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Full Length Research Paper

Presence of Viral dsRNA molecules in the Spanish population of *Gremmeniella abietina*

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***Gremmeniella abietina* var. *abietina* has commonly been pointed out as a species complex, which includes different races and biotypes. Among them, the Spanish population seems to be a unique population derived from type A. Furthermore, *G. abietina* is known to harbour infections and co-infections of putative mycoviruses belonging to different families. In particular, *G. abietina* type A harbours putative members of families *Totiviridae* and *Partitiviridae* but also *Narnaviridae*, with members of genus *Mitovirus.* In case of *G. abietina* type B, a novel putative virus with endornavirus affinities has also been identified. Different types of *G. abietina* seem to host a divergent virus community. So, ninety-one isolates of the Spanish *G. abietina* were analyzed to check out the presence of viral dsRNA molecules. Thus, the 89% of Spanish isolates presented at least one dsRNA molecule, which is a significant frequency. Overall, eight dsRNA banding patterns were detected, suggesting the occurrence of putative members of different virus genera as *Partitivirus*, *Mitovirus* and *Totivirus*. This is the first approach to the study of fungal mycoviruses in the Spanish population of *G. abietina*.**

Key words: *Gremmeniella*, mycovirus, host, dsRNA, taxonomy.

INTRODUCTION

Mycoviruses are obligate parasites of fungi that generally produce cryptic infections because they are transmitted intracellularly by spores (sexual and asexual) and through anastomosis (Buck, 1986; Ghabrial, 1998). They are usually isometric particles of 25 to 50 nm in diameter and their genome is composed of single-stranded (ss) or double-stranded (ds) DNA or RNA (Van Regenmortel et al., 2000). Furthermore, retrovirus-like elements made of (+) ssRNA have also been found incorporating their genome into the host as dsRNA (Peterson-Burch and Voytas, 2002). In general, dsRNA genome, which codes for a RNA dependent RNA polymerase (RDRP), is the one most commonly associated with viral parasitism in fungi.

Classification of fungal viruses is based on virion morphology, genome organization, method of replication and the number and size of structural and non-structural viral proteins (Van Regenmortel et al., 2000; Bamford et al., 2002). According to the present taxonomical classification, eight families and one genus that is not related to any specific family, are recognized to infect fungi (Van Regenmortel et al., 2000; Ghabrial, 2009; Mayo, 2002). Thus, within dsRNA viruses, there are four families: The family, *Totiviridae,* which contains a single linear uncapped dsRNA molecule with a size of 4.6 to 7.0 kb. Family, *Partitiviridae*, whose genome contains two linear dsRNA segments of 1.4 to 3.0 kb. Family, *Chrysoviridae*, which members carry three to four linear dsRNA molecules with a length of 2.8 to 3.6 kb (Caston et al., 2003). Finally, the family, *Hypoviridae* with a linear genome that codifies a dsRNA molecule of 9 to 13 kb. To date, two families have been characterized within (+) ssRNA viruses, the families *Narnaviridae* and *Barnaviridae*. Members of *Narnaviridae* lack of true virions, and particularly, the genus *Mitovirus* has a linear genome of approximately 2.5 kb (Hong et al., 1999). Thus, the family *Barnaviridae* has only one member that infects to *Agaricus bisporus* and its genome has a length

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of 4.0 kb (Revill et al., 1994). Within Retrovirus-like elements are two families: Family *Metaviridae* with a genome of (+) ssRNA (4 to 10 kb) as well as the family *Pseudoviridae* (Peterson-Burch and Voytas, 2002). Finally, dsDNA viruses are represented by genus *Rhizidiovirus* whose only member contains one linear dsDNA molecule of 25.5 kb in size (Hausner et al., 2000).

Consequently, as in Tuomivirta et al. (2002), the existence of different kinds of viruses in *G. abietina* isolates is widely explained, the aims of this study were: (i) to investigate if dsRNA particles were commonly isolated in the Spanish population of *G. abietina* and (ii) to try to identify which type of viruses they could correspond to.

MATERIALS AND METHODS

Isolates

Ninety-one Spanish isolates of *G. abietina* from *Pinus halepensis* stands located in Valle de Cerrato, Hontoria, Villalba de los Alcores and Astudillo (Botella et al., 2010).

DsRNA isolations

DsRNAs molecules were extracted following a modification of the protocol of Morris and Dodds (1979), which is based on the capability of modified CF-11 fibrose cellulose to bind specifically to dsRNA molecules.

All the samples were cultivated on MOS plates complemented with cellophane membrane to avoid negative interferences of the agar on the dsRNA isolation. Approximately, 1 to 1.5 g of mycelia was put into falcon tubes of 50 ml to be disrupted with 2.5 ml of lysis buffer (50 mM Tris-HCl pH 8.0 (ICN Biomedicals, Ohio, USA; J.T. Baker, Deventer, Holland), 50 mM EDTA (Riedel-de Häen, Seeize, Germany), 3% sodium dodecyl sulphate (SDS) (Acros Organics, Geel, Belgium), and 1% β-mercaptoethanol (YA-Kemia Oy, Helsinki, Finland)) and with a ULTRA-TURRAX® TP-18/10 (Janke & Kunkel GmbH & co KG IKA-Werk, Staufen, Germany) homogenizer. Then, 3 ml of phenol: Chlorophorm-isoamylalcohol (25: 24: 1) (Amresco, Ohio, USA; Tamro Medlab Oy, Vantaa, Finland; YA-Kemia Oy, Helsinki, Finland) were added to develop the extraction of total nucleic acids. Afterwards, 30 min of centrifugation (3000 rpm) was carried out, followed by a last extraction with chlorophorm:isoamylalcohol (24:1). Then, the samples treated with 30 µl NaCl and ethanol (16.5%) were kept during, at least, 20 min at -20°C. After this period, 50 mg of CF-11 was added into the tube and mixed during 10 min on ice. The following steps consisted of washing the samples with Tris/Sucrose/EDTA (TSE) buffer (10 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA) supplemented with 15% ethanol through handmade columns of 5 ml pipette tips and a cotton plug. Thus, the samples were transferred to the columns and washed 14 times with 1.2 ml of TSE buffer each time. Then, dsRNA molecules bound to CF-11 column were eluted with 400 µl TSE buffer without ethanol followed by 30 µl 5M NaCl and 1100 µl absolute ethanol for precipitation of dsRNA. Finally, after an incubation of 20 min at -20°C and 20 min centrifugation, the precipitate was dried up under vacuum and re-suspended in 10 µl Tris/EDTA (TE) (6 mM Tris-HCl pH 8.0, 1 mM EDTA). Albeit CF-11 cellulose is considered to bind specially dsRNA, isolations were repeated twice for positive isolates in order to discard the possibility of being artefacts or DNA.

DsRNA gel electrophoresis

DsRNA banding patterns were checked by electrophoresis in order to identify the possible virus molecules hosted in the Spanish isolates of *G. abietina*. Thereby, 6 µl of sample were pipetted into 2 µl of tracking dye supplemented with sucrose to be loaded in a 1% agarose gel containing 1 x TAE buffer with 50 µl of ethidium bromide during 60 min at 120 V. The size of the dsRNA was determined comparing the size with a Gene Ruler™ 1 kb DNA Ladder Plus (Fermentas). After running, the gel was observed under UV light and photographed.

RESULTS AND DISCUSSION

In this study, dsRNA molecules were found in the 89% of the Spanish isolates studied. This result not only confirms the presence of mycoviruses in *G. abietina* in Spain but also supports the common high dsRNA occurrence found in previous studies (Tuomivirta et al., 2002). Furthermore, 8 different dsRNA banding patterns were observed (Figure 1). According to the molecular size of the bands members of genus *Mitovirus*, *Partitivirus*, *Totivirus* and *Hypovirus* could be hosted in Spanish *G. abietina* isolates.

The existence of dsRNA molecules in the genome of fungi is very common in nature, as it has been demonstrated over the last years (Pearson et al. 2009). Basidiospores of *Heterobasidion annosum* are also often infected (Ihrmark et al., 2001, 2002), *Cryphonectria parasitica* strains normally infected by genus *Hypovirus*, *Mitovirus* in *Ophiostoma novo-ulmi* (Hong et al., 1999), *Totivirus* in *Sphaeropsis sapinea* (Preisig et al., 1998), Finnish *G. abietina* var. *abietina* type A (Tuomivirta et al., 2002) etc. In particular, *G. abietina* biotype A was shown to harbour dsRNA in 44% of all tested isolates (Tuomivirta et al., 2002) and even if samples originating from ascospores were excluded, the percentage increased until 55% of isolates. Thus, such high infection rate in the Spanish isolates of *G. abietina* is in accordance with *Discula destructive*, which hosts dsRNA molecules in 79% of its isolates (Rong et al., 2001) but it contrasts with the frequency of isolation of other species as *S. sapinea* with a rate of 32% (Steenkamp et al., 1998).

Although sequencing is the most reliable method to identify viruses, the molecular weight of the bands and the banding pattern can propose the type of virus. In this case, each banding pattern corresponded to an isolate and as it is observed in the Figure 1, more than one virus could be hosted per isolate.

Thus, according to Figure 1, the first banding pattern, which consisted of one band of about 3 kb, could refer to genus *Chrysovirus*, however, as it consist of only one band, it is more possible to belong to *Mitovirus* or *Narnavirus*. This band appeared in the banding patterns numbers 5, 6 and 7 as well. Then, the pattern number 8 could be the genus *Totivirus* (~6 kb) because it consists of a single molecule of about 4 to 7 kb as it is described in the literature. This band also appeared in 2 and 5.

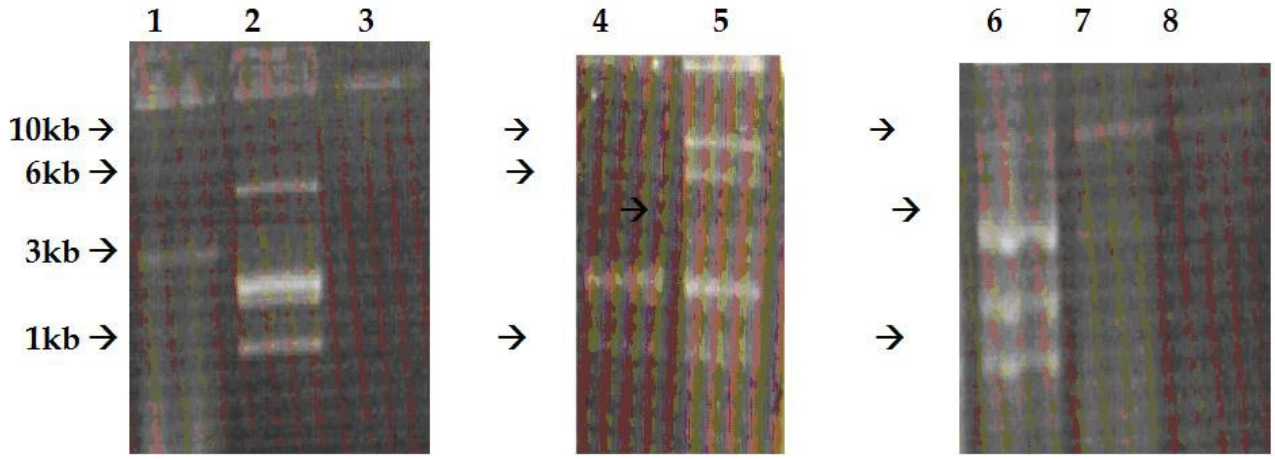

Figure 1. Common banding patterns found in the Spanish population of *G. abietina*.

Pattern number 4 has two similar linear molecules of 1.4 to 3.0 kb and one of about 1 kb, so it could correspond to *Partitiviridae*, which can also be observed in numbers 2, 5 and 6. In addition, banding pattern number 5 has a band over 10 kb that could be a member of the family *Hypoviridae*. However, as this family has not previously been confirmed in *G. abietina*, sequencing would be completely necessary. Finally, number 3 does not harbour any dsRNA molecule.

Conclusions

This study confirms that the Spanish population of *G. abietina* hosts dsRNA viral molecules at a significant high rate. Therefore, these results are interesting because they establish the bases for further phylogenic studies of the history of *G. abietina* and they open the possibility of finding hypovirulent strains of *G. abietina* that could behave as biological control agents.

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