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Polyphyletic viruses of *Gremmeniella abietina* type A, a major pathogenic fungus of coniferous trees

Tero Tuomivirta

VANTAAN TUTKIMUSKESKUS – VANTAA RESEARCH CENTRE

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Polyphyletic viruses of *Gremmeniella abietina* type A, a major pathogenic fungus of coniferous trees

Doctoral Thesis
by
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Vantaa Research Centre

Academic dissertation in Plant Pathology
Faculty of Agriculture and Forestry
University of Helsinki

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To my family

Preface

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Vantaa, September, 2004

Tero Tuomivirta

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Abbreviations used

aa	amino acid
bp	base pair
DdV1	*Discula destructiva* virus 1
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
CP	coat protein
CsCl	cesium chloride
ds	double stranded
FsV1	*Fusarium solani virus 1*
GaMRV-S	*Gremmeniella abietina* mitochondrial RNA virus S
GaRV-L	*Gremmeniella abietina* RNA virus L
GaRV-MS	*Gremmeniella abietina* RNA virus MS
HvV190S	*Helminthosporium victoriae virus 190S*
kb	kilobase
kbp	kilobasepair
kDa	kilodalton
LTR	long terminal repeat
LTT	large tree type
MBV	*Mushroom bacilliform virus*
mRNA	messenger RNA
nt	nucleotide
OMV4	*Ophiostoma mitovirus 4*
OMV5	*Ophiostoma mitovirus 5*
OMV6	*Ophiostoma mitovirus 6*
ORF	open reading frame
PcV	*Penicillium chrysogenum virus*
PCR	polymerase chain reaction
RAMS	random amplified microsatellite
RdRp	RNA-dependent RNA polymerase
RNA	ribonucleic acid
RNase	ribonuclease
ss	single stranded
STT	small tree type
TEM	transmission electrone microscope
UTR	untranslated region
VLP	virus-like particle

List of Original Publications

This thesis is based on the following publications, which will be referred to in the text in Roman numerals (I-IV). The published papers are reprinted with the permission from the publishers.

I **Tuomivirta, T.T.**, Uotila, A. and Hantula, J. 2002. Two independent double-stranded RNA patterns occur in the Finnish *Gremmeniella abietina* var. *abietina* type A. For Pathol. 32, 197-205.

II **Tuomivirta, T.T.** and Hantula, J. 2003. Two unrelated double-stranded RNA patterns in *Gremmeniella abietina* type A code for putative viruses of the families *Totiviridae* and *Partitiviridae*. Arch. Virol. 148, 2293-2305.

III **Tuomivirta, T.T.** and Hantula, J. 2003. *Gremmeniella abietina* mitochondrial RNA virus S1 is phylogenetically related to the members of the genus *Mitovirus*. Arch. Virol. 148, 2429-2436.

IV **Tuomivirta, T.T.** and Hantula, J. 2004. Three unrelated viruses occur in a single isolate of *Gremmeniella abietina* var. *abietina* type A. Manuscript submitted to Virus Research.

Author's contribution

The contribution of Tero Tuomivirta is presented here. The following text has been compiled from documents where each of authors' contribution to the above mentioned publications were stated and signed.

Paper I
Original idea of the work and the initial observation of double-stranded RNA in *Gremmeniella abietina* type A was made by Jarkko Hantula. JH requested *G. abietina* isolates from colleagues listed in the paper. Antti Uotila planned, executed and analyzed the results of the pathogenicity tests. Tero T. Tuomivirta and JH also participated to the pathogenicity tests. Ultracentrifugation experiment was performed by TTT who had also the main responsibility on the writing process. AU and JH also participated in the writing process and discussions of the results.

Papers II, III and IV
The original idea for the work appeared in a brainstorming process conducted by Tero T. Tuomivirta and Jarkko Hantula. Isolates C5 and HR2 (paper II) were requested from colleagues. Isolates Luumäki 7 (paper III) and SurS4 (paper IV) used in the studies were collected and isolated by JH. The work was planned mainly and executed almost entirely by TTT who had also the main responsibility on the writing process. JH also participated in the writing process and discussions of the results.

Abstract

Fungal viruses are obligate parasites transmitted via intracellular routes. They are usually cryptic (i.e. there are no associated symptoms) but also phenotypic changes associated with viruses have been reported among plant-pathogenic fungi. Fungal viruses have been classified into eight recognized families and one genus not associated with any specific family, but their taxonomy is not strictly associated with the effect on the host phenotype.

Gremmeniella abietina is an ascomycetous fungus causing Scleroderris canker in coniferous trees. Two types (A and B) of this fungus with different pathogenic properties occur in Finland. *G. abietina* type A is capable of seriously damaging grown-up trees whereas type B occurs in seedlings.

This thesis comprises of experiments on three different double-stranded (ds)RNA patterns found in *G. abietina* type A. In total 44% of isolates contained dsRNA, but no firm link between the occurrence of dsRNA and pathogenicity of the fungus towards *Pinus sylvestris* could be established. All three different dsRNA patterns were found in a single mycelium, and they could be separated in isopycnic ultracentrifugation. The co-existence of all three different dsRNA patterns suggested that they are not probably maintained by using exactly the same mechanisms. Altogether six dsRNA patterns were completely sequenced and based on BLAST searches they encoded putative viruses of the families *Narnaviridae*, *Partitiviridae* and *Totiviridae*. The analysis of their putative RNA-dependent RNA polymerase sequences suggested polyphyletic origin for these viruses. All three dsRNA patterns showed effective transmission via conidia.

1. Introduction

1.1 Fungal viruses

Viruses are obligate parasites that infect all kinds of organisms from simple bacteria to mammals (van Regenmortel et al., 2000). Therefore it is not surprising that viruses occur also in fungi (Buck, 1986). Fungal viruses seem to lack life-cycle outside the cell and apparently are transmitted only by intracellular routes (Buck, 1986). These viruses are usually cryptic (i. e. there are no associated symptoms), which is probably a major reason for their late discovery (Buck, 1986). The genomes of viruses may be composed of DNA or RNA, and the nucleic acid may be either single-stranded (ss) or double-stranded (ds) (van Regenmortel et al, 2000). Only dsRNA, dsDNA, and positive (+) ssRNA viruses are found in recognized members of the virus families infecting fungi (van Regenmortel et al, 2000). In addition to these viruses, also retrovirus-like elements made of +ssRNA are found and they have the capacity to incorporate their genome into the host genome as dsDNA (Buck, 1986).

Early observations on fungal viruses or virus-like particles (VLPs) were made using transmission electron microscopy (TEM) (Buck, 1986), which as a method is simple. TEM, however requires a relative high virus concentration in the sample and it is easy to misinterpret electron micrographs as host specimens may contain structures resembling VLPs or the VLPs may remain unrecognized. Therefore, fungal viruses have more recently been screened by testing for the occurrence of dsRNA in mycelium as dsRNA is usually associated with viral infection. The dsRNA isolation can be conducted by utilizing specific binding properties of different cellulose types (Morris and Dodds, 1997) or by precipitating dsRNA with lithium chloride (Diaz-Ruiz and Kaper, 1978). dsRNA isolation can be used to detect viruses with dsRNA or also +ssRNA genomes as the latter ones form dsRNA as their replicative forms. However, viruses with negative (-) ssRNA genomes or DNA genomes cannot be detected, and therefore the current methodology based solely on dsRNA isolation may give a biased overall picture of the viral diversity in fungi as all virus types can not be detected.

1.2 Taxonomy of fungal viruses

Taxonomy tries to classify organisms according to a phylogenic framework, in which the evolutionary relationship between different virus species can be deduced. To classify virus families, a number of discriminating characteristics can be used, such as virion morphology, genome organization, method of replication and the number and size of structural and non-structural viral proteins (van Regenmortel et al., 2000). Nature does not necessarily follow such man made classification, as only survival and propagation are needed for a successful future of a virus species.

The current taxonomy of fungal viruses consists of eight recognized families: *Totiviridae*, *Partitiviridae, Chrysoviridae*, *Hypoviridae*, *Narnaviridae*, *Barnaviridae*, *Metaviridae* and *Pseudoviridae*) and one genus (*Rhizidiovirus*) not associated with any specific family (van Regenmortel et al., 2000; Ghabrial, 2001; Mayo, 2002).

1.2.1 dsRNA viruses

1.2.1.1 Family *Totiviridae*

Members of the family *Totiviridae* usually cause cryptic infections in fungi and protozoa (Van Regelmortel et al., 2000). Viruses found in fungi belong to the genus *Totivirus* and those found in protozoa belong to the genera *Giardiavirus* and *Leishmaniavirus* (Ghabrial, 2001). Virion buoyant density in CsCl is 1.33–1.43 g/cm^3. Isometric particles of 30–40 nm in diameter are found and they contain a single linear uncapped dsRNA molecule, 4.6–7.0 kbp in size. Defective and satellite dsRNAs may be present. mRNAs for capsid and RNA-dependent RNA polymerase (RdRp) are produced via conservative mechanisms. Two major open reading frames (ORFs) are present in the genomes of totiviruses. The 5'-proximal end encodes the capsid protein (CP) and 3'-proximal end the RdRp. In the translation of RdRp three basic mechanisms have been observed among the members of the family *Totiviridae*. The first is a hypothetical ribosomal hopping mechanism observed in *Leishmania RNA virus 2-1* (Scheffter et al., 1995) to produce a CP-RdRp fusion protein. The second mechanism is based on different types of frameshifts. *Giardia lamblia virus* (Wang et al., 1993) and *Saccharomyces cerevisieae virus L-A* (Dinman et al., 1991) seem to translate the CP-RdRp fusion protein via a -1 translational frameshift mechanism. An opposite situation to the preceding frameshift is a +1 ribosomal frameshift probably used in *Leishmania RNA virus 1-1* (Stuart et al., 1992) and *Trichomonas vaginalis* virus strain T1 (Tai and Ip, 1995). Both of these frameshift mechanisms involve a consensus heptameric slippery site and pseudoknot structures. The third mechanism used to translate unfused RdRp is a hypothetical reinitiation mechanism found in *Helminthosporium victoriae virus 190S* (Soldevila and Ghabrial, 2000). About twenty viral sequences with RdRps similar to totiviruses can be found in the GenBank.

1.2.1.2 Family *Partitiviridae*

Members of the family *Partitiviridae* usually cause cryptic infections in fungi and plants. Currently three genera are assigned to this family and those infecting fungi are members of the genera *Partitivirus*. The members of the genera *Alphacryptovirus* and *Betacryptovirus* are found in plants. Viruses of these three genera contain two linear dsRNA segments (1.4–3.0 kbp in size) and the two segments of individual viruses have almost the equal length. Both of the dsRNA molecules contain one major ORF. The buoyant density in CsCl is 1.34–1.39 g/cm^3 in these viruses. Isometric particles of 30–40 nm in diameter are found and they contain at least two separate capsid types containing one dsRNA molecule each. The larger dsRNA encodes the RdRp and the smaller one the CP and their *in vitro* transcription and replication uses a semi-conservative mechanism. Satellite or defective dsRNAs may be present. Partitiviruses have been hypothesized to have originated from the genus *Totivirus* (Ghabrial, 1998). About twenty viral sequences with RdRps similar to partitiviruses can be found in the GenBank.

1.2.1.3 Family *Chrysoviridae*

The members of the family *Chrysoviridae* (genus *Chrysovirus*) are found solely in fungi and are former members of the family *Partitiviridae*. The buoyant density of

these viruses in CsCl is 1.35 g/cm^3 and they are 35–40 nm in diameter. These viruses are composed of three to four linear dsRNA molecules. All dsRNA molecules contain one large ORF and two of them encode the RdRp and CP. The dsRNA molecules are 2.8–3.6 kbp in length. The functions of other dsRNA molecules are unknown but earlier they were suspected to be satellite or defective dsRNAs. Lately, research made on *Penicillium chrysogenum virus* (PcV) revealed that all four dsRNA molecules found in PcV code for virion-associated proteins (Castron et al., 2003). Therefore, the members of this family were recently removed from the family *Partitiviridae* (Mayo, 2002) after the sequencing of the two members of the genus *Chrysovirus*. RdRps of the members of the family *Chrysoviridae* resemble those of totiviruses (*Helminthosporium victoriae virus 145S*; Ghabrial et al., 2002).

1.2.1.4 Family *Hypoviridae*

The members of the family *Hypoviridae* (genus *Hypovirus*) lack true virions and the linear viral genome (9–13 kbp) is enclosed inside a pleomorphic vesicle made from host-derived lipids. The genome of hypoviruses contains one or two ORFs. The buoyant density of the vesicle in CsCl is 1.27–1.3 g/cm^3 and they possess RdRp activity. Only few formal members of this family have been sequenced and they are solely found in *Cryphonectria parasitica*, which is the causative agent of chestnut blight in chestnut trees. Some members of the *Hypoviridae* reduce the virulence of the fungus towards its host causing hypovirulence. Hypovirulence can be introduced via anastomosis (Anagnostakis, 1982) or *in vitro* (Chen el. al., 1994; Chen and Nuss, 1999) to virus-free isolates of *C. paracitica* or to a completely new fungal species by *in vitro* transformation of full-length complementary DNA (van Heerden et al., 2001; Sasaki et al., 2002). Putative protease, RdRp and helicase motifs of polyprotein of hypoviruses are more similar to the plant virus *Barley yellow mosaic virus* of the genus *Bymovirus* of the family *Potyviridae* (Koonin et al., 1991) than to other fungal families.

1.2.2 +ssRNA viruses

1.2.2.1 Family *Narnaviridae*

Members of the family *Narnaviridae* infect solely fungi and they lack true virions. The linear genomes of these viruses are approximately 2.5 kb in size and they contain only one major ORF encoding for RdRp. There are two genera in the family *Narnaviridae*, which differ considerably from each other. The five members of the genus *Mitovirus* have a GC-poor (approximately 30%) genome and they are located and translated in the mitochondria (Cole et al., 2000). This is in contrast to the two members of the genus *Narnavirus* infecting *Saccharomyces cerevisiae* (Rodrigues-Cousiño, et al. 1991; Esteban et al., 1992), which have a GC-rich (approximately 60%) genome and can be found as ribonucleoprotein (+ssRNA–RdRp) complexes (Solórzano et al., 2000) in cytoplasma. Moreover, the putative RdRps of the members of the genus *Mitovirus* are more similar to a number of translated open reading frames found in the mitochondrion of *Arabidopdis thaliana* than to RdRps of the members of the genus *Narnaviridae* (Hong et al., 1998). Some members of genus *Mitovirus* are suspected of causing hypovirulence (Deng et al., 2003).

1.2.2.2 Family *Barnaviridae*

Only one member infecting *Agaricus bisporus* is assigned to the family *Barnaviridae* (genus *Barnavirus*). The virions of *Mushroom bacilliform virus* (MBV) contain a linear +ssRNA genome of 4.0 kb in length. The genome of MBV contains four major and three minor ORFs. Two of the major ORFs encode for putative RdRp and capsid proteins, which have similarities with certain plant luteoviruses and carmoviruses (Revill et al., 1994).

1.2.3 Retrovirus-like elements

1.2.3.1 Family *Metaviridae*

The family *Metaviridae* contains two genera (*Metavirus* and *Errantivirus*) and they are morphologically poorly characterized retrotransposons found in fungi, plants and invertebrates. Five recognized members are identified from fungi. Virions containing different intermediates with different lengths composed of RNA and DNA may be found. Long terminal repeats (LTRs) are positioned at both ends of their genome mainly consisting of +ssRNA (4–10 kb) and the 3' end of the genome is polyadenylated. The members of the family *Metaviridae* are related to the members of the family *Retroviridae* infecting vertebrates by amino acid sequences of their putative reverse transcriptase (Peterson-Burch and Voytas, 2002) as well as the family *Pseudoviridae.*

1.2.3.2 Family *Pseudoviridae*

The members of the family *Pseudoviridae* are morphologically poorly characterized retrotransposons found in fungi, plants and invertebrates and are commonly referred to as LTR retrotransposons of the SceTy1V/DmeCopV family (Peterson-Burch and Voytas, 2002). The main difference between members of the family *Pseudoviridae* and *Metaviridae* is in their genome organization. The members of *Metaviridae* encode the putative viral capsid, nucleocapsid, protease, integrase, and reverse transcriptase/ RNase H. Again, the members of the families *Metaviridae* and *Retroviridae* encode the integrase downstream to reverse transcriptase/ RNase H. LTRs are positioned at both ends of their genome consist of +ssRNA (5–6 kb) and the 3' end is polyadenylated. All members of the family *Pseudoviridea* infecting fungi are isolated from *Saccharomyces cerevisiae.*

1.2.4 dsDNA viruses

1.2.4.1 Genus *Rhizidiovirus*

The single member of the genus *Rhizidiovirus* is not associated with any specific family. The virions are 60 nm in diameter and they contain one linear dsDNA molecule of 25.5 kbp in size. No sequences are available. The buoyant density of virions is 1.31 g/cm^3. Virions have been found only in *Rhizidiomyces*, which phylogenetically belong to the kingdom *Stramipila* (Hausner et al., 2000) and therefore are not considered to belong to the kingdom *Fungi.* Therefore it is questionable to consider the member of the genus *Rhizidiovirus* a mycovirus.

1.2.5 Other viruses

There are a number of viral sequences available in the GenBank, which can not be classified as members of the virus families listed above. These sequences are generally cloned from dsRNA molecules and they are usually associated with isometric particles.

1.3 Transmission of dsRNA in ascomycetes

Fungal viruses are not always considered "true" viruses as they do not lyse their host and are apparently transmitted only by intracellular routes. This may seem to be an ineffective way to spread but about 30% of fungal species contain viruses (Buck, 1986). One obvious intracellular route is the growth of hyphae during somatic replication and thus persistent infection can be maintained in a single fungal isolate.

Anastomosis is a special feature among fungi, in which hyphae from different fungal individuals are able to make cell to cell contacts. These contacts offer a way for viral dispersal. The anastomosis, however, is restricted by vegetative incompatibility. Strains carrying the same or nearly the same alleles of *vic* genes (Liu and Milgroom, 1996) are capable of conducting anastomosis. However, fungal strains with different alleles are vegetatively incompatible, and therefore no anastomosis occurs between them. Studies on the anastomosis of *G. abietina* have not been reported.

In ascomycetes transmission of dsRNA (presumably inside virions if dsRNA is associated inside virion in hyphae) into conidia (asexual spores) is common in most cases, but usually dsRNA is not found in ascospores (sexual) (Buck. 1986). Exceptions to this common rule are the members of the genus *Mitovirus*: when a strain with *Cryphonectria mitovirus 1* acts as the mother during meiosis, the dsRNA will be passed to ascospores (Polashock and Hillman, 1994; Polashock et al., 1997). In contrast to ascospores, basidiospores of *Heterobasidion annosum* are frequently infected (Ihrmark et al., 2002; 2004).

1.4 *Gremmeniella abietina*

Gremmeniella abietina (Lagerb.) M. Morelet var. *abietina* is an ascomycetous fungus causing Scleroderris canker on coniferous trees. In Finland, two types of this fungus have been observed on Scots pine (*Pinus sylvestris* L.): type A (or large tree type, LTT) strains cause symptoms on both large trees and seedlings, whereas type B (or small tree type, STT) strains are found only in seedlings or shoots covered with snow during the winter (Uotila, 1983; Kaitera et al., 1998). The types can be identified by morphological criteria (Uotila, 1983), fatty acid and sterol profiles (Müller and Uotila, 1997), immunoblotting (Petäistö et al., 1996), or by using various genetic fingerprinting methods (Hellgren and Högberg, 1995; Hamelin et al., 1996; Hantula and Müller, 1997) and sequence-specific PCR (Hamelin et al., 2000). Isolates belonging to A and B types are able to produce artificial hybrids with low fitness (Uotila et al., 2000). *G. abietina* type A has also been introduced to North America, where it is causing increasing destruction among conifers (Laflamme and Lachance, 1987; Hamelin et al., 1996). In addition to the types observed in Finland, two other types of *G. abietina* occur in North America and Central Europe. They have been designated as the North

American race and Alpine type, respectively, and their pathogenic properties are similar to type B.

In Finland, severe Scleroderris canker epidemics during the 1980s were caused by *G. abietina* type A (Kaitera et al., 1998) and the latest outbreak of *G. abietina* took place in Sweden where as much as 300 000 hectares of pine forest were damaged (Wulff and Walheim, 2002). The amplitude of damage caused by *G. abietina* to conifers is dependent on several factors. Provenance and thus the genetic makeup of the tree affects its sensitivity probably via influencing the process leading to dormancy (Uotila, 1985). Low total sunlight radiation, summer frost, a low temperature sum (Uotila, 1988), and probably also mild winters as well as high stem density (Niemelä, 1992) increase the risk of Scleroderris canker. Topography, and thus the microclimate, in the growing site also has an effect on damages caused by *G. abietina* as trees in large water divides, low-lying plateaus and low relative elevation (kettle holes) were found to be more susceptible to *G. abietina* in Southern Finland (Uotila, 1988; Nevalainen 2002). In nurseries chemical fungicides can be used to control *G. abietina*. In practical forestry, seed from southern provenance should be avoided, and in risk sites Scots pine should not be grown. In already infected sites heavy thinning and removal of infected trees are advisable. Global climate change is believed to result in increasing rainfall in Finland. As this is considered to be beneficial for *G. abietina* (Kellomäki *et al.*, 1988), the number of epidemics caused by this fungus may increase in the future. Thus, attempts are needed, and have been made (Jacobi *et al.*, 2000), to use novel control strategies against the fungus.

2. Aims of the study

The widespread presence of dsRNA molecules (viruses) in fungi has been known for many years. Fortunately, an increasing number of these molecules have been cloned, sequenced and deposited into databanks in the recent years. This makes it possible to make comprehensive analyses between different virus species. Furthermore, the possible practical applications of fungal viruses to control damages made by plant pathogenic fungi enhance the interest towards these molecules.

The aim of the study was to characterize dsRNA molecules of *Gremmeniella abietina* type A in different ways. Several hypotheses were made:

- The presence of dsRNA patterns in *G. abietina* type A reduces its pathogenicity towards *Pinus sylvestris* (I).
- Three different dsRNA pattern types found in *G. abietina* type A code for virus genomes (I, II, III, IV).
- The three different dsRNA patterns (viruses) of *G. abietina* type A are not derived from each other but represent different viruses (II, III, IV).
- Mycoviruses of *G. abietina* may have a polyphyletic origin (IV).

3. Materials and methods

The total number of *G. abietina* isolates examined for the occurrence of dsRNA was 25 (Table 1). The type of *G. abietina* isolates were confirmed to be type A using random amplified microsatellite (RAMS) fingerprints (I and IV; Hantula and Müller 1997; Kaitera et al. 1998).

dsRNA was isolated (I–IV) with modifications of the method of Morris and Dodds (1979) based on specific binding of dsRNA to fibrous cellulose powder (CF–11). dsRNA was visualized on an agarose gel (I). Enzymatic analyses of dsRNA molecules were conducted with DNase 1 and RNAase treatments (Pryor and Boelen 1987) as described in I and III. Transmission of dsRNA molecules to conidia were tested with isolate SurS4 as described in IV. Two of the three dsRNA pattern types were also used in the pathogenesis experiments as described in I. Two ultracentrifugation experiments

Table 1. Occurrence of the dsRNA molecules in *Gremmeniella abietina* type A isolated mainly from *Pinus sylvestris*.

Isolate	Origin	Culture type	Collector	6000 bp dsRNA	2600 bp dsRNA	1800, 1600 and 1200 bp dsRNA
HR1	Symptomless tree	Mycelial isolate	Hanna Ranta	no	no	no
HR2	Symptomless tree	Mycelial isolate	Hanna Ranta	yes	no	no
HR3	Symptomless tree	Mycelial isolate	Hanna Ranta	yes	no	yes
A26	Symptomless shoot	Mycelial isolate	Juha Kaitera	yes	no	yes
B21	Symptomless shoot	Mycelial isolate	Juha Kaitera	yes	no	yes
C23	Symptomless shoot	Mycelial isolate	Juha Kaitera	yes	no	yes
Viheriäistenneva	Symptomatic shoot	Ascospore isolate	Antti Uotila	no	no	no
HU 1.6	Symptomatic shoot	Ascospore isolate	Antti Uotila	no	no	no
Kankaanranta	Symptomatic shoot	Ascospore isolate	Antti Uotila	no	no	no
MH 1.6	Symptomatic shoot	Ascospore isolate	Antti Uotila	no	no	no
Oulanka	Symptomatic shoot	Ascospore isolate	Antti Uotila	no	no	no
ANY1	Symptomatic shoot	Unkown	Anneli Ylimartimo	no	no	no
A1	Symptomatic shoot	Mycelial isolate	Juha Kaitera	no	no	yes
B1	Symptomatic shoot	Mycelial isolate	Juha Kaitera	yes	no	yes
B13	Symptomatic shoot	Mycelial isolate	Juha Kaitera	no	no	yes
C5	Symptomatic shoot	Mycelial isolate	Juha Kaitera	no	no	yes
C8	Symptomatic shoot	Mycelial isolate	Juha Kaitera	no	no	no
Luumäki 2	Symptomatic shoot	Mycelial isolate (type not identified)	Jarkko Hantula	no	no	no
Luumäki 7	Symptomatic shoot	Mycelial isolate	Jarkko Hantula	no	yes	no
Luumäki 14	Symptomatic shoot	Mycelial isolate (type not identified)	Jarkko Hantula	no	no	no
Luumäki 15	Symptomatic shoot	Mycelial isolate (type not identified)	Jarkko Hantula	no	no	no
SurC1 (*Pinus contorta*)	Symptomatic shoot	Mycelial isolate (type not identified)	Jarkko Hantula	no	no	no
SurS2	Symptomatic shoot	Mycelial isolate (type not identified)	Jarkko Hantula	no	no	no
SurS3	Symptomatic shoot	Mycelial isolate (type not identified)	Jarkko Hantula	no	no	no
SurS4	Symptomatic shoot	Mycelial isolate	Jarkko Hantula	yes	yes	yes

were conducted: rate-zonal centrifugation was performed with isolate HR3 and isopycnic centrifugation with isolate SurS4 to determine the buoyant densities of different dsRNA molecules or patterns as described in I and IV, respectively. Furthermore, fractions were taken from the SurS4 ultracentrifugation experiment to determine by phenol extraction if the dsRNA molecules were somehow enclosed, as described in IV. Altogether ten dsRNA molecules found in *G. abietina* type A were cloned and sequenced as described in II–IV. For a general overview of the cloning process, see Figure 1. Sequences were compiled, analyzed and aligned with sequences obtained from the GenBank with the Vector NTI Suite 2 software package (InforMax Inc.). For alignment the CLUSTAL W algorithm (Thompson et al., 1994) with default parameters was used. The MEGA 2.1 program was used for phylogenetic analyses (Kumar et al., 2001). Amino acid sequences were searched through protein BLAST (Altscul et al., 1997) and "BLAST 2 Sequences" (Tatusova and Madden 1999) search engines of the National Center for Biotechnology Information (NCBI) or Baylor College of

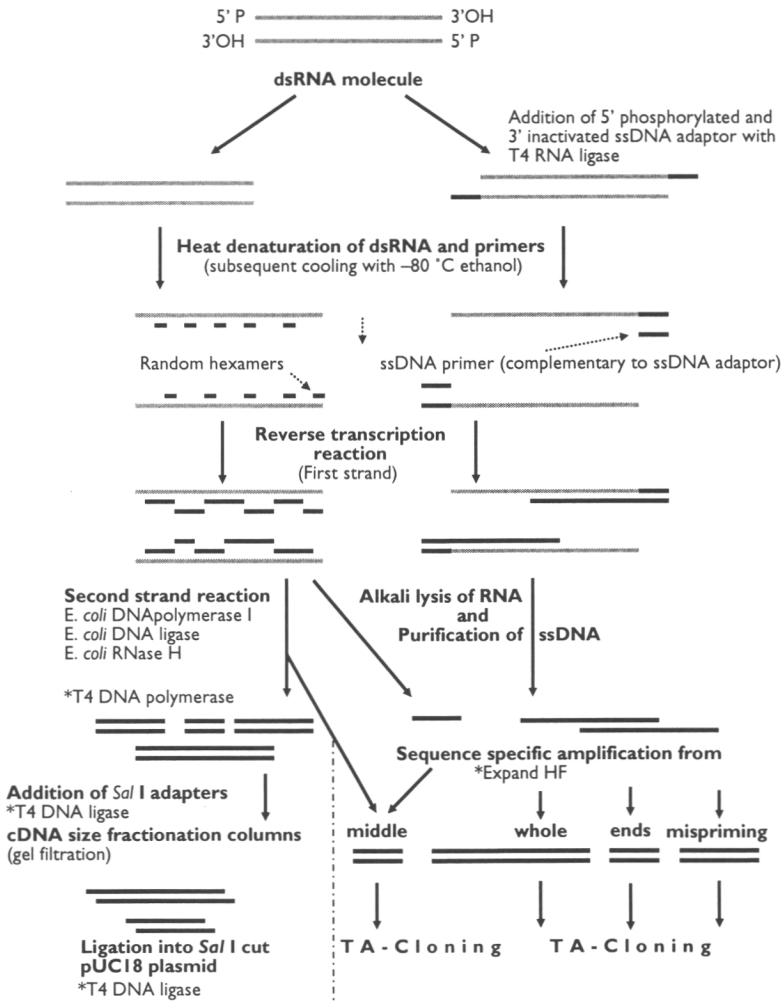

Fig.1. General overview of the cloning processes. For details, see II-IV.

Medicine HGSC (BCM) Search Launcher. Secondary structures were predicted with the RNA structure 3.6 program (Mathews et al., 1999).

4. Results and discussion

4.1 Identification of dsRNA patterns

Eleven isolates, identified to be *G. abietina* type A by RAMS fingerprints, contained dsRNA molecules (Table 1) based on binding to CF-11. Among these isolates three different dsRNA patterns (I, II and III) were found and they could be visualized in an agarose gel electrophoresis. The patterns were composed of one, three and one molecules with apparent sizes of 6000 bp; 1800, 1600 and 1200 bp; and 2600 bp, respectively. These patterns were treated with RNase (from bovine pancreas) and DNase I, which confirmed that the patterns were composed of dsRNA (not shown). All three dsRNA patterns were found to inhabit the SurS4 isolate of *G. abietina* type A (IV) and they were named as GaRV-L (L for lone), GaRV-MS (MS for multisegment) and GaMRV-S (S for single), respectively. All three dsRNA patterns could be isolated from conidia (N=15), thus indicating efficient transmission of all dsRNA patterns. This suggests that these three patterns are not maintained using the exactly same mechanisms as they seem not to disturb each other. The results of this study show clearly that dsRNA is common among isolates of *G. abietina* type A. In total 44% of all tested isolates harbored dsRNA and if all samples originating from ascospores (which are not expected to contain dsRNA molecules in ascomycetous fungi except mitoviruses) are excluded, 55% of isolates harbored dsRNA. This is not surprising as dsRNA has been found in many fungal species (Nuss and Koltin, 1990). dsRNA frequencies can wary considerably between species, as only 32% of *Sphaeropsis sapinea* (Steenkamp et al. 1998) isolates harbored dsRNA compared to 79% of *Discula destructiva* (Rong et al., 2001)

4.2 Effect of GaRV-L and GaRV-MS dsRNA patters on the pathogenicity of *G. abietina* type A

Two pathogenicity tests with *G. abietina* isolates harboring GaRV-L and GaRV-MS dsRNA patterns were performed (Table 2, in I), but no firm link between the presence of dsRNA and the pathogenicity of *G. abietina* towards *Pinus sylvestris* could be established. All isolates were pathogenic, but both dsRNA-containing and dsRNA-free isolates were found among the most pathogenic isolates. There was no difference between the pathogenicity of isolates containing GaRV-L and GaRV-MS patterns. It should be noticed, however, that the pathogenicity tests conducted could not be carried out with isogenic isolates with and without dsRNA, that the test trees were genetically different, and that the pathogenicity test setup used measured only how mycelium is able to grow in phloem after inoculation. However, the same test setup had previously been utilized successfully in a study of two biotypes of *G. abietina* (Terho and Uotila,

1993). It should also be noted that there was considerable difference in the canker lengths in experiments 1 and 2 (I), which was probably due to weather differences in different years, or because of the considerable difference in the sizes of the trees used in the two experiments. So, no firm conclusion can be drawn on the effect on dsRNA viruses on the pathogenicity of *G. abietina* type A.

4.3 Ultracentrifugation of dsRNA patterns

In the rate-zonal ultracentrifugation experiments on gently broken cells of isolate HR3 (Fig. 1 in I) GaRV-L and GaRV-MS patterns were separated (Fig. 2 in I). In a CsCl gradient of gently broken cells of isolate SurS4 three patterns could be separated (Fig. 1 in IV): dsRNA patterns GaMRV-S, GaRV-MS and GaRV-L were found in fractions 1, 7, and 8, respectively. The buoyant densities of fractions 7 and 8 were 1.37 and 1.42 g/cm^3, respectively, and phenol extraction was needed for successful isolation of all dsRNA molecules. This suggested that all three dsRNA patterns were somehow enclosed into compartments. There is no direct evidence about the nature of these compartments but the following speculation can be made. The film on top of the centrifuge tube containing the GaMRV-S pattern could contain lipid vesicles or organelles (mitochondria) with low buoyant density. The buoyant densities of fractions containing GaRV-MS and GaRV-L dsRNA patterns are typical of the members of the families *Partitiviridae* and *Totiviridae*. The virions of members in these two families are composed of dsRNA genome and protein capsid.

4.4 Sequences of GaMRV-S patterns from isolates Luumäki 7 and SurS4 (III and IV)

Altogether two GaMRV-S dsRNA patterns were sequenced from isolates Luumäki 7 and SurS4. The lengths of the dsRNA molecules were 2572 bp (GenBank sequence accession AF534641) and 2578 bp (AY615209), respectively. The GC content of these two molecules was 31% and they showed 94% nucleotide (nt) identity. The sequences of these two GaMRV-S patterns did not contain long open reading frames (ORFs) when using a normal translation table. However, when using a mitochondrial translation table, in which UGA codes for tryptophan, a long ORF could be identified in both patterns. These ORFs encoded for putative protein in isolates Luumäki 7 and SurS4 starting at positions 254 and 269, respectively. Both ORFs could potentially yield a protein of 741 amino acids (aa) with a predicted molecular mass of 85.4 kDa. These two proteins had 96% aa sequence similarity and both contained the conserved motifs of RNA-dependent RNA polymerase-like (RdRp) proteins encoded by mitochondrial viruses and related RNAs (Hong et al., 1999). As it became evident that the GaMRV-S patterns described here coded for putative viruses of *G. abietina* type A, the patterns were designated as *Gremmeniella abietina* mitochondrial RNA virus S (GaMRV-S). Isolates Luumäki 7 and SurS4 harbored strains 1 (GaMRV-S1) and 2 (GaMRV-S2), respectively. Other highly similar RdRps based on BLAST searches made on GaMRV-S2 were *Ophiostoma mitovirus 4* (OMV4; Hong et al., 1999; aa similarity 37%), *Ophiostoma mitovirus 6* (OMV5; Hong et al., 1999; aa similarity 34%) and *Ophiostoma mitovirus 6* (OMV6; Hong et al., 1999; aa similarity 37%) (Fig. 2 in IV). These viruses

are recognized members of the genus *Mitovirus*. This indicates that GaMRV-S1 and GaMRV-S2 are putative members of the genus *Mitovirus*. The members of the genus *Mitovirus* have a +ssRNA genome but they replicate via a dsRNA intermediate (Ghabrial, 2001), which was isolated in this study.

The putative initiation codons of RdRp of both GaMRV-S strains were located in an AU-rich context surrounded by regions of relatively high CG content. The same feature can also be found in a number of similar viruses (III and IV), albeit no conserved nt sequences were found. The function of such regions is unknown, but they should have a thermodynamically lower melting temperature compared to other regions in the genome. Another interesting feature in GaMRV-S viruses was that the ends of these two strains were not exact, as length and sequence variations occurred in both ends (Fig. 2 for GaMRV-S1; II, IV). Such a feature has not previously been found in

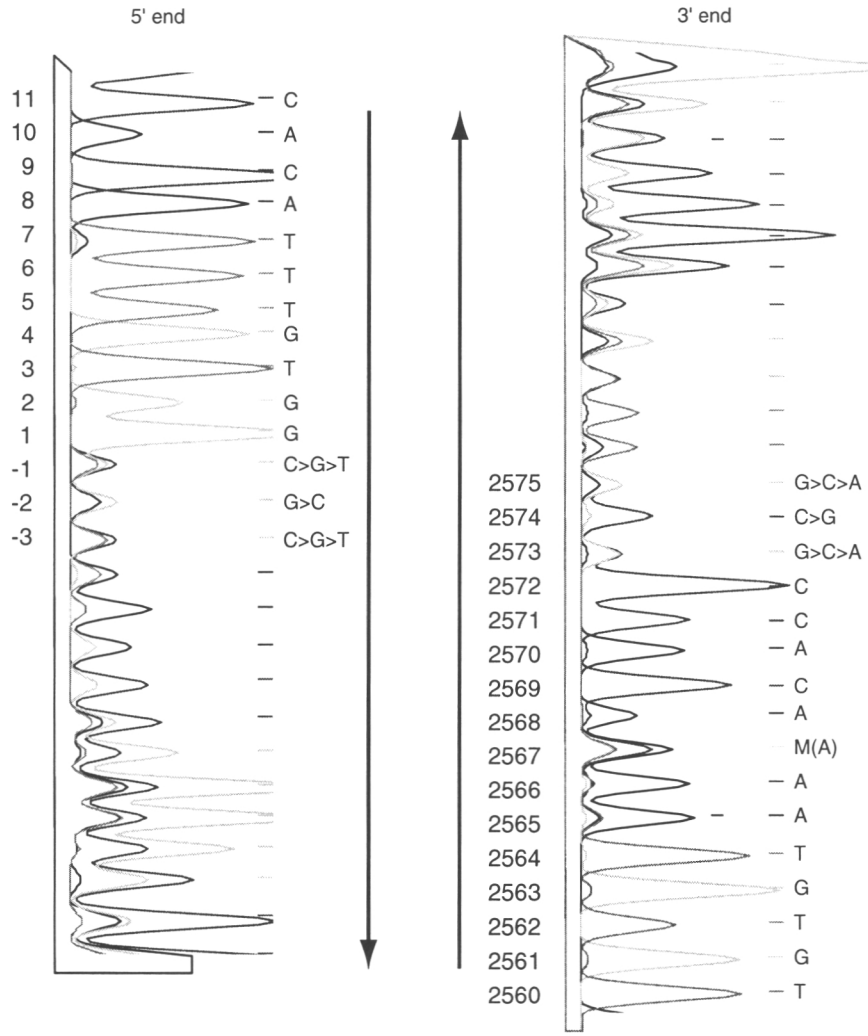

Fig.2. Direct sequencing experiment of the ends of GaMRV-S1. The arrows indicate the direction of sequencing reaction and the running numbers indicate the base position on the GaMRV-S1 genome.

similar viruses in fungi (II and IV). Panhandle and stem-loop structures were found in both untranslated regions (UTRs) of GaMRV-S1 genome (III). Similar structures were found in genomes of OMV4 and OMV6 but not in GaMRV-S2, *Ophiostoma mitovirus 3a* (Hong et al., 1998), OMV5 and *Sclerotinia homoeocarpa* mitovirus 1 (Deng et al., 2003), which contained only a 3'UTR stemloop structure. Despite this difference these findings together suggest that the viruses discussed here might have a recent common ancestor and therefore would be closely related to each other.

4.5 Sequences of GaRV-MS patterns from isolates C5 and SurS4 (II and IV)

Altogether six dsRNA molecules from two *G. abietina* type A isolates harboring GaRV-MS dsRNA pattern were sequenced from isolates C5 and SurS4. The lengths of these molecules were 1782 bp (AY089993), 1586 bp (AY089994), 1186 bp (AY089995) in isolate C5 and 1781 bp (AY615211), 1586 bp (AY615212) and 1186 bp (AY615213) in isolate SurS4, respectively. The corresponding molecules had nucleotide identities of 98%, 98% and 97%, respectively. An ORF could be identified in all molecules starting at nt 63 (539 aa, 62.1 kDa), 100 (433 aa, 47.1 kDa), 348 (237 aa, 26.6 kDa), 63 (539 aa, 62.1 kDa), 100 (433 aa, 47.1 kDa), and 348 (237 aa, 26.5 kDa), respectively. The corresponding putative proteins had similarities of 98%, 99.5% and 97%, respectively. The largest dsRNA molecules in both *G. abietina* isolates coded for RdRp as they contained the conserved motifs III, IV, V and VI found in the RdRps of dsRNA viruses infecting lower eukaryotes (Bruenn, 1993). Also two new possible conserved motifs (VIIa and VIIa) unique for certain partitiviruses were identified (Fig. 3 in IV). As it became evident that the GaRV-MS patterns described here coded for putative viruses of *G. abietina* type A, the patterns were designated as *Gremmeniella abietina* RNA virus MS (GaRV-MS). Isolates C5 and SurS4 harbored strains 1 (GaRV-MS1) and 2 (GaRV-MS2), respectively. Based on BLAST searches made on the putative RdRp of GaRV-MS2, other highly similar RdRps could be found in *Penicillium stoloniferum* virus S (Kim et al., 2003; aa similarity 71%), *Discula destructiva* virus 1 (DdV1) (Rong et al., 2002; 64%), *Discula destructiva* virus 2 (Rong et al., 2002;71 %) and *Fusarium solani virus 1* (FsV1) (Nogawa et al., 1996; 60%), of which FsV1 is a recognized member of the genus *Partitivirus* (Fig. 3 in IV). This shows that GaRV-MS1 and GaRV-MS2 are putative members of the genera *Partitivirus.* A BLAST search suggested that the 1586 bp molecule in both isolates would code for a putative coat protein (CP). The smallest molecule in both isolates showed some similarity with the putative protein of RNA3 in DdV1 (Rong et al., 2002). Besides sharing similarities in their coding region, also sequence similarities between these viruses were identified in their UTRs (Fig. 3 in II; IV). These findings suggest that all viruses discussed here might have a recent common ancestor and therefore would be closely related to each other.

4.6 Sequences of GaRV-L patterns from isolates HR2 and SurS4 (II and IV)

Altogether two GaRV-L dsRNA molecules were sequenced from isolates HR2 and SurS4. The lengths of the dsRNA molecules were 5122 bp (AF337175) and 5129 bp (AY615210), respectively, and they showed 90% identity. dsRNA molecules in isolates HR2 and SurS4 contained two large partially overlapping ORFs. The first ORF started at nt positions 276 (776 aa, 80.5 kDa) and 272 (776 aa, 80.4 kDa), respectively. Starting nucleotides for the second ORFs were 2603 (825 aa, 90.0 kDa) and 2559 (825 aa, 90.1 kDa), respectively. These two putative protein pairs showed 97% similarity to analogous predicted proteins described above. The protein encoded by second ORFs contained all eight conserved motifs of RdRps of viruses infecting lower eukaryotes (Bruenn, 1993). As it became evident that the GaRV-L patterns described here coded for putative viruses of *G. abietina* type A, the patterns were designated as *Gremmeniella abietina* RNA virus L (GaRV-L). Isolates HR2 and SurS4 harbored strains 1 (GaRV-L1) and 2 (GaRV-L2), respectively. Based on comparisons of RdRp by BLAST made on GaRV-L2, other highly similar viruses were *Sphaeropsis sapinea* RNA virus 2 (Preisig et al., 1998; aa similarity 50%), *Coniothyrium minitans* RNA virus (Cheng et al., 2003; 50%), *Helicobasidium mompa* Totivirus 1–17 (Nomura et al., 2003; 35%), *Sphaeropsis sapinea* RNA virus 1 (Preisig et al., 1998; 35%) and *Helminthosporium victoriae virus 190S* (HvV190S) (Huang and Ghabrial 1996; 35%) of which HvV190S is a recognized member of the genus *Totivirus* (Fig. 4 in IV). Therefore it can be concluded that GaRV-L1 and GaRV-L2 are putative members of the genus *Totivirus*. The first ORF in both isolates coded for putative CP, as BLAST searches indicated high similarity with analogous proteins of the viruses described above. Besides sharing similarities in their coding regions, sequence similarities between these viruses were also identified in their 5' UTR regions (Fig. 2 in II; IV) approximately 55 nt downstream from the CP starting godon. All viruses discussed here have partially overlapping ORFs to code CP and RdRp and the juncture point of ORFs contains an overlapping start/stop tetramer **AUGA** speculated to be a facilitator of reinitiation mechanism (Soldevila and Ghabrial, 2000) for the production of RdRp. These findings suggest that all viruses discussed here might have a recent common ancestor and therefore would be closely related to each other.

4.7 Phylogeny of RNA virus families with members found in *G. abietina*

An analysis of virions should answer the fascinating question about the evolution of viruses and their possible relationships. Research made on this topic shows that virions from different families share similarities in their virion structure (Bamford et al., 2002) and aa sequences of different proteins (Koonin et al., 1989; Koonin et al., 1991; Bruenn, 1991; Koonin, 1992; Koonin et al., 1993; Gibbs et al., 2000; Ahn a Lee, 2001). Of these studies Koonin et al. (1989; 1991; 1992; 1993), Gibbs et al. (2000), and Ahn and Lee (2001) are in favor of polyphyletic origin of dsRNA viruses whereas Bruenn (1991) favored a monophyletic origin.

Partitiviruses have been hypothesized to have originated from the genus *Totivirus* (Ghabrial, 1998). This hypothesis is not supported by the general picture of conserved motifs of RdRps of putative viruses of *G. abietina* (Figs. 2–4 in IV). Also the phylogenic analysis made on the RdRps of some putative members of the families *Totiviridae* and *Partitiviridae* (Fig. 1 in II) suggest that such a hypothesis is inaccurate. This investigation supports the theory of polyphyletic origin for GaMRV-S, GaRV-MS and GaRV-L viruses as their conserved motifs of RdRps are more similar to viruses or putative ORFs of non-fungal origin (Figs. 2–4 in IV). The RdRp of GaMRV-S is more similar to ORFs found in mitochondria of the plants *Arabidopsis thaliana* (Unseld et al., 1997) and *Brassica napus* L (Handa, 2003) than to the two other viruses found in *G. abietina*. Also the RdRp of GaRV-L was found to be more similar to Cucurbit yellows-associated virus (Coffin and Coutts, 1994), isolated from the plant *Cucumis sativus* L., than to the RdRps of the GaMRV-S and GaRV-MS. Finally, replicases of *Sweet potato feathery mottle virus* (Sakai et al., 1997) and *Sorghum mosaic virus* (Yang and Mirkov, 1997) of the genus *Potyvirus* were found to be somewhat similar to RdRp of GaRV-MS.

5. Concluding remarks

dsRNA molecules of polyphyletic origin, probably encoding viruses belonging to families *Narnaviridae*, *Partitiviridae* and *Totiviridae*, were identified in *G. abietina* type A. All three putative viruses were found from a single isolate of *G. abietina*. These viruses showed efficient transmission via conidia which probably is the main route for virus dispersal if the normal somatic hyphal growth is excluded. The co-existence of these viruses indicates that they are not probably maintained by using exactly the same mechanisms. In centrifugation experiments it was found that the RNAs genomes of these viruses were enclosed albeit the exact composition of these compartments was not identified. Unfortunately, the number of fungal isolates used in this study was quite small as isolates from symptomless trees, which were in priority in study, were hard to find. The viruses described here do not seem to induce hypovirulence in *G. abietina* type A. More isolations from symptomless trees could be done in order to find hypovirulent strains of *G. abietina*. One possibility to induce hypovirulence in *G. abietina* would be *in vitro* transfection of a *C. parasitica* hypovirus but such work involves major questions of ethics and biohazards.

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Paper I

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Two independent double-stranded RNA patterns occur in the Finnish *Gremmeniella abietina* var. *abietina* type A

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Summary

The occurrence of double-stranded RNA (dsRNA) among isolates of *Gremmeniella abietina* var. *abietina* type A was studied. Nine out of the 17 isolates investigated contained dsRNA, but none of them was of ascospore origin. Two fairly different dsRNA patterns were found and they occurred independently of each other. The first was composed of a single approximately 6000 bp long dsRNA molecule. The second was a multisegmented dsRNA pattern composed of three dsRNA molecules with apparent sizes of 1800 1600 and 1200 bp, respectively. In pathogenicity experiments no major differences were observed between dsRNA-containing and dsRNA-free isolates.

1 Introduction

Gremmeniella abietina (Lagerb.) Morelet var. *abietina* is the causative agent of Scleroderris canker on coniferous trees. In Finland two types of this fungus have been observed on Scots pine (*Pinus sylvestris* L.): type A (or large tree type, LTT) strains cause symptoms on both large trees and seedlings, whereas type B (or small tree type, STT) strains are found only in seedlings or shoots covered with snow during the winter (Uotila 1983; Kaitera et al. 1998). Isolates belonging to A and B types are able to produce artificial hybrids with low fitness (Uotila et al. 2000).

During the 1980s Finland suffered from severe Scleroderris canker epidemics, which were caused by *G. abietina* type A (Kaitera et al. 1998). It is anticipated that global climate change in Finland will result in increasing rainfall, which is considered to be beneficial for *G. abietina* (Kellomäki et al. 1988), and so the number of epidemics caused by this fungus may increase in future. In addition to being a problem in Finland and other parts of Europe, *G. abietina* type A has been introduced to North America, where it is causing increasing destruction among the conifers (Laflamme and Lachance 1987; Hamelin et al. 1996). Thus, attempts are needed, and some have already been made (Jacobi et al. 2000), to use novel control strategies against the fungus.

The occurrence of dsRNA is usually linked to virus infection in fungi. These virus-like-particles are not true viruses because they do not have extracellular life cycles. The three dsRNA virus families found among fungi are *Totiviridae*, *Partitiviridae* and *Hypoviridae* (van Regenmortel et al. 2000). Virions in *Totiviridae* have a single 4.6–7.0 kbp long and uncapped dsRNA molecule that codes for a coat protein and RNA-dependent RNA polymerase (RDRP). Members of *Totiviridae*, which infect fungi, belong to genus *Totivirus*. Members of *Partitiviridae*, which infect fungi, are divided to two genera: *Partitivirus* and *Chrysovirus*. Virions of *Partitiviridae* contain two unrelated linear dsRNA segments from 1.4

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to 2.2 kbp, which are separately encapsidated. The two segments of individual viruses have very similar sizes. The smaller segment usually codes for capsid protein and the larger for RDRP. *Chrysovirus* virions contain three to four unrelated and separately encapsidated dsRNA segments of about 3 kbp each. *Hypoviridae* family has only one genus, *Hypovirus* and they infect *Cryphonectria parasitica* fungus. These pleomorphic vesicles contain linear dsRNA which is about 9–13 kbp long. All three virus families may have additional segments (satellite and defective) present (VAN REGENMORTEL et al. 2000).

DsRNAs have also been observed in another pine pathogenic fungus, *Sphaeropsis sapinea* (STEENKAMP et al. 1998) and in a number of different plant pathogenic fungi (NUSS and KOLTIN 1990; ZHANG et al. 1994; NUSS 1996; MCCABE et al. 1999). DsRNA elements may have a significant effect to the phenotypes of their hosts as has been shown in the cases of *Cryphonectria parasitica* (Murrill.) Barr. (DAY et al. 1977) and *Ophiostoma ulmi* (Buism.) Nannf. (BRASIER 1983). Due to this capability the dsRNA of *C. parasitica* has been used as a biological control agent against chestnut blight. On most other pathogens, however, the effects have been less clear or non-existent (ZHANG et al. 1994; STEENKAMP et al. 1998).

In this study the occurrence of dsRNA was tested among Finnish isolates of *G. abietina* var. *abietina* type A. Furthermore, the effect of dsRNA on the pathogenecity of *G. abietina* was examined.

2 Materials and methods

2.1 Fungal isolates

The 17 fungal isolates examined are listed in Table 1. Three of them originated from studies on endophytic fungi, and thus have been isolated from healthy trees in areas where no epidemics prevailed. Three other isolates originate from areas with serious infections, but from symptomless shoots. The remaining 11 isolates originate from shoots showing symptoms. All isolates were grown on modified orange serum agar (MOS) (MÜLLER et al. 1994) at 15–20°C.

2.2 Identification of the *G. abietina* type

The type of most isolates has been previously identified. During this study the type of isolates HR1, HR2 and HR3 was identified using random amplified microsatellite (RAMS) fingerprints, which contain markers specific for each type. For details of identification, see HANTULA and MÜLLER (1997) and KAITERA et al. (1998).

2.3 DsRNA isolations

DsRNA was isolated by a modification of the method of MORRIS and DODDS (1979). In short, the fungal mycelium was disrupted and homogenized by quartz sand in a lysis buffer [50 mM Tris-HCl pH 8.0, 50 mM EDTA, 3% sodium dodecyl sulphate (SDS) and 1% β-merkaptoethanol]. After the disruption two phenol-chloroform (1 : 1) extractions followed by one chloroform-isoamylalcohol (24 : 1) extraction were carried out. The obtained material was ethanol-precipitated, and resuspended in TSE buffer (10 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA) containing 15% ethanol, and mixed with CF-11 cellulose for 10 min on ice. The material was transferred to a column, and washed with TSE buffer containing 15% ethanol. Finally the dsRNA was eluted by TSE buffer, precipitated with ethanol, dried under a vacuum and resuspended in TE (6 mM Tris-HCl pH 8.0, 1 mM EDTA).

2.4 DNase 1 treatment

The DNase treatment (30 min at 37°C) was carried out using 10 units of DNase 1 (Boehringer Mannheim, Mannheim, Germany) in a DNase buffer (4 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$). The common cloning vector pUC18 was used as a positive control.

2.5 RNase treatment

The RNase treatment (30 min at 37°C) was carried out using 0.1 μg RNase (from bovine pancreas, Boehringer Mannheim) in 1 × SSC (0.15 M NaCl – 0.15 M sodium citrate, pH 7.0) or in 0.01 × SSC. For control, a subsample from a dsRNA isolation sample was taken before the CF-11 column. In this subsample both DNA and single-stranded RNA (ssRNA) were still present.

2.6 Electrophoresis

The material from DNase 1 and RNase treatments were analysed in 1% agarose gels (FMC BioProducts, Rockland, ME, USA) using *Pst*I-, *Eco*RI- and *Xho*I-digested bacteriophage λ DNA as a size control. When necessary, agarose gels supplemented with 1% synergel (Diversifield Biotech, Boston, MA, USA) were used. The electrophoreses was carried out in TAE-buffer (40 mM Tris-acetate pH 8.0, 1 mM EDTA).

2.7 Ultracentrifugations

Isolates HR2, HR3 and C5 were used for ultracentrifugation experiments. Sample preparation for ultracentrifugation was performed with ULTRA-TURRAX® TP-18/10 (Janke and Kunkel GmbH and Co KG IKA-Werk, Staufen, Germany) homogenizer at 4°C using 50 ml test tubes. Mycelia (2–3 g) grown on MOS agar for 4–8 weeks at 20°C were homogenized in 2 ml of osmotic stabilizer (OS) buffer (0.6 M NaCl in phosphate buffer pH 6.0) (PHILLIPS 1993) for 30 s. The homogenized mycelia were pelleted with Labofuge GL (Heraeus Christ GmbH, Osterode, Germany) centrifuged for 10 s at RCF_{max} = 2800 **g**. The homogenization and centrifugation steps were then repeated. The supernatant was stored on ice until used in the centrifugations.

The 34–68% sucrose gradient ($RCF_{average}$ = 71 000 **g**, 20 h) centrifugations were performed with Sorvall® Discovery™ 100 (Sorvall Products, L.P Newtown, CT, USA) ultracentrifuge using TH-641 rotor (Dupont; Sorvall Products) at 4°C. The gradients were fractionated and the occurrence of dsRNA in fractions was tested electrophoretically as described above.

2.8 Pathogenesis of type A *G. abietina* on *Pinus sylvestris*

The pathogenicity of dsRNA harbouring fungal isolates was tested at Hyytiälä Forestry Field Station (University of Helsinki) as described by TERHO and UOTILA (1999). Inoculations of experiment 1 were done in October 1997 on Scots pine trees, 2 m in height, derived from seeds. Five trees were inoculated twice with the same isolate. *Gremmeniella abietina* type A isolates HR3, C23, MH 1.6 and type B isolate Siika 1.1 were used. Thus 10 replicates were made with each isolate. The mycelia were put into the phloem of the trees. The holes in the tree stem were made using a 4 mm cork borer and the bark was replaced over the mycelium. The instruments used were sterilized between inoculations with 70% ethanol. Six control inoculations without mycelia were also carried out. In June 1998 the pathogenicity of each isolate was estimated by

Table 1. **Occurrence of the dsRNA molecules in *G. abietina* isolates obtained from symptomless shoots or from shoots showing typical symptoms of Scleroderris canker**

Isolate	Origin	Culture type	Collector	6000 bp dsRNA	1800, 1600 and 1200 bp dsRNA
HR1	Symptom-free tree	Mycelial isolate	Dr Hanna Ranta	no	no
HR2	Symptom-free tree	Mycelial isolate	Dr Hanna Ranta	yes	no
HR3	Symptom-free tree	Mycelial isolate	Dr Hanna Ranta	yes	yes
A26	Symptom-free shoot	Mycelial isolate	Dr Juha Kaitera	yes	yes
B21	Symptom-free shoot	Mycelial isolate	Dr Juha Kaitera	yes	yes
C23	Symptom-free shoot	Mycelial isolate	Dr Juha Kaitera	yes	yes
Viheriäistenneva	Symptomatic shoot	Ascospore isolate	Dr Antti Uotila	no	no
HU 1.6	Symptomatic shoot	Ascospore isolate	Dr Antti Uotila	no	no
Kankaanranta	Symptomatic shoot	Ascospore isolate	Dr Antti Uotila	no	no
MH 1.6	Symptomatic shoot	Ascospore isolate	Dr Antti Uotila	no	no
Oulanka	Symptomatic shoot	Ascospore isolate	Dr Antti Uotila	no	no
ANY1	Symptomatic shoot	Unkown	Dr Anneli Ylimartimo	no	no
A1	Symptomatic shoot	Mycelial isolate	Dr Juha Kaitera	no	yes
B1	Symptomatic shoot	Mycelial isolate	Dr Juha Kaitera	yes	yes
B13	Symptomatic shoot	Mycelial isolate	Dr Juha Kaitera	no	yes
C5	Symptomatic shoot	Mycelial isolate	Dr Juha Kaitera	no	yes
C8	Symptomatic shoot	Mycelial isolate	Dr Juha Kaitera	no	no

measuring the length of the lesion under the bark. Inoculations of experiment 2 in October 1999 were carried out in a similar manner in the field as described above on Scots pines, 0.5 m tall, derived from seeds. The fungal isolates used were C5, C23, HR2, HR3, MH 1.6 and Valkea 1.2, and 12 replicates were made. Ten control trees were punctured with a sterile cork borer. The pathogenicity was measured in May 2000 as described above. The results were analysed with non-parametric Kruskall–Wallis test, because the variances were not the same between isolates. *Gremmeniella abietina* was isolated from the infected tissue of the first and last tree of each set and tested for their RAMS fingerprints.

3 Results

3.1 Identification of dsRNA

Only isolates confirmed to be *G. abietina* type A by RAMS fingerprinting were studied. Nine isolates contained material which was bound to CF-11 cellulose and formed bands in an agarose gel. Although CF-11 cellulose is considered to bind specifically dsRNA, the nature of these bands as dsRNA was further tested by treatments with DNaseI and RNase (from bovine pancreas) as described by PRYOR and BOELEN 1987). The DNase treatment did not have any effect on the obtained material in conditions in which 100 ng of plasmid DNA was completely digested. The obtained material was also resistant to RNase treatment in conditions of high salt concentration where ssRNA was digested. However, in conditions of low salt concentration both ssRNA and dsRNA were digested. Based on these experiments we concluded that the obtained material was dsRNA.

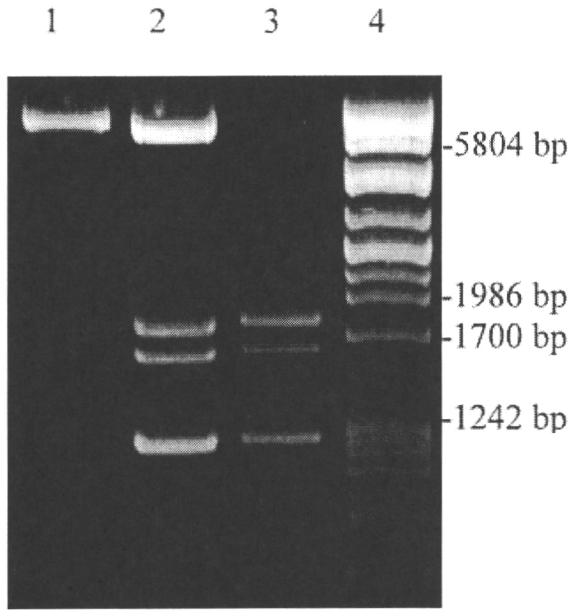

Fig. 1. The common dsRNA patterns observed in *G. abietina* type A. The samples are as follows: (1) dsRNA from isolate HR2; (2) dsRNA from isolate HR3; (3) dsRNA from isolate C5 and (4) is λ DNA digested with restriction enzymes *Pst*I, *EcoR*I and *Xho*I. The essential band sizes of DNA marker are marked on the right

Table 2. **The pathogenicity of *G. abietina* isolates in two inoculation experiments. Isolate C5 harboured 1800, 1600 and 1200 bp dsRNA molecules. Isolates HR3 and C23 harboured 6000, 1800, 1600 and 1200 bp dsRNA molecules. Isolate HR2 harboured a 6000 bp dsRNA molecule. Other isolates did not contain dsRNA**

	Canker length (mm)	
Isolate	Mean	Standard deviation
Experiment 1		
HR3	102.3	31.8
C23	89.7	54.1
MH 1.6	108.1	28.3
Siika 1.1	86.3	14.8
Control	17.8	2.0
Experiment 2		
C5	40.6	11.9
C23	52.2	14.9
HR2	53.8	10.0
HR3	40.3	4.5
MH 1.6	43.8	8.0
Valkea 1.2	52.8	7.0
Control	6.4	0.7

3.2 DsRNA molecules in type A *G. abietina*

Four molecular weight classes of dsRNA were observed (Fig. 1; Table 1). The apparent sizes of these molecules were 6000, 1800, 1600 and 1200 bp when compared to a DNA standard.

The 6000 bp band was observed in six isolates (Table 1). The 1800, 1600 and 1200 bp coexisted in eight isolates, five of which also contained the 6000 bp molecule. The 6000 bp molecule was, however, observed alone in the isolate HR2.

No dsRNA was observed in cultures derived from single ascospores. In mycelial isolates 1800, 1600 and 1200 bp molecules occurred in four out of the six isolates obtained from symptom-free shoots and in four out of the six isolates from shoots showing symptoms of Scleroderris canker. Thus, the distribution of these molecules did not correlate to symptoms. In contrast, the 6000 bp molecule was observed in five out of six isolates obtained from symptom-free shoots, but only in one isolate originating from a shoot showing symptoms. Thus, there was a statistically significant difference from a random distribution ($p = 0.0400$, Fisher's exact test).

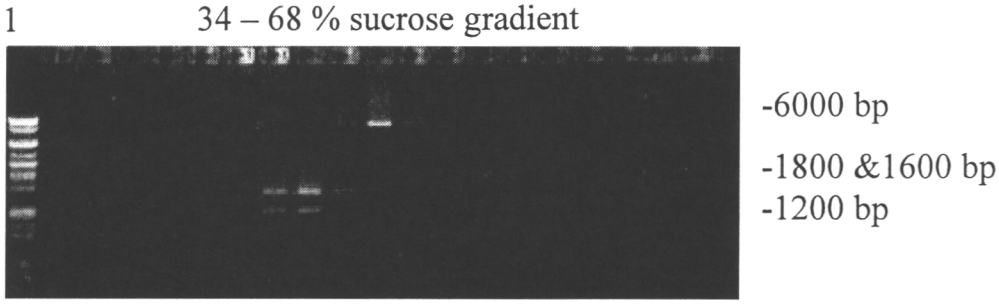

Fig. 2. Ultracentrifugation of disrupted mycelium of isolate HR3. The first sample is λ DNA digested with restriction enzymes *Pst*I, *Eco*RI and *Xho*I. The other samples show the occurrence of dsRNA in the fractions as the density of the sucrose gradient increases from left to right (34–68%). The 1800 and 1600 bp bands have not yet been separated

Ultracentrifugation was performed on gently broken cells in order to test the separation of different dsRNA molecules within the mycelium. The migration rate of 6000 bp dsRNA was different from that of 1800, 1600 and 1200 bp dsRNA molecules (Fig. 2).

3.3 The effect of dsRNA on the pathogenicity of *Gremmeniella abietina* type A

Pathogenicity tests were performed in order to obtain information about the effect of the presence of dsRNA on the fungus. There was (Table 2), however, no obvious link between the presence of dsRNA in fungi and the pathogenicity of *G. abietina* type A towards *P. sylvestris* in the present test conditions. It seems that the variation in canker size was caused by other factors than the presence of dsRNA in *G. abietina* type A. All isolates were pathogenic when compared with controls ($p < 0.0001$). There were also differences between isolates in canker size ($p < 0.01$ in experiment 1), but both dsRNA-containing and dsRNA-free isolates were observed among the most pathogenic isolates. No differences were found between isolates containing either the 6000 bp or the three other dsRNA molecules. The original dsRNA patterns were found from mycelia re-isolated from cankers in all cases in which this was tested.

4 Discussion

The results of this study show that dsRNA is common among isolates of *G. abietina* type A as, in total, 53% of all tested isolates contained dsRNA. If the samples originating from ascospores were excluded, then the natural proportion of dsRNA-containing isolates is 82%. This is not surprising, as dsRNA is known from a large number of fungi (Nuss and Koltin 1990), and as for example most of the studied *Puccinia* isolates contain dsRNA (Zhang et al. 1994). Thus, the present finding supports the idea that dsRNA would be a common genetic element among fungi.

Four dsRNA molecules were observed among *G. abietina* type A isolates in Finland. Molecules with sizes of 1800, 1600 and 1200 bp were linked to each other as they co-occurred in the same isolates and they had similar mobility during ultracentrifugation. However, the 6000 bp molecule was found in isolates with and without the other three dsRNA molecules and it had unique mobility characteristics in centrifugation. Furthermore, the 1800, 1600 and 1200 bp molecules were found in isolates with and without the 6000 bp molecule. Based on these observations it was concluded that two separate dsRNA patterns occur in type A *G. abietina*. They were designated as GaRV-L (L for lone) and GaRV-MS (MS for multisegment). Based on what is known about dsRNA in fungi GaRV-L might be a member of the *Totiviridae* and GaRV-MS the *Partitiviridae* family, although sequence data is needed to confirm this. The finding of two different dsRNA patterns in the same fungal species is not unique, as many other similar cases are also known (e.g. Sanderlin and Ghabrial 1978).

The dsRNA pattern composed of only the 6000 bp molecule was correlated with the origin of isolates from symptomless shoots. This might suggest that GaRV-L would reduce the virulence of its host. We tested this hypothesis by pathogenicity experiments, which however, did not support our hypothesis. Therefore it was concluded that dsRNA does not seem to have a major effect on the pathogenicity of *G. abietina*. It should, however, be pointed out that it was not possible to carry out these tests using isogenic isolates with and without virus infection, the experiment trees had different genotypes and that the pathogenicity experiments conducted during the present study tested only how the mycelium can grow in phloem after penetration. The same method has previously been successfully used, for example, to examine differences in pathogenicity between two *G. abietina* biotypes (Terho and Uotila 1999). In the present study considerable differences in canker length were observed between experiments 1 and 2, which probably

were due to weather changes from year to year or differences in the tree sizes used in the two experiments.

The main conclusions of this study are that (i) dsRNA is common among isolates of *G. abietina* type A in Finland, and (ii) two fairly different dsRNA patterns were found.

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Résumé

Deux profils indépendants d'ARN doubles brins sont présents chez le Gremmeniella abietina var. abietina de type A de Finlande

L'existence d'ARN doubles brins (dsRNA) a été étudiée chez des isolats finlandais de *Gremmeniella abietina* var. *abietina* de type A. Parmi dix-sept isolats examinés, neuf contenaient un dsRNA, mais aucun d'entre eux n'était d'origine ascosporée. Deux profils nettement différents de dsRNA ont été trouvés, indépendants l'un de l'autre. Le premier était constitué d'une molécule unique d'environ 6000 pb. Le second présentait un profil en plusiers fragments de tailles apparentes de 1800, 1600 et 1200 pb respectivement. En test de pouvoir pathogène, nous n'avons pas observé de différence notable entre les isolats contenant ou pas d'ARN doubles brins.

Zusammenfassung

Vorkommen von zwei voneinander unabhängigen doppelsträngigen RNS in Gremmeniella abietina var. abietina Typ A aus Finnland

Bei 17 Isolaten von *Gremmeniella abietina* var. *abietina* Typ A wurde das Vorkommen von doppelsträngiger RNS (dsRNS) untersucht. Dabei enthielten neun Isolate dsRNS, jedoch keines von ihnen stammte aus Ascosporen. Es wurden zwei deutlich unterschiedliche dsRNS-Muster gefunden, die unabhängig voneinander vorkamen. Das eine dsRNS-Muster bestand aus einem einzigen, ungefähr 6000 bp langen Molekül. Das zweite war multisegmentiert und bestand aus drei dsRNS-Molekülen mit der ungefähren Grösse von jeweils 1800 bp, 1600 bp und 1200 bp. In Pathogenitätstests waren keine grösseren Unterschiede zwischen Isolaten mit und ohne dsRNS zu beobachten.

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Paper II

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II

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Two unrelated double-stranded RNA molecule patterns in *Gremmeniella abietina* type A code for putative viruses of the families *Totiviridae* and *Partitiviridae**

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Summary. Two double stranded (ds) RNA molecule patterns, probably of viral origin, were sequenced from *Gremmeniella abietina* var. *abietina* type A. The genome of *Gremmeniella abietina* RNA virus L1 (GaRV-L1) from isolate HR2 was 5133 bp and contained two open reading frames (ORFs). The 5′-proximal ORF coded for a putative coat protein (CP) and the 3′-proximal ORF encoded putative RNA-dependent RNA polymerase (RdRp). GaRV-L1 had sequence similarities with a previously described totivirus (*Helminthosporium victoriae 190S virus*) and two unclassified members of family *Totiviridae* (*Sphaeropsis sapinea* RNA virus 1 and *Sphaeropsis sapinea* RNA virus 2). The genome of *Gremmeniella abietina* RNA virus MS1 (GaRV-MS1) from isolate C5 was composed of three dsRNA molecules coding for a putative RdRp (dsRNA1), a putative CP (dsRNA2) and protein of unknown function (dsRNA3). The lengths of these dsRNA molecules were 1782, 1586 and 1181 bp, respectively. Sequence comparisons indicated that the GaRV-MS1 dsRNA pattern comprises a putative virus that is highly similar to *Discula destructiva* virus 1, *Discula destructiva* virus 2 and *Fusarium solani virus 1* of the family *Partitiviridae*.

Introduction

The occurrence of double stranded (ds) RNA in fungi is usually associated with a virus infection. The four fungal dsRNA virus families are *Chrysoviridae*,

*The GenBank accession numbers of the sequences of GaRV-L1 and GaRV-MS1 (composed of dsRNA1, dsRNA2 and dsRNA3) reported in this paper are AF337175, AY089993, AY089994 and AY089995, respectively.

Hypoviridae, *Partitiviridae* and *Totiviridae* [16, 36]. Members of the family *Partitiviridae* are isometric cytoplasmic viruses with a genome composed of two linear 1.4–3.0 kbp dsRNA segments [8]. The smaller RNA codes for a coat protein (CP) and the larger RNA for a virion-associated RNA-dependent RNA polymerase (RdRp) [8]. Additional satellite RNA or defective RNA segments may be present. Transcription and replication are based on a semi-conserved mechanism [8]. Family *Partitiviridae* was divided into four genera. *Partitivirus* and *Chrysovirus* for viruses that infect fungi, and *Alphacryptovirus* and *Betacryptovirus* for viruses that infect plants [7]. Recently the genus *Chrysovirus* has been taken out of the family *Partitiviridae* and is placed into the new family *Chrysoviridae* [16]. All these viruses cause latent infections and may have originated from totiviruses [6]. Members of the family *Totiviridae* are isometric dsRNA viruses with single, linear, uncapped 4.6–7 kbp dsRNA genomes [39]. The mRNA for CP and RdRp proteins is produced via a conserved mechanism [39]. Lower eukaryotes (fungi and protozoa) often carry latent infections of totiviruses, which may have shared an archetypical ancestor before fungi and protozoa diverged [3]. The effect of dsRNA varies but usually no symptoms on the host are observed.

Gremmeniella abietina (Lagerb.) M. Morelet var. *abietina* is the causative agent of Scleroderris canker on coniferous trees. In Finland, two variants of this fungus occur on Scots pine (*Pinus sylvestris* L.): type A (or large tree type, LTT) strains cause symptoms on both large trees and seedlings, whereas type B (or small tree type, STT) strains are found only in seedlings or shoots covered with snow during the winter [12, 33]. The Alpine type, present in Central Europe, and the endemic North American (NA) type are two additional *G. abietina* variants. The two variants of *G. abietina* in Finland should probably be considered as closely related, but distinct species [34].

The occurrence of two cryptic (i.e. no associated symptoms) dsRNA patterns designated as GaRV-L and GaRV-MS in *G. abietina* type A was recently reported [32]. In this study a representative of each pattern was cloned and sequenced.

Methods

Fungal isolates and large scale purification of dsRNA

The GaRV-L1 (*G. abietina* RNA virus L, strain 1) and GaRV-MS1 (*G. abietina* RNA virus MS, strain 1) dsRNA patterns to be cloned and sequenced were derived from *G. abietina* type A isolates HR2 and C5, respectively [32]. The GaRV-L1 pattern was composed of a single approximately 6000 bp dsRNA molecule. GaRV-MS1 was a multisegmented dsRNA pattern composed of three dsRNA molecules with apparent lengths of 1800 bp (dsRNA1), 1700 bp (dsRNA2) and 1200 bp (dsRNA3), respectively. The isolates were grown on modified orange serum agar [18] covered with a cellophane membrane at 15–20 °C.

A large scale modification of the method of Morris and Dodds [17] based of CF-11 (Whatman, Maidstone, England) was developed in order to purify large amounts of dsRNA. Mycelia (40 g) were homogenized in lysis buffer (50 mM Tris-HCl pH 8.0, 50 mM EDTA,

3% sodium dodecyl sulphate and 1% β-mercaptoethanol) twice with a ULTRA-TURRAX® TP-18/10 (Janke & Kunkel GmbH & co KG IKA-Werk, Staufen, Germany) homogenizer, and extracted with water saturated phenol (pH 8.0). Hexadexyltrimethylammonium bromide (CTAB) (Sigma H-6269) and NaCl were added to final concentrations of 1% and 425 mM, respectively, and samples were incubated at 65 °C for 10 min. Phenol:chloroform:isoamylalcohol (25:24:1) extraction was followed with two cloroform:isoamylalcohol (24:1) extractions. After the supplementation of ethanol (16.5%) and CF-11, samples were mixed and centrifuged. Supernatants were discarded and CF-11 pellets were suspended to TSE buffer (10 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA) supplemented with 15% ethanol. Columns were loaded with CF-11 sample suspension and washed with 100 ml of TSE + 15% ethanol buffer. The dsRNA bound to CF-11 was eluted with TSE buffer without ethanol and divided to fractions. Samples from each fraction were assayed for dsRNA in an agarose gel. Fractions containing dsRNA were pooled and precipitated with ethanol. The dsRNA was finally purified by electrophoresis in an agarose LM-MP gel (Boehringer Mannheim GmbH, Mannheim, Germany). Each dsRNA band was cut out with scalpel and extracted from LGT-agarose with the "RNaid Kit" (Bio 101, CA, U.S.A.) as described by the manufacturer. Finally, the purified dsRNA was precipitated with ethanol and dissolved into diethylpyrocarbonate (DEPC) treated water. The concentration of purified dsRNA was determined in an agarose gel using DNA standards of known concentration. With this method yields from isolates HR2 and C5 were 25 μg and 1 μg per dsRNA molecule, respectively.

Cloning of viral cDNA with random primers

The cloning of random cDNA from dsRNA molecules was performed with the "SuperScript™ Plasmid System for cDNA Synthesis and Plasmid Cloning" kit (Life technologies inc., Rockville, MD, U.S.A.). The cDNA synthesis was performed as described by the manufacturer except that 5 μg of $pD(N)_6$ Random Hexamers (Amersham Pharmacia Biotech, Uppsala, Sweden) were used as primers and denaturation was done by boiling 0.4 to 1.5 μg of dsRNA (dissolved into DEPC treated water) with primer mixture for 10 min. The denaturated sample was rapidly cooled at −80 °C. After the first- and second strand synthesis, and the addition of linkers, the dsDNA was ligated to pUC18 (Amersham Pharmacia Biotech, Uppsala, Sweden) cut with *SalI* (Boehringer Mannheim GmbH, Mannheim, Germany). Ligated plasmids were transformed into *Escherichia coli* DH5α (Life technologies inc., Rockville, MD, U.S.A.) and screened for inactivity of β-galactosidase. Plasmids were isolated with QIAprep® Miniprep (Qiagen GmbH, Hilden, Germany), and the size of the cloned cDNA was estimated by electrophoresis of PCR amplified inserts. For this purpose M13 reverse and forward (−20) primers of "TOPO TA Cloning" kit (Invitrogen corp., CA, U.S.A.) were used with DyNAzyme™ II (Finnzymes OY, Espoo, Finland) thermostable polymerase as described by the manufacturers.

Cloning of the 3′ ends

The 3′ ends of the viral dsRNAs were cloned as described by Lambden et al. [15] using 5′ phosphorylated and 3′ inactivated T4 RNA ligase adaptor (GCATTCGACCCGGGTT) (Amersham Pharmacia Biotech, Uppsala, Sweden). Ligation was performed as described by the manufacturer using 0.4–5 μg of dsRNA and 3.5 μg of T4 RNA ligase adaptor combined on ice and ten units of T4 RNA ligase (Fermantas, Vilnius, Lithuania), except that a final concentration of 10% of dimethyl sulfoxide (DMSO) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was used and the ligation reaction was performed at 4 °C for 20 h. To remove free adaptors, the "High pure PCR product purification kit" (Boehringer Mannheim GmbH, Mannheim,

Germany) was used as described by the manufacturer. SuperScriptII RT (Life Technologies inc., Rockville, MD, U.S.A.) enzyme was used in the first strand reaction as described by the manufacturer except that dsRNA (1 μg) and RT primer (AACCCGGGTCGTATGC) (1 μg) were denaturated by boiling (10 min) and subsequent cooling at −80 °C. One μl of 500 mM EDTA (pH 8.0) was added to stop the RT reaction. RNA was hydrolyzed by adding 3 μl of 1 M NaOH and incubating the reaction for 60 min at 65 °C. The reaction was neutralized by adding 10 μl of 500 mM Tris-HCl (pH 6.9) and 3 μl of 1 M HCl. Short ssDNA fragments were removed with the "High pure PCR product purification kit" as described by the manufacturer. The eluted samples were used as templates in PCR. Specific primers, based on sequences obtained from primary clones, were designed to anneal to each end and were used separately with the RT primer to amplify the ends. For amplification "Expand^{TM} High fidelity PCR system" (Boehringer Mannheim GmbH, Mannheim, Germany) was used as described by the manufacturer (initial denaturation 95 °C 10 min, followed by 20–25 cycles of amplification 95 °C 1 min, 62 °C 1 min, 68 °C 2 min and final extension (68 °C 4 min)). The PCR products were purified with "High pure PCR product purification kit", cloned with "TOPO TA Cloning" kit and screened as described above.

Sequencing and computer aided analyses

Cloned viral cDNAs were sequenced with a 4200L-2 NEN Global IR^2 System (LI-COR inc., Lincoln, NE, U.S.A.) using the SequiTherm EXCELII sequencing kit (Epicentre, Madison, WI, U.S.A.) with IRD700 labelled M13 Forward and IRD800 labelled M13 Reverse oligonucleotides as described by the manufacturer. Sequences were compiled, analysed and aligned (with sequences from Genebank) using Vector NTI Suite 2 (InforMax inc., MD, U.S.A.) software package. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 [14]. Complete and partial nucleic acid sequences were screened through "BCM Search Launcher: Nucleic Acid Sequence Searches" (Baylor College of Medicine HGSC, http://searchlauncher.bcm.tmc.edu). Complete amino acid (aa) sequences were screened trough protein BLAST [2] and "BLAST 2 Sequences" [31] search engines of National Center for Biotechnology Information (NCBI).

Results

Cloning and genome organization of GaRV-L1 dsRNA pattern

The uttermost 3′ ends of linear dsRNA molecule were independently cloned twice and identical clones were obtained from each end. The remainder of the genome was cloned and sequenced independently three to eight times. The dsRNA genome of GaRV-L1 (accession number AF337175) was 5133 base pairs (bp) with a GC content of 57%. Both strands terminated at the 3′-end with UGC triplet. The genome contained two large open reading frames (ORFs) on the same strand and which overlapped by a single nucleotide. ORF1 encoded a putative CP (according to BCM Search Launcher the smallest sum probability values were lowest with members of family *Totiviridae* CPs). ORF1 started at nt 276 (AUG) and ended at nt 2604 (UGA), thus encoding a 776 amino acid (aa) protein with a predicted molecular mass of 80.5 kDA. The second ORF2 started at nt 2603 (AUG) and ended at nt 5078 (UGA), thus encoding a 825 aa protein with a predicted molecular mass of 90.0 kDA. This second ORF encodes a putative RdRp, in which all

eight conserved motifs of RdRps from dsRNA viruses of lower eukaryotes [3] were found (data not shown). In comparison with sequences in Genbank, ORF1 (putative CP) and ORF2 (putative RdRp) had highest similarities with homologous proteins of an unclassified member of the family *Totiviridae*, *Sphaeropsis sapinea* RNA virus 2 (SsRV2) [23], with aa sequence similarities of 60% and 50%, respectively. Based on BLAST searches other similar RdRp and CPs were those of *Sphaeropsis sapinea* RNA virus 1 (SsRV1) [23] and *Helminthosporium victoriae virus 190S* (HvV190S) [9]. These four putative viruses shared a proline rich C-terminus (130 amino acid) of their CP (containing 26, 25, 21, 23% of proline, respectively).

Cloning and genome organization of GaRV-MS1 dsRNA pattern

The complete dsRNA1 sequence (accession number AY089993) was derived from twenty independently produced clones, with each nucleotide position determined from at least five independent sequencing reactions. The length of dsRNA1 was 1782 bp with a GC content of 50%. DsRNA1 included one ORF spanning 91% of the molecule. This ORF started at nt 63 and ended at nt 1680, thus encoding a 539 aa protein with a predicted molecular mass of 62.1 kDA. This putative protein contained conserved RdRp motifs 3, 4, 5, and 6 from dsRNA viruses infecting lower eukaryotes [3] and, therefore, probably coded for the viral RdRp (data not shown). The RdRp was most similar to the corresponding protein of an unclassified member of the family *Partitiviridae*, *Discula destructiva* virus 1 (DdV1) with identity of 65% [24].

DsRNA2 sequence (AY089994) was compiled from twelve independently produced clones, with each nucleotide position determined from at least three sequencing reactions. DsRNA2 was 1586 bp with a GC content of 52%, containing one ORF spanning 84% of the molecule. This ORF started at nt 100 and ends at nt 1429, thus encoding a 433 aa protein with a predicted molecular mass of 47.1 kDa. This putative CP had lowest sum probability values with CPs of viruses in the family *Partitiviridae* according to BMC Search Launcher. The CP had highest similarity (51%) with the corresponding protein of an unclassified member of the family *Partitiviridae*, *Discula destructiva* virus 2 (DdV2) [24].

DsRNA3 sequence (AY089995), was compiled from nine independently produced clones with each nucleotide position determined from at least six reactions. DsRNA3 was 1186 bp with a GC content of 47%. This segment contained one ORF spanning only 64% of the dsRNA3. This ORF started from nt 348 (AUG) and ended at nt 1059 (UAG), thus encoding a 237 aa protein with a predicted molecular mass of 26.6 kDa. Surprisingly, no similarities with known proteins were found when this putative protein was screened through BCM Search Launcher and NCBI Protein BLAST search engines. However, placement and size of this ORF resembled the putative ORF of DdV1 RNA3 [24]. As "BLAST 2 Sequences" [31] analysis showed significant similarity (expect value 1e-18) between these two putative proteins, we deposited the gene product of dsRNA3 into GenBank. As

protein coded by dsRNA3 was now available as a protein in Genbank, similar protein found from RNA3 of DdV1 was used to screen protein BLAST search engine at NCBI. The best match was ORF of dsRNA3 of GaRV-MS1 and the "expect value" between these two putative proteins was 7e-17, which was indicative for a relatively high similarity.

Phylogenetic relationships of GaRV-L1 and GaRV-MS1 dsRNA patterns

Based on multiple alignments of available complete putative aa sequences of RdRp phylogenetic tree was constructed (Fig. 1). The tree showed that GaRV-L1 grouped together with SsRV2, a virus from a pine pathogenic fungus *Sphaeropsis sapinea* [27]. Other closely related RdRps originated from fungal (SsRV1 and HvV190S) or protozoal [*Eimeria brunetti* RNA virus 1 (EbRV1) (AF356189), *Leishmania RNA virus 1* (LRV1-1) [29] and *Leishmania RNA virus 2-1* (LRV2-1) [25]] hosts. The same phylogenetic analysis showed that GaRV-MS1 grouped together with DdV1, DdV2, and *Fusarium solani virus 1* [19], of which FsV1 is a classified member of the genus *Partitivirus*.

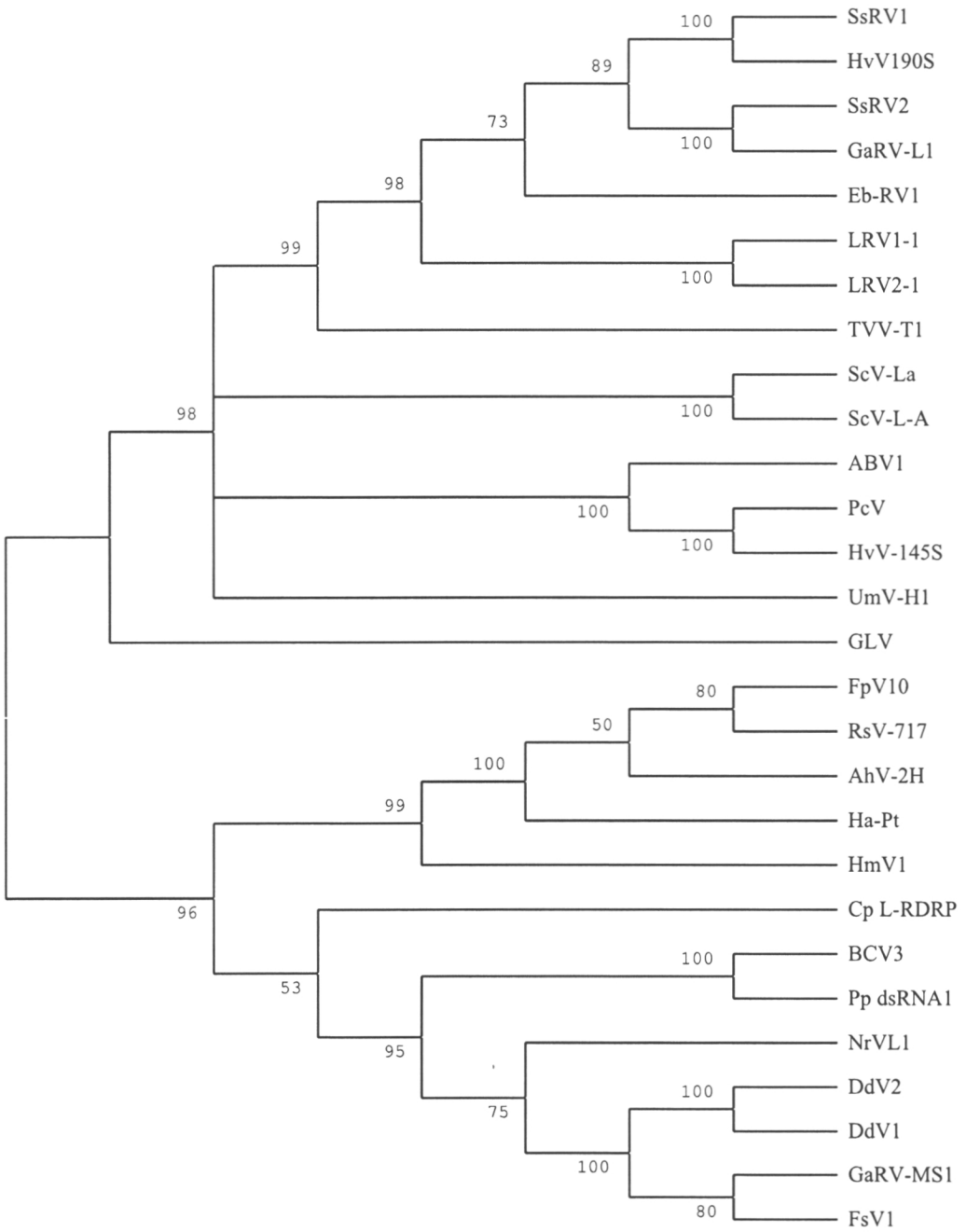
The noncoding regions of the GaRV-L1 and GaRV-MS1 dsRNA patterns

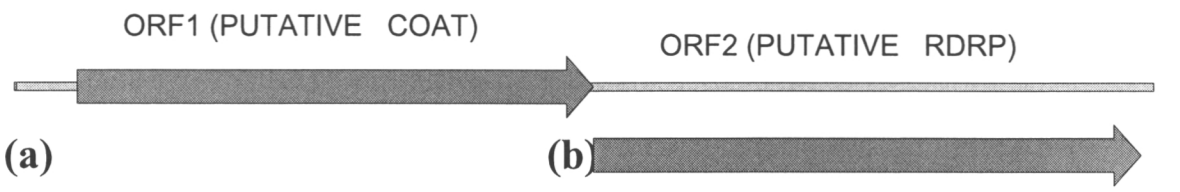
Both dsRNA patterns included relatively long noncoding regions. Upstream of GaRV-L1 ORF1 there was a noncoding region (275 bp) that shares a 14 nts

Fig. 1. Unrooted, condensed (50% cutt-off) neighbor joining tree [14] of different putative dsRNA viruses based on the alignment of their available complete putative RNA-dependent RNA polymerase amino acid sequences. The tree is based on sequences of AbV1, *Agaricus bisporus* virus [35]; AhV-2H, *Atkinsonella hypoxylon virus* (isolate 2H) [20]; BVC-3, *Beet cryptic virus 3* [40]; Cp L-dsRNA, Larger dsRNA of *Cryptosporidium parvum* [13]; DdV1, *Discula destructiva* virus 1 [24]; DdV2, *Discula destructiva* virus 2 [24]; EbRV1, *Eimeria brunetti* RNA virus 1 (AF356189); FpV10, *Fusarium poae virus* [4]; FsV1, *Fusarium solani virus 1* [19]; GLV, *Giardia lamblia virus* [37]; GaRV-L1, *Gremmeniella abietina* RNA virus L1 (this study); GaRV-MS1, *Gremmeniella abietina* RNA virus MS1 (this study); HmV1, *Helicobasidium mompa* dsRNA mycovirus (strain V70) [22]; HvV-145S, *Helminthosporium victoriae virus 145S* (AF297176); HvV190S, *Helminthosporium victoriae virus 190S* [9]; Ha-Pt, *Heterobasdion annosum* P-type partitivirus [11]; LRV1-1, *Leishmania RNA virus 1-1* [29]; LRV2-1, *Leishmania RNA virus 2-1* [24]; NrVL1, *Nectria radicicola* virus L1 [1]; PcV, Penicillium chrysogenum virus (AF296439); Pp dsRNA1, dsRNA1 of *Pyrus pyrifolia* [21]; RsV-717, *Rhizoctonia solani virus 717* [28]; ScV-La, *Saccharomyces cerevisiea virus La* [3]; ScV-L-A *Saccharomyces cerevisiea virus L-A* [10]; SsRV1, Sphaeropsis sapinea *RNA virus 1* [23]; SsRV2, Sphaeropsis sapinea *RNA virus 2* [23]; TVV-T1, *Trichomonas vaginalis* virus strain T1 [30]; UmV-H1, Ustilago maydis virus H1 [3]. Bootstrap values are indicated at the branch points

sequence with SsRV2 (Fig. 2a). The same region had sequence similarities also with SsRV1 and HvH190S. No sequence similarities were found among 3′-untranslated regions (UTRs) of these molecules.

The sizes of 5′ UTRs of dsRNA1 and dsRNA2 were 62 and 99 bp, respectively. The lengths of 3′ UTRs of the same molecules were 100 and 155 bp. The lengths





GaRV-L1
5133 basepairs

(a)

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GaRV-L1        (201)UUGUUAUAGUGGUUGGGCGAGGGUUCCGAAGACCCCUAAACUACAACAA(249)

SsRV2          (214)GGUGUCGUAAUGGGGCGAGGAGGGUUCCGAAGAUCCAAACCAUUACACACC(264)
                                     ********** *****
SsRV1          (152)CAAUCGUCAUUGUUCGGGGGGAGGGUUCCGUUGAUCCCCAACACGACGAUUG(203)

Hv190SV  (182)AAGGGUGGUGUUGGUUGGCGAGGGGGGGCUUCGGCCUUCCGUUGACCCAACUGAUAUCACACUU(245)

Consensus                                GRGGNUUCSG
  OR
Hv190SV   (182)AAGGGUGGUGUUGGUUGGCGAGGGGGGGCUUCGGCCUUCCGUUGACCCAACUGAUAUCACA (245)

Consensus                                GRGGGYUYCG
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(b)

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GaRV-L1    (2600) U CAAUGA UUGA (2610)
Hv190SV    (2602) A CAAUGA GUGA (2612)
SsRV1      (2571) C CAAUGA AUAA (2581)
SsRV2      (2655) G CAAUGA GUAA (2665)
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Fig. 2. Genomic similarities between GaRV-L1, *Gremmeniella abietina* RNA virus L1 (this study); SsRV1, Sphaeropsis sapinea *RNA virus 1* [23]; SsRV2, Sphaeropsis sapinea *RNA virus 2* [23]; HvV190S, *Helminthosporium victoriae virus 190S* [9]. **a** The gray box refers to the identical bases between GaRV-L1 and SsRV2. The asterisks refers to identical bases between SsRV1 and SsRV2. The two possible consensus sequences between these four putative viruses are determined. **b** The overlap of ORF1 and ORF2 of GaRV-L1, Hv190SV, SsRV1 and SsRV2. The putative start codon for ORF2 is bolded. The putative stop codon for ORF1 is underlined. The consensus bases in the overlap region are boxed

of UTRs of RNA1 and RNA2 of DdV1 and DdV2 [24] were very similar to those of GaRV-MS1, and conserved motifs could be found between these six molecules (Fig. 3a–d). Furthermore, additional conserved motifs could be found among these three viruses (Fig. 3c–d). Neither of the noncoding regions of GaRV-MS1 dsRNA3 had sequence similarities with other molecules, except for a GCAAA motif at the beginning of the 5′ UTR.

```
(a)
Alignment based on the beginning of the strands
GaRV-MS1 RdRp (1)  CGCAAAAUAUACGAU-AUUUUUA (22)
DdV1 RdRp     (1) GCGCAAAAGCCUCGCG--CUUUUC (22)
DdV2 RdRp     (1) GCGCAAAACG---ACCGAGUUUUA (21)
GaRV-MS1 Coat (1)  CGCAAAAUU-UGACA-AAUUUUG (21)
DdV1 Coat     (1)  CGCAAAAUUCUGGCUGAAUUUUG (23)
DdV2 Coat     (1) GCGCAAAAGU---GGU-ACUUUUG (20)
Consensus          CGCAAAA           UUUU

(b)
Alignment based on putative start codons
GaRV-MS1 Coat(71) AGAAUUC--AAGCCUCCAUCACGUUCUGGUC--AUGUCUG (106)
GaRV-MS1 RdRp(35) UGAUUUU--C-GCCUCCUUCAUUUCAGUGUC--AUGUCCG (69)
DdV1 Coat    (73) UUAUUU-----GCCUCCUUCAUCUUGAGACC--AUGGCCG (105)
DdV1 RdRp    (33) AGAGUUUCCU-GCCUCCUUCAUCUCUUGAGCGAAUGGAAG (71)
DdV2 Coat    (72) AGAAUUUCUAAGCCUCCUUCUUUCUCGAGCC--AUGGCUG (109)
DdV2 RdRp    (32) AGGGUUGA---GCCUACUUCAUAUCUUGAGCGAAUGGAAG (68)
Consensus             UU     GCCUcCuUCau uy        AUGgc G

(c)
Putative RdRp 3'UTR
GaRV-MS1   (1717) AACUAGAACCAUUUCUGUUUAUCAGGUGGUUGUGAAACAGGUC-CAACCUGUG----------ACUAUUAACAA-UCA end
DdV1       (1710) AACUGGCAACA-UUCUGUGUAUACAG-GGAUGUGAUUUAAAUAACGAUGUGUGUAAAACAUGUCGUGCAGUAAAAACUCC end
DdV2       (1708) AACGGGCAAUC-UUCUGUGUAUACAG-UGAUGUGAAACAA-UAACGUUAUGUG--AAACAUAAUGUGCAGUAA-AACUCA end
Consensus         AAC         UUCUGU UAU        UGUGA              UGUG                  UAA AA UC

(d)
Putative coat 3' UTR
GaRV-MS1   (1497) CUUG-------CUGUUUACCAGUGAG---UGACCCUCAAAUUCUUUGGUUGAGAACA (1543)
DdV1       (1483) CUUA-----ACCUGUUUACCAGGUAGCCCUGUUUAUCAGGCGCUCAUUUUCAAAAUU (1534)
DdV2       (1508) GUUACAAACACCUGUUUAUCAGGGAGUCCUGUUCAUCAGGCACUCAUUCUUUUAAA- (1563)
Consensus          UU        CUGUUUA CAG

GaRV-MS1  (1544)  ----UAACAGUGUAAAC-----UGUGUGUG-AGUGGUACAAUA-ACCAACCUCU end
DdV1      (1535)  CUUUUGAGUGGGUAACC-CAGCUGUGUAUACAGCGG-GUGAUC-AUAAACCUCC end
DdV2      (1563)  -----GAGUGGGUAACUUCUUCCGU-UACACGAAGGAGUGAACCAUAAAACUCC end
Consensus                 GUAA                                AA CUC
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Fig. 3. Aligned sequences of UTRs of RdRp and coat segments of GaRV-MS1, *Gremmeniella abietina* RNA virus MS1 (this study); *Discula destructiva* virus 1 [24]; DdV2, *Discula destructiva* virus 2 [24]. **a** The 5′ UTR alignments based on the beginning of the strands and **b** on the putative start codon. **c** The 3′ UTR alignments based on the putative RdRp and **d** on the putative coat strands

Discussion

The cloning strategy used in this investigation resulted in a complete sequence of two dsRNA viral genomes. The genomes of GaRV-L1 and GaRV-MS1 dsRNA molecule patterns resembled those of SsRV2 and DdV1, respectively. Both patterns contained ORFs possibly coding for putative RdRp and putative CPs. The origin of dsRNA3 of GaRV-MS1 is unknown but because it was more similar to RNA3 in DdV1 than to any sequence in GaRV-MS1 itself, it is not a defective segment but may be a satellite RNA. This, however, is not clear, as dsRNA3 has never been observed separately from dsRNA1 and dsRNA2, and all three segments migrate together in rate zonal centrifugation [32]. Based on aa sequence similarities it is clear that dsRNA3 and RNA3 of DdV1 share a common origin, although the functions of the putative proteins encoded by these dsRNA molecules are unknown.

Overlapping region of ORF1 to ORF2 of GaRV-L1 dsRNA pattern

In the family *Totiviridae* three basic mechanisms have been observed in the translation of RdRp. The first is a hypothetical ribosomal hopping mechanism used in LRV2-1 [25] for the production of coat-RdRp fusion protein. The second mechanism is based on different types of frameshifts. *Giardia lamblia virus* [37] and *Saccharomyces cerevisiea virus L-A* (ScV-L-A) [5] seem to produce coat-RdRp fusion protein via a -1 translational frameshift mechanism. In contrast, LRV1-1 and *Trichomonas vaginalis* virus strain T1 [30] probably use $+1$ ribosomal frameshift mechanisms [6]. Both of these mechanisms involve consensus heptameric slippery sites and pseudoknot structures, which are not found in GaRV-L1. In addition, the possible change of frame in GaRV-L1 should take place in a region of only 15 nts. Therefore, these mechanisms are probably not used for the translation of RdRp in GaRV-L1. The third mechanism used to translate RdRp is a hypothetical coupled termination-reinitiation mechanism found in Hv190SV [26]. As GaRV-L1 and Hv190SV (as well as SsRV1 and SsRV2, Fig. 2b) share a highly conserved motif in the start codon of RdRp it is possible that a coupled termination-reinitiation mechanism also is used in GaRV-L1.

Taxonomic considerations

Phylogenetic analysis show clearly that the two unrelated dsRNA molecule patterns of *Gremmeniella abietina* type A code for viral genomes of the families *Totiviridae* and *Partitiviridae*. The taxonomy of dsRNA viruses similar to GaRV-L1 and GaRV-MS1 is somewhat confusing. HvV190S is a member of the genus *Totivirus*, whereas LRV1-1 and LRV2-1 are members of the genus *Leishmaniavirus*. EbRV1 is an unclassified member of the family *Totiviridae* as are SsRV1 and SsRV2. On the other hand ScV-L-A and *Saccharomyces cerevisiea virus La* (ScV-La) [3] also belong to the genus *Totivirus* [39], despite being grouped separately from HvV190S in phylogenetic analysis.

The dendogram analysis of dsRNA molecules similar to GaRV-MS1 does not entirely support the current taxonomy of the family *Partitiviridae*. The dsRNA1 found in *Pyrus pyrifolia* [21], an unencapsidated seed- and pollen-transmitted cryptic linear dsRNA molecule, groups together with BCV-3 [40], a member of the genus *Alphacryptovirus*. These two putative RdRp's form a firm group and they split the currently known putative members of the family *Partitiviridae* infecting fungi into two separate groups. The recent removal of *Penicillium chrysogenum virus* (PcV; AF296439-AF296442) and *Helminthosporium victoriae virus 145S* (HvV145S; AF297176-AF297179) from the family *Partitiviridae* to a new family *Chrysoviridae* [16] is well justified, as the representatives of this new family are clustering separately from the family *Partitiviridae* in our phylogenetic analysis. Their RdRp seems to be similar to the members of the family *Totiviridae*, and all eight conserved motifs from RdRps of dsRNA viruses of lower eukaryotes [3] can be found (data not shown).

Based on our phylogenetic analysis (and other aspects) two distinct groups, those similar to GaRV-L1 (including SsRV1, SsRV2 and Hv190SV) and GaRV-MS1 (including DdV1, DdV2 and FSV1), were identified. These dsRNA molecule clusters, however, do not represent all members of the genera *Totivirus* and *Partitivirus*. Therefore, we conclude that taxonomy of the families *Totiviridae* and *Partitiviridae* should be further clarified in the future.

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Paper III

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III

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Gremmeniella abietina mitochondrial RNA virus S1 is phylogenetically related to the members of the genus *Mitovirus**

Brief Report

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Summary. A double stranded (ds) RNA genome of *Gremmeniella abietina* mitochondrial RNA virus S1 (GaMRV-S1) was sequenced. The length of the genome was 2572 base pairs, it had a very low GC content (30.6%), and sequence and length variations occurred in both ends of it. The genome coded for a putative 741 amino acid long RNA-dependent RNA polymerase (RdRp) using a mitochondrial translation code. Comparison of the putative amino acid sequences suggested that GaMRV-S1 is a putative member of the genus *Mitovirus*.

*

Viruses of fungi have genomes composed of double stranded (ds)RNA or positive-sense single-stranded RNA [(+)ssRNA] [7]. Viruses with ssRNA(+) genome belong to the families *Narnaviridae* or *Barnaviridae* [7]. Members of the family *Narnaviridae* have been divided in two genera, *Narnavirus* and *Mitovirus*. These viruses do not form true virions and their genomes code only for an RNA-dependent RNA polymerase (RdRp) [2, 5]. Some of the conserved motif domains of the putative viral RdRps resemble those found in the β subunit of coliphage replicases [9, 24]. Viruses of the genus *Narnavirus* reside in the cytoplasm where the RdRp is translated [33], and their genomes are GC rich [3, 29]. In contrast,

*The Genbank accession number of the sequence of GaMRV-S1 reported in this paper is AF534641.

viruses of the genus *Mitovirus* are located in mitochondria, in which the RdRp is translated, and their genomes are GC poor [9, 11].

Gremmeniella abietina (Lagerb.) M. Morelet var. *abietina* is the causal agent of Scleroderris canker on coniferous trees. In Finland, two variants of this fungal pathogen occur on Scots pine (*Pinus sylvestris* L.). Strains of type A (designated also as the 'large tree type', LTT) cause symptoms on large trees and seedlings, whereas strains of type B ('small tree type', STT) are found only in seedlings or shoots covered with snow during the winter [12, 31]. These two variants of *G. abietina* in Finland probably should be considered as closely related but distinct species [32]. We have recently shown that two unrelated dsRNA patterns code for putative viruses in *G. abietina* type A [28, 29]. In this study, we report the occurrence and genomic sequence of a third dsRNA pattern in the type A strains of *G. abietina*. The name *Gremmeniella abietina* mitochondrial RNA virus S1 (GaMRV-S1) is proposed for this novel virus.

The type of the dsRNA-containing isolate luumäki 7 of *G. abietina*, obtained from a pine branch showing typical symptoms of Scleroderris canker, was identified using random amplified microsatellite (RAMS) fingerprints as described in Hantula and Müller [8] and Kaitera et al. [12]. For DNA and dsRNA extraction, the fungal isolate was grown at 15–20 °C on modified orange serum agar (MOS) [19] covered with a cellophane membrane. dsRNA was isolated using a previously described method [18, modified as in 29] based on CF-11 cellulose (Whatman, Maidstone, England). The nature of nucleic acids bound to and eluted from CF-11 were characterized using DNase 1 and RNase treatments. The DNase treatment (30 min at 37 °C) was carried out using 10 units of DNase 1 (Boehringer Mannheim, Mannheim, Germany) in 40 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$. The cloning vector pUC18 (Amersham Pharmacia Biotech, Uppsala, Sweden) was used as a positive control. The RNase treatment (30 min at 37 °C) was carried out using 0.1 ug RNase (from bovine pancreas, Boehringer Mannheim, Mannheim, Germany) in 2 × SSC (0.3 M NaCl – 0.3 M sodium citrate, pH 7.0) or in 0.01 × SSC.

cDNA synthesis was based on the ligation of adapters to the 3′ ends of dsRNA molecules [15], allowing cDNA synthesis from the 3′ ends of both template strands of the dsRNA molecule as described in [29] except, that the following modifications were applied. In the ligation reaction using T4 RNA ligase (Fermantas, Vilnius, Lithuania), 2 μg of dsRNA and 3.5 μg of 5′ phosphorylated and 3′ inactivated ligase adaptor (5′-GCATTCGACCCGGGTT-3′, Amersham Pharmacia Biotech, Uppsala, Sweden) were used. dsRNA was denatured by boiling the sample (including the primers) for 10 min, and 320 ng of dsRNA was used for the reverse transcription (RT) with RevertAid H minus M-MuLV reverse transcriptase (Fermantas, Vilnius, Lithuania). The cDNA sample was purified with "High Pure PCR product purification Kit" (Boehringer Mannheim GmbH, Mannheim, Germany) and used as a (1 μl) template in polymerase chain reaction (PCR) (28 cycles; Expand High Fidelity PCR System, Boehringer Mannheim GmbH, Mannheim, Germany) with a RT oligonucleotide (5′-AACCCGGGTCGTATGC-3′, complementary to the adaptor sequence). With this method, only incomplete

amplification products were initially obtained, but the reduction of the dsRNA denaturation time down to two minutes resulted in a single PCR product corresponding to the size of the dsRNA molecule. Only 25 cycles of amplification was needed. PCR products were cloned and sequenced using previously described methods [29]. In addition, four primers designed during the study were used: 5′-GATTACTACTTTATCTGGTGTTG-3′, 5′-ATCTCGTACCATTGGCA-3′, 5′-TCTATTAAGAGATAAGTAATAATGTTT-3′ and 5′-ACTGTCGAGGGGTGAA-3′. These labeled primers were based on the preliminary sequences obtained from the first sequencing reactions and used in the cycle sequencing reactions as described for M13 primers [29] except that the annealing temperature was 47 °C instead of 50 °C. Sequences were compiled, aligned and analyzed using the Vector NTI Suite 2 software package (InforMax inc., MD, U.S.A). Phylogenetic analyses were conducted using MEGA version 2.1 [13]. Complete nucleic acid and putative amino acid (aa) sequences were screened using protein BLAST [1] search engine of the National Center for Biotechnology Information (NCBI). The RNAstructure 3.6 program [16] was used to predict secondary structures.

The RAMS fingerprinting analysis of Luumäki 7 isolate indicated that it belongs to *G. abietina* type A (not shown). The isolate contained a ~2500 bp nucleic acid molecule (not shown). This molecule was resistant to DNase 1 as well as to RNase in high salt concentration, but in low salt concentration it was degraded by RNase (not shown). Thus, we conclude that the molecule was composed of dsRNA [20, 22] and probably represented the genome of dsRNA virus or a replicating form of ssRNA(+) virus. The molecule was later designated as *Gremmeniella abietina* mitochondrial RNA virus S (strain S1) (GaMRV-S1), and we use this name hereon.

The GaMRV-S1 sequence (accession number AF534641) was assembled from the sequences of twenty independently amplified, overlapping PCR products. Any portion of the final sequence was verified from at least four independent clones. Furthermore, the 5′- and 3′-ends of the GaMRV-S1 genome were cloned independently ten and five times, respectively. The length of the GaMRV-S1 genome was 2572 bp and its GC content was 30.6%. Using the universal genetic code there were no long ORFs in the genome of GaMRV-S1. However, using the mitochondrial translation table, in which UGA codes for tryptophan, GaMRV-S1 was found to contain a single large ORF spanning 86% of the genome. The putative start codon for this ORF was AUG (position 254) and the stop codon was UAA (position 2477), potentially yielding a protein of 741 amino acids with a predicted molecular mass of 85.4 kDa. This putative protein contains conserved motif domains of RdRp-like proteins encoded by mitochondrial viruses and related RNAs [11] (Fig. 1).

The most common sizes of 5′ and 3′ untranslated regions (UTRs) of GaMRV-S1 were 253 and 122 bp, respectively. The UTRs could potentially form stem-loop and panhandle structures (data not shown). These structures were predicted to be stable even when a genomic region of up to 300 bp was used for analysis. The GaMRV-S1 and related sequences in Genbank (see below) did not share long conserved nucleic acid motifs in their UTRs. However, the putative start codon

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                                  Domain I                                    Domain II
GaMRV-S1 231 LGSLSLIYDPECKVRIVAMLDYTTQLFLRPIHNDLFKLLKKLPQDRTFTQN (10) SFWSIDLTAATDRFPI (38)
    OMV4 226 LGKLSIVHDPELKERVIAMVDYTTQFALRPIHNILLNNLSKLPCDRTFTQD (11) RYHSLDLSAATDRFPI (37)
    OMV5 241 FGKISIVKDPELKMRVIAMVDYHSQFVLKKIHNSLFNKLKLIKSDRTFTQD (10) RFWSMDLSAATDRFPI (37)
    OMV6 238 IRRLSIVHDPECKERVIAIFDYGSQMVLKPIADVLFDLLRNIPSDRTFTQS (12) KFWSIDLSSATDRFPI (37)
   OMV3a 260 LGKLAIKEEAAGKARVFAMADSITQSVMAPLNSWVFSKLKDLPMDGTFNQQ (18) EFYSYDLSSATDRLPM (36)
    SMV1 261 LGKLAIKEEAAGKARVFAMADSITQSVMAPLNSWVFSKLKGLPMDGTFNQQ (18) EFYSYDLSSATDRLPM (37)
    CMV1 327 MGKLSVVYDQAGKARIVAITNSWIQTAFYSLHLHVFKLLKNIDQDGTFDQE (15) KFYGFDLTAATDRLPI (35)
    RVM2 335 LSQFALKEEAAGKIRLFALMDSITQSVMSPLHDYMFAILRNIPNDGTFDQE (14) KAFSYDLTAATDRLPV (47)
             . ..:: .:   K R:.A:..   Q  .  :   :.  L. :  D TF Q.      . .. DL::ATDR.P:


                      Domain III                      Domain IV                    Domain V    Domain IV
GaMRV-S1 346 YSVGQPMGAYSSWPAFTLSHHLVVHWCA (7) FKDYIILGDDIVIHNDNIAKKYIEIM (2) LGVGLSNSKTHVS (4) EFAKR
    OMV4 341 YSVGQPMGAYTSWAAFTLTHHLVVHWAA (7) FKDYIILGDDIVIKNNKVAQIYINLM (2) WGVDISLSKTHVS (4) EFAKR
    OMV5 355 YKVGQPMGAYSSWAAFTLTHHLVVFYSA (7) FTNYILLGDDIVINNDKVAKYYIRTM (2) LGVELSMNKTHVS (4) EFAKR
    OMV6 354 YNCGQPMGAQSSWPMFTLAHHVIVRVAA (7) FDKYIILGDDIVINNDNVALKYMEIM (2) FKVEISRNKTHVS (4) EFAKR
    SMV1 382 YSVGQPMGALSSWAMLALSHHVIVQIAA (7) FTNYALLGDDIVIADKAVATSYHMIM (2) LGVEINLSKSLVS (4) ELAKR
   OMV3a 381 YSVGQPMGALSSWAMLALSHHVIVQIAA (7) FTNYALLGDDIVIADKAVATSYHMIM (3) LGVEINLSKSLVS (4) EFAKR
    CMV1 444 YAVGQPMGAYSSFAMLALTHHVIVQVAA (8) FTDYCILGDDIVIAHDTVASEYLKLM (2) LGLSISSGKSVIS (4) EFAKK
    RVM2 463 YEVGQPMGALSSWPGLALTHHWIVQVAA (9) NTEYEILGDDIVIFNELIAQEYLNIM (2) IGCEINLNKSISS (6) EFAKR
             Y. GQPMGA :S:  ..L.HH :V  .A       .Y :LGDDIVI .. :A  Y   M         :. .K:  S     E.AK:
```

Fig. 1. The conserved amino acid sequence motif domains in the RNA-dependent RNA polymerase-like proteins encoded by mitochondrial viruses and related RNAs. GaMRV-S1, *Gremmeniella abietina* RNA virus S1 (this study); OMV3a, *Ophiostoma mitovirus 3a* [9]; OMV4,*Ophiostoma mitovirus 4* [11]; OMV5,*Ophiostoma mitovirus 5* [11]; OMV6,*Ophiostoma mitovirus 6* [11]; CMV1, *Cryphonectria mitovirus 1* [20]; SMV1, *Sclerotinia homoeocarpa* mitovirus 1 (AY172454); and RVM2, *Rhizoctonia virus M2* [14]. The alignment is based on Clustal W algorithm [27]. Conserved amino acids are indicated below the alignment. Colons (:) and points (.) indicate higher and lower levels of chemical similarity, respectively, as defined by the Multiple Sequence Alignment program. Numbers in parentheses indicate the number of amino acid residues between the domains

of GaMRV-S1 occurred in an AU rich context, which in turn was surrounded by regions with high GC content (data not show). The putative start codons of *Ophiostoma mitovirus 3a* (OMV3a; [9], *Ophiostoma mitovirus 4* (OMV4; [11], *Ophiostoma mitovirus 5* (OMV5; [11], *Ophiostoma mitovirus 6* (OMV6; [11] and *Sclerotinia homoeocarpa* mitovirus 1 (SMV1; AY172454) also are located within an AU rich context within GC-rich genomic regions.

The genome of GaRV-MS1 resembles viral genomes of the members of the genus *Mitovirus* found in *Ophiostoma novo-ulmi*. The putative RdRp contained amino acid sequence motif domains I-VI characteristic of the RdRp-like proteins encoded by mitochondrial viruses and related RNAs [11]. The uttermost ends of untranslated regions formed several stable stem-loop and panhandle structures. These structures resemble those found in the members of genus *Mitovirus* infecting *Ophiostoma novo-ulmi* [9, 11] and their satellite viruses [10]. Despite these similarities in genomic structure and amino acid sequence, no extensive nucleic acid sequence similarities were found between the untranslated regions of GaMRV-S1 and these viruses. Also the lack of nucleic acid sequence similarity at the close proximity of the putative start codon was unexpected. These features of GaMRV-S1 differ from the two previously described (unrelated) viruses of *G. abietina* and their closest hypothetical relatives which are located and translated in the cytoplasm of the their host cells [29].

There was length variation in the primary clones obtained from the very uttermost ends of both strands. Signatures of length and sequence variation also were observed in direct sequencing reactions towards both ends of a complete PCR product of GaMRV-S1 (data not shown). Therefore, it is highly probable that length and sequence variation occurred in the ends of the RNA molecule (within a single strain of *G. abietina*). We consider this observation reliable, because the border between the clear and unclear sequence was precise in direct sequencing experiment. The length of this variation, however, was not possible to determine conclusively here, but it seems that the panhandle structures in the ends of the single RNA-molecules were not disrupted due to these variations. Such variation has not previously been reported for other members of the genus *Mitovirus*, either because they do not exist or because they have not been looked for. If the latter alternative is true, it will be interesting to see if similar variations also occur in other mitoviruses.

A phylogenetic analysis (Fig. 2) based on multiple alignments of the putative RdRp amino acid sequences grouped GaMRV-S1 together with OMV4 and OMV5, OMV6. Other similar putative RdRps, based on blast searches, were those found from *Cryphonectria mitovirus 1* (CMV1; [20], *Rhizoctonia virus M2* (RVM2; [14], OMV3 and SMV1. Also, the amino acid sequences deduced from ORF204 (expect value 2e-19), ORF251 (expect value 1e-17) of the mitochondrion [30] and chromosome 2 (locus At2g07749; NM_147269; expect value 2e-15) DNA of *Arabidopsis thaliana* were found to have similarities with the putative RdRp of GaMRV-S1. GaMRV-S1, OMV4, OMV5 and OMV6 formed a group supported by a high bootstrap value. It is remarkable that OMV3a was excluded from this cluster and was rather grouped with SMV1 and RVM2.

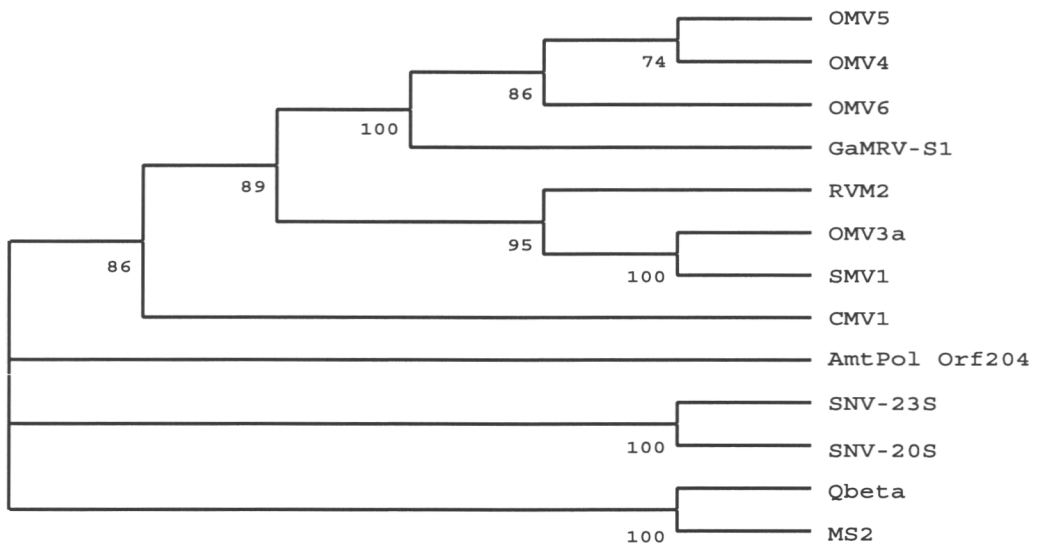

Fig. 2. Condensed (70% cutt-off) neighbor joining tree [13] based on the complete amino acid sequences of the putative RNA-dependent RNA polymerases aligned. The tree is based on the sequences of GaMRV-S1, *Gremmeniella abietina* RNA virus S1 (this study); OMV3a, *Ophiostoma mitovirus 3a* [9]; OMV4, *Ophiostoma mitovirus 4* [11]; OMV5,*Ophiostoma mitovirus 5* [11]; OMV6, *Ophiostoma mitovirus 6* [11]; CMV1, *Cryphonectria mitovirus 1* [20]; RVM2, *Rhizoctonia mitovirus 2* [14]; SMV1, *Sclerotinia homoeocarpa* mitovirus 1 (AY172454); SNV-20S, *Saccharomyces narnavirus 20S RNA* [24]; SNV-23S, *Saccharomyces narnavirus 23S RNA* [3]; MS2, *Enterobacteria phage MS2* [4]; Qbeta, *Enterobacteria phage Qβ* [17]; AtmPol ORF204, RdRp-like gene in *Arabidopsis thaliana* mitochondrial DNA [30]. Bootstrap values are indicated at the branch points

The evolution and coexistence of mitoviruses has been discussed by Hong et al. [11]. Fungal viruses do not have an extracellular phase in their infection cycle, and it has been suggested that they may co-evolve with their hosts [6]. On the other hand, horizontal transmission of viruses between different fungal species may occur, as discussed by Hong et al. [9] and Preisig et al. [21], or replication of different mitoviruses in the same cell may require sufficient sequence divergence [11]. The data available does not conclusively favor either of these two hypotheses [21, 25, 29] and the finding of a putative mitovirus in *G. abietina* type A reported here adds a new piece of this puzzle of fungal virus evolution, and supports the view that the diversity of fungal viruses does not completely follow the evolution of their hosts.

There have been many reports on multiple dsRNA molecules occurring in fungi [23, 26, 28]. Now, it has become evident that *G. abietina* type A is infected with a number of different virus species [29; this study]. To our knowledge, this is the first case where a single fungal species is known to host viruses representing three major fungal virus families (*Totiviridae*, *Partitiviridae* and *Narnaviridae*).

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Paper IV

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IV

Three unrelated viruses occur in a single isolate of *Gremmeniella abietina* var. *abietina* type A

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Summary

Five enclosed double stranded (ds)RNA bands in electrophoresis, probably of viral origin, were found from a single isolate (SurS4) of *Gremmeniella abietina* var. *abietina* type A. Analysis of the dsRNAs revealed that they represented three different viruses, named as *Gremmeniella abietina* mitochondrial RNA virus S2 (GaMRV-S2), *Gremmeniella abietina* RNA virus MS2 (GaRV-MS2) and *Gremmeniella abietina* RNA virus L2 (GaRV-L2). The genome of GaMRV-S2 was 2587 base pairs (bp) long and had a very low GC content (31%). Sequence variations occurred at both ends. The genome coded for a putative RNA-dependent RNA polymerase (RdRp) under a mitochondrial translation code. The GaRV-MS2 genome was composed of three dsRNA molecules (1781 bp, 1586 bp and 1186 bp). They coded for putative a RdRp, a coat protein (CP) and a protein with an unknown function, respectively. The GaRV-L2 genome was 5129 bp long and contained two ORFs. The 5'-proximal ORF coded for a putative CP, whereas the 3'-proximal ORF encoded for a putative RdRp. The buoyant density of GaRV-MS2 and GaRV-L2 were 1.37 and 1.42 g/ml, respectively. GaMRV-S2, GaRV-MS2 and GaRV-L2 were closely related to the previously described viruses GaMRV-S1, GaRV-MS1 and GaRV-L1, respectively and are putative members of the genera *Mitovirus*, *Partitivirus* and *Totivirus*, respectively. This is the first report on the occurrence of viruses of all these different genera in a single fungal host, and in a single fungal isolate.

Keywords: *Gremmeniella abietina*, dsRNA, *Mitovirus*, *Partitivirus*, *Totivirus*

Introduction

Closer examination has revealed that viruses are of frequent occurrence in mycelia of different fungi (Buck, 1986). Usually they do not form extracellular particles, but are dispersed via cellular contacts during anastomosis. The viral genomes usually consist of double stranded RNA (dsRNA) segment(s), sometimes associated with a protein capsid or membrane structures (Ghabrial, 2001). Based on the sequences available in GenBank, the three most common genera of fungal viruses are *Mitovirus*, *Partitivirus* and *Totivirus*. The viruses of these genera usually cause minor or no detectable effects. In contrast, the members of genus *Hypovirus* are less common but may significantly reduce the virulence of their hosts that are plant pathogens (McCabe et al., 1999).

Members of the genus *Mitovirus* are positive-sense single-stranded RNA [(+)ssRNA] viruses that replicate via replicative dsRNA intermediate (Ghabrial, 2001). Mitoviruses, located and translated in mitochondria, do not form true virions (Cole *et al.*, 2000), have a low CG content and only a single open reading frame (ORF) coding for a putative RNA dependent RNA-polymerase (RdRp) (Hong *et al.*, 1998; 1999) can be found. On the other hand, members of the genus *Partitivirus* have isometric cytoplasmic virions and the genome is composed of two 1.4–3.0 kbp dsRNA segments, of which the smaller one codes for a coat protein (CP) and larger for a RdRp (Ghabrial *et al.*, 2000). Defective and satellite RNA segments may also be present (Ghabrial *et al.*, 2000). Partitiviruses have been proposed to have originated from the genus *Totivirus* (Ghabrial, 1998), which also have isometric cytoplasmic particles and contain a single 4.6–7 kbp dsRNA coding for a CP and a RdRp (Wickner *et al.*, 2000).

There are few reports on the occurrence of multiple viruses in single fungal isolates and which have been verified by sequencing of the observed dsRNAs. In *Sphaeropsis sapinea*, which is a known pathogen of pines throughout the world, two different totiviruses (Preisig *et al.*, 1998) and in *Ophiostoma novo-ulmi*,

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infecting Dutch elm, four mitoviruses (Hong *et al.*, 1998; Hong *et al.*, 1999) were found in single fungal isolates. Furthermore two viruses belonging to families *Totiviridae* (Huang and Ghabrial, 1996) and *Chrysoviridae* (Ghabrial *et al.*, 2002) were found in the mycelium of a single isolate of *Helminthosporium victoriae* that infects victoria variety oats. A single isolate of *Rhizoctonia solani*, which causes economically important diseases on numerous crop, turfgrass and tree species, contained two dsRNA molecules, which were related to plant bromoviruses (Jian, *et al.*, 1998) and mitoviruses (Lakshman *et al.*, 1998).

Gremmeniella abietina (Lagerb.) M. Morelet var. *abietina* is the causative agent of Scleroderris canker on coniferous trees. *G. abietina* causes severe epidemics which are mainly dependent on annual weather conditions (Uotila, 1988). The latest outbreak of *G. abietina* occurred in Sweden in year 2001 (Wulff and Walheim, 2002) when more than 50000 hectares of pine forests was severely affected. In northern Europe, two variants of this fungus occur on Scots pine (*Pinus sylvestris* L.): type A (or large tree type, LTT) strains cause symptoms on both large trees and seedlings, whereas type B (or small tree type, STT) strains are found only in seedlings or shoots covered with snow during the winter (Uotila, 1983; Kaitera *et al.*, 1998). The two types of *G. abietina* in northern Europe should probably be considered as closely related, but distinct species (Uotila *et al.*, 2000). The Alpine type, present in Central Europe, and the endemic North American (NA) type are two additional *G. abietina* variants. We have recently described viruses of genera *Totivirus*, *Partitivirus* and *Mitovirus* in isolates of *G. abietina* type A (Tuomivirta and Hantula, 2003a; 2003b).

The aim of the study was to report the occurrence of three different viruses in a single *G. abietina* type A isolate. We also discuss about phylogenic relationship of these viruses based on the conserved motifs of their putative RNA-dependent RNA polymerase amino acid sequences.

Methods

Fungal isolate

Isolate SurS4 of *G. abietina* type A was obtained from a pine branch, that showed typical symptoms of Scleroderris canker in Nummi-Pusula, Southern Finland (60° 34', 23° 53'). The type of *G. abietina* was determined using random amplified microsatellite (RAMS) fingerprints as described in Hantula and Müller (1997) and Kaitera *et al.* (1998).

Nucleic acid isolation and electrophoresis

Isolate SurS4 was grown on modified orange serum agar (MOS) (Müller *et al.*,1994) covered with cellophane membrane at 20 °C. dsRNA was isolated from the mycelium with the method of Morris and Dodds (1979), but modified as in Tuomivirta *et al.*, (2002; 2003a). The protocol is based on the specific binding of dsRNA to CF-11 cellulose (Whatman, Maidstone, England) dsRNA was detected by electrophoresis as described before (Tuomivirta *et al.*, 2002).

Single spore isolations

Conidiospores were produced by cultivating SurS4 isolate in a 150 ml Erlenmyer flask containing autoclaved barley grain and milled pine needles (Uotila 1990) at 15 °C, until conidiospores were produced. Spores were collected from the flask by rinsing with sterile water and plated on a MOS agar plate. After germinating, single spore isolations were made under a microscope and isolates were plated for cultivation on MOS agar.

Determination of buoyant densities

The buoyant density of dsRNA containing particles was determined in a CsCl-gradient. Samples for this analysis were prepared as follows. Mycelia (14 g) were grown on MOS agar for 4-8 weeks at 20 °C and cells were disrupted using ULTRA-TURRAX® TP-18/10 (Janke & Kunkel GmbH & co KG IKA-werk, Staufen, Germany) homogenizer at 4 °C in 25 ml of osmotic stabilizer (OS) buffer (0.6 M NaCl in phosphate buffer pH 6.0) (Phillips 1993). The homogenized mycelia were pelleted with Megafuge 1.0 (Kendro Laboratory Products, Osterode, Germany) centrifuge using 3360 rotor and homogenization was repeated. Finally, homogenate was centrifuged for 10 minutes at 3000 rpm. CsCl was added to supernatant ($\delta = 1{,}35$ g/ml) and stored in –80 °C until used in ultracentrifugation. Isopycnic ultracentrifugation (29000 rpm, 96h) was performed with Sorvall® Discovery™ 100 (Sorvall products, L.P Newtown, Connecticut, U.S.A.) ultracentrifuge using TH-641 rotor (Dupont, Sorvall products, Newtown, Connecticut, U.S.A) at 6 °C. Gradients were divided into 10 fractions, and the occurrence of dsRNA in each fraction was tested by dsRNA isolation using CF-11 column as described above. In order to test whether the dsRNA in fractions was free or somehow encapsidated, the fractions were treated or not treated with phenol:chlorophorm extrac-

tion prior to the dsRNA isolation in CF-11 column. Also the densities of the fractions were measured.

Sequencing of dsRNA molecules

cDNA synthesis and cloning of different dsRNA pattern types was performed as described before (Tuomivirta and Hantula 2003a; 2003b). Sequencing was performed with a 4200L-2 NEN Global IR^2 using the SequiTherm EXCELII sequencing kit (Epicentre, Madison, WI, USA) with IRD700 labelled M13 Forward and IRD800 labelled M13 Reverse oligonucleotides as described by the manufacturer. Also six specific primers were used (the sequences may be requested from the corresponding author). Sequences were compiled, analysed and aligned (with sequences from GeneBank) using Vector NTI Suite 2 (InforMax inc., MD, U.S.A) software package. BLAST (Altschul *et al.*, 1997) searches based on nucleic acid (na) and complete amino acid (aa) sequences were conducted on sequences in GenBank and the RNAstructure 3.6 program (Mathews *et al.*, 1999) was used to predict secondary structures.

Results

The RAMS fingerprint analysis of SurS4 isolate confirmed that it belonged to the *G. abietina* type A (data not shown). SurS4 contained five dsRNA molecules with apparent molecular weights of 5000, 2500, 1800, 1600 and 1200 bp as determined by electrophoresis. These sizes were similar to those of the dsRNA molecules previously reported for viruses of *G. abietina* type A (Tuomivirta and Hantula, 2003a; 2003b). Purification of dsRNA for cloning purposes yielded approximately 1µg dsRNA per pattern type when using 40 g of mycelia. Five dsRNA molecules were separated in an ultracentrifugation experiment (Fig. 1) in which dsRNA molecules peaked in fractions one (2500 bp), seven (1800 bp, 1600 bp, 1200 bp) and eight (5000 bp). Phenol extraction was needed for the binding to CF-11 and thus for successful dsRNA isolation (data not shown). The buoyant densities of fractions seven and eight were 1.37 and 1.42 g/ml, respectively.

Fraction one on the top of the gradient contained a dsRNA molecule with a sequence length of 2587 bp (GenBank sequence accession no. AY615209). The GC content of this molecule was 31%, and it showed 94% nucleotide (nt) identity to the previously described *G. abietina* mitochondrial RNA virus S1 (GaMRV-S1) (Tuomivirta and Hantula, 2003b). Therefore, the new putative virus was designated as *Gremmeniella abietina* mitochonrial RNA virus S strain 2 (GaMRV-S2). No plausible open reading frame (ORF) was found in GaMRV-S2 when the universal genetic code was used. However, using the mitochondrial translation table (in which UGA codes for trytophan) a long ORF starting at nt 269 (741 aa, 85.4 kDa) was identified. This putative protein had a similarity of 96% with the putative RdRp of GaMRV-S1 and it shared conserved motifs with RNA-dependent RNA polymerase-like (RdRp) proteins encoded by mitochondrial viruses and related

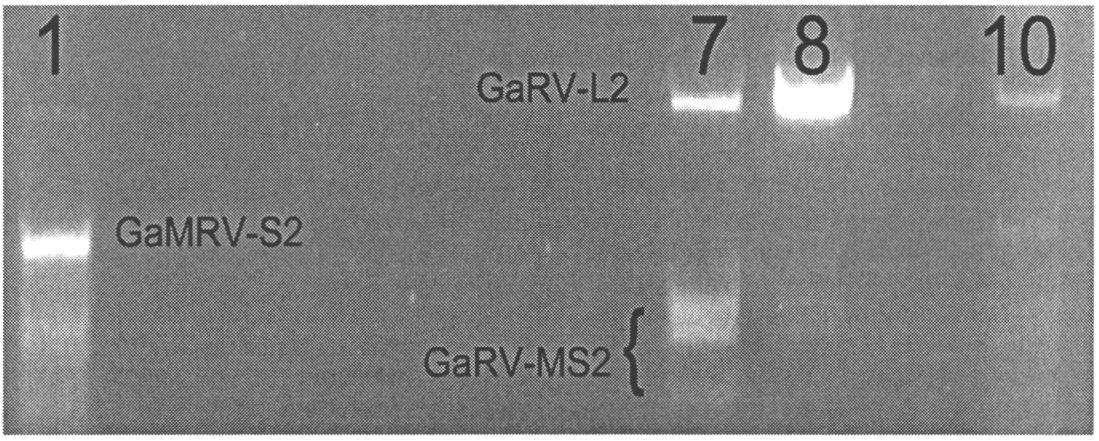

FIG.1. The occurrence of dsRNA in fractions of the isopycnic CsCl-gradient made on the disrupted mycelia of isolate SurS4 of *Gremmeniella abietina* type A. The numbers of fractions containing dsRNA are marked above and the names of dsRNA patterns are marked next to the patterns. The buoyant densities of fractions seven and eight were 1.37 and 1.42 g/ml, respectively.

4

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                                          Domain I                                  Domain II
                          . .LS:: DPE K R::A:.DY :Q. L: I . L.. L. :  DRTFTQ.        : S:DL::ATDRFPI
         GaMRV-S2 231 LGSLSLIYDPECKVRIVAMLDYTTQLFLRPIHNDLFKLLKKLPQDRTFTQN (10) SFWSIDLTAATDRFPI (38)
         GaMRV-S1 231 LGSLSLIYDPECKVRIVAMLDYTTQLFLRPIHNDLFKLLKKLPQDRTFTQN (10) SFWSIDLTAATDRFPI (38)
             OMV4 226 LGKLSIVHDPELKERVIAMVDYTTQFALRPIHNILLNNLSKLPCDRTFTQD (11) RYHSLDLSAATDRFPI (37)
             OMV5 241 FGKISIVKDPELKMRVIAMVDYHSQFVLKKIHNSLFNKLKLIKSDRTFTQD (10) RFWSMDLSAATDRFPI (37)
             OMV6 238 IRRLSIVHDPECKERVIAIFDYGSQMVLKPIADVLFDLLRNIPSDRTFTQS (12) KFWSIDLSSATDRFPI (37)
             SMV1 261 LGKLAIKEEAAGKARVFAMADSITQSVMAPLNSWVFSKLKGLPMDGTFNQQ (18) EFYSYDLSSATDRLPM (37)
            OMV3a 260 LGKLAIKEEAAGKARVFAMADSITQSVMAPLNSWVFSKLKDLPMDGTFNQQ (18) EFYSYDLSSATDRLPM (36)
             CMV1 327 MGKLSVVYDQAGKARIVAITNSWIQTAFYSLHLHVFKLLKNIDQDGTFDQE (15) KFYGFDLTAATDRLPI (35)
             RVM2 335 LSQFALKEEAAGKIRLFALMDSITQSVMSPLHDYMFAILRNIPNDGTFDQE (14) KAFSYDLTAATDRLPV (47)
Bn-mtDNA ORF448 78 SGRLAQSLEGAGKRRLFVIGNWFKQRLLYPVHVWGMSVLRRIPQDGTFHQE (12) FIASFDLSAATDRWPV (42)
At-mtDNA ORF251  1                                MSVLRRIPQDGTFHQE (12) FIASFDLSAATDRWPV (42)
                            ..:    :   K R:..: .   Q ..  :    .  L. :  D TF Q.           ..DL::ATDR.P:


                          Domain III                      Domain IV                    Domain V    Domain VI
                       Y. GQPMGA SSW   FTL:HHL:V.  .A       F..YIILGDDIVI.H..:A  Y:. M      . V :S .KTHVS      EFAKR
         GaMRV-S2 348 YSVGQPMGAYSSWPAFTLSHHLVVHWCA (7)  FKDYIILGDDIVIHNDKVAKKYIEIM (2) LGVGLSDSKTHVS (4) EFAKR
         GaMRV-S1 346 YSVGQPMGAYSSWPAFTLSHHLVVHWCA (7)  FKDYIILGDDIVIHNDNIAKKYIEIM (2) LGVGLSNSKTHVS (4) EFAKR
             OMV4 341 YSVGQPMGAYTSWAAFTLTHHLVVHWAA (7)  FKDYIILGDDIVIKNNKVAQIYINLM (2) WGVDISLSKTHVS (4) EFAKR
             OMV5 355 YKVGQPMGAYSSWAAFTLTHHLVVFYSA (7)  FTNYILLGDDIVINNDKVAKYYIRTM (2) LGVELSMNKTHVS (4) EFAKR
             OMV6 354 YNCGQPMGAQSSWPMFTLAHHVIVRVAA (7)  FDKYIILGDDIVINNDNVALKYMEIM (2) FKVEISRNKTHVS (4) EFAKR
             SMV1 382 YSVGQPMGALSSWAMLALSHHVIVQIAA (7)  FTNYALLGDDIVIADKAVATSYHMIM (2) LGVEINLSKSLVS (4) ELAKR
            OMV3a 381 YSVGQPMGALSSWAMLALSHHVIVQIAA (7)  FTNYALLGDDIVIADKAVATSYHMIM (3) LGVEINLSKSLVS (4) EFAKR
             CMV1 444 YAVGQPMGAYSSFAMLALTHHVIVQVAA (8)  FTDYCILGDDIVIAHDTVASEYLKLM (2) LGLSISSGKSVIS (4) EFAKK
             RVM2 463 YEVGQPMGALSSWPGLALTHHWIVQVAA (9)  NTEYEILGDDIVIFNELIAQEYLNIM (2) IGCEINLNKSISS (6) EFAKR
 Bn-mDNA ORF448 199 FVAGQPLGYYGSWALFALSHHAIVWLAA (10) FLDYALLGDDIVIADRSVAKEYRSLL (2) LQVDISDAKSIVS (5) EFAKR
 At-mDNA ORF251  87 FVAGQPLGYYGSWALFALSHHAIVWLAA (10) FLDYALLGDDIVIADRSVAKEYRSLL (2) LQVDISDAKSIVS (5) EFAKR
                       :. GQP:G   .S:   ..L:HH :V   .A        .Y :LGDDIVI .. :A  Y   :         .:. .K:  S      E.AK:
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FIG.2. The conserved amino acid sequence motif domains in the RNA-dependent RNA polymerase-like proteins encoded by mitochondrial viruses and related RNAs (Hong *et al.*, 1999) GaMRV-S2, *Gremmeniella abietina* RNA virus S2 (this study); GaMRV-S1, *Gremmeniella abietina* RNA virus S1 (Tuomivirta and Hantula, 2003b); OMV4, *Ophiostoma mitovirus 4* (Hong *et al.*, 1999); OMV5, *Ophiostoma mitovirus 5* (Hong *et al.*, 1999), OMV6, *Ophiostoma mitovirus 6* (Hong *et al.*, 1999); OMV3a, *Ophiostoma mitovirus 3a* (Hong *et al.*, 1998); SMV1, *Sclerotinia homoeocarpa* mitovirus 1 (Deng *et al.*, 2003); CMV1, *Cryphonectria mitovirus 1* (Polashock and Hillman, 1994); RVM2, *Rhizoctonia virus M2* (Lakshman *et al.*, 1998); Bn-mtDNA ORF448, *Brassica napus* mitochondrial DNA open reading frame (ORF) 448 (Handa, 2003); and At-mtDNA ORF251, *Arabidopsis thaliana* mitochondrial DNA ORF 251 (Unseld *et al.*, 1997) . The alignment is based on Glustal W algorithm (Thompson *et al.*, 1994). Conserved amino acids are indicated below the alignment. Colons (:) and points (.) indicate higher and lower levels of chemical similarity, respectively, as defined by the Multiple Sequence Alignment program. Conserved amino acids between GaMRV-S2, GaMRV-S1, OMV4, OMV5 and OMV6 are indicated on the top of the alignment as described above. Numbers in parentheses indicate the number of amino acid residues between the domains.

RNAs (Hong *et al.*, 1999) (Fig. 2). Other highly similar viruses based on a BLAST search were *Ophiostoma mitovirus 4* (OMV4; Hong *et al.*, 1999; aa similarity 37%), *Ophiostoma mitovirus 5* (OMV5; Hong *et al.*, 1999; 34%) and *Ophiostoma mitovirus 6* (OMV6; Hong *et al.*, 1999; 37%), which are recognized members of genus *Mitovirus.*

Three dsRNA molecules (AY615211-AY615213) occurred in fraction 7. The lengths of the dsRNA molecules determined by sequencing were 1781 bp, 1586 bp and 1186 bp, and their nt sequence identities to those of the three *G. abietina* RNA virus MS1 (GaRV-MS1) segments (Tuomivirta and Hantula, 2003a) were 98%, 98% and 97%, respectively. It was therefore a new strain of *G. abietina* RNA virus MS, designated as strain 2 (GaRV-MS2). A single ORF for putative protein was found on each of the three molecules at nt 63 (539 aa, 62.1 kDa), 100 (443 aa, 47.1 kDa) and 348 (237 aa, 26.5 kDa). The putative proteins had similarities of 98%, 99.5% and 97% with the putative RdRp, CP and unknown proteins of GaRV-MS1, respectively. The putative RdRp of GaRV-MS2 contained the conserved RdRp motifs III, IV, V and VI (Fig. 3) found in dsRNA viruses infecting lower eukaryotes (Bruenn, 1993). Also two new possible conserved motifs (VIIa and VIIIa) unique for partitiviruses were identified (Fig. 3). Other viruses with highly similar RdRp based on a BLAST search were *Penicillium stoloniferum* virus S (PsV-S) (Kim *et.al.,* 2003; aa similarity 71%), *Discula destructiva* virus 1 (DdV1) (Rong *et al.*, 2002; 64%), *Discula destructiva* virus 2 (DdV2) (Rong *et al.*, 2002; 71%) and *Fusarium solani virus 1* (FsV1) (Nogawa *et.al.*, 1996; 60%), of which FsV1 is a recognized member of the genus *Partitivirus.*

Fraction 8 contained a dsRNA molecule (AY615210) with the length of 5129 bp. The sequences at both ends were verified with direct sequencing. The identity of its nt sequence was 90% to the previously described *G. abietina* RNA virus L1 (GaRV-L1) (Tuomivirta and Hantula, 2003a). Therefore we designated this new putative virus as *G. abietina* RNA virus L, strain 2 (GaRV-L2). Two partially overlapping ORFs starting at nt 272 (776 aa, 80.4 kDa) and 2599 (825 aa, 90.1 kDa) were found and the corresponding putative proteins had similarities of 97% with CP and RdRp proteins of GaRV-L1. The putative RdRp of GaRV-L2 contained all eight conserved RdRp motifs (Fig. 4) from dsRNA viruses infecting lower eukaryotes (Bruenn, 1993). Based on comparisons of RdRp by BLAST, other highly similar viruses were *Sphaeropsis sapinea* RNA virus 2 (SsRV2) (Preisig *et al.*, 1998; aa similarity 50%), *Coniothyrium minitans* RNA virus (CmRV) (Cheng et.al., 2003; 50%), *Helicobasidium mompa* Totivirus 1-17 (HmTV1-17) (Nomura *et al.*, 2003; 35%), Sphaeropsis *sapinea* RNA virus 1 (SsRV1) (Preisig *et al.*, 1998; 35%) and *Helminthosporium victoriae 190S virus* (HvV190S) (Huang and Ghabrial 1996; 35%) of which HvV190S is a recognized member of the genus *Totivirus.* A conserved GGUUCC motif observed in 5´UTR of GaRV-L1 (Tuomivirta and Hantula, 2003a) was found also in GaRV-L2 55 nts downstream of the putative start codon of CP. The same motif occurs also in approximately the same position in SsRV2, CmRV, TmTV1-17, SsRV1 and HvV190S. In HvV190S the corresponding sequence was GGCCUUCC.

Conidiospore isolations

Single conidiospore isolations were made to test the transmission of dsRNA molecules to asexual spores. All 15 mycelial cultures derived from single spores contained the same five dsRNA molecules as the parental culture (Fig. 5), indicating efficient transmission for all three viruses to asexual spores.

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                    Domain III              Domain IV                          Domain V
                PKTRL.W:YP:EML :EG      L:G:DFS.FD:KVP:WLI      VW:  :MV:YFINTPIL PDGRMF RK RGVPSGSW:TQ::DSVVN IL   :.Y L
GaRV-MS2    219 PKTRLVWVYPAEMLVVEGQ (39) LYGLDFSSFDTKVPSWLI (31) VWD-GMVWYFINTPILNPDGRMF-RKRRGVPSGSWWTQMVDSVVNYIL---VEY-L (10)
GaRV-MS1    219 PKTRLVWVYPAEMLVVEGQ (39) LHGLDFSSFDTKVPSWLI (31) VWD-GMVWYFINTPILMPDGRMF-RKRRGVPSGSWWTQMVDSVVNYIL---VEY-L (10)
PsV-S       218 PKTRLVWIYPAEMLAIEGF (39) LYGLDFSGFDTKVPAWLI (31) VWD-GMVWYFINTPILMPDGRMF-RKYRGVPSGSWFTQMIDSVVNYIL---VDY-L (10)
DdV1        218 PKTRLVWIYPAEMLVVEGF (39) LYGIDFSAFDSKVPAWLI (31) VWE-AMVWYFINTPILMPDGRMF-RKYRGVPSGSWWTQIIDSVVNNIL---IDY-L (10)
DdV2        218 PKTRLIWIYPAEMLCVEGF (39) LYGIDFSAFDSKVPAWLI (31) VWD-AMVWYFINTPILMPDGRMF-RKYRGVPSGSWWTQIIDSVVNNIL---IDY-L (10)
FsV1        199 PKTRLAWIYPSEMLMVEGL (39) LYGLDFSSFDTKVPAWLI (31) VWD-GMVYYFINTPILMPDGRMF-RKRRGVPSGSWWTQLVDSVVNWIL---VKY-L (10)
BCV3        193 TKVRGVWGRAFHYILIEGT (40) LYALDWSSFDSSVTRFEI (21) FEL-SRIL-FKHKKLAAPDGNIY-MIHKGIPSGSYYTSIVGSVVNRLR---IEY-I (11)
Pp dsRNA1   193 TKIRNVWGRAFHYILIEGT (39) LYAIDWKQFDATVSRFEI (29) FEL-SRQL-FIHKKIAAPDGYIY-WSHKGIPSGSYFTSIIGSIINRLR---IEY-L (11)
Cp-L-RdRp   163 NKPRLIWAYPGYLSIIENQ (40) IAQIDFSSFDATVRTELI (19) MLD-QLRHYFINTPILFYDKIIV--KNRGIPSGSAFTQIIGTIVNMIACQYASL-R (11)
HmMV        223 TKIRVVFGVSKRHVLPSAM (45) FVMVDWSSFDLRSLFSII (36) LWN-WVCEACFQMPHRLPDGNVYKRLFRGIPSGLFTTQFLDSFYNMIMI--LTI-L (11)
Ha-St        18 NKLRTIWGVSKLWIISEAQ (44) YITIDWSRFDKRAYFWLI (35) LWE-WTIEAFFDSPIVLPNGHVYKRRFAGIPSGLFITQLMDSWYNYVM---LCSIL (11)
OmIV-II     258 PKVRLVFGAPFTLLTAELI (45) VATLDWSGFDRYARHTVI (43) LWN-WMTDAVLTVPLLMPDGTMIRFTHSGIYSGYMQTQILDSLYNMVMI--FTM-V (11)
Ha-Pt       296 LKVRPVYNAPMLFLMLEAM (39) FMGFDYSRFDQLAP-FTI (72) ILS-FIWWWYVFMVFITPDGYGYVRLLAGVPSGIFMTQILDSFVNLFIF--VDA-L (14)
RsV-717     336 LKVRPVYAVDELFLDLECM (39) FATIDWSGYDQRLP-WFI (41) LINFTATWYF-NMVFLSADGFAFRRQFAGVPSGMLLTQFLDSFGNLYLI--IDS-L (14)
AhV-2H      269 LKVRPVYNAPMIYIRIECM (39) FLLIDWSRFDHLAP-FTI (63) LISFLERWYR-DMVFVTPDGFAYRRTHAGVPSGILMTQFIDSFVNLTIL--LDG-L (14)
FpV10       305 LKQRPVYAVDDLFLTIESM (39) FFTIDWSGFDQRLP-WVI (42) LITFLATWYF-NMVFVTADGFSYVREHAGVPSGMLNTQFLDSFGNLFLL--IDG-L (14)
CpV         273 LKVRPVNNAPMIFLRIECM (39) FMMIDWSKFDQTVP-FTL (63) LLKFINTWFK-EMVYITPDGFAYSRTFAGVPSGILCTQLIDSFVNLVVL--IDS-L (14)
SMV         185 NKTRTFTAAPLETLLGGKV (41) YCDADGSQFDTSLTPYLI (23) LYT-EIVY----TPIATPDGSVI-KKFKGNNSG-QPSTVVDNTLMVIIA--FNY-T (16)
SPFMV       184 NKTRTFTAAPLDTLLGGKV (41) YCDADGSQFDSSLSPYLI (23) LYT-EIVY----TPILTPDGTIV-KKFKGNNSG-QPSTVVDNTLMVVLA--VHY-T (16)
                  K R                       .D . :D        :      .          .       .        G  SG    : .:..         .

                 Domain VI                   Domain  VIIa                     Domain VIIIa
                .VLGDDS  AF          .M :. EK. . E    D . .KLLG..    YR. .            :R.T.EWF.LALYPE .
GaRV-MS2    386 LRVLGDDS--AF (18) GMILKPEKCEKTE---DPSDFKLLGTT---YRECH---------PHRDTNEWFKLALYPE-S
GaRV-MS1    386 LRVLGDDS--AF (18) GMILKPEKCEKTE---DPSDFKLLGTT---YRGCH---------PHRDTNEWFKLALYPE-S
PsV-S       385 LRVLGDDS--AF (18) NMILHPEKCEKTK---DPTAFKLLGTT---YRNGR---------PHRETNEWFKLALYPE-S
DdV1        385 LKVLGDDS--AF (18) GMVIKPEKCERTE---DPNDFKLLGTK---YRDGR---------VYRSTDEWFTLALYPE-S
DdV2        385 PEVLGDDS--AF (18) GMVIRPEKCEKTE---DPAEFKLLGTK---YRSGR---------VHRSTDEWFSLALYPE-S
FsV1        366 LRVLGDDS--AF (18) GMDLSDEKSISVE---DATELKLLGVR---YRDGH---------AFRETEEWFKLALYPE-G
BCV3        351 CYTQGDDS--LI (18) GWIMNPDKTEYST---DPGYVTFLGRT----AHGF-M-------NARSLDKCLRLLMFPE--
Pp dsRNA1   350 CYTQGDDS--LS (18) GWVLNPEKTEYST---IPSEVHFLGRT---MLGGL---------NTREIKRCLRLLIYPE--
Cp-L-RdRp   322 CWL-GDDSFLNF (20) GLNVSIDKTHYTTRFIDDFEVRFKGVRPYVKFLGKQIDILLDLTFHNDLDKLDAQMALPEKE
HmMV        404 IRVQGDDS-LIR (25) DSVARPEKTHITN---NPNEINALGYD---YPNGY---------PHRDWRKLLAQLLHPRST
Ha-St       197 IKVQGDDS-IIR (25) GAIISMDKSELRN---SIENCEVLSYR---HFMGL---------PYRDLLKMLAQFYHTKAR
OmIV-II     446 IKVQGDDS--IF (25) GAVLSDKKSEIRD---SLEHAEVLKYR---NRNGI---------PYRDPIALLAQLRHPERS
Ha-Pt       509 LFIQGDDN-LVF (23) HMIVSPDKSWITR---LRTKIEVLGYT---NANGM---------PHRDVSKLIATLAYPERT
RsV-717     518 LFIMGDDNS-IF (13) NMHLSKTKSVITT---LRSKIETLSYR---CNFGK---------PRRDVEKLIAQLVYPEHG
AhV-2H      473 VFIMGDDN-VIF (23) GMVINISKSAVTS---IRRKIEVLGYT---NNYGF---------PTRSISKLVGQLAYPERH
FpV10       488 LFIMGDDNS-AF (23) GMVLSKTKSIITT---LRHKIETLSYQ---CNFGH---------PRRPIGKLVAQLCFPERG
CpV         477 ILLMGDDN-VVF (23) NMKVNVDKSIFTT---LRRKIEILGYT---NNYGM---------PVRSLSKLIGQLAYPERH
SMV         348 MYANGDDLLLAI (18) GLNF--DFTSRTR---DRTELWFMSTRG-IKIDNMYI-------PKLEQERIVAILGWDR-S
SPFMV       347 FFANGDDLLLAM (18) GLNY--DFSSRTS---NKEELWFMSHRG-LKRDGIFI-------PKLEPERIVSILEWDR-S
                  . GDD                        .             .                              .
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FIG.3. Four out of the eight conserved RdRp motifs domains (III-VI) from dsRNA viruses infecting lower eukaryotes (Bruenn, 1993) and two additional (VIIa and VIIIa) motifs observed during this study. GaRV-MS2, *Gremmeniella abietina* RNA virus MS2 (this study); GaRV-MS1, *Gremmeniella abietina* RNA virus MS1 (Tuomivirta and Hantula, 2003a); PsV-S, *Penicillium stoloniferum* virus S (Kim *et.al.,* 2003); DdV1, *Discula destructiva* virus 1 (Rong *et al.*, 2002); DdV2, *Discula destructiva* virus 2 (Rong *et al.*, 2002); FsV1, *Fusarium solani virus 1* (Nogawa, *et al.*, 1996); BVC-3, *Beet cryptic virus 3* (Xie *et al.*, 1993); Pp dsRNA1, dsRNA1 of *Pyrus pyrifolia* (Osaki *et al.*, 1998); Cp L-dsRNA, Larger dsRNA of *Cryptosporidium parvum* (Khramtsov *et al.*, 1997); HmMV, *Helicobasidium mompa* mycovirus (Osaki *et al.*, 2002); Ha-St, *Heterobasdion annosum* S-type partitivirus (Ihrmark et.al., 2001); OmIV-II, Oyster mushroom isometric virus II (AY308801); Ha-Pt, *Heterobasdion annosum* P-type partitivirus (Ihrmark 2001b); RsV-717, *Rhizoctonia solani virus 717* (Strauss *et al.*, 2000); AhV-2H, *Atkinsonella hypoxylon virus* (isolate 2H) (Oh and Hillman 1995); FpV10, *Fusarium poae virus* (Compel *et al.*, 1999); CpV, *Ceratocystis polonica* partitivirus (AY247204); SMV, *Sorghum mosaic virus* (Yang and Mirkov, 1997); and SPFMV, of *Sweet potato feathery mottle virus* (Sakai *et al.*, 1997). The alignment is based on Glustal W algorithm (Thompson *et al.*, 1994). Conserved amino acids are indicated below the alignment. Colons (:) and points (.) indicate higher and lower levels of chemical similarity, respectively, as defined by the Multiple Sequence Alignment program. Conserved amino acids between GaRV-MS2, GaRV-MS1, PsV-S, DdV1, DdV2 and FsV1 are indicated on the top of the alignment as described above. Numbers in parentheses indicate the number of amino acid residues between the domains.

Discussion

This is the first report, in which the occurrence of three putative viruses of the families *Narnaviridae*, *Partitiviridae* and *Totiviridae* has been confirmed in a single fungal isolate. Furthermore, the data showed that these viruses are transmitted via conidia. Viruses of these families are common in fungi, but mixed infections with them have not been reported. Our data suggests that these three viruses use differing mechanisms of replication, transcription and translation sufficiently, so that they can be replicated in the same cells. The high identity of sequences of GaMRV-S2 to GaMRV-S1 showed that they are strains of the same virus. Equally, GaRV-MS2 and GaRV-MS1 would be strains of the same virus as well as GaRV-L2 and GaRV-L1.

There were interesting similarities and dissimilarities between strains GaMRV-S2 and GaMRV-S1. In both viruses a putative start codon occurred in an AU rich context surrounded by regions with high GC content and the signatures of length and sequence variations at the uttermost ends of the genome was found in direct sequencing experiment (see Tuomivirta and Hantula, 2003b). However, the lengths and sequence of uttermost end of the 5'untranslated region (UTR) was different in the two GaMRV-S strains. A panhandle structure was not found in the 5' UTR of GaMRV-S2. This suggests that it might not be as important as suggested previously for GaMRV-S1 (Tuomivirta and Hantula, 2003b), OMV4 (Hong *et al.*, 1999) and OMV6 (Hong *et al.*, 1999). This conclusion is supported by the facts that *Ophiostoma mitovirus 3a* (Hong *et al.*, 1998a), OMV5 (Hong *et al.*, 1999) and *Sclerotinia homoeocarpa* mitovirus 1 (Deng *et al.*, 2003) do not contain predicted panhandle structures. The ends of 3' UTRs of GaMRV-S2 and GaMRV-S1 were, on the other hand, highly similar and a stable stem-loop structure was predicted using 300 bp of the 3'-UTR for analysis.

GaRV-MS1 (Tuomivirta and Hantula, 2003a) and GaRV-MS2 (this study) were highly identical in their nt sequences and no differences were found in the conserved motifs of their UTR sequences. This suggests for important role for these ends for the survival of the virus. Some of these conserved motifs (Tuomivirta and Hantula, 2003a) originally found from GaRV-MS1, DdV1 and DdV2 occur also in the 5'-UTRs of the recently sequenced virus PsV-S (Kim *et.al.,* 2003). A highly unexpected feature was the highly conserved and very long UTR sequence of the 1186 bp long dsRNA molecule in GaRV-MS2, which was called dsRNA3 in GaRV-MS1 (Tuomivirta and Hantula, 2003a). The UTR sequences were over three times more conserved than the nt sequence of the putative ORF of the same dsRNA. This suggests an important role for the UTR in the replication, transcription and/or translation of this molecule. The role of this dsRNA molecule in the partitiviruses of *G. abietina* is unknown.

The UTRs of the two totiviruses of *G. abietina* were highly similar, except that 5' UTR of GaRV-L2 was four nucleotides shorter in the uttermost end than that of GaRV-L1. In addition, a motif observed in 5'UTR of GaRV-L1 (Tuomivirta and Hantula, 2003a) can also be found in GaRV-L2 and in SsRV2, CmRV, TmTV1-17, SsRV1 and HvV190S. This suggests that in GaRV-L the uttermost end of 5'-UTR is not critical for the survival of the virus. On the other hand 3'-UTR in GaRV-L seems to be very conserved and thus may have very important role in the virus. The ORFs of CP and RdRp were partially overlapping and this region contained an

8

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                      Domain I                     Domain II                                      Domain III
                 . E.: L GR     D: .E           .. W. RW W.VNG: ...          T :S:: KLE GK RAIFA DT . .         :.  .  WR. :::L.PG GG
GaRV-L2     216 LCESNVLLGRAAGPADLSEE (46) YQEHWNDRWAWAVNGA-HSG (38) TFVSASPKLEAGKTRAIFACDTVNYLAFEHLLAPVEKRWRNSKVILDPGRGG (19)
GaRV-L1     216 LCESNVLLGRAAGPADLSEE (46) YQEHWNDRWAWAVNGA-HSG (38) TFVSASPKLEAGKTRAIFACDTVNYLAFEHLLAPVEKRWRNSKVILDPGRGG (19)
SsRV2       215 LVEAQTLQGRAVQPADMHSE (46) LQEHWDSRWLWAVNGS-QSG (39) TYVSGSPKLEHGKTRAIFACDTLNYLAFEHLLASVESRWRGERVVLNPGRGG (19)
CmRV        218 MVETNVLLGRDVAPIDLVEE (47) LEDHWATRWQWAVNGA-HSG (39) TYVSASPKLEHGKTRAIFAWDTINSLAMEHLMSTVEANWRGERVILNPGKGG (19)
HmTV1-17    238 LVEAQSLQGRMTGACDLAEE (43) LDDFWSRRWLWCVNGS-HTN (40) TFVSATQKLEHGKTRAIFACDTQNYFRVSHLLGPVQNRWRNERILLDPGEGG (19)
SsRV1       235 LCEAQTLLGRAVSTIDLAHE (42) LDSWWSSRWLWCVNGS-QND (40) TNISASPKLEHGKTRAIFACDTRSYFAFEWLLGATQKAWRNHRVLLDPGGGG (19)
HvV190S     236 FVEANTLQGRYDRTLDMDHE (42) EDEWWTSRWLWCVNGS-QNA (35) TSVSPSVKLENGKDRAIFACDTRSYFAFTYWLTPIEKKWRGARVILNPGEGG (19)
Eb-RV1      262 LCELAVLQGRGVNRLDLLED (41) SDDFFRRRYAWCVGGA-HNL (41) VRVSVAEKVEQGKGRAIYSCDTLSYTAFSWLLEAVEKEWASRSVILNPGKGG (21)
LRV1-4      244 LVEMKCLLGRGVSEIDVELE (40) ICDFWSSRWLWAANGS-HSR (37) VYVTPSAKLEHGKTRLLLACDTLSYMWFEYALRPVERIWENSNVILDPGSIG (21)
LRV1-1      272 LVEMKCLLGRGVNEIDVETE (40) ICDFWSSRWLWAANGS-HSR (37) VYVTPSAKLEHGKTRLLLACDTLSYMWFEYALRPVERIWENSNVILDPGSMG (21)
LRV2-1      290 YVELKCLRGRGIAELDVIAE (42) KDDFWSSRWLWAANGG-HSR (37) VYVTPSQKLEHGKSRLLLACDTLSYLWFEYYLKPVETAWLNKRTLLNPGVVN (29)
TVV2        221 LTELNTLQGRGVTSSDAKRD (40) FKQHVFTSPLWVKKGA-HH- (28) VYITQAPKLEHGKTRFIYNCDTVSYIYFDYILNYIESVWSNKHVLLNPDYMN (13)
TVV3        153 LTECNTLIGRGVKSSDPSAD (41) FKLHLITSPLWCKAGS-HH- (28) VFITQAEKLEHGKTRYIYNCDTXSYLFFDYILHYVECVWSNESVLLNPAAMS (16)
TVV-T1      154 FIELPTLLGRGAIPGDAALD (39) FEHHLEERLLWSRSGS-HY- (34) VFIRQSRKLEHGKERFIYNCDTISYVYFDFVLKLFESGWQDSEAILSPGDYS (15)
ScV-L-A     164 LFELAVLMNRGVGHVSWQAE (38) WDDYTANRWEWVPGGSVHSQ (38) VRAWTSTKYEWGKQRAIYGTDLRSTLITNFAMFRCEDVLTH--KFPVGDQAE (18)
ScV-La      236 LFELNVLENGVDEEVDWEKE (39) WQEYWRQRAVIMPGGSVHSQ (39) IHAYTSTKYEWGKVRALYGCDFSSHTMADFGLLQCEDTFPG--FVPTGSYAN (17)
Cy-aV           --------------------  36  IGAVRVSKRVWA---D--Y- (15) EMASASEKYENGKPRALYGVEPVHYVLSTYATKGLEDGNAEVEGFEKGGSNF (21)
                  E   L .     .  :                 G. .               . : K E GK R :.  : .            .    .    .    .

                      Domain IV                         Domain V               Domain VI            Domain VII          Domain VIII
                MLD:D.FNS.H: ..    :          GT:MSGHR T . .SV N .Y:         :H.GDDVY          R:N  KQS:G  . EFLR :       . G  Y..R::AS :SG.W .
GaRV-L2     410 MLDYDDFNSHHSTRAMQILFQ (36) GTLMSGHRGTTFINSVLNKAYL (14) LHVGDDVYF (20) RMNRMKQSVGHVSTEFLRNA (5) TYG--YFARAVASTVSGNWVN
GaRV-L1     410 MLDYDDFNSHHSTRAMQMLFQ (36) GTLMSGHRGTTFINSVLNKAYL (14) LHVGDDVYF (20) RMNRMKQSVGHVSTEFLRNA (5) TYG--YFARAVASTVSGNWVN
SsRV2       410 MLDYDDFNSHHSIRAMQILFE (36) GTLMSGRRGTTYISSVLNEVYL (14) IHVGDDVYL (20) RMNPMKQSVGHTSTEFLRLA (5) TYG--YLARAVASTISGNWVT
CmRV        414 MLDYDDFNSHHTTRTMQIVVE (36) GTLMSGHRLTTYINSVCNEAYL (14) LHVGDDVYL (20) RMNPAKQSVGHVTTEFLRVA (5) SYG--YLARAVASITSGSWVN
HmTV1-17    431 MLDYDDFNSHHATETQKILFE (36) GTMMSGHRGTMFINSVLNAVYI (14) LHAGDDVYA (20) RMNSTKQSVGVVCGEFLRMA (5) AIG--YVCRSIASFISGNWST
SsRV1       427 MLDFDDFNSHHSLNSQRMLFG (35) GTLPSGHRGTTIVNSVLNAAYI (14) LHTGDDVYI (20) RINPAKQSVGFGTGEFLRMA (5) TRG--YLARSVASFVSGNWTN
HvV190S     423 MLDYDNFNSQHSNETMAALYE (35) GTLMSGHRATTFTNSVLNAAYI (14) LHAGDDVYL (20) RMNPTKQSIGYTGAEFLRLG (5) AIG--YLCRAIASLVSGSWTS
Eb-RV1      456 MLDYSDFNSQHSNEAMRIVIE (37) GSLMSGHRATTYWNSVLNAAYV (14) FHVGDDVLI (20) TLQRSKQSVGIGGYEFLRVA (6) VGG--YVARSVAGLVSGSWVS
LRV1-4      433 AVDYDDFNSQHTLLSQKIVFE (36) GTLMSGHRATSFINSVLDRAYI (10) MHVGDDILM (19) RLNASKQVFSKTSGEFLRVA (5) SHG--YLARVVSSAVSGNWVS
LRV1-1      461 AVDYDDFNSQHTLMSQKIVFE (36) GTLMSGHRATSFINSVLNRAYI (10) MHVGDDILM (19) RLNASKQVFSKTSGEFLRVA (5) SHG--YLARVISSAVSGNWVS
LRV2-1      489 SLDFEDFNSQHSLAAQKMVFE (36) GTLMSGHRATTFINTILNTAYL (10) YHVGDDVIF (19) RCNPHKQCASEYSGEFLRVA (5) STG--YVARAIASCVSGNWVS
TVV2        392 MLDYTDFNSQHSIESMKQVFL (33) -TLPSGHRATTFINSVLNRAYL  (8) FHTGDDVLL (15) ELNKTKQSFGP-SAEFLRLH (4) VSG--YPARAISSLVSGNWLS
TVV3        325 MIDYTDFNSQHSLESQKLVFE (33) -TLPSGHRATTFINSVLNKAYL  (8) FHCGDDVLL (15) ELNKSKQSFGP-NAEFLRLH (4) VIG--YPSRAVSSLVSGNWLS
TVV-T1      332 MLDYTDFNSQHTIQSMRLIFE (34) -TLPSGHRATTFINTVLNWCYT  (9) MCAGDDVIL (16) KFNPSKQSTGT-RGEFLRKH (4) VFA--YPCRAIASLVSGNWLS
ScV-L-A     348 CFDYDDFNSQHSIASMYTVLC (42) GTLLSGWRLTTFMNTVLNWAYM (14) VHNGDDVMI (20) RAQPAKCNLFS-ISEFLRVE (6) GLGAQYLSRSCATLVHSRIES
ScV-La      421 CYDFDDFNSQHSKEAMQAVID (42) GTLFSGWRLTTFFNTALNYCYL (12) LHNGDDVFA (20) RANTTKMNIGT-IAEFLRVD (4) STGSQYLTRGIATFTHSRVES
Cy-aV       138 MMDYADFNVQIRPKAQALVHK (43) QGMFSGTKATDLLNTLLNKAYF (21) VHQGDDVWI (20) VFQPEKQLFGVGTGEYLRIL (5) ARG--YLQRSVANYLLRQVQD
                 .D  .FN .    .   :           : SG : T . .:. . .Y.          GDD:.             : K  .    E:LR             :  Y  R. :.  .  .  .
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FIG.4. Conserved RdRp motifs domains from dsRNA viruses infecting lower eukaryotes (Bruenn, 1993). GaRV-L2, *Gremmeniella abietina* RNA virus L2 (this study); GaRV-L1, *Gremmeniella abietina* RNA virus L1 (Tuomivirta and Hantula 2003a); SsRV2, Sphaeropsis sapinea *RNA virus 2* (Preisig *et al.*, 1998); CmRV, *Coniothyrium minitans* RNA virus (Cheng et.al., 2003); HmTV1-17, *Helicobasidium mompa* Totivirus 1-17 (Nomura *et al.*, 2003); SsRV1, Sphaeropsis *sapinea* RNA virus 1 (Preisig *et al.*, 1998); HvV190S, *Helminthosporium victoriae virus 190S* (Huang and Ghabrial 1996); Eb-RV1, *Eimeria brunetti* RNA virus 1 (AF356189); LRV1-4, *Leishmania RNA virus 1-4* (Scheffter *et al.*, 1994); LRV1-1, *Leishmania RNA virus 1-1* (Stuart *et al.*, 1992); LRV2-1, *Leishmania RNA virus 2-1* (Scheffter *et al.*, 1995); TVV2, *Trichomonas vaginalis* virus II (Bessarab *et al.*, 2000); TVV3, *Trichomonas vaginalis* virus 3 (AF325840); TVV-T1, *Trichomonas vaginalis* virus-T1 (Tai and Ip, 1995); ScV-L-A, *Saccharomyces cerevisiea virus L-A* (Icho and Wickner 1989); ScV-La , *Saccharomyces cerevisiea virus La* (Bruenn 1993); and Cy-av, Cucurbit yelows-assosiated virus (Coffin and Coutts, 1995). The alignment is based on Glustal W algorithm (Thompson *et al.*, 1994). Conserved amino acids are indicated below the alignment. Colons (:) and points (.) indicate higher and lower levels of chemical similarity, respectively, as defined by the Multiple Sequence Alignment program. Conserved amino acids between GaRV-L2, GaRV-L1, SsRV2, CmRV, HmTV1-17, SsRV1 and HvV190S are indicated on the top of the alignment as described above. Numbers in parentheses indicate the number of amino acid residues between the domains.

overlapping start/stop tetramer **AUGA** speculated to be a facilitator of the reinitiation mechanism (Soldevila and Ghabrial, 2000) for the production of RdRp among the viruses discussed above.

It was not possible to collect dsRNA from fractions of ultracentrifugation gradients using CF-11 unless the fractions were pretreated with phenol:chlorophorm. This suggests that the dsRNA molecules were enclosed in some kind of compartments. Although we do not have direct evidence on the nature of these compartments the following suggestions can be made. The film on the top of the tube after ultracentirifugation probably contained mainly GaMRV-S2 dsRNA molecules entrapped or enclosed inside lipid vesicles or organelles (mitochondria) with a low buoyant density. The pellet probably contained free RNA and DNA from disrupted compartments. The buoyant densities of GaRV-MS2 and GaRV-L2 were typical to members of the families *Partitiviriae* and *Totiviridae* and suggested that these two particle types were composed of dsRNA and protein.

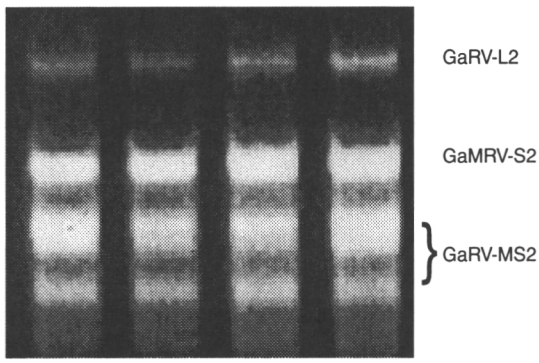

FIG.5. dsRNA isolations of mycelia of *Gremmeniella abietina* type A from isolate SurS4 conidiospores. Names of the corresponding dsRNA patterns are marked on the right.

Partitiviruses have been proposed to have originated from the genus *Totivirus* (Ghabrial, 1998). Therefore comparison of conserved motifs of RdRps of these three viruses in *G. abietina* and their supposed relatives should also shed light on the evolutionary origin of dsRNA viruses. Some studies favor monophyletic origin for the dsRNA viruses (Bruenn, 1991), whereas others favor polyphyletic origin (Koonin *et al.*, 1989; Koonin *et al.*, 1991; Koonin, 1992; Koonin and Dolja, 1993; Gibbs *et al.*, 2000; Ahn and Lee, 2001). This investigation supports polyphyletic origin for ssRNA and dsRNA viruses GaMRV-S, GaRV-MS and GaRV-L. The critical findings include (i) conserved motifs of RdRps of the three viruses reported here differentiate considerably from each other. (ii) GaMRV-S2 contains all typical conserved motifs of RNA dependent RNA polymerase-like (RdRp) proteins encoded by mitochondrial viruses and related RNAs (Hong *et al.*, 1999). Moreover, GaMRV-S2 aa sequence was more similar to putative ORFs found from mitochondrial genomes of plants *Arabidopsis thaliana* (Unseld *et al.*, 1997) and *Brassica napus* L (Handa, 2003) than to the two other viruses found in the SurS4 isolate of *G. abietina*. (iii) In GaRV-L2, eight conserved RdRp motifs from dsRNA viruses infecting lower eukaryotes (Bruenn, 1993) were found. These same motifs have also been found from Cucurbit yellows-associated virus dsRNA virus (Coffin and Coutts, 1994), isolated from plant *Cucumis sativus* L., but not from GaRV-MS2 as only motifs III, IV, V, and VI could be identified. (iv) Instead, new motifs VIIa and VIIIa were identified in GaRV-MS2, which could not be found in GaRV-L2, but could be identified from other putative partitiviruses. The RdRp of GaRV-MS2 was found to be more similar to replicases of *Sweet*

potato feathery mottle virus (Sakai *et al.*, 1997) and *Sorghum mosaic virus* (Yang and Mirkov, 1997) of the genus *Potyvirus* than to RdRp of GaRV-L2.

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