothirium minitans* virus, EfV-1: *Epichloë festucae* virus 1; SsRV: *Sphaeropsis sapinea* viruses 1 and 2; HmV17: *Helicobasidium mompa* virus, Hv190SV: *Helminthosporium victoriae* 190S virus; CeRV1: *Chalara elegans* virus 1; LRV: *Leishmania* viruses 1-1, 2-1, and 1-4; GLV: *Giardia lamblia* virus; UmVH1: *Ustilago maydis* virus H1, *Saccharomyces cerevisiae* viruses L-A and L-BC (Romo *et al.* 2007).

a



b



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