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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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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

Contents

Abbreviations used 6

List of Original Publications 7

Abstract 8

1. Introduction 9

1.1 Fungal viruses 9

1.2 Taxonomy of fungal viruses 9

1.2.1 dsRNA viruses 10

1.2.1.1 Family *Totiviridae* 10

1.2.1.2 Family *Partitiviridae* 10

1.2.1.3 Family *Chrysoviridae* 10

1.2.1.4 Family *Hypoviridae* 11

1.2.2 +ssRNA viruses 11

1.2.2.1 Family *Narnaviridae* 11

1.2.2.2 Family *Barnaviridae* 12

1.2.3 Retrovirus-like elements 12

1.2.3.1 Family *Metaviridae* 12

1.2.3.2 Family *Pseudoviridae* 12

1.2.4 dsDNA viruses 12

1.2.4.1 Genus *Rhizidiovirus* 12

1.2.5 Other viruses 13

1.3 Transmission of dsRNA in ascomycetes 13

1.4 *Gremmeniella abietina* 13

2. Aims of the study 14

3. Materials and methods 15

4. Results and discussion 17

4.1 Identification of dsRNA patterns 17

4.2 Effect of GaRV-L and GaRV-MS dsRNA patters on the pathogenicity of *G. abietina* type A 17

4.3 Ultracentrifugation of dsRNA patterns 18

4.4 Sequences of GaMRV-S patterns from isolates Luumäki 7 and SurS4 (III and IV) 18

4.5 Sequences of GaRV-MS patterns from isolates C5 and SurS4 (II and IV) 20

4.6 Sequences of GaRV-L patterns from isolates HR2 and SurS4 (II and IV) 21

4.7 Phylogeny of RNA virus families with members found in *G. abietina* 21

5. Concluding remarks 22

6. References 22

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

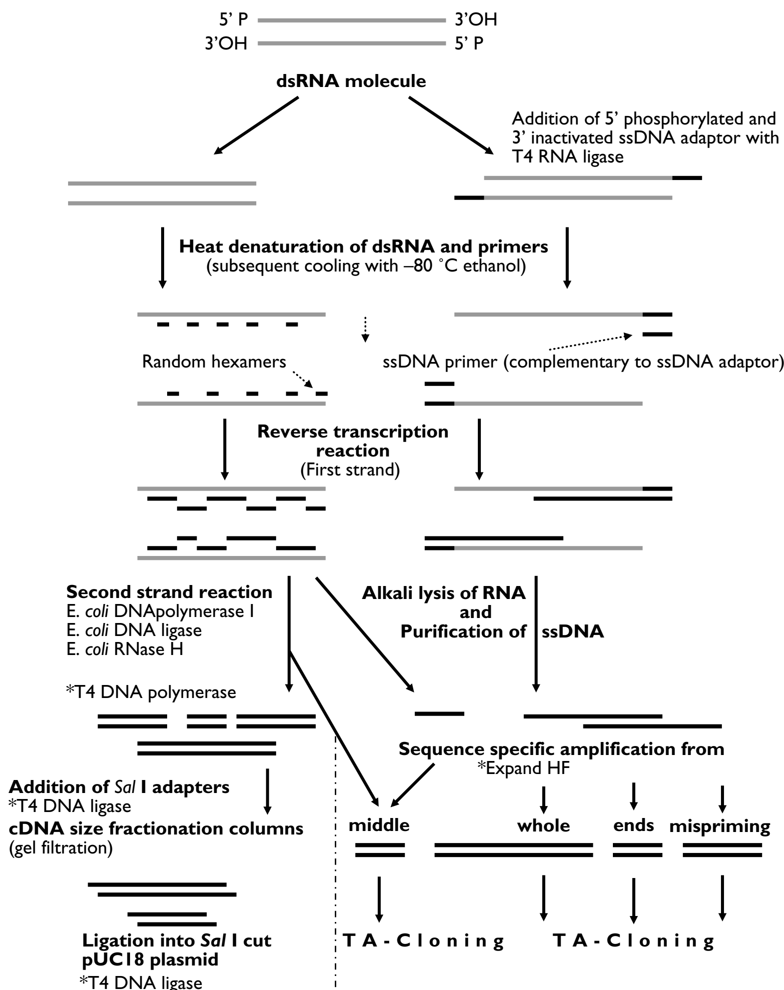

Fig.1. General overview of the cloning processes. For details, see II-IV.

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

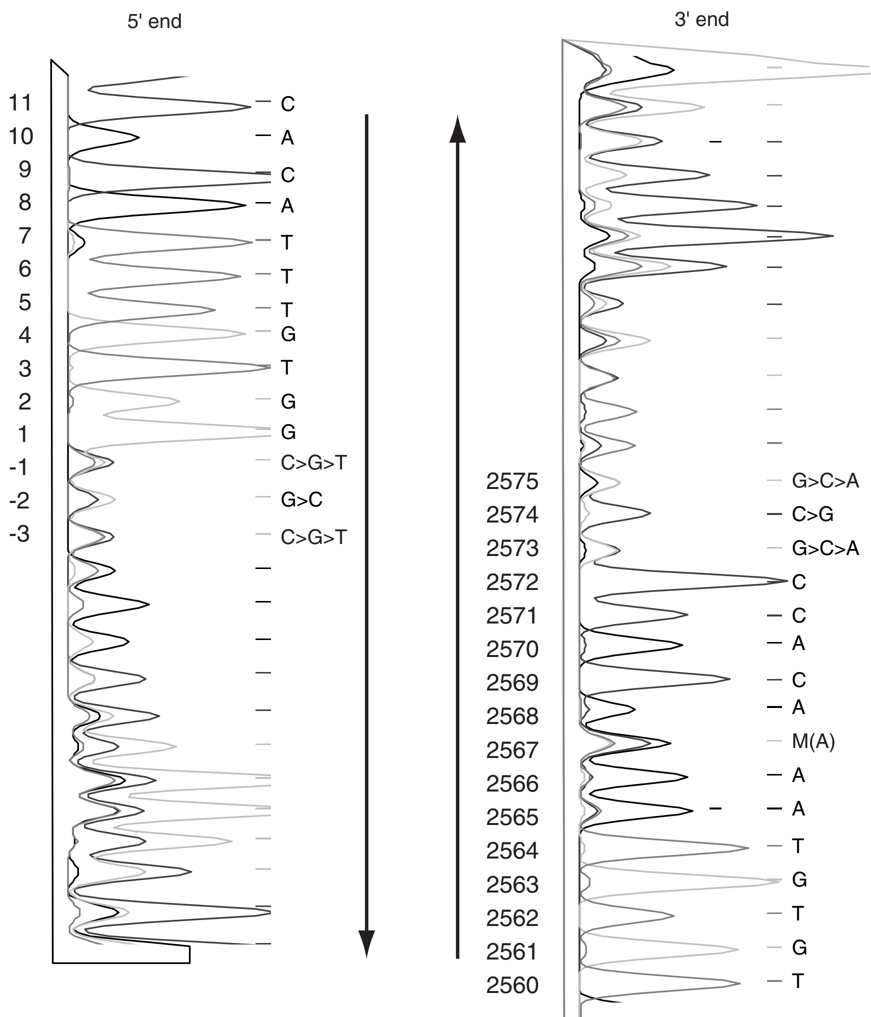

Fig.2. Direct sequencing experiment of the ends of GaMRV-S1. The arrows indicate the direction of sequencing reaction and the running numbers indicate the base position on the GaMRV-S1 genome.

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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