

## INVITED REVIEW

**VIRUS-INDUCED HYPOVIRULENCE IN *CRYPHONECTRIA PARASITICA*:  
STILL AN UNRESOLVED CONUNDRUM****M. Turina and L. Rostagno***Istituto di Virologia Vegetale del CNR, Strada delle Cacce 73, Torino 10135, Italy***SUMMARY**

In this paper we review some of the most recent literature about the interactions between *Cryphonectria parasitica*, the ascomycete fungus that causes chestnut blight, and viruses in the species *Cryphonectria hypovirus 1* (family *Hypoviridae*), which, when present in the fungus, render the fungus incapable of causing the severe disease that is normally the result of infection of European and American chestnut trees (*Castanea sativa* and *C. dentata*, respectively). We provide an overview of the findings accumulated over the last few decades, in an attempt to link them to the technological advances made during this time. In particular, we focus on what is known about the molecular aspects of the virus-fungus interaction, detailing, and critically reviewing some recent findings and their significance in understanding the phenomenon of virus-induced hypovirulence. After describing the diversity of virus species that infect *C. parasitica*, we detail various aspects of the effects the presence of virus has on secretory pathways, and on various elements of signal transduction pathways. We also discuss the recent finding of a silencing suppressor encoded by the genome of *Cryphonectria hypovirus 1*, and recent results from studies of differential expression profiling using cDNA microarray analysis.

*Key words:* Chestnut blight, hypovirulence, hypovirus, silencing, signal transduction, secretion.

**INTRODUCTION**

Since the discovery of hypovirulence as a natural phenomenon that limited the extent of chestnut blight epidemics in Italy in the mid fifties (Biraghi, 1953), a number of laboratories have made attempts to understand the biological nature of the phenomenon at molecular, cellular, physiological and ecological levels. A

group of French researchers in particular was able to show that the natural phenomenon described by Biraghi was not associated with resistance by the chestnut tree but to fungal hypovirulence. Moreover, the same researchers suggested, and were able to support their hypothesis with evidence, that the determinant for hypovirulence was inherited cytoplasmically (Grente and Sauret, 1969a; 1969b). Thirty years ago a milestone was set in the clarification of the chestnut blight hypovirulence phenomenon when, by using auxotrophic mutants as nuclear markers, hypovirulence was found to be a fungal trait, inherited maternally, and transmissible through hyphal anastomosis (Van Alfen *et al.*, 1975). This demonstrated the potential of hypovirulence for the biocontrol of chestnut blight. A further advance was the discovery that transfection with biologically active cDNA clones of the strain 713 of *Cryphonectria hypovirus 1* (CHV-1-713) made the fungus hypovirulent on chestnut cuttings (Choi and Nuss, 1992; Chen *et al.*, 1994). Since then, a number of studies have tried to focus on the molecular details of this virus-fungus interaction. Some of these studies were pioneering and ground-breaking in their characterization of a number of molecular pathways that lead to a greater understanding of the cellular biology of filamentous fungi. Less convincing is the overall relevance of each of the various fungal molecules so far studied to virus-induced hypovirulence. In our opinion there is still no clear cause-effect connection between virus infection, specific molecular targets, and hypovirulence.

Three excellent papers have recently reviewed the molecular and the ecological aspects of hypovirulence, and the diversity of the viruses present in *Cryphonectria parasitica* (Hillman and Suzuki, 2004; Milgroom and Cortesi, 2004; Nuss, 2005). In particular, critical analyses of the successes and failures of the various attempts at using virus-caused hypovirulence for biocontrol of chestnut blight in various parts of the world have stressed the importance of as yet unknown environmental and ecological factors, that go beyond the mere introduction of the right molecular tool (the right hypovirus). This review will not address studies of the characterization of mitochondrial genetic material and the characterization of plasmid and viral elements inside mitochondria,

which have also been studied in *C. parasitica*, and which in at least one case have been linked to a hypovirulent phenotype (Bell *et al.*, 1996; Baidyaroy *et al.*, 2000; Gobbi *et al.*, 1990, 1997, 2002, 2003; Mahanti and Fulbright, 1995; Monteiro-Vitorello *et al.*, 1995, 2000).

## VIRUSES IN *C. PARASITICA*

A thorough review was written recently on this subject (Hillman and Suzuki, 2004). But for a better understanding of the details of virus-induced hypovirulence, it is necessary to summarize briefly some biological and molecular features of the diversity of viruses present in *C. parasitica* and more particularly of those virus species that belong to the family *Hypoviridae*.

In Table 1 we display the main taxonomical features of a number of viruses found in *C. parasitica*: details on their molecular and biological features can also be found in the 8<sup>th</sup> ICTV Report (Fauquet *et al.*, 2005). Before concentrating on species in the family *Hypoviridae*, which are by far the most studied among the viruses that infect *C. parasitica*, it is of some interest to outline the basic features of those in other taxonomic groups. Two viruses in the new virus genus *Mycoreovirus* were discovered in the early 1990s. Both viruses have 11 segments of dsRNA in the fungal cytoplasm (Enebak *et al.*, 1994). The viruses resemble those in the family *Reoviridae*, and phylogenetic analysis confirms this taxonomic placement (Suzuki *et al.*, 2004). In particular, the virus *Cryphonectria mycoreovirus 1* strain 9B21 induces hypovirulence without interfering with other developmental processes such as pigmentation and sporulation. It could become of some interest as an alternative model to CHV1 in virus-fungus interaction since 9B21 can establish infection following the introduction of purified particles into fungal protoplasts (Hillman *et al.*, 2004).

Another virus isolated from New Jersey in the early 1990s, recently denominated *Cryphonectria mitovirus 1*, strain NB631 (Fauquet *et al.*, 2005), was shown to be a mitochondrial virus belonging to a newly established family of dsRNA viruses, the *Narnaviridae* (Polashock and Hillman, 1994). In this case, a mildly hypovirulent phenotype could not be attributed unequivocally to the presence of the virus, since the mitochondrial DNA could also account for the phenotype (Polashock *et al.*, 1997). Viruses belonging to the families *Partitiviridae* and *Chrysoviridae* are also found in *C. parasitica*, but their biological and molecular characterization is not fully resolved (Hillman and Suzuki, 2004).

Four distinct virus species in the family *Hypoviridae* are well characterized. They are taxonomically related but have different genome organizations. The most common such viruses in Europe are isolates of *Cryphonectria hypovirus 1* (CHV-1). A similar virus, *Cryphonectria hypovirus 2* (CHV-2), was found in New Jersey at the end of the 1980s (Hillman *et al.*, 1992). *C. parasitica* strains containing CHV-2 are brown and debilitated when inoculated on chestnut stems (Chung *et al.*, 1994) resulting in a hypovirulent phenotype. The commonest viruses in *C. parasitica* in North America are *Cryphonectria hypovirus 3* (CHV-3) and *Chryphonectria hypovirus 4* (CHV-4). Both have a monocistronic genome. CHV-3 was isolated in Michigan in the early 1980s (Fulbright and Garrod, 1984), but its genome characterization was completed only later (Smart *et al.*, 1999). CHV-4 is similar but its presence in *C. parasitica* does not induce hypovirulence (Linder-Basso *et al.*, 2005; Peever *et al.*, 1997).

CHV-1, and particularly its CHV-1/EP713 strain is the most thoroughly studied among the viruses belonging to the family *Hypoviridae*. It was introduced through anastomosis from a French hypovirulent fungal strain to an American virulent strain (Anagnostakis and

**Table 1.** Taxonomic position of viruses infecting *C. parasitica*.

Virus taxonomical placement	Species	Abbreviations	Strains	Virulence*	Relevant references
Family <i>Hypoviridae</i>	<i>Cryphonectria hypovirus 1</i>	CHV1	EP713	-	Shapira <i>et al.</i> , 1991
Genus <i>Hypovirus</i>			EURO7	-	Chen and Nuss, 1999
	<i>Cryphonectria hypovirus 2</i>	CHV2	NB58	-	Hillman <i>et al.</i> , 1992
	<i>Cryphonectria hypovirus 3</i>	CHV3	GH2	-	Smart <i>et al.</i> , 1999
	<i>Cryphonectria hypovirus 4</i>	CHV4	SR2	+	Linder-Basso <i>et al.</i> , 2005
Family <i>Reoviridae</i>	<i>Cryphonectria mycoreovirus 1</i>	CpMYRV1	9B21	-	Hillman <i>et al.</i> , 2004
Genus <i>Mycoreovirus</i>	<i>Cryphonectria mycoreovirus 2</i>	CpMYRV2	C18	-	Enebak <i>et al.</i> , 1994
Family <i>Narnaviridae</i>	<i>Cryphonectria mitovirus 1</i>	CMV1	NB631		Polashock and Hillman, 1994
Genus <i>Mitovirus</i>					
Family <i>Partitiviridae</i>			FEN	+	Peever <i>et al.</i> , 1997
Family <i>Crysoviridae</i>			OB5	+	Hillman and Suzuki, 2004

\* Virulence estimate is based on comparison with a wild type strain (EP155 or EP67) measured as canker size diameter: (+) refers to virus strains that do not affect virulence, whereas (-) refers to viruses causing hypovirulence in *C. parasitica*.

Day, 1979). It was not until the early 1990s that its sequence and genomic organization became available (Shapira *et al.*, 1991). Phylogenetic comparison of available sequences showed closest homology of these viruses to the ssRNA genome of plant pathogenic viruses belonging to the family *Potyviridae* (Koonin *et al.*, 1991). It was then assumed that even though dsRNA accumulates abundantly in the cytoplasm, indeed, CHV-1 is to be considered a positive strand RNA viruses (even though ICTV still classifies *Hypoviridae* as viruses with dsRNA genome). This is indeed confirmed by the fact that the positive strand RNA transcripts are able to start an infectious cycle in the host cell (Choi and Nuss, 1992), whereas purified dsRNA transfected into protoplasts cannot start the infectious cycle. The CHV-1 genome is 12.7 kb long and contains two major open reading frames, ORF A and ORF B (Shapira *et al.*, 1991). It has a 495 bp 5' untranslated region (UTR) which encodes a few small ORFs: similarity with other picornaviruses suggested that the first AUG is an internal ribosome entry site (IRES), but no data have been published yet to unequivocally substantiate such hypothesis (Shapira *et al.*, 1991). Not much is known functionally about the 3' UTR trailer region. The genome ends with a poly(A) tail. Another question not yet fully resolved is the expression strategy of the two ORFs: the most likely hypothesis is that a termination/re-initiation of translation mechanism is in act, as shown for other viruses and retrotransposons (Horvath *et al.*, 1990; Kojima *et al.*, 2005), and as hypothesized also for CHV-2 (Hillman *et al.*, 1994).

Since a biologically active infectious clone of CHV-1 has been available for some time, a number of details of the molecular features of its genome and genome-encoded proteins are available. ORF A, encodes a 69 kDa protein, that is processed *in cis* to originate p29 and p40 (Choi *et al.*, 1991). The p29 protein is a papain-like proteinase shown to be related to potyvirus helper component protein (HC-Pro) (Koonin *et al.*, 1991). Only the first 72 amino acids are necessary for virus replication, whereas the rest of the protein is dispensable (Craven *et al.*, 1993). The amino terminal part is involved in symptom expression, whereas the carboxy terminal part of the protein encodes the conserved catalytic domain for self cleavage from the 69 kDa precursor (Choi *et al.*, 1991; Craven *et al.*, 1993). This protein also functions as a symptom determinant (low sporulation and pigmentation) both in *cis* and *trans* (Craven *et al.*, 1993b; Suzuki *et al.*, 1999). Recently p29 was shown to be an integral membrane protein that co-localizes with dsRNA in *trans* Golgi network vesicles (Jacob-Wilk *et al.*, 2006). Its ability to suppress silencing is discussed later in this review. Less is known about the protein p40, also resulting from proteolytic processing of ORF A. It is a highly basic protein, that is dispensable for viral replication (Suzuki *et al.*, 2000), and thought to be a *cis* replication

enhancer (possibly through facilitating ORF B expression) (Suzuki and Nuss, 2002).

The functions of ORF B are less understood. This ORF codes for an amino-terminal protease which self cleaves into a 48 kDa protein (Choi *et al.*, 1991). The remaining portion of ORF B has the signature domains of a helicase and a RNA dependent RNA polymerase (Koonin *et al.*, 1991), but so far it is not known to have specific proteolytic sites. Antibodies to the polymerase domain were shown to react with a specific 87 kDa band in vesicle preparations that showed polymerase activity *in vitro* (Fahima *et al.*, 1994). More recently, vesicle preparations containing protease inhibitors showed that the same antibodies against helicase and polymerase domains reacted with a higher molecular weight (circa 220 kDa) protein (Jacob-Wilk *et al.*, 2006). A role of ORF B in determining the severity of the hypovirulent phenotype was determined through chimeric studies between two different hypovirus strain, CHV-1/EP73 and CHV-1/EURO7 (Chen and Nuss, 1999; Chen *et al.*, 2000). With these studies, canker size and the quantity of stomatal pustules were both associated with specific domains in ORF B.

#### PERTURBATION OF THE SECRETORY PATHWAY

Two different lines of evidence turned the attention to the influence of virus infection on fungal vesicular secretory pathways: on one side, ultrastructural studies showed that virus-infected hypovirulent strains contained electron dense material (possibly dsRNA) in the vesicles (Dodds, 1980; Newhouse *et al.*, 1990); on the other, it was shown that the accumulation of a number of proteins secreted through vesicles is down-regulated in virus-infected fungal strains (McCabe and Van Alfen, 1999).

Viruses in the family *Hypoviridae* do not seem to have a capsid protein, as suggested by a number of studies. An initial protocol for viral purification through polyethyleneglycol (PEG) precipitation and differential centrifugation yielded club-shaped "particles" with absorbance features of nucleoproteins, which appeared to be specific to virus-infected strains (Dodds, 1980). The author, however, could not support the morphological association of such structures with dsRNA and discussed the possibility that the structures he isolated were cellular sites of dsRNA replication, rather than pleiomorphic virus particles (Dodds, 1980).

Other authors, who approached the question of virus structure using freeze-substituted hyphae and conidia and examining ultrathin sections, found virus-like particles (VLP) 50-90 nm in diameter, possibly derived from Golgi bodies (defined only on the basis of structural features) (Newhouse *et al.*, 1983, 1990). These authors also suggested that the VLP are derived from host mem-

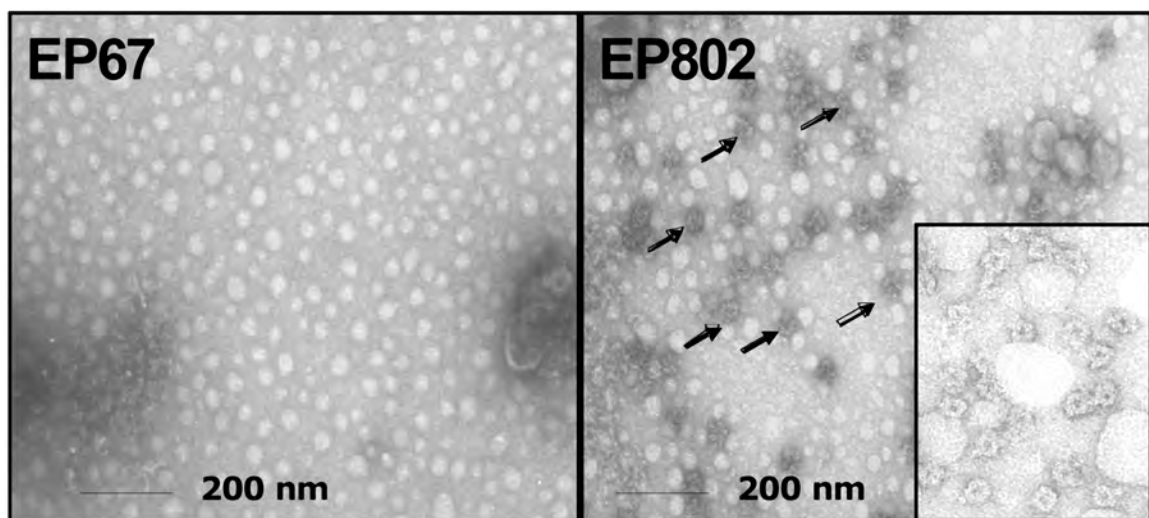
branes (double membranes) engulfing dsRNA. Bernhard's RNA staining showed a granular core and treatment with sodium methoxide-methanol benzene resulted in the removal of the membrane surrounding each VLP (Newhouse *et al.*, 1990). At the same time, a biochemical study on the nature of the purified vesicles (PEG precipitation and differential ultracentrifugation) confirmed the absence of a major coat protein (Hansen *et al.*, 1982, 1985). In particular, these authors detected only the presence of lipids, dsRNA, and carbohydrates, but could not isolate any significant protein band (Hansen *et al.*, 1982, 1985). Of particular interest is the fact that the dsRNA was protected from RNase treatment inside the vesicles and breaking the vesicles exposed the dsRNA to degradation (Hansen *et al.*, 1985). Further work on these vesicles showed associated RNA polymerase activity and characterized the nature and orientation of the molecules synthesized *in vitro* by the vesicle fraction (Fahima *et al.*, 1993, 1994).

In the last few years, researchers have looked at better protocols to characterize subsets of vesicle populations. One such protocol derived from coated vesicle purification in peas (Lin *et al.*, 1992) was adapted to *C. parasitica*. The main differences between this protocol and the ones used previously were the presence of antioxidant and protease inhibitors in the extraction buffer, and the use of a ficoll-D<sub>2</sub>O gradient that exerts less osmotic pressure than sucrose on the purified vesicles. Fig. 1 shows electron microscope pictures of the gradient fraction that contained dsRNA stained in uranyl acetate. A granular, somewhat geometrical, round shaped structure *ca.* 30-40 nm in diameter was specific to the virus-infected strain. To better characterize this gradient fraction, a number of genes coding for structural components of the vesicle pathway, specific to

the trans-Golgi network (TGN) or of the endoplasmic reticulum (ER) vesicle network, were cloned and antibodies were raised to the proteins they encode. Although in the virus-infected strain there was a higher accumulation of all the chosen markers, only those related to trans-Golgi network (middle component of the adaptor protein 1 complex and Kex2p) co-purified with vesicle fractions that contained dsRNA, and a protein reacting with antibodies specific to the polymerase and helicase domain of CHV-1 (Jacob-Wilk *et al.*, 2006). Based on these results, the authors concluded that the virus does not co-localize with ER-derived membranes, as most potyviruses do, but to TGN-derived vesicles in agreement with molecular marker studies and ultrastructural work done in the late eighties.

So far we have only discussed possible structural interactions between CHV-1 and a particular subset of viral vesicles, but possibly the interactions are not merely structural but also functional, i.e. the virus actively interferes with vesicular secretion of cargo proteins through as yet unknown mechanisms.

Apart from the structural studies that seem to place virus replication in TGN vesicles, the suggestion that CHV-1 could be involved in interference with secretory pathways came from early work that was aimed at identifying proteins or messenger RNA down-regulated by CHV-1. In particular, in the mid-eighties and early-nineties, differential expression studies relied on polyacrylamide gel electrophoresis (PAGE) and/or two dimensional (2D) gel electrophoresis for identifying proteins (Powell *et al.*, 1986; Powell and Van Alfen, 1987a), and on subtractive hybridization (Powell and Van Alfen, 1987b) or later, differential display of mRNA (Chen *et al.*, 1996), for investigating differences in the expression at the mRNA level. Among the proteins ini-



**Fig. 1.** Electron micrographs of negatively stained preparation of the vesicle fraction from D<sub>2</sub>O Ficoll gradients. EP67 is a virus-free wild type strain, EP802 is the isogenic strain harbouring the hypovirus CHV-1. Arrows point at electron dense particulate structures of a diameter of 30-40 nm.

tially identified by such studies, a laccase (Rigling and Van Alfen, 1991, 1993), a pheromone-encoding gene (Zhang *et al.*, 1998), and cryparin, a cell wall hydrophobin (Powell and Van Alfen, 1987a; Carpenter *et al.*, 1992; Zhang *et al.*, 1994), were all shown to be down-regulated in the presence of CHV-1. The three proteins share a striking commonality: they have molecular domains typical of secretory proteins, such as a signal-peptide domain cleaved by a signal peptidase and a pro-protein domain possibly cleaved by a Kex2p analogue. For this reason some authors started constructing a working model in which the virus hijacks a subset of the secretory vesicles for its own replication and protection, and slows or stops the secretion of such vesicles resulting in vesicle build-up. Such vesicles would be recognized by a pathway that would send a message back to the nucleus inhibiting transcription of the same secretory genes. It was shown through nuclear runoff assays that the down-regulation of transcription for the genes down-regulated when the virus is present, occurs at the nuclear level (Kazmierczak *et al.*, 1996).

Among the three gene products thought to be secreted in vesicles, cryparin seemed to be the most amenable to further studies. Cryparin is an abundant hydrophobin whose function in the fungus infection cycle was recently identified because its absence prevented the eruption of stromal pustules through the bark (Kazmierczak *et al.*, 2005). Due to its abundant accumulation in culture media, and the availability of specific antibodies for its detection, cryparin is easily studied in its various intermediates during the secretion pathway. In this respect, it was the only secreted protein for which an effect of the virus on its secretion could be thoroughly investigated (McCabe and Van Alfen, 1999). The results of a pulse-chase experiment showed that cryparin is secreted more slowly by virus-infected strains than by wild type strains, showing a possible functional link between the presence of virus and perturbation of secretion (N.K. Van Alfen, personal communication). Experiments with a fusion protein of green fluorescent protein (GFP) and cryparin showed the accumulation of GFP in discrete bodies, allowing speculation about the presence of cryparin in secretory vesicles (P. Kazmierczak, personal communication). Overall, the evidence of a viral perturbation of the secretory pathway is robust, but what is missing is a causal link between secretion and virulence (therefore hypovirulence). Furthermore, co-localization of markers in a gradient fraction does not give proof of the equivalence between secretory vesicles and those that harbour virus. Only morphological studies with specific marker antibodies, or confocal microscopy studies using fluorescent markers could prove that vesicles containing viral markers (p29, helicase or polymerase) are secretory vesicles containing TGN markers.

## VIRAL EFFECTS ON SIGNAL TRANSDUCTION ELEMENTS

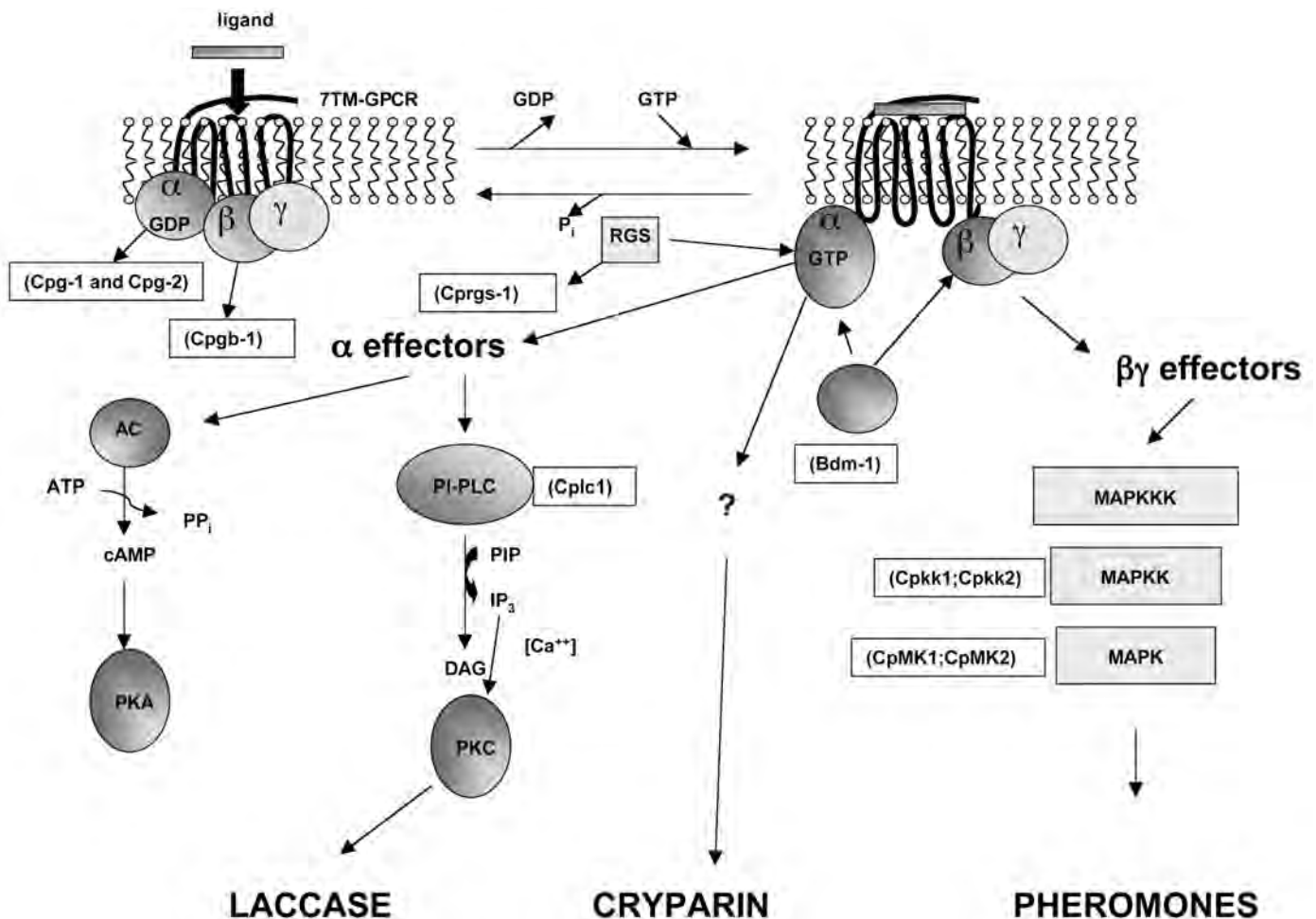
Signal transduction components have been the target of research in a number of laboratories. That signal transduction elements might be effectors for virus-induced hypovirulence was suggested by the observation that some of the proteins down-regulated following virus infection are controlled by signal transduction pathways. One group has focused for more than 10 years on the elucidation of possible interference of virus infection with G-protein signalling (Nuss, 1996). Other work has touched on different elements of signal transduction, mostly those involving kinase proteins (Kim *et al.*, 2002, 2004; Park *et al.*, 2004; Choi *et al.*, 2005; Turina *et al.*, 2006).

**Studies on signal transduction pathways involving G-protein.** The importance of G-proteins in mediating signal transduction from the environment to the nucleus is demonstrated by the wealth of literature on the subject. The relevance and the structural features of G-protein-mediated signal transduction in various model organisms, and in particular in filamentous fungi, has been recently reviewed (Oldham and Hamm, 2006; Yu, 2006).

In order to respond to environmental cues, a cell uses a number of signal transduction elements. Among them, signalling mediated by heterotrimeric guanine nucleotide-binding proteins (G-protein) goes through a cycle of activation and deactivation (Fig. 2). A simplified model is that activation is stimulated when an extracellular ligand binds to a seven transmembrane domain receptor (7TM-GPCR). Upon binding, it alters its conformation acting on the  $\alpha$  subunit of the  $\alpha\beta\gamma$  complex of protein (bound to each other and to the 7TMR intracellularly when not stimulated) forcing it to release GDP. Upon stimulus, the  $\alpha$  subunit protein binds GTP (activated state) and becomes free from the  $\beta\gamma$  complex. Both  $\alpha$  and  $\beta\gamma$  can transduce signals independently to a series of effectors. The cycle ends when the intrinsic phosphatase activity of the  $\alpha$  subunit causes the hydrolysis of GTP to GDP. GDP-bound  $\alpha$  subunits bind to the  $\beta\gamma$  subunits to reform the complex with the 7TM-GPCR (Oldham and Hamm, 2006).

The effectors of GTP- $\alpha$  subunit are adenylyl cyclase (AC), which upon stimulation acts on protein kinase A (PKA) cascades, cBMP phosphodiesterase, phosphoinositide-specific phospholipase C (PI-PLC), which upon stimulation acts on protein kinase C, and the small G-protein RhoA through RhoGEFs. The G- $\beta\gamma$  effectors include G-protein-activated inwardly rectifying K<sup>+</sup> (GIRK) channels and mitogen activated protein (MAP) kinase cascades (Fig. 2) (McCudden *et al.*, 2005).

The idea of an involvement of signal transduction elements in the virus-fungus interaction, springs from an early observation that *lac-1*, a laccase gene encoded by



**Fig. 2.** Signal transduction pathways relevant to studies on CHV-1-caused hypovirulence in *C. parasitica*. Three molecules shown to be down-regulated by virus infection (laccase, cryparin, and pheromones) are under the control of signal transduction pathways depending from  $\alpha$  or  $\beta\gamma$  effectors of G protein signalling. The acronyms are detailed in the text. In parenthesis the specific genes cloned and characterized in *C. parasitica*.

*C. parasitica*, is down-regulated following virus infection (Choi *et al.*, 1992); moreover, *lac-1* is under the control of a positive regulatory pathway involving calmodulin and calcineurin (Larson *et al.*, 1992; Larson and Nuss, 1994). Since calmodulin and calcineurin were known to be molecules involved in signal transduction pathways, much effort has focused on looking for elements of signal transduction pathways that could be influenced by virus infection and whose perturbation would show some link to the hypovirulent phenotype (Fig. 2). Initial attention was on G protein components: in particular two different  $\alpha$  subunits, *cpg-1* and *cpg-2* (Choi *et al.*, 1995; Gao and Nuss, 1996). Initial observation linked the down-regulation of *cpg-1* and *cpg-2* to cAMP accumulation (Chen *et al.*, 1996). Phenotypically, *cpg-1* disruption showed that this gene is required for efficient hyphal growth, orange pigmentation, conidiation and sexual reproduction, whereas *cpg-2* did not interfere with any of the above mentioned developmental processes (Gao and Nuss, 1996). At the time, the fact that

cAMP could play a key role in hypovirulence, fitted well with reports that cAMP-mediated signalling was implicated in morphogenetic changes related to the infection cycle in *Magnaporthe grisea* (Lee and Dean, 1993; Mitchell and Dean, 1995) and *Ustilago maydis* (Gold *et al.*, 1994). In the following years, more effort was put into finding a correlation between other G-protein-linked alterations of signal transduction, virus infection and hypovirulence. In particular, a  $\beta$  subunit of the heterotrimeric G-protein, *cpgb-1*, was characterized and again, its involvement in processes linked to hypovirulence was shown and, in this case, growth was not affected (Kasahara and Nuss, 1997).

Another protein with similarity to mammalian phosphatidylinositol 3-kinase was shown to regulate G-protein-mediated signal transduction and it was named *bdm-1* (beta disruption mimic factor): this protein possibly interacts with the  $\beta\gamma$  complex (Kasahara *et al.*, 2000). Furthermore a regulator of G-protein signalling (RGS) was cloned and characterized, and its involvement in specific *cpg-1* regula-

tion, conidiation, virulence and cryparin synthesis was shown (Segers *et al.*, 2004).

A recent study has linked laccase production unequivocally to the control of a inositol-specific phospholipase C (Fig. 2), whose gene was disrupted in *C. parasitica* (Chung *et al.*, 2006). But no specific effect on this gene or on its protein product was linked to the presence of virus. Lack of virulence of the *cplc1*-null mutant was attributed to its strong growth deficiency (Chung *et al.*, 2006).

Overall, a number of interesting aspects of *C. parasitica* biology were elucidated in these studies and some of them pioneered interest in G-protein-mediated signal transduction in filamentous fungi. However, it is still not clear if the effect on signal transduction is a primary or a secondary effect of virus infection and, as yet, there is no evidence of a direct interaction between virus-encoded proteins and any element of signal transduction. Moreover it would be interesting to address the issue of which specific environmental cues trigger the elements of signal transduction studied so far (possibly in relation to plant infection).

**Studies involving kinase proteins.** Among the genes initially isolated in a subtractive hybridization study between virus-infected and wild type *C. parasitica* strains were pheromone genes shown to be involved in the sexual reproduction cycle (Turina *et al.*, 2003; Zhang *et al.*, 1998, 1993). Since pheromone gene regulation in *Saccharomyces cerevisiae*, the model organism for pheromone-mediated signal transduction, is under the control of a mitogen activated protein (MAP) kinase cascade, we pursued the characterization of key elements of the MAP kinase cascades present in *C. parasitica* genomes (Fig 2). So far, two of the three mitogen-activated protein kinase kinase (MAPKK) have been cloned and sequenced (Cpkk1 and Cpkk2), whereas Cpkk3 is currently undergoing characterization. Of the two MAPKK sequenced, only Cpkk1 was shown to be present in higher amounts and in different phosphorylation states in virus-infected hypovirulent strains than in uninfected strains (Turina *et al.*, 2006). But no evidence of a direct effect of virus infection on the various hyperphosphorylation states of Cpkk1 and on its turn-over could be shown (Turina *et al.*, 2006). The authors argue the possibility that hyperphosphorylation and up-regulation are signatures of a juvenile state and that an as yet unknown key element controlling development is the primary target for CHV-1 infection.

Another group of researchers has chosen to study a number of kinases in the hope to identify molecules involved in hypovirulence and regulated by CHV-1 infection (Kim *et al.*, 2002, 2004; Park *et al.*, 2004; Choi *et al.*, 2005). CpMK1, is a *Hog 1* homologue from *C. parasitica* (Park *et al.*, 2004). This gene could be disrupted and in part the hypovirulent phenotype could be repro-

duced, particularly in relation to pigmentation, conidiation, laccase production and cryparin expression. Moreover, a direct or indirect influence on its phosphorylation pattern was shown in CHV-1/713-infected strains in hyperosmotic conditions. The same group of researchers also characterized another MAP kinase from *C. parasitica*, CpMK2 (Choi *et al.*, 2005). CpMK2, is closely related to yeast FUS3, a kinase that is regulated by the pheromone-dependent MAP kinase cascade. Again, a *cpmk2* gene disruption reproduces some of the phenotypic changes associated with virus infection, but no effect on the phosphorylation level of CpMK2 could be observed. The significantly smaller canker size caused by *cpmk2* mutants, is probably the indirect effect of a strong deficiency in growth on solid media (Choi *et al.*, 2005).

A *C. parasitica* Ser/Thr protein kinase was shown to be up-regulated by viral infection (Kim *et al.*, 2002). Also, induced up-regulation caused symptoms that mimicked those of virus infection (Kim *et al.*, 2002). A recent disruption of the same gene showed its dramatic effect on colony morphology (only microcolonies could arise from null mutant conidia), affecting hyphal growth and differentiation (Kim *et al.*, 2004).

Overall these results show how most of the elements of signal transduction cascades are influenced by virus infection. Often, disruption or overexpression of the same signal transduction elements reproduce some of the CHV-1/713 phenotype, including sometimes hypovirulence itself. But again, the lack of any proof of direct interaction between the virus and any of these signal transduction elements raises doubts about the approach so far pursued.

### C. PARASITICA, HYPOVIRUS INFECTION, SILENCING AND SILENCING SUPPRESSORS

Silencing and silencing-related phenomena are the focus of much interest in many laboratories working on various biological model systems across kingdoms in eukaryotic organisms. The possible therapeutic applications of RNA interference lead to the award of the 2006 Nobel prize for medicine for the seminal work on RNA interference in the animal model system *Caenorhabditis elegans* (Fire *et al.*, 1998). Previous work on plants and filamentous fungi contributed (and somewhat preceded) the work on *C. elegans* (Napoli *et al.*, 1990; Vanderkrol *et al.*, 1990; Romano and Macino, 1992). In particular the studies on quelling in *Neurospora crassa*, due to its genetic amenability, showed the importance of some of the key elements of the post-transcriptional gene silencing (PTGS) pathway (Cogoni and Macino, 1997, 1999a, 1999b). In plants, a link between virus defence and PTGS was established (Ratcliff *et al.*, 1997). Such a link could not be established in *N. crassa*, where

virus infection is not a well established experimental system although a recent study showed a role for PTGS elements in repressing the activity of a retrotransposon (Nolan *et al.*, 2005). *C. parasitica*, is phylogenetically very close to *N. crassa*, and since a virus infection system is well characterized and amenable to a reverse genetic approach, this organism seemed to be an ideal host to study the relationship between PTGS pathways and virus defence. Before the awareness of a specific silencing pathway, transgenic suppression was indeed used in a reverse genetic study in *C. parasitica*, uncovering the potential of such a system for gene function analysis (Choi *et al.*, 1995).

More recently, PTGS was used in targeted functional studies by using hairpin constructs and the potential and limitations of such an approach in this fungal species have been discussed (Gullusci and Turina, 2007). The most interesting aspect though, relates to the possibility that the CHV-1 genome expresses a suppressor of gene silencing. The most likely candidate seems to be the papain-like protease p29, a protein which retains homology to potyvirus HC-Pro, a well studied suppressor of gene silencing (Kasschau and Carrington, 1998; Burgyan, 2006). Moreover, p29 seems to affect dsRNA accumulation (Suzuki *et al.*, 2003) and like potyvirus HC-Pro, it seems to be necessary and sufficient for the synergistic effect between CHV-1 and a mycoreovirus infecting *C. parasitica* (Sun *et al.*, 2006) mimicking analogous effects in plant potyvirus were a synergistic effect between a potyvirus and a second virus infecting the same plant was studied (Pruss *et al.*, 1997). Indeed a recent paper shows evidence that p29 is a suppressor of gene silencing in *C. parasitica* and in plants (Segers *et al.*, 2006). However, no link was established between p29 suppression activity and the specific role in suppression of RNA silencing during CHV1 infection. Furthermore, p29 is not required for CHV-1 infection in *C. parasitica* (like for some plant virus silencing suppressors) and the redundancy of p29 mutant in a host that lacks a small RNA pathway was not shown, and this seems to be the suggested experimental approach to demonstrate that p29 suppression activity is important during the virus life cycle in its host (Li and Ding, 2006).

A series of concerns related to the experimental system used are not well addressed in this paper, e.g. p29 was previously shown to maintain a juvenile phenotype arresting developmental processes such as pigmentation and conidia production (Craven *et al.*, 1993). Such juvenile phenotype is also associated with a high metabolic activity with a great accumulation of host membrane fractions. It is therefore difficult to distinguish an increase in GFP expression due to a general increase in metabolic activity (even in a silenced strain) from a direct effect on GFP expression due to silencing suppression. Furthermore, no specific effect on siRNA or siR-

NA pathway components (as we know them from *N. crassa* system) was shown following p29 expression. Reversion of a host gene silenced phenotype upon CHV-1 infection (or p29 expression) were not performed in this study (Segers *et al.*, 2006) and this test, even if not substantial for showing suppression activity, would be of practical relevance, since it would help in reverse genetic studies entailing silencing of a specific host gene. Our own preliminary attempt with such an experimental approach in *C. parasitica* failed (Gullusci and Turina, 2007). The same study also shows that p29 has suppression activity in plants: in fact, the specific mechanism seems to involve the interference with long distance spread of RNA silencing (Segers *et al.*, 2006), whereas no activity was shown in suppression of the local silencing signal. But long distance in plants has a whole different anatomical and physiological meaning than long distance spread in filamentous fungi, and conservation of such suppression activity could imply that p29 effect as a silencing suppressor in plants is only a secondary effect of the protein with no real biological meaning. Moreover it is known that dsRNA of CHV-1 is protected from the cytoplasm from membranous vesicles (Hansen *et al.*, 1982), and possibly this is a further mechanism that allows CHV-1 replication to escape the host siRNA defence pathway.

#### **CRYPHONECTRIA-HYPOVIRUS INTERACTIONS IN THE POST-GENOMIC ERA**

Possibly in the next few years the complete nucleotide sequence of *C. parasitica* will be known, and this milestone will add a new tool to further understanding of the biology of virus-induced hypovirulence. Meanwhile, a comprehensive approach to expression studies has been already outlined in a number of studies published in the last few years.

*C. parasitica* has been a model organism among filamentous fungi for applications of techniques aimed at elucidating differentially expressed genes or proteins. We have already reviewed initial studies with this intent (Powell *et al.*, 1986; Powell and Van Alfen, 1987b). But these studies only could identify a small number of up- or down-regulated genes. A larger number of genes was identified by differential display and ordered differential display studies (Chen *et al.*, 1996; Kang, 2000). But a much more sensitive and extensive approach to the identification of differentially expressed genes was the use of cDNA microarray analyses technique.

The first of these studies (Allen *et al.*, 2003) compared the expression of virus-infected EP713 strain to the wild type strain EP155, and 20% of the 2200 genes present in the cDNA library spotted on the microchip were shown to be up- or down-regulated. The authors discussed the possibility that some of the up- or down-

regulated genes could be key elements of the fungal response to virus infection or in virus-induced hypovirulence: in particular the authors drew attention to a limited number of host stress response genes such as a heat shock protein 70 (HSP70), and a glutathione S-transferase (GST), and genes involved in the activated methyl cycle, or transcriptional regulator factors. But no follow-up work was published on the functional characterization of these genes in particular supporting their direct relevance to the viral infection cycle or virus-induced hypovirulence.

Another study from the same group compared the differential expression between a mild and a severe strain of CHV-1 (Allen and Nuss, 2004b). This study outlined a number of genes that are selectively up- or down-regulated by each of the two viruses, and a number of genes that are co-regulated by the two virus strains (80 out of 2200). Generally, the mild strain altered the expression of 10% of the genes when compared to the isogenic virus-free strain, whereas half of the genes were altered by the severe strain. The authors discussed the new results particularly outlining the commonality and differences for the three classes of genes described in the previous study (Allen *et al.*, 2003). Indeed, not all of the genes previously outlined are up- or down-regulated by both virus strains. However, as no link was established to the hypovirulence character of each of the two strains, the results shed no new light on virus-induced hypovirulence.

A further study using the same cDNA array microchip addressed the possible expression commonalities between what has been defined as mitochondrial hypovirulence (Monteiro-Vitorello *et al.*, 1995) and virus-induced hypovirulence (Allen and Nuss, 2004a). This study found that 47% of the genes differentially expressed by CHV1-EP713, were also differentially expressed in strain EP155/*mit2*, the strain carrying the mtDNA mutation responsible for hypovirulence. The authors drew conclusions about a molecular linkage between viral hypovirulence and mitochondrial hypovirulence, but no evidence was shown for such a relationship: virus infection and mtDNA mutation were shown to have a common set of genes differentially expressed, but no causal link to their respective hypovirulence was shown.

A final study by the same group of researchers looked for commonalities between CHV-1 infection and the expression of genes in strains lacking the G $\alpha$  subunit (CPG-1) or the G $\beta$  (CPGB-1) subunit (Dawe *et al.*, 2004). A number of genes were co-regulated in the two knock out strains and a subset of these genes was also co-regulated in virus-infected strains, but again, at this time, it is impossible to link any of these changes to hypovirulence induced by CHV-1, since these changes could be co-regulated because of some other virus-induced phenotype variation, such as lack of pigmentation,

conidiation or sexual development. A few of the limitations common to these expression studies are to be outlined. The cDNA library used represented around 2200 genes out of the likely 10,000 genes potentially encoded by the *C. parasitica* genome (Dawe, 2003). Interestingly a number of genes shown to be up- or down-regulated by other research groups with northern blot analysis are not discussed in any of the papers where expression is studied through cDNA microarray analysis.

It is difficult to evaluate the significance of the changes in expression shown by the various studies summarized in the previous paragraphs. The relevance to the hypovirulence phenotype might be made more clear by controls such as a comparison between the expression profile induced by CHV-1 and CHV-4 (a non-hypovirulent species), or between CHV-1 and a CHV-1 mutant unable to cause hypovirulence. Along the same line of reasoning, a comparison of expression profile changes induced by CHV-1 and the micoreovirus 9B21, both of which induce hypovirulence, but the latter with minimal phenotype-associated symptoms, would help focusing on hypovirulence itself.

Furthermore, some researchers feel that the general effect of CHV-1 infection is the maintenance of a juvenile state with a delay in the onset of development. A comparison of the expression profile of two different time points from the same virulent wild type strain EP155 would help understand what is the subset of genes that alter their expressions secondarily, and not because of their relevance to the hypovirulence phenotype (being EP155 virulent independently from the time point of sampling). Another aspect to be underlined is the limit imposed by the number of genes represented in the cDNA library. The library contains an over representation of metabolic and structural genes, whereas it is under-represented for other more specific and more developmentally and environmentally regulated genes.

More importantly, there is one final limitation that all the studies so far carried out fail to address: virulence (and therefore hypovirulence) are phenomena linked to the growth of the fungus on its plant host, the chestnut tree. All the studies so far considered, look at differential expression of protein and of RNA in a nutrient rich liquid culture or in mycelia grown on agar plates: we might be looking at a whole change in expression that has no relevance to what really happens during the plant infection cycle, where a completely different set of genes are likely to be turned on, given the very different structural and physiological features of the fungus when it causes cankers on the chestnut tree. *In vivo* and *in planta* expression studies are particularly difficult to carry out, but technology in this field is developing, and a more focused approach at fungal differential expression *in planta* would give us a completely new perspective, possibly more linked to the virulence-hypovirulence phenomenon.

## CONCLUDING REMARKS

We have tried to give a comprehensive overview of the research carried out in laboratories around the world in order to shed light on the molecular mechanism of virus-induced hypovirulence although brevity and conciseness was required when presenting molecular details. We have the impression though, that the wealth of information provided by the many competing laboratories is not really improving our understanding of the natural phenomenon that occurs in chestnut stands in some parts of Europe. The complexity of the interactions at play in naturally occurring biocontrol by hypovirulent strains is possibly too complex for simple answers through experiments in controlled environments. Furthermore, we think that thirty years of research focusing on the understanding of molecular details has somewhat driven away the attention from a biological and ecological understanding of hypovirulence. An example of how a better understanding of some basic biological concepts would help to interpret the molecular data is the definition of hypovirulence itself. We are aware that any debilitation in growth can possibly result in a "hypovirulent" phenotype during a virulence experiment in chestnut cuttings, but the hypovirulence measured in such a way has little to do with a hypovirulent phenotype that can be used in biocontrol experiments in natural infections in chestnut stands and forests, which is the main interest of public funding for *C. parasitica* research.

An example of such misunderstanding is the failure to establish natural biocontrol using CHV-1/EP713 strain (Anagnostakis *et al.*, 1998), a strain that seemed to be well equipped for treating single cankers caused by virulent strains of the same vegetative compatibility (VC) group, but that probably lacked qualities contributing to the overall fitness when released in open environment. The failure to establish biocontrol is even more disappointing since the transgenic strategy ensured transmission to ascospore progeny overcoming the VC barriers of virus spread through anastomosis (Anagnostakis *et al.*, 1998). Concerns raised by the failure in establishing biocontrol through one single release of strain CHV-1/EP713 are being addressed by the use of a multiple release strategy or the use of different CHV-1 strains, such as CHV-1-EURO7 (Chen and Nuss, 1999), and we are looking forward to some positive results.

As for the two main molecular mechanisms of virus-induced hypovirulence so far pursued, we notice that they are not mutually exclusive, and theoretically a unifying model can be envisioned since G-protein signalling can also be receptor independent (Cismowski, 2006). Furthermore, there are a number of reports showing the interaction of G-proteins with Golgi vesicles (Stow *et al.*, 1991; Aronin and Difiglia, 1992; Maier *et al.*, 1995; Denker *et al.*, 1996; Martin *et al.*, 1999).

New challenges still await scientists studying virus-induced hypovirulence: surprisingly, no direct interaction was shown between any of the proteins encoded by the virus genome and host proteins. However, initial failures could prove to be successes in the long run, since the technology of protein-protein interaction and protein complex study is improving. For example, it would be interesting to use the most modern proteomic approaches for the analysis of the viral vesicles separated with the new protocol. Another approach that we think was not sufficiently pursued is an extensive mutagenesis screen, looking for mutations that do not change the phenotype of the fungus grown on artificial media, but that specifically impair the ability of the fungus in establishing a successful infection on chestnut stems or for mutations in the fungal host that maintain the competence for virus replication but impair the expression of the virulent phenotype. Mutagenesis approaches provided a wealth of information on various biological systems, and were particularly successful in studies of some model filamentous fungi. Surprisingly the *C. parasitica* conundrum was never seriously tackled through this labor-intensive technique. Possibly the application of "tilling" techniques could make the mutagenesis approach less labour intensive and improve the chances for success (Till *et al.*, 2003; Lamour and Finley, 2006).

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## REFERENCES

- Allen T.D., Dawe A.L., Nuss D.L., 2003. Use of cDNA microarrays to monitor transcriptional responses of the chestnut blight fungus *Cryphonectria parasitica* to infection by virulence-attenuating hypoviruses. *Eukaryotic Cell* **2**: 1253-1265.
- Allen T.D., Nuss D.L., 2004a. Linkage between mitochondrial hypovirulence and viral hypovirulence in the chestnut blight fungus revealed by cDNA microarray analysis. *Eukaryotic Cell* **3**: 1227-1232.
- Allen T.D., Nuss D.L., 2004b. Specific and common alterations in host gene transcript accumulation following infection of the chestnut blight fungus by mild and severe hypoviruses. *Journal of Virology* **78**: 4145-4155.
- Anagnostakis S.L., Day P.R., 1979. Hypovirulence conversion in *Endothia parasitica*. *Phytopathology* **69**: 1226-1229.
- Anagnostakis S.L., Chen B.S., Geletka L.M., Nuss D.L., 1998. Hypovirus transmission to ascospore progeny by field-released transgenic hypovirulent strains of *Cryphonectria parasitica*. *Phytopathology* **88**: 598-604.
- Aronin N., Difiglia M., 1992. The subcellular-localization of the G-Protein-G (I-Alpha) in the basal ganglia reveals its

- potential role in both signal transduction and vesicle trafficking. *Journal of Neuroscience* **12**: 3435-3444.
- Baidyaroy D., Huber D.H., Fulbright D.W., Bertrand H., 2000. Transmissible mitochondrial hypovirulence in a natural population of *Cryphonectria parasitica*. *Molecular Plant-Microbe Interactions* **13**: 88-95.
- Bell J.A., Monteiro-Vitorello C.B., Hausner G., Fulbright D.W., Bertrand H., 1996. Physical and genetic map of the mitochondrial genome of *Cryphonectria parasitica* Ep155. *Current Genetics* **30**: 34-43.
- Biraghi A., 1953. Possible active resistance to *Endothia parasitica* in *Castanea sativa*. *Report Congress International Union Forest Research Organization* **11**: 149-157.
- Bugyan J., 2006. Virus-induced RNA silencing and suppression: defence and counter defence. *Journal of Plant Pathology* **88**: 233-244.
- Carpenter C.E., Mueller R.J., Kazmierczak P., Zhang L., Vilalón D.K., Van Alfen N.K., 1992. Effect of a virus on accumulation of a tissue-specific cell-surface protein of the fungus *Cryphonectria (Endothia) parasitica*. *Molecular Plant-Microbe Interactions* **5**: 55-61.
- Chen B., Geletka L.M., Nuss D.L., 2000. Using chimeric hypoviruses to fine-tune the interaction between a pathogenic fungus and its plant host. *Journal of Virology* **74**: 7562-7567.
- Chen B., Nuss D.L., 1999. Infectious cDNA clone of hypovirus CHV1/Euro7: a comparative virology approach to investigate virus-mediated hypovirulence of the chestnut blight fungus *Cryphonectria parasitica*. *Journal of Virology* **73**: 985-992.
- Chen B.S., Choi G.H., Nuss D.L., 1994. Attenuation of fungal virulence by synthetic infectious hypovirus transcripts. *Science* **264**: 1762-1764.
- Chen B.S., Gao S.J., Choi G.H., Nuss D.L., 1996. Extensive alteration of fungal gene transcript accumulation and elevation of G-protein-regulated cAMP levels by a virulence-attenuating hypovirus. *Proceedings of the National Academy of Sciences USA* **93**: 7996-8000.
- Choi E.S., Chung H.J., Kim M.J., Park S.M., Cha B.J., Yang M.S., Kim D.H., 2005. Characterization of the ERK homologue CpMK2 from the chestnut blight fungus *Cryphonectria parasitica*. *Microbiology-Sgm* **151**: 1349-1358.
- Choi G.H., Chen B.S., Nuss D.L., 1995. Virus-mediated or transgenic suppression of a G-protein alpha-subunit and attenuation of fungal virulence. *Proceedings of the National Academy of Sciences USA* **92**: 305-309.
- Choi G.H., Larson T.G., 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. *Molecular Plant-Microbe Interactions* **5**: 119-128.
- Choi G.H., Nuss D.L., 1992. Hypovirulence of chestnut blight fungus conferred by an infectious viral cDNA. *Science* **257**: 800-803.
- Choi G.H., Shapira R., Nuss D.L., 1991. Co-translational autoprolysis involved in gene expression from a double-stranded RNA genetic element associated with hypovirulence of the chestnut blight fungus. *Proceedings of the National Academy of Sciences USA* **88**: 1167-1171.
- Chung H.J., Kim M.J., Lim J.Y., Park S.M., Cha B.J., Kim Y.H., Yang M.S., Kim D.H., 2006. A gene encoding phosphatidylinositol-specific phospholipase C from *Cryphonectria parasitica* modulates the lac1 expression. *Fungal Genetics and Biology* **43**: 326-336.
- Chung P.H., Bedker P.J., Hillman B.I., 1994. Diversity of *Cryphonectria parasitica* hypovirulence-associated double-stranded RNAs within a chestnut population in New Jersey. *Phytopathology* **84**: 984-990.
- Cismowski M.J., 2006. Non-receptor activators of heterotrimeric G-protein signaling (AGS proteins). *Seminars in Cell and Developmental Biology* **17**: 334-344.
- Cogoni C., Macino G., 1997. Isolation of quelling-defective (qde) mutants impaired in posttranscriptional transgene-induced gene silencing in *Neurospora crassa*. *Proceedings of the National Academy of Sciences USA* **94**: 10233-10238.
- Cogoni C., Macino G., 1999a. Gene silencing in *Neurospora crassa* requires a protein homologous to RNA-dependent RNA polymerase. *Nature* **399**: 166-169.
- Cogoni C., Macino G., 1999b. Posttranscriptional gene silencing in *Neurospora* by a RecQ DNA helicase. *Science* **286**: 2342-2344.
- Craven M.G., Pawlyk D.M., Choi G.H., Nuss D.L., 1993. Papain-like protease-P-29 as a symptom determinant encoded by a hypovirulence-associated virus of the chestnut blight fungus. *Journal of Virology* **67**: 6513-6521.
- Dawe A.L., 2003. An ordered collection of expressed sequences from *Cryphonectria parasitica* and evidence of genomic microsynteny with *Neurospora crassa* and *Magnaporthe grisea*. *Microbiology-Sgm* **149**: 2373-2384.
- Dawe A.L., Segers G.C., Allen T.D., McMains V.C., Nuss D.L., 2004. Microarray analysis of *Cryphonectria parasitica* G alpha- and G beta gamma-signalling pathways reveals extensive modulation by hypovirus infection. *Microbiology-Sgm* **150**: 4033-4043.
- Denker S.P., McCaffery J.M., Palade G.E., Insel P.A., Farquhar M.G., 1996. Differential distribution of alpha subunits and beta gamma subunits of heterotrimeric G proteins on Golgi membranes of the exocrine pancreas. *Journal of Cell Biology* **133**: 1027-1040.
- Dodds J.A., 1980. Association of type-1 viral-like dsRNA with club-shaped particles in hypovirulent strains of *Endothia parasitica*. *Virology* **107**: 1-12.
- Enebak S.A., MacDonald W.L., 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.
- Fahima T., Kazmierczak P., Hansen D.R., Pfeiffer P., Van Alfen N.K., 1993. Membrane-associated replication of an unencapsidated double-strand RNA of the fungus *Cryphonectria parasitica*. *Virology* **195**: 81-89.
- Fahima T., Wu Y., Zhang L., Van Alfen N.K., 1994. Identification of the putative RNA polymerase of *Cryphonectria hypovirus* in a solubilized replication complex. *Journal of Virology* **68**: 6116-6119.

- Fire A., Xu S. Q., Montgomery M. K., Kostas S.A., Driver S.E., Mello C. C., 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**: 806-811.
- Fauquet C.M., Mayo M.A., Maniloff J., Desselberger U., Ball L.A., 2005. Virus Taxonomy. Eight Report of the International Committee on Taxonomy of Viruses. Elsevier/Academic Press, London, UK.
- Fullbright D.W., Garrod S.W., 1984. Double-Stranded-RNA (dsRNA) banding-pattern changes in hypovirulent *Endothia parasitica*. *Phytopathology* **74**: 801-801.
- Gao S.J., Nuss D.L., 1996. Distinct roles for two G protein alpha subunits in fungal virulence, morphology, and reproduction revealed by targeted gene disruption. *Proceedings of the National Academy of Sciences USA* **93**: 14122-14127.
- Gobbi E., Carpanelli A., Firrao G., Locci R., 1997. The *Cryphonectria parasitica* plasmid pUG1 contains a large ORF with motifs characteristic of family B DNA polymerases. *Nucleic Acids Research* **25**: 3275-3280.
- Gobbi E., Firrao G., Carpanelli A., Locci R., Van Alfen N.K., 2003. Mapping and characterization of polymorphism in mtDNA of *Cryphonectria parasitica*: evidence of the presence of an optional intron. *Fungal Genetics and Biology* **40**: 215-224.
- Gobbi E., Rekab D., Locci R., 2002. Mitochondrial plasmids of the pCp family are spread worldwide in *Cryphonectria parasitica* populations. *Mycological Research* **106**: 1408-1416.
- Gobbi E., Wang Y., Martin R.M., Powell W.A., Van Alfen N.K., 1990. Mitochondrial - DNA of *Cryphonectria parasitica*, lack of migration between vegetatively compatible strains. *Molecular Plant-Microbe Interactions* **3**: 66-71.
- Gold S., Duncan G., Barrett K., Kronstad J., 1994. Cyclic AMP regulates morphogenesis in the fungal pathogen *Ustilago maydis*. *Genes and Development* **8**: 2805-2816.
- Grente J., Sauret S., 1969a. Control of so-called exclusive hypovirulence by cytoplasmic determinants. *Comptes Rendus Hebdomadaires des Seances de l'Academie des Sciences, Serie D* **268**: 3173-3176.
- Grente J., Sauret S., 1969b. Exclusive hypovirulence an original phenomenon in plant pathology. *Comptes Rendus Hebdomadaires des Seances De l'Academie des Sciences, Serie D* **268**: 2347-2350.
- Gulluscii M., Turina M., 2007. Silencing of cryparin, a cell wall hydrophobin, in *Cryphonectria parasitica*. *Journal of Plant Pathology* **89**: 99-105.
- Hansen D.R., Gillies K., Van Alfen N.K., 1982. Unusual packaging of a naked viral genome associated with hypovirulence of *Endothia parasitica*. *Phytopathology* **72**: 957-957.
- Hansen D.R., Van Alfen N.K., Gillies K., Powell W.A., 1985. Naked dsRNA associated with hypovirulence of *Endothia parasitica* is packaged in fungal vesicles. *Journal of General Virology* **66**: 2605-2614.
- Hillman B.I., Halpern B.T., Brown M.P., 1994. A viral dsRNA element of the chestnut blight fungus with a distinct genetic organization. *Virology* **201**: 241-250.
- Hillman B.I., Supyani S., Kondo H., Suzuki N., 2004. A reovirus of the fungus *Cryphonectria parasitica* that is infectious as particles and related to the *Coltivirus* genus of animal pathogens. *Journal of Virology* **78**: 892-898.
- Hillman B.I., Suzuki N., 2004. Viruses of the chestnut blight fungus, *Cryphonectria parasitica*. *Advances in Virus Research* **63**: 423-472.
- Hillman B.I., Tian Y., Bedker P.J., Brown M.P., 1992. A north American hypovirulent isolate of the chestnut blight fungus with European isolate-related dsRNA. *Journal of General Virology* **73**: 681-686.
- Horvath C.M., Williams M.A., Lamb R.A., 1990. Eukaryotic coupled translation of tandem cistrons. Identification of the influenza B virus bm2 polypeptide. *Embo Journal* **9**: 2639-2647.
- Jacob-Wilk D., Turina M., Van Alfen N.K., 2006. Mycovirus cryphonectria hypovirus 1 elements cofractionate with trans-golgi network membranes of the fungal host *Cryphonectria parasitica*. *Journal of Virology* **80**: 6588-6596.
- Kang H.S., 2000. Ordered differential display from. *Cryphonectria parasitica*. *Plant Pathology Journal* **16**: 142-146.
- Kasahara S., Nuss D.L., 1997. Targeted disruption of a fungal G-protein beta subunit gene results in increased vegetative growth but reduced virulence. *Molecular Plant-Microbe Interactions* **10**: 984-993.
- Kasahara S., Wang P., Nuss D.L., 2000. Identification of bdm-1, a gene involved in G-protein [beta]-subunit function and [alpha]-subunit accumulation. *Proceeding of the National Academy of Sciences USA* **97**: 412-417.
- Kasschau K., Carrington J., 1998. A counterdefensive strategy of plant viruses: suppression of post-transcriptional gene silencing. *Cell* **95**: 461-470.
- Kazmierczak P., Kim D.H., Turina M., Van Alfen N.K., 2005. A hydrophobin of the chestnut blight fungus, *Cryphonectria parasitica*, is required for stromal pustule eruption. *Eukaryotic Cell* **4**: 931-936.
- Kazmierczak P., Pfeiffer P., Zhang L., Van Alfen N. K., 1996. Transcriptional repression of specific host genes by the mycovirus *Cryphonectria hypovirus 1*. *Journal of Virology* **70**: 1137-1142.
- Kim M.J., Choi J.W., Park S.M., Cha B.J., Yang M.S., Kim D.H., 2002. Characterization of a fungal protein kinase from *Cryphonectria parasitica* and its transcriptional upregulation by hypovirus. *Molecular Microbiology* **45**: 933-941.
- Kim M.J., Park S.M., Kim Y.H., Cha B.J., Yang M.S., Kim D.H., 2004. Deletion of a hypoviral-regulated cpgk1 gene in a chestnut blight fungus, *Cryphonectria parasitica*, results in microcolonies. *Fungal Genetics and Biology* **41**: 482-492.
- Kojima K.K., Matsumoto T., Fujiwara H., 2005. Eukaryotic translational coupling in UAAUG stop-start codons for the bicistronic RNA translation of the non-long terminal repeat retrotransposon SART1. *Molecular and Cellular Biology* **25**: 7675-7686.
- Koonin E.V., Choi G.H., Nuss D.L., Shapira R., 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. *Proceedings of the National Academy of Sciences USA* **88**: 10647-10651.

- Lamour K., Finley L., 2006. A strategy for recovering high quality genomic DNA from a large number of Phytophthora isolates. *Mycologia* **98**: 514-517.
- Larson T.G., Choi G.H., Nuss D.L., 1992. Regulatory pathways governing modulation of fungal gene expression by a virulence-attenuating mycovirus. *EMBO Journal* **11**: 4539-4548.
- Larson T.G., Nuss D.L., 1994. Altered transcriptional response to nutrient availability in hypovirus-infected chestnut blight fungus. *EMBO Journal* **13**: 5616-5623.
- Lee Y.H., Dean R.A., 1993. Camp regulates infection structure formation in the plant pathogenic fungus *Magnaporthe grisea*. *Plant Cell* **5**: 693-700.
- Li F., Ding S.W., 2006. Virus counterdefense: Diverse strategies for evading the RNA-silencing immunity. *Annual Review of Microbiology* **60**: 503-531.
- Lin H.B., Harley S.M., Butler J.M., Beevers L., 1992. Multiplicity of clathrin light-chain-like polypeptides from developing pea (*Pisum sativum* L.) cotyledons. *Journal of Cell Science* **103**: 1127-1137.
- Linder-Basso D., Dynek J.N., Hillman B.I., 2005. Genome analysis of *Cryphonectria hypovirus 4*, the most common hypovirus species in North America. *Virology* **337**: 192-203.
- Mahanti N., Fulbright D.W., 1995. Detection of mitochondrial-DNA transfer between strains after vegetative contact in *Cryphonectria parasitica*. *Molecular Plant-Microbe Interactions* **8**: 465-467.
- Maier O., Ehmsen E., Westermann P., 1995. Trimeric G-protein alpha-subunits of the Gs and Gi families localized at the Golgi membrane. *Biochemical and Biophysical Research Communications* **208**: 135-143.
- Martin M.E., Hidalgo J., Vega F.M., Velasco A., 1999. Trimeric G proteins modulate the dynamic interaction of PKAII with the Golgi complex. *Journal of Cell Science* **112**: 3869-3878.
- McCabe P.M., Van Alfen N.K., 1999. Secretion of cryparin, a fungal hydrophobin. *Applied and Environmental Microbiology* **65**: 5431-5435.
- McCudden C.R., Hains M.D., Kimple R.J., Siderovski D.P., Willard F.S., 2005. G-protein signaling: back to the future. *Cellular and Molecular Life Sciences* **62**: 551-577.
- Milgroom M.G., Cortesi P., 2004. Biological control of chestnut blight with hypovirulence: A critical analysis. *Annual Review of Phytopathology* **42**: 311-338.
- Mitchell T.K., Dean R.A., 1995. The Camp-dependent protein-kinase catalytic subunit is required for appressorium formation and pathogenesis by the rice blast pathogen *Magnaporthe grisea*. *Plant Cell* **7**: 1869-1878.
- Monteiro-Vitorello C.B., Baidyaroy D., Bell J.A., Hausner G., Fulbright D.W., Bertrand H., 2000. A circular mitochondrial plasmid incites hypovirulence in some strains of *Cryphonectria parasitica*. *Current Genetics* **37**: 242-256.
- Monteiro-Vitorello C.B., Bell J.A., Fulbright D.W., Bertrand H., 1995. A cytoplasmically transmissible hypovirulence phenotype associated with mitochondrial DNA mutations in the chestnut blight fungus *Cryphonectria parasitica*. *Proceedings of the National Academy of Sciences USA* **92**: 5935-5939.
- Napoli C., Lemieux C., Jorgensen R., 1990. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. *Plant Cell* **2**: 279-289.
- Newhouse J.R., Hoch H.C., Macdonald W.L., 1983. The ultrastructure of *Endothia parasitica*. Comparison of a virulent with a hypovirulent isolate. *Canadian Journal of Botany* **61**: 389-399.
- Newhouse J.R., Macdonald W.L., Hoch H.C., 1990. Virus-like particles in hyphae and conidia of European hypovirulent (dsRNA-containing) strains of *Cryphonectria parasitica*. *Canadian Journal of Botany* **68**: 90-101.
- Nolan T., Braccini L., Azzalin G., De Toni A., Macino G., Cogoni C., 2005. The post-transcriptional gene silencing machinery functions independently of DNA methylation to repress a LINE1-like retrotransposon in *Neurospora crassa*. *Nucleic Acids Research* **33**: 1564-1573.
- Nuss D.L., 1996. Using hypoviruses to probe and perturb signal transduction processes underlying fungal pathogenesis. *Plant Cell* **8**: 1845-1853.
- Nuss D.L., 2005. Hypovirulence: Mycoviruses at the fungal-plant interface. *Nature Reviews Microbiology* **3**: 632-642.
- Oldham W.M., Hamm H.E., 2006. Structural basis of function in heterotrimeric G proteins. *Quarterly Reviews of Biophysics* **39**: 117-166.
- Park S.M., Choi E.S., Kim M.J., Cha B.J., Yang M.S., Kim D.H., 2004. Characterization of HOG1 homologue, CpMK1, from *Cryphonectria parasitica* and evidence for hypovirus-mediated perturbation of its phosphorylation in response to hypertonic stress. *Molecular Microbiology* **51**: 1267-1277.
- Peever T.L., Liu Y.C., Milgroom M.G., 1997. Diversity of hypoviruses and other double-stranded RNAs in *Cryphonectria parasitica* in North America. *Phytopathology* **87**: 1026-1033.
- Polashock J.J., Bedker P.J., Hillman B.I., 1997. Movement of a small mitochondrial double-stranded RNA element of *Cryphonectria parasitica*: ascospore inheritance and implications for mitochondrial recombination. *Molecular and General Genetics* **256**: 566-571.
- Polashock J.J., Hillman B.I., 1994. A small mitochondrial double-stranded (ds) RNA element associated with a hypovirulent strain of the chestnut blight fungus and ancestrally related to yeast cytoplasmic T-dsRNA and W-dsRNA. *Proceedings of the National Academy of Sciences USA* **91**: 8680-8684.
- Powell W.A., Gobbi E., Van Alfen N.K., 1986. 2-D gel analysis of polypeptides from virulent and hypovirulent strains of *Endothia parasitica*. *Phytopathology* **76**: 1128-1128.
- Powell W.A., Van Alfen N.K., 1987a. Two nonhomologous viruses of *Cryphonectria (Endothia) parasitica* reduce accumulation of specific virulence-associated polypeptides. *Journal of Bacteriology* **169**: 5324-5326.
- Powell W.A., Van Alfen N.K., 1987b. Differential accumulation of poly(a) +RNA between virulent and double-stranded RNA-induced hypovirulent strains of *Cryphonectria*

- (*Endothia*) *parasitica*. *Molecular and Cellular Biology* **7**: 3688-3693.
- Pruss G., Ge X., Shi X.M., Carrington J.C., Vance V.B., 1997. Plant viral synergism: The potyviral genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses. *Plant Cell* **9**: 859-868.
- Ratcliff F., Harrison B.D., and Baulcombe D.C., 1997. A similarity between viral defence and gene silencing in plants. *Science* **276**: 1558-1560.
- Rigling D., Van Alfen N.K., 1991. Regulation of laccase biosynthesis in the plant pathogenic fungus *Cryphonectria parasitica* by double-stranded RNA. *Journal of Bacteriology* **173**: 8000-8003.
- Rigling D., Van Alfen N.K., 1993. Extracellular and intracellular laccases of the chestnut blight fungus *Cryphonectria parasitica*. *Applied and Environmental Microbiology* **59**: 3634-3639.
- Romano N., Macino G., 1992. Quelling: Transient inactivation of gene expression in *Neurospora crassa* by transformation with homologous sequences. *Molecular Microbiology* **6**: 3343-3353.
- Segers G.C., Regier J.C., Nuss D.L., 2004. Evidence for a role of the regulator of G-protein signaling protein CPRGS-1 in got subunit CPG-1-mediated regulation of fungal virulence, conidiation, and hydrophobin synthesis in the chestnut blight fungus *Cryphonectria parasitica*. *Eukaryotic Cell* **3**: 1454-1463.
- Segers G.C., van Wezel R., Zhang X.M., Hong Y.G., Nuss D.L., 2006. Hypovirus papain-like protease p29 suppresses RNA silencing in the natural fungal host and in a heterologous plant system. *Eukaryotic Cell* **5**: 896-904.
- Shapira R., Choi G.H., Nuss D.L., 1991. Virus-like genetic organization and expression strategy for a double-stranded RNA genetic element associated with biological control of chestnut blight. *EMBO Journal* **10**: 731-739.
- Smart C.D., Yuan W., Foglia R., Nuss D.L., Fulbright D.W., Hillman B.I., 1999. *Cryphonectria hypovirus 3*, a virus species in the family *Hypoviridae* with a single open reading frame. *Virology* **265**: 66-73.
- Stow J.L., Dealmeida J.B., Narula N., Holtzman E.J., Ercolani L., Ausiello D.A., 1991. A Heterotrimeric G-protein, G-alpha-I-3, on Golgi membranes regulates the secretion of a heparan-sulfate proteoglycan in Llc-Pk1 epithelial cells. *Journal of Cell Biology* **114**: 1113-1124.
- Sun L.Y., Nuss D.L., Suzuki N., 2006. Synergism between a mycoreovirus and a hypovirus mediated by the papain-like protease p29 of the prototypic hypovirus CHV1-EP713. *Journal of General Virology* **87**: 3703-3714.
- Suzuki N., Chen B., Nuss D.L., 1999. Mapping of a hypovirus p29 protease symptom determinant domain with sequence similarity to potyvirus HC-Pro protease. *Journal of Virology* **73**: 9478-9484.
- Suzuki N., Geletka L.M., Nuss D.L., 2000. Essential and dispensable virus-encoded replication elements revealed by efforts to develop hypoviruses as gene expression vectors. *Journal of Virology* **74**: 7568-7577.
- Suzuki N., Maruyama K., Moriyama M., Nuss D.L., 2003. Hypovirus papain-like protease p29 functions in trans to enhance viral double-stranded RNA accumulation and vertical transmission. *Journal of Virology* **77**: 11697-11707.
- Suzuki N., Nuss D.L., 2002. The contribution of protein p40 to hypovirus-mediated modulation of fungal host phenotype and viral RNA accumulation. *Journal of Virology* **76**: 7747-7759.
- Suzuki N., Supyani S., Maruyama K., Hillman B.I., 2004. Complete genome sequence of Mycoreovirus-1/Cp9B21, a member of a novel genus within the family *Reoviridae*, isolated from the chestnut blight fungus *Cryphonectria parasitica*. *Journal of General Virology* **85**: 3437-3448.
- Till B.J., Reynolds S.H., Greene E.A., Codomo C.A., Enns L.C., Johnson J.E., Burtner C., Odden A.R., Young K., Taylor N.E., Henikoff J.G., Comai L., Henikoff S., 2003. Large-scale discovery of induced point mutations with high-throughput TILLING. *Genome Research* **13**: 524-530.
- Turina M., Prodi A., Van Alfen N.K., 2003. Role of the Mf1-1 pheromone precursor gene of the filamentous ascomycete *Cryphonectria parasitica*. *Fungal Genetics and Biology* **40**: 242-251.
- Turina M., Zhang L., Van Alfen N.K., 2006. Effect of *Cryphonectria hypovirus 1* (CHV1) infection on Cpk1, a mitogen-activated protein kinase kinase of the filamentous fungus *Cryphonectria parasitica*. *Fungal Genetics and Biology* **43**: 764-774.
- Van Alfen N.K., Jaynes R.A., Anagnostakis S.L., Day P.R., 1975. Chestnut blight biological control by transmissible hypovirulence in *Endothia parasitica*. *Science* **189**: 890-891.
- Vanderkrol A.R., Mur L.A., Delange P., Mol J.N.M., Stuitje A.R., 1990. Inhibition of flower pigmentation by antisense Chs genes: Promoter and minimal sequence requirements for the antisense effect. *Plant Molecular Biology* **14**: 457-466.
- Yu J. H., 2006. Heterotrimeric G protein signaling and RGSs in *Aspergillus nidulans*. *Journal of Microbiology* **44**: 145-154.
- Zhang L., Baasiri R.A., Van Alfen N.K., 1998. Viral repression of fungal pheromone precursor gene expression. *Molecular and Cellular Biology* **18**: 953-959.
- Zhang L., Churchill A.C.L., Kazmierczak P., Kim D.H., Van Alfen N.K., 1993. Hypovirulence-associated traits induced by a mycovirus of *Cryphonectria parasitica* are mimicked by targeted inactivation of a host gene. *Molecular and Cellular Biology* **13**: 7782-7792.
- Zhang L., Villalon D., Sun Y., Kazmierczak P., Van Alfen N.K., 1994. Virus-associated down-regulation of the gene encoding cryparin, an abundant cell-surface protein from the chestnut blight fungus *Cryphonectria parasitica*. *Gene* **139**: 59-64.