Mycobiology 29(2): 65-72 (2001)

Viral Infection Regulates Fungal Pathogenicity

Il-Pyung Ahn*

National Institute of Agricultural Science and Technology, Rural Development Administration, Suwon 441-707, Korea

Double-stranded RNAs

What is the double-stranded RNA? Double-stranded RNA (dsRNA) is a RNA duplex, that is, two complementary strands stabilized their structure by hydrogen bonds between them and hydrophobic interaction from environment (Buck, 1986; Hollings, 1982). They are resistant to RNaseA under the high ionic strength because their duplex structure is very stable in such conditions. The dsRNAs reported up to now showed distinguishable elution pattern in cellulose CF-11 column (Dodds *et al.*, 1984). They are retained in 15~16.5% ethanol-buffered column and eluted in the presence of ethanol-free buffer. The elution pattern and resistance to RNaseA have been exploited in recent years with the intention to develop a technique for detection and diagnosis of RNA viruses from plants and dsRNA infection of fungal tissues (Valverde *et al.*, 1990). The dsRNAs have been reported in bacteria, flowering plants, vertebrates, invertebrates, algae, and fungi (Buck, 1986). The dsRNAs were also reported as replicative form of single-stranded RNA viruses pathogenic to plants and satellite RNAs, genome of cryptic (temperate) and phytoreoviruses (Boccardo *et al.*, 1983; Jordan *et al.*, 1983; Rice *et al.*, 1989; Valverde and Dodds, 1986; Zelcer *et al.*, 1981).

It is widely believed that dsRNA is unable to take a role as a messenger RNA (mRNA) in host cells and sometimes inhibits the host protein synthesis (Choi *et al.*, 1991; Fire *et al.*, 1998; Gao and Nuss, 1996; Giardina *et al.*, 1995; Lindberg, 1960; Marcus *et al.*, 1986; Rigling *et al.*, 1989; Rigling and Van Alfen, 1991). Recently, there were reports about the silencing of specific genomic RNA expression by the transfection of synthesized dsRNA in *Caenorabditis elegans* (Fire *et al.*, 1998).

For effective *in vivo* transcription and replication of dsRNA, viral genome-coded RNA polymerases are necessary and such polymerases have been demonstrated in a number of mycoviruses (Koonin *et al.*, 1991; Poch *et al.*, 1989). There are two strategies of dsRNA replication in fungi, semi-conservative and conservative. PsV-S is the mycovirus of *Penicillium stoloniferum* and responsible for inteferon-like substance production. Its dsRNA genome showed *in vitro* replicase activity, and give rise to dsRNA progeny molecules that remain unfolded during the whole replication (Banks *et al.*, 1968). This is a typical example of conservative replication. A typical example for semi-conservative mode is found in the replication of L-A dsRNA virus of *Saccharomyces cerevisiae*. In this mode, two types of RNA polymerase activities, ds → ss RNA by transcriptase and ss → dsRNA by RNA polymerase, were required (Garfinkel *et al.*, 1985). These activities would permit L-A dsRNA to replicate asynchronously.

DsRNAs in plant pathogenic fungi. DsRNAs are widespread in plant pathogenic fungi (Hollings, 1982). The presence or absence of different sizes and number of dsRNAs detected on agarose or polyacrylamide gel electrophoresis has been used to characterize the field isolates of several fungal pathogens (Morris and Dodds, 1979). DsRNAs can be used not only in analyzing cytoplasmic genetic elements but also as a genetic marker in fungi (Michelmore and Hulberts, 1987; Newhouse *et al.*, 1992; Valverde *et al.*, 1990).

Numerous correlative evidences and conversion tests in various kinds of organisms supported the inevitable association of virulence and/or virulence-related phenotypes with dsRNAs (Boland, 1992; Brasier, 1983; Castanho *et al.*, 1978; Elliston, 1985; Finkler *et al.*, 1985; Fulbright, 1984; Hammar *et al.*, 1989; Kousik *et al.*, 1994; McFadden *et al.*, 1983; Rigling and Van Alfen, 1991; Smit *et al.*, 1996; Zhang *et al.*, 1993). These indirect evidences indicate the value of dsRNA because of their potential for pollution-free control of fungal diseases. However, most of them remain as speculation since Kochs postulates have not been satisfied.

Ophiostoma (*Ceratocystis*) *ulmi* is the causal agent of Dutch elm disease. "Diseased" isolates of the pathogen displayed a slow growth rate, reduced viability of conidia, and impairment of sexual reproduction (Rogers *et al.*, 1986). The diseased state was transmitted by extrachromosomal elements referred as d-factors. Multiple dsRNA segments have been found in both healthy and diseased strains of *O. ulmi* and were efficiently transmitted between strains and to its conidia. Transmission of the diseased state coincided with the transmission of a specific set of dsRNA segments. Healthy single spore-derived isolates recovered from a diseased strain lacked dsRNAs. Further work showed that the dsRNA segments in d^2-

*Corresponding author <E-mail: jinhyung@rda.go.kr>

infected isolates were co-purified with mitochondria, and mitochondria isolated from such diseased isolates were deficient in cytochrome aa_3 (Brasier, 1983).

The causal agent of wheat take-all, *Gaeumanomyces graminis* var. *tritici*, is another pathogen in which cytoplasmic elements have been implicated in the pathogenicity variation found in natural populations (McFadden *et al.*, 1983). Results from a long-term analysis of a few hypovirulent strains revealed the presence of a complement of nine segments of dsRNA. Conidial isolates resulting from mitotic division in such strains are segregated to virulent and hypovirulent cultures. The virulent segregates were free of viral dsRNAs whereas the hypovirulent isolates contained viral dsRNAs.

Castanho and Butler (1978) reported the Rhizoctonia decline due to dsRNAs. Pairing of healthy (dsRNA-free) isolate with diseased (dsRNA-containing) one resulted in the conversion of virulence into hypovirulence. Subsequent researches revealed that the presence of dsRNA did not correlate with hypovirulence (Bharathan and Tavantzis, 1990, 1991; Kousik *et al.*, 1994; Zanzinger *et al.*, 1984). Recently, Jian *et al.* (1997) reported that specific dsRNA was responsible for modulation of fungal virulence and its effect was counteracted with another dsRNA molecule in the same thallus.

Helminthosporium victoriae, the causal agent of victoria oat blight, produces a host specific toxin, victorin, and induced enormous yield losses in a oat variety with resistant gene to the oat crown rust in USA in 1947 and 1948. Lindberg (1960) reported the correlative evidence about the relationship between the mycelial disease, inhibition of victorin production and cytoplasmic dsRNAs in *H. victoriae*. Ghabrial (1986) reported that the mycelial disease of *H. vitoriae* is induced by the 145 S virus because 190 S virus was detected from some healthy isolates. In addition, 145 S virus did not occur alone (Sanderin and Ghabrial, 1978). This means that 145 S might be dependent upon their maintenance and replication on the product of 190 S.

The first clear indication of the physical nature of a cytoplasmic element associated with transmissible hypovirulence in *Cryphonectria* (*Endothia*) *parasitica* was provided by a report in which several hypovirulent strains were shown to harbor dsRNA molecules not present in virulent strains (Anagnostakis, 1979; Kuhlman, 1983). It was subsequently shown that the conversion of a virulent strain to a hypovirulent phenotype coincided with transmission of dsRNAs. Since the dsRNAs were not reintroduced into fungal strains in a cell-free form that was subsequently able to replicate, there was no demonstration of the direct cause and effect relatioship between dsRNA and hypovirulence. Choi and Nuss (1992) reported that transformation of virulent *C. parasitica* strains with a full length complementary DNA copy of a hypovirulence-associated viral dsRNA conferred the complete hypovirulence phenotype. Cytoplasmic dsRNA was resurrected from the chromosomally integrated cDNA copy and was apparently responsible for the converting virulent strains to hypovirulent. Further work showed that hypovirulence was coupled with associated traits such as slow growth rate and lack of pigmentation. Also, *in vitro* synthesized full-length transcripts of the hypovirus-coding strand were infectious when introduced into fungal sphaeroplasts. Hypovirus infections were readily established in *C. parasitica* and in related fungal species not previously reported to harbor the particular dsRNA.

Mycelial degeneration, attenuation of virulence or pathogenicity, inhibition of conidiogenesis, or poor pigmentation were observed in the dsRNA-infected isolates of several plant pathogens. These include *Chalara elegans* (*Thielaviopsis basicola*) (Bottacin *et al.*, 1994), the causal agent of tobacco root rot, *Diaporthe ambigua* (Smit *et al.*, 1996), the pathogen of soybean dieback, *Leucostoma persoonii* (Hammar *et al.*, 1989; Snyder *et al.*, 1989), the agent of cytospora disease, *Sclerotinia sclerotiorum* (Boland, 1992), the causal agent of zoysiagrass white mould, and *S. homoeocarpa* (Zhou and Boland, 1997), the pathogen of turf grass dollor spot.

Hypovirulence by dsRNA in *Cryphonectria parasitica*. *Cryphonectria parasitica*, the causal agent of American chestnut blight, has occurred in North America and caused severe devastation all over the forests (Anagnostakis, 1981). Hypovirulent isolates of *C. parasitica* have been found in infected European and American chestnut trees. These dsRNA-infected isolates are usually characterized by reduction in virulence, mycelial growth, pigmentation, sporulation, oxalic acid accumulation, and intra- and extracellular laccase activities compared with normal, dsRNA-free isolates (Anagnostakis, 1979; Day *et al.*, 1977; Fulbright, 1984). These characteristics and dsRNA can be transmitted correspondingly from hypovirulent isolates to virulent one following hyphal anastomosis (Chen *et al.*, 1994).

There have been many reports about the effect of biological control using these dsRNA-containing isolates (Chung *et al.*, 1994; Enebak *et al.*, 1994). If conversion takes place in a canker on a chestnut host, the canker is often limited and healing tissue grows again (Fulbright, 1984).

Transmission of hypovirulence determinants and the resulting conversion occur following hyphal anastomosis between hypovirulent and virulent isolates (Anagnostakis, 1979). The frequency and stability of anastomoses between different isolates of *C. parasitica* are determined by several *vic* genes that govern vegetative incompatibility. As in other Asocmycetes, vegetative incompatibility occurs between paired homokaryotic mycelia that have different alleles at one or more of the controlling loci, and it results in the death of fused cells (Anagnostakis, 1981). Field

observations suggest that vegetative incompatibility can restrict conversion (Kuhlman, 1983).

Three kinds of dsRNA molecules, L, M, and S, were detected and the L-dsRNA was responsible for the fungal hypovirulence. All three kinds of dsRNAs showed sequence homology (Rae *et al.*, 1989). Shapira *et al.* (1991) demonstrated that M and S dsRNAs are the deletion product of L-dsRNA using RT-PCR-mapping. One strand of L-dsRNA contains a 3' polyadenylate [poly (A)] tail that is base-paired to a stretch of polyuridine [poly (U)] present at the 5 terminus of the complementary strand (Hiremath *et al.*, 1988). The molecule consists of 12,712 base pairs, excluding the poly(A) : poly(U) homopolymer domain. Genetic organization and expression strategies of the L-dsRNA are similar to those of several plant viral genomes and have an apparent evolutionary relationship to the plant potyvirus (Koonin *et al.*, 1991). Choi and Nuss (1992) reported that introduction of full-length cDNA clone of L-dsRNA into virulent isolate by DNA-mediated transformation resulted in the acquisition of hypovirulence phenotypes. In addition, complete L-dsRNA was resurrected from the chromosomally integrated cDNA copy because transcript of infectious clone integrated into chromosome could function as mRNA as well as a template for RNA-dependent RNA polymerase (Chen *et al.*, 1994).

L-dsRNA contains two large open reading frames (ORFs) within the poly(A) strand that were designated ORF A (622 codons) and ORF B (3,165 codons). *In vitro* translation products of ORF A were p29, the papain-like cystein protease and p40 (Choi *et al.*, 1991). Those of ORF B were RNA-dependent RNA polymerase and helicase. Both are necessary for the replication of L-dsRNA. Transformation of virulent isolates with ORF A-disrupted infectious clones resulted in the reduced pigmentation, laccase activities, and sporulation, but there was no reduction of virulence (Choi *et al.*, 1992), indicating the above characteristics and virulence were not always synchronously regulated by L-dsRNA.

Many investigations have been performed to characterize the hypovirulence mechanisms induced by L-dsRNA including regulation of fungal gene expression in the transcriptional level and pharmacological experiments about the signal transduction pathways (Larson *et al.*, 1992). Rigling and Van Alfen (1993) reported that intra- and extracellular laccase activities were distinguishably down-regulated in L-dsRNA-containing hypovirulent isolates. They also demonstrated that this is due to the reduced expression of laccase gene in the hypovirulent isolates (Kazmierczak *et al.*, 1996). Zhang *et al.* (1993) revealed that hypovirus-related traits were also mimicked in the virulence gene, *vir2*-disrupted healthy isolates. These results indicated that dsRNA infection causes a significant and persistent alteration of fungal gene expression/transcript accumulation. Subsequent studies identified numerous fungal genes under the control of the dsRNA in this fungus. These genes include a gene encoding a putative hormone (Zhang *et al.*, 1993); *cbh-1*, a gene encoding the cellobiohydrolase I CBH-1 (Wang and Nuss, 1995); and *cpg-1*, a gene encoding G protein alpha subunit, CPG-1 (Choi *et al.*, 1995).

There are two different and opposing regulatory pathways appearing to govern laccase gene, *lac-1* transcript level in dsRNA-free isolates; a stimulatory pathway was found to be dependent on the inositol triphosphate (IP_3) and calcium second messenger systems, especially including calmodulin (Larson *et al.*, 1992; Larson and Nuss, 1993). The second pathway limiting transcript accumulation was shown to require ongoing protein synthesis. Changes in the *lac-1* transcript accumulation were related to modulation of promoter activity, and this activity was suppressed in the dsRNA-infected, hypovirulent isolates. The dsRNA interferes with transduction of an IP_3-calcium-dependent signal that is required for stimulation of *lac-1* transcription. Recent reports proved that more than 300 fungal gene expression was down-regulated by L-dsRNA by the differential display-polymerase chain reaction (DD-PCR) (Nuss, 1996). Among them, more than 65% genes were also down-regulated in the disruption transformant of *cpg-1*, the gene encoding GTP-binding protein Gi_{α}-subunit. This mutant also showed all hypovirulence-related traits including reduced virulence, pigmentation, and sporulation, and diseased cultural morphology on culture media. In addition, both L-dsRNA infection and disruption of *cpg-1* elevate the cyclic AMP (cAMP) levels 3 to 5 folds (Chen *et al.*, 1996). They also reported that it was possible to mimic the effect of virus infection and disruption of *cpg-1* on transcript accumulation for representative fungal genes by drug-indued elevation of cAMP level. These results suggest a role for Gi_{α} protein-regulating cAMP accumulation in dsRNA-mediated alteration of fungal gene expression. On the other hand, disruption of G_{β} subunit did not cause any noticible phenotpic changes except the inhibitory effect on myceial growth (Kasahara and Nuss, 1997).

It is believed that there are more than seven vegetative incompatibility genes (*vic* genes) in *C. parasitica* (Rigling *et al.*, 1989) and as a result, this fungal species contained 27 different VCGs that are not able to fuse with each other. Vegetative incompatibility is indeed a significant barrier to successful interaction of virulent and hypovirulent isolates in the field, resulting in the failure of biological control. Nuss (1996) suggested that infectious clone-integrated transformants could be used as more superior biological control agent to hypovirulent isolates harboring cytoplasmic L-dsRNA because the transformants might overcome the vegetative incompatibility barriers in nature by transmission of infectious clones through sexual recombination, following hyphal anastomosis and infectious clone-

Table 1. Double-stranded RNAs in plant pathogenic fungi

Fungi	Proposed role of dsRNA	References
Cryphonectria parasitica	Hypovirulence	Choi and Nuss (1992)
Gaeumannomyces graminis var. *tritici*	Reducing virulence	Blanche *et al.* (1981)
Ophiostoma ulmi	Mycelial disease Inhibition of cytochrome aa_3	Brasier (1983)
Rhizoctonia solani	Enhancing virulence	Finkler *et al.* (1985) Jian *et al.* (1997)
	Reducing virulence	Castanho *et al.* (1978) Jian *et al.* (1997)
Helminthosporium victoriae	Mycelial disease Inhibition of victorin production	Lindberg (1960) Ghabrial (1986)
Ustilago maydis	Killer toxin	Koltin *et al.* (1978)
Phytophthora infestans	Enhancing virulence Genetic marker	Tooley *et al.* (1989) Newhouse *et al.* (1992)
Magnaporthe grisea	Cryptic	Chun and Lee (1997)
Chalara elegans	Reducing virulence	Bottacin *et al.* (1994)
Diaporthe ambigua	Reducing virulence	Smit *et al.* (1996)
Leucostoma persoonii	Reducing virulence	Hammer *et al.* (1989)
Sclerotinia sclerotiorum	Reducing virulence	Boland (1993)
S. homoeocarpa	Reducing virulence	Zhou and Boland (1997)

containing conidia.

All these results paved the way of using engineered dsRNA-mediated hypovirulence for biological control strategies in other fungal pathogen-plant host systems.

Mycoviruses

Many fungal species are infected by dsRNA viruses, however, mycoviruses with DNA or single-stranded RNA (ssRNA) genomes are also known (Hollings, 1982). These mycoviruses classified so far are in the families Barnaviridae and Totiviridae or the genus *Rhizidiovirus*. Unclassified viruses are known to infect fungi in the genera *Agaricus*, *Allomyces*, *Aspergillus*, *Colletotrichum*, *Gaeumannomyces*, *Helminthosporium*, *Lentinus* and *Periconia* (Buck, 1986). Most fungal viruses induce only inapparent effects, that is, cryptic, except some notable effect of dieback in cultivated mushrooms (Hollings, 1982). Some plant viruses are transmitted to new host plants by association with root-infecting fungi, and the vector phase is the motile zoospores in *Olpidium* sp. transmitting *Tombusvirus* and *Necrovirus* spp., the unclassified lettuce big vein and tobacco stunt virus (Campbell, 1996). Important viruses infecting fungi are described in Table 2.

Most fungal viruses are isometric and contained dsRNA(s) as their genome and mainly classified into two groups, Totiviridae and Partiviridae. The family Partiviridae occupies much larger portion than Totiviridae. These are the most distinguishable characteristics different from viruses infecting plants and animals. More than 90% of plant-infecting viruses have (+) ssRNA as their genomes and their virion shape are comparatively diverse. Physicochemical and genomic characteristics of animal-infecting viruses are even more diverse than those of plant viruses. Until now, very few mycoviruses have been adequately characterized, and most are known only as virus-like particles (VLPs) in electron micrographs of partially purified extracts from fungi or, sometimes, in thin-section studies (Buck, 1986).

The viral disease of the cultivated mushroom (*Agaricus bisporus*), La France disease, has been recorded almost all over the countries where it is grown, and is the

Table 2. Classification of mycoviruses and their characteristics

Family	Genus	Virion	Genome	Host
Barnaviridae	Barnavirus	Bacilliform	(+)ssRNA	*Agaricus* spp.
Partiviridae	Crysovirus	Isometric	Segemented dsRNAs	*Penicillium* spp.
	Partivirus	Isometric	Segmented dsRNAs	*Agabicus*, *Aspergillus*, *Gaeumanomyces*, *Penicillium*, *Rhizoctonia*, *Ophiostoma* etc.
Hypoviridae	Hypovirus	No	Non-segmented linear dsRNA	*Cryphonectria parasitica*
Totiviridae	Totivirus	Isometric	Non-segmented dsRNA	*Helminthosporium*, *Saccharomyces*, *Ustilago*, *Aspergillus*
No	Rhizidiovirus	Isometric	25 kbp dsRNA	*Rhizidomyces*

first evident example of mycovirus. Hollings (1962) isolated three kinds of virus particles from diseased mushrooms. This disease is spread by viable infected mycelium and by spores from infected mushrooms. The various symptoms, which are most damaging to mushroom yield, have been reported. In cell-free preparations from diseased mushrooms, stained with phosphotungstic acid, usually three types of virus particles were observed, often in combination (Harmsen *et al.*, 1989). Isometric particles are 25 and 34 nm in diameter, and bacilliform particles with 50×19 nm (Passmore and Frost, 1979). Diseased mushrooms also contain up to 10 segments of dsRNA fragments ranging 0.8~3.6 kbp which do not show sequence similarity among them. On the other hand, healthy tissues do not contain any virions and dsRNA except L6 dsRNA (Harmsen *et al.*, 1989). Injection of a cell-free virus preparation into young mushrooms and subsequent reisolation of the three types of virus particles from fruiting bodies showing symptom proved that these particles caused the disease. The 34 nm isometric virus, La France isometric virus (LIV), has a distinct hexagonal outline, three kinds of proteins, 63, 66, and 129 kDa, and 9 segments of dsRNAs (Goodin *et al.*, 1992). The *Mushroom bacilliform virus* (MBV) contained no protein and 4.4 kbp ssRNA. The titer of 25 nm virus particle is relatively lower than other particles.

Because the rareness of RNA-dependent polymerization processes encoded by their host, the RNA viruses were forced to develop very specific polymerase activities for the replication of their own RNA genome. Two main types of polymerase are encoded on the viral genome; RNA-dependent RNA polymerases leading to a strictly RNA life cycle and RNA-dependent DNA polymerases (reverse transcriptases) in which the RNA genome represents a transient state leading to DNA and possible integration in the host genome. RNA-dependent RNA polymerases (RDRPs) are involved in the replication processes of plus- and minus- ssRNA, and dsRNA viruses, and RDRPs are involved in the replication of retroid-elements including retroviruses, the transposable integrated elements (non-viral and viral retrotransposons), and some DNA viruses including hepadnaviruses.

Despite wide variations in morphology, genome organization and sequences of their structural proteins among viruses, the RNA-dependent RNA polymerase sequences have revealed the conservation of large peptide regions ranging 120 to 210 amino acids, and there are four consensus sequences (Koonin *et al.*, 1991). Interviral relationships across wide evolutionary distances may also exist. One of the most conserved regions of the RDRP, GDD span, are also present in RNA-dependent DNA polymerases as well as DNA-dependent DNA polymerases. Koonin *et al.* (1991) indicated the more close phylogenetic relationship between potyvirus and L-dsRNA in *C. parasitica* rather than relationship between L-dsRNA and dsRNA mycoviruses or cryptic viruses based on the deduced amino acid sequences for conserved cDNA sequences of RDRPs. On the other hand, RDRPs in mitochondrial dsRNA in *C. parasitica* NB631 showed a close relationship with yeast W- and T-dsRNA (Polashock *et al.*, 1997). In spite of the co-existence of 6.4 and 3.6 kbp dsRNAs in *Rhizoctonia solani*, 6.4 kbp dsRNA showed close relationship with *Broad bean mottle virus* (BBMV) and 3.6 kbp dsRNA with Potyvirus (Jian *et al.*, 1997; Lakshman *et al.*, 1998).

Phylogenetic analyses data also unveiled the origin of some mycoviruses in plant pathogenic fungi (Nogawa *et al.*, 1996). They interact with their host continuously through infection, the plant-microbe interaction, in the nature. It is presumptive that the fungus might have been acquired ancestral viruses from plant viruses or provided them to plants. It is attractive to assume the evolutionary relatinships between mycoviruses and plant viruses, however, more of dsRNA sequences will be necessary for elucidation of this hypothesis.

Nectria radicicola (anamorph : *Cylindrocarpon destructans* (Zins.) Scholten) is a homothallic Ascomycetous fungus, and cause root rot or vascular wilts of a wide variety of trees and crops all over the world (Booth, 1966; Chung, 1975; Matuo and Miyazawa, 1969). Nectria root rot of ginseng caused by this fungus is one of the major obstacles for stable ginseng production (Chung, 1975; Reeleder and Brammall, 1994). This disease is manifested in young and mature plants throughout ginseng growing regions, since this crop requires fertile soil, rich in humus, and continuous shade for six years.

Chemical treatments on soil were not effective and resistant germplasm is not available yet (Kwon *et al.*, 1998; Miyazawa, 1966). Although biological control promoting soil suppressiveness has been conducted and shed some light on the contol of ginseng root rot, the fortified understanding about the expression mechanism of virulence and virulence-related fators are required to establish novel strategies to control this disease (Chung and Kim, 1978).

Nectria root rot has also been known as one of the most important factors for replanting problem in ginseng cultivation. However, the mechanisms responsible for replanting problem are not clearly understood. It has been explained that replanting problem is induced by the increase of pathogenic populations proportion among soil microbial community during continuous cropping in the same field (Jacobson and Gordon, 1988; Ziezold *et al.*, 1998).

Dissecting the genetic and biochemical mechanisms regulating virulence in *N. radicicola* might lead to the development of biological control or other pollution-free methods of plant disease control. In this study, we report

that a 6.0 kbp dsRNA in *N. radicicola* is responsible for upregulation of fungal virulence. An RNA-dependent RNA polymerase gene present in this dsRNA is related closely to those of plant cryptic viruses containing dsRNAs as their genome. Biochemical analyses indicated that the 6.0 kbp dsRNA also is involved in the regulation of signal-transduction pathways of the host fungal cells.

We analyzed dsRNAs from *Nectria radicicola*, the causal fungus of ginseng root rot. Four distinct sizes of dsRNAs, 6.0, 5.0, 2.5, and 1.5 kbp, were detected from 24 out of the 81 strains tested. Curing tests of individual dsRNAs suggested that the presence of 6.0 kbp dsRNA was associated with high levels of virulence, sporulation, laccase activity, and pigmentation in this fungus. The 6.0 kbp dsRNA-cured strains completely lost virulence-related phenotypes. This 6.0 kbp dsRNA was reintroduced by hyphal anastomosis to a dsRNA-cured strain marked with hygromycin resistance, which resulted in the restoration of virulence-related phenotypes. These results strongly suggest that 6.0 kbp dsRNA upregulates fungal virulence in *N. radicicola*. Sequencing of several cDNA clones derived from 6.0 kbp dsRNA revealed the presence of RNA-dependent RNA polymerase (RDRP) gene. Phylogenetic analysis showed that this gene is closely related to those of plant cryptic viruses. Biochemical analyses suggested that the 6.0 kbp dsRNA may regulate fungal virulence through signal transduction pathways involving both cAMP-dependent protein kinase and protein kinase C.

References

Anagnostakis, S. L. 1979. Hypovirulence conversion in *Endothia parasitica*. *Phytopathology* **69**: 1226-1229.

_____. 1981. Stability of double-stranded RNA components of *Endothia parasitica* through transfer and subculture. *Exp. Mycol.* **5**: 236-242.

Banks, G. T., Buck, K. W., Chain, E. B., Himmelweit, F. and Marks, J. E. 1968. Viruses in fungi and interferon stimulation. *Nature* **218**: 542-545.

Bharathan, N. and Tavantzis, S. M. 1990. Genetic diversity of double-stranded RNA from *Rhizoctonia solani*. *Phytopathology* **80**: 631-635.

_____ and _____. 1991. Assessment of genetic relatedness among double-stranded RNAs from isolates of *Rhizoctonia solani* from diverse geographic origins. *Phytopathology* **81**: 411-415.

Boccardo, G., Lisa, V. and Milne, R. G. 1983. Cryptic viruses in plants. pp. 425-430. *In*: R. W. Compans and D. H. L. Bishop eds. Double-stranded RNA Viruses. Elsevier, New York.

Boland, G. J. 1992. Hypovirulence and double-stranded RNA in *Sclerotinia sclerotiorum*. *Can. J. Plant Pathol.* **14**: 10-17.

Booth, G. 1966. The genus *Cylindrocarpon*. *Commonw. Mycol. Papers* **104**: 1-56.

Bottacin, A. M., Levesque, C. A. and Punja, Z. K. 1994. Characterization of dsRNA in *Chalara elegans* and effects on growth and virulence. *Phytopathology* **84**: 303-312.

Brasier, C. M. 1983. A cytoplasmically transmitted disease of *Ceratocystis ulmi*. *Nature* **305**: 220-222.

Buck, K. W. 1986. Fungal Virology - an Overview. pp. 1-84 *In*: K. W. Buck ed. Fungal Virology. CRC Press, Boca Raton, FL.

Campbell, R. N. 1996. Fungal transmission of plant viruses. *Annu. Rev. Phytopathol.* **34**: 87-108.

Castanho, B., Butler, E. E. and Shepherd, R. J. 1978. The association of double-stranded RNA with Rhizoctonia decline. *Phytopathology* **68**: 1515-1519.

Chen, B., Choi, G. H. and Nuss, D. L. 1994. Attenuation of fungal virulence by synthetic infectious hypovirus transcripts. *Science* **264**: 1762-1264.

_____, Gao, S., Choi, G. H. and Nuss, D. L. 1996. Extensive alteration of fungal gene transcript accumulation and elevation of G-protein-regulated cAMP levels by a virulence-attenuating hypovirus. *Proc. Natl. Acad. Sci. USA* **93**: 7996-8000.

Choi, G. H., Chen, B. and Nuss, D. L. 1995. Virus-mediated or transgenic suppression of a G-protein a subunit and attenuation of fungal virulence. *Proc. Natl. Acad. Sci. USA* **92**: 305-309.

_____, Larson, T. G. and Nuss, D. L. 1992. Molecular analysis of the laccase gene from the chestnut blight fungus and selective suppression of its expression in an isogenic hypovirulent strain. *Mol. Plant-Microbe Interact.* **5**: 119-128.

_____ and Nuss, D. L. 1992. Hypovirulence of chestnut blight fungus conferred by an infectious viral cDNA. *Science* **257**: 800-803.

_____, Shapira, R. and Nuss, D. L. 1991. Cotranslational autoproteolysis involved in gene expression from a double-stranded RNA genetic element associated with hypovirulence of the chestnut blight fungus. *Proc. Natl. Acad. Sci. USA* **88**: 1167-1171.

Chung, H. S. 1975. Studies on *Cylindrocarpon destructans* (Zins.) Scholten causing root rot of ginseng. *Rept. Tottori Mycol. Inst. (Japan)* **12**: 127-138.

_____ and Kim, C. H. 1978. Biological control of ginseng root rots with soil amendments. pp. 67-74. *In*: Proc. 2nd Int. Ginseng Symp. Korea Ginseng Research Institute, Seoul, Korea

Chung, P., Bedker, P. J. and Hillman, B. I. 1994. Diversity of *Cryphonectria parasitica* hypovirulence-associated double-stranded RNAs within a chestnut population in New Jersey. *Phytopathology* **84**: 984-990.

Day, P. R., Dodds, J. A., Elliston, J. E., Jaynes, R. A. and Anagnostakis, S. L. 1977. Double-stranded RNA in *Endothia parasitica*. *Phytopathology* **67**: 1393-1396.

Dodds, J. A., Morris, T. J. and Jordan, R. L. 1984. Plant viral double-stranded RNA. *Annu. Rev. Phytopathol.* **22**: 151-168.

Elliston, J. E. 1985. Characteristics of dsRNA-free and dsRNA-containing strains of *Endothia parasitica* in relation to hypovirulence. *Phytopathology* **75**: 151-158.

Enebak, S. A., MacDonald, W. L. and Hillman, B. I. 1994. Effect of dsRNA Associated with isolates of *Cryphonectria parasitica* from the central Appalachians and their relatedness to other dsRNAs from North America and Europe. *Phytopathology* **84**: 528-534.

Finkler, A., Koltin, Y., Barash, I., Sneh, B. and Pozniak, D. 1985. Isolation of a virus from virulent strains of *Rhizoctonia solani*. *J. Gen. Virol.* **66**: 1221-1232.

Fire, A., Xu, S. Q., Montgomery, M. K., Kostas, S. A., Drirver, S. E. and Mello, C. C. 1998. Potent and specific genetic interference by double-stranded RNA in *Chaenorabditis elegans*.

Nature **391**: 806-811.

Fulbright, D. W. 1984. Effect of eliminating dsRNA in hypovirulent *Endothia parasitica*. *Phytopathology* **74**: 722-724.

Gao, S. and Nuss, D. L. 1996. Distinct roles for two G protein alpha subunits in fungal virulence, morphology, and reproduction revealed by targeted gene disruption. *Proc. Natl. Acad. Sci. USA* **93**: 14122-14127.

Garfinkel, D. J., Bocke, J. D. and Fink, G. R. 1985. Ty element transposition reverse transcriptase and virus-like particles. *Cell* **42**: 507-517.

Ghabrial, S. A. 1986. A transmissible disease of *Helminthosporium victoriae*-evidence for a viral etiology. pp. 163-176 *In*: K. W. Buck ed. Fungal Virology. CRC Press, Boca Raton, FL.

Giardina, P., Cannio, R., Martirani, L., Marzullo, L., Parmieri, G. and Sannia, G. 1995. Cloning and sequencing of a laccase gene from the lignin-degrading Basidiomycete *Pleurotus ostreatus*. *Appl. Environ. Microbiol.* **61**: 2408-2413.

Goodin, M. M., Schlagnhaufer, B. and Romaine, C. P. 1992. Encapsidation of the La France disase-specific double-stranded RNAs in 36 nm isometric virus-like particles. *Phytopathology* **82**: 285-290.

Hammar, S., Fulbright, D. W. and Adams, G. C. 1989. Association of double-stranded RNA with low virulence in an isolate of *Leucostoma persoonii*. *Phytopathology* **79**: 568-572.

Harmsen, M. C., van Griensven, L. J. and Wessels, J. G. H. 1989. Molecular analysis of *Agaricus bisporus* double-stranded RNA. *J. Gen. Virol.* **70**: 1613-1616.

Hiremath, S. T., Nuss, D. L., Ghabrial, S. A. and Rhoads, R. E. 1988. Molecular structure of hypovirulence-associated dsRNAs in the chestnut blight fungus *Endothia parasitica*. *Nucleic Acid Res.* **14**: 9877-9896.

Hollings, M. 1962. Viruses associated with a dieback diseases of cultivated mushrooms. *Nature* **196**: 962-965.

_____. 1982. Mycoviruses and plant pathology. *Plant Dis.* **66**: 1106-1112.

Jacobson, D. J. and Gordon, T. R. 1988. Vegetative compatibility and self-incompatibility within *Fusarium oxysporum* f. sp. *melonis*. *Phytopathology* **78**: 668-672.

Jian, J., Lakshman, D. K. and Tavantzis, S. M. 1997. Association of distinct double-stranded RNAs with enhanced or diminished virulence in *Rhizoctonia solani* infecting potato. *Mol. Plant-Microbe Interact.* **10**: 1002-1009.

Jordan, R. L., Dodds, J. A. and Ohr, H. D. 1983. Evidence for viruslike agents in avocado. *Phytopathology* **73**: 1130-1135.

Kasahara, S. and Nuss, D. L. 1997. Targeted disruption of a fungal G-protein beta subunit gene results in increased vegetative growth but reduced virulence. *Mol. Plant-Microbe Interact.* **10**: 984-993.

Kazmierczak, P., Pfeiffer, P., Zhang, L. and Van Alfen, N. K. 1996. Transcriptional repression of specific host genes by the mycovirus Cryphonectria hypovirus 1. *J. Virol.* **70**: 1137-1142.

Koonin, E. V., Choi, G. H., Nuss, D. L., Shapira, R. and Carrington, J. C. 1991. Evidence for common ancestry of a chestnut blight hypovirulence- associated double-stranded RNA and a group of positive-strand RNA plant viruses. *Proc. Natl. Acad. Sci. USA* **88**: 10647-10651.

Kousik, C. S., Snow, J. P. and Valverde, R. A. 1994. Comparison of double-stranded RNA components and virulence among isolates of *Rhizoctonia solani* AG-1IA and AG-1IB. *Phytopathology* **84**: 44-49.

Kuhlman, E. G. 1983. Effects of hypovirulence in *Cryphonectria parasitica* and of secondary blight infections on dieback of American chestnut trees. *Phytopathology* **73**: 1030-1034.

Kwon, W.-S., Chung, C.-M., Kim, O.-T., Lee, M.-G. and Choi, K.-T. 1998. Breeding process and characteristics of KG101, a superior line of *Panax ginseng* C. A. Meyer. *Kor. J. Ginseng Sci.* **22**: 11-17.

Lakshman, D. K., Jian, J. and Tavantzis, S. M. 1998. A double-stranded RNA element from a hypovirulent strain of Rhizoctonia solani occurs in DNA form and is genetically related to the pentafunctional AROM protein of the shikimate pathway. *Proc. Natl. Acad. Sci. USA* **95**: 6425-6429.

Larson, T. G., Choi, G. H. and Nuss, D. L. 1992. Regulatory pathways governing modulation of fungal gene expression by a virulence-attenuating mycovirus. *EMBO J.* **11**: 4539-4548.

_____ and Nuss, D. L. 1993. Cyclophilin-dependent stimulation of transcription by cyclosporin A. *Proc. Natl. Acad. Sci. USA* **90**: 148-152.

Lindberg, G. D. 1960. Reduction in pathogenicity and toxin production in diseased *Helminthosporium victoriae*. *Phytopathology* **50**: 457-460.

Marcus, L., Barash, I., Sneh, B., Koltin, Y. and Finkler, A. 1986. Purification and characterization of pectolytic enzymes produced by virulent and hypovirulent isolates of Rhizoctonia solani Kuhn. *Physiol. Mol. Plant Pathol.* **29**: 325-336.

Matuo, T. and Miyazawa, Y. 1969. On *Cylindrocarpon panacis* sp. Nov. causing root rot of ginseng. *Trans. Mycol. Soc. Japan.* **9**: 109-112.

McFadden, J. J. P., Buck, K. W. and Rawlinson, C. J. 1983. Infrequent transmission of double-stranded RNA virus particles but absence of DNA proviruses in single ascospore cultures of *Gaeumannomyces graminis*. *J. Gen. Virol.* **64**: 927-937.

Michelmore, R. W. and Hulberts, S. H. 1987. Molecular markers for genetic analysis of phytopathogenic fungi. *Annu. Rev. Phytopathol.* **25**: 383-404.

Miyazawa, Y. 1966. Studies on control of ginseng root rot. *Nogyo Oyobi Engei* **45**: 101-105.

Morris, T. J. and Dodds, J. A. 1979. Isolation and analysis of double-stranded RNA from virus infected plant and fungal tissue. *Phytopathology* **69**: 854-858.

Newhouse, J. P., Tooley, P. W., Smith, O. P. and Fishel, R. A. 1992. Characterization of double-stranded RNA in isolates of *Phytophthora infestans* from Mexico, the Netherlands, and Peru. *Phytopathology* **82**: 164-169.

Nogawa, M., Kageyama, T., Nakatani, A., Taguchi, G., Shimosaka, M. and Okazaki, M. 1996. Cloning and characterization of mycovirus double-stranded RNA from the plant pathogenic fungus, *Fusarium solani* f. sp. *robiniae*. *Biosci. Biotech. Biochem.* **60**: 784-788.

Nuss, D. L. 1996. Using hypoviruses to probe and perturb signal transduction processes underlying fungal pathogenesis. *Plant Cell* **8**: 1845-1853.

Passmore, E. L. and Frost, R. R. 1979. The detection and occurrence of virus-like particles in extracts of mushrooms sporophores. *Phytopathol. Z.* **80**: 85-87.

Poch, O., Sauvaget, I., Delarue, M. and Tordo, N. 1989. Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. *EMBO J.* **8**: 3867-3874.

Polashock, J. J., Bedker, P. J. and Hillman, B. I. 1997. Movement of a small mitochondrial double-stranded RNA element of *Cryphonectria parasitica*: ascospore inheritance and implications for mitochondrial recombination. *Mol. Gen. Genet.* **256**: 566-

571.

Rae, B. P., Hillman, B. I., Tartaglia, J. and Nuss, D. L. 1989. Characterization of double-stranded RNA genetic elements associated with biological control of chestnut blight: organization of terminal domains and identification of gene products. *EMBO J.* **8**: 657-663.

Reeleder, R. D. and Brammall, R. A. 1994. Pathogenicity of *Pythium* species, *Cylindrocarpon destructans*, and *Rhizoctonia solani* to ginseng seedlings in Ontario. *Can. J. Plant Pathol.* **16**: 311-316.

Rice, A. P., Kostura, M. and Mathews, M. B. 1989. Identification of a 90-kDa polypeptide which associates with adenovirus VA RNAI and is phosphorylated by the double-stranded RNA-dependent protein kinase. *J. Biol. Chem.* **264**: 20632-20637.

Rigling, D., Heiniger, U. and Hohl, H. 1989. Reduction of laccase activity in dsRNA-containing hypovirulent strains of *Cryphonectria (Endothia) parasitica*. *Phytopathology* **79**: 219-223.

_____ and Van Alfen, N. K. 1991. Regulation of laccase biosynthesis in the plant pathogenic fungus *Cryphonectria parasitica* by double-stranded RNA. *J. Bacteriol.* **173**: 8000-8003.

_____ and _____. 1993. Extra- and intracellular laccase of the chestnut blight fungus, *Cryphonectria parasitica*. *Appl. Environ. Microbiol.* **59**: 3634-3639.

Rogers, H. J., Buck, K. W. and Brasier, C. M. 1986. Transmission of double-stranded RNA and a disease factor in *Ophiostoma ulmi*. *Plant Pathol.* **35**: 277-287.

Sanderin, R. S. and Ghabrial, S. A. 1978. Physicochemical properties of two distinct types of virus-like particles from *Helminthosporium victoriae*. *Virology* **87**: 142-151.

Shapira, R., Choi, G. H., Hillman, B. I. and Nuss, D. L. 1991. The contribution of defective RNAs to the complexity of viral-encoded double-stranded RNA populations present in hypovirulent strains of the chestnut blight fungus *Cryphonectria parasitica*. *EMBO J.* **10**: 741-746.

Smit, W. A., Wingfield, B. D. and Wingfield, J. H. 1996. Reduction of laccase activity and other hypovirulence-associated traits in dsRNA-containing strains of *Diaporthe ambigua*. *Phytopathology* **86**: 1311-1316.

Snyder, B. A., Adams, G. C. and Fulbright, D. W. 1989. Association of a viruls-like particle with a diseased isolate of *Leutostoma persoonii*. *Mycologia* **81**: 241-247.

Tavantzis, S. M., Romaine, C. P. and Smith, S. H. 1980. Purification and partial characterization of a bacilliform virus from *Agaricus bisporus*. *Virology* **105**: 94-102.

Valverde, R. A. and Dodds, J. A. 1986. Evidence for a satelite RNA associated naturally with the U5 strain and experimentally with the U1 strain of tobacco mosaic virus. *J. Gen. Virol.* **67**: 1875-1884.

_____, Nameth, S. T. and Jordan, R. L. 1990. Analysis of double-stranded RNA for plant virus diagnosis. *Plant Dis.* **74**: 255-258.

Wang, P. and Nuss, D. L. 1995. Induction of a *Cryphonectria parasitica* cellobiohydrolase I gene is suppressed by hypovirus infection and regulated by a GTP-binding- protein-linked signaling pathway involved in fungal pathogenesis. *Proc. Natl. Acad. Sci. USA* **92**: 11529-11533.

Zanzinger, D. H., Bandy, B. P. and Tavantzis, S. M. 1984. High frequency of finding double-stranded RNA in naturally occurring isolates of *Rhizoctonia solani*. *J. Gen. Virol.* **65**: 1601-1605.

Zelcer, A., Weaber, K., Balazs, E. and Zaitlin, M. 1981. The detection and characterization of viral-related double-stranded RNAs in tobacco mosaic virus-infected plants. *Virology* **113**: 417-427.

Zhang, L., Churchill, A. C., Kazmierczak, P., Kim, D. H. and Van Alfen, N. K. 1993. Hypovirulence-associated traits induced by a mycovirus of *Cryphonectria parasitica* are mimicked by targeted inactivation of a host gene. *Mol. Cell Biol.* **13**: 7782-7792.

Zhou, T. and Boland, G. J. 1997. Hypovirulence and double-stranded RNA in *Sclerotinia homoeocarpa*. *Phytopathology* **87**: 147-153.

Ziezold, A. M., Reeleder, R. D., Hall, R. and Proctor, J. T. A. 1998. Seedborne fungi and fungicide seed treatment to of ginseng. *J. Ginseng Res.* **22**: 229-236.