Plant Pathol. J. 19(1) : 30-33 (2003)

Symposium

The 5th Molecular Plant-Microbe Interactions

December 6, 2002, Daejeon, Korea

Modulation of a Fungal Signaling by Hypovirus

Dae-Hyuk Kim[1]*

[1]Institute of Molecular Biology and Genetics, Basic Science Research Institute, Chonbuk National University, Chonbuk, Chonju 561-756, Korea

(Received on November 12, 2002; Accepted on January 18, 2003)

The chestnut blight fungus, *Cryphonectria parasitica*, and its hypovirus are a useful model system in the study of the mechanisms of hypoviral infection and its consequences, such as a biological control of fungal pathogens. Strains containing the double-stranded (ds) RNA viruses *Cryphonectria hypovirus* 1 show characteristic symptoms of hypovirulence and display hypovirulence-associated changes, such as reduced pigmentation, sporulation, laccase production, and oxalate accumulation. Interestingly, symptoms caused by hypoviral infection appear to be the result of aberrant expression of a number of specific genes in the hypovirulent strain. Several viral regulated fungal genes are identified as cutinase gene, *Lac*1, which encodes an extracellular laccase, *Crp*, which encodes an abundant tissue-specific cell-surface hydrophobin that mediates physical strength, and *Mf*2/1 and *Mf*2/2, which encode pheromone genes involved in poor sporulation in the presence of hypovirus. Since the phenotypic changes in the fungal host are pleiotropic, although coordinated and specific, it has been suggested that the hypovirus disturbs one or several regulatory pathways (Nuss, 1996). Accordingly, several studies have shown the implementation of a signal transduction pathway during viral symptom development. Although further studies are required, hypovirulence and its associated symptom development due to the hypoviral regulation of a fungal heterotrimeric G-protein have been suggested. In addition, recent studies have shown the presence of a novel protein kinase gene *cppk*1 and its transcriptional upregulation by hypovirus. In this review, the presence of important components in signal transduction pathway, their putative biological function, and viral-specific regulation will be addressed.

Keywords **:** *Cryphonectria parasitica*, hypovirulence, signal transduction pathway.

*Corresponding author.
Phone) +82-63-270-3440, FAX) +82-63-270-4312
E-mail) dhkim@moak.chonbuk.ac.kr

Chestnut blight, caused by *Cryphonectria parasitica* (Murr.) Barr, resulted in the almost complete destruction of chestnut forests and orchards in North America since the discovery of the disease in 1904. However, the disease discovered in Europe in 1938 has been less destructive than that discovered in North America. This low level of disease severity could be the result of the greater blight resistance of European chestnut and the apparent widespread occurrence of hypovirulence, which has a transmissible (through hyphal anastomosis) and blight-curative (biological control) properties associated with dsRNA Cryphonectria hypoviruses (Van Alfen et al., 1975). The direct cause-effect relationship between hypovirulence and mycovirus has been proven by transforming the virus-free strain with an infectious cDNA copy of mycovirus followed by the recovery of hypovirulence, as well as dsRNA hypovirus particles (Chen et al., 1994).

In addition to hypovirulence, fungal strains harboring hypoviruses may exhibit an altered colony morphology that includes reduced sporulation and changes in pigmentation. Reduction in levels of enzymes such as laccase and cutinase, or of metabolites such as oxalate may also occur in hypovirulent strains.

The chestnut blight fungus, *C. parasitica*, and its hypovirus are one of the best-documented examples of a naturally occurring form of biological control for plant disease and fungal gene regulation by mycovirus. Previous reports have demonstrated that the expression of a number of specific genes is altered in the hypovirulent strain, which is identified as cutinase gene (Varley et al., 1992), *Lac*1, which encodes an extracellular laccase (Choi and Nuss, 1992; Rigling and Van Alfen, 1991; Kim et al., 1995), *Crp*, which encodes an abundant tissue-specific cell-surface hydrophobin that mediates physical strength (Zhang et al., 1994), and *Mf*2/1 and *Mf*2/2, which encode pheromone genes involved in poor sporulation in the presence of hypovirus (Zhang et al., 1993; Zhang et al.,

1998). Theses studies all suggest that hypovirus modulates fungal gene expression in a specific manner. Since the hypovirus causes diverse phenotypic changes, but in a coordinated and specific way, that slow the development of the fungus without killing it, it has been suggested that the hypovirus disturbs one or several regulatory pathways. Therefore, it can be said that the signal transduction pathway in *C. parasitica* appears to be the main target of hypoviruses.

Eukaryotic organisms recognize and respond to various external environments through a variety of signal transduction pathways, which involve protein phosphorylation at some critical step. This is a highly evolved and sophisticated process that is critical for nearly all aspects of physiological regulation in eukaryotes, including filamentous fungi. In filamentous fungi, signaling pathways that act via phosphoryation may be involved in the following: 1) response to extracellular signals; 2) regulation of cell cycle-related processes; and 3) response to nutritional and environmental stresses (Dickman and Yarden, 1999). Filamentous fungi have signaling systems that are similar to those of the higher eukaryotes. Theses systems include G-proteins, mitogen activated protein kinases (MAPK), protein kinase C (PKC), phospholipase C (PLC), and protein kinase. Several genes involved in *C. parasitica* signal transduction have been identified: *cpg-1, cpg-2, cpgb-1, bdm-1,* and *cppk1.* These genes encode two $G_{i\alpha}$ sub-units, a G_{β} sub-unit, a beta disruption mimic factor, and a novel Ser/Thr protein kinase, respectively (Choi et al., 1995; Kasahara and Nuss, 1997; Kasahara et al., 2000; Kim et al., 2002). However, among many regulatory genes of *C. parasitica*, not many of them were both viral-regulated and specific symptom-related i.e., they were either viral-regulated but reproduce all symptoms (Choi et al., 1995; Gao and Nuss, 1996), or specific symptom-related but viral independent (Wang et al., 1998). Accordingly, it is not easy to depict the details of molecular hierarchy explaining how mycovirus regulates the fungal gene expression to result in specific viral symptoms.

This review explored whether the hypoviruses affect a fungal signal transduction pathway and what the biological function of a specific pathway might be in terms of symptom development.

Does the hypovirus affect the G protein-mediated signal transduction in *C. parasitica*?

Several studies have shown the existence of genes encoding major components of signal transduction pathway. These include two G_{α} sub-unit genes (*cpg-1* and *cpg-2*) and a G_{β} sub-unit gene (*cpgb-1*), which seem to be among the very

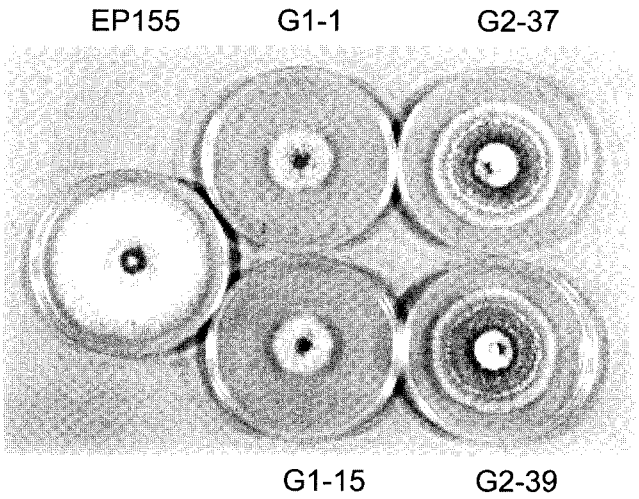

Fig. 1. Colony morphology of wild type strain EP155/2, *cpg-1* disruption mutants G1-1 and G1-15, and *cpg-2* disruption mutants G2-37 and G2-39. When grown on PDA, disruptants G1-1 and G1-15 grew more slowly than the control strain EP155/2, whereas G2-37 and G2-39 had only slightly reduced growth rate. Orange pigmentation was also reduced for *cpg-1* disruptants G1-1 and G1-15, but the *cpg-2* disruptants G2-37 and G2-39 had slightly increased orange pigmentation in comparison with EP155 (Gao and Nuss, 1996).

first G protein genes cloned from filamentous fungi. The deletion of *cpg-1* showed a set of phenotypic changes similar to, but more severe than, those associated with hypovirus infection (Gao and Nuss, 1996). Changes included a marked reduction in fungal growth rate and loss of virulence, asexual sporulation, female sterility, and transcriptional induction of laccase gene (*lac*-1). However, *cpg-2* disruption resulted in slight reductions in growth rate and sporulation. No other phenotypes were distinctive. These results provide definitive confirmation of a requirement of CPG-1-linked signaling for a number of fungal processes, including virulence and reproduction, while demonstrating that a second G_{α}, CPG-2, is dispensable for these processes (Fig. 1). However, the viral regulation of this gene is still cumbersome even if there was a subsequent report that the CPG-1 protein was reduced in virus-containing strain. Moreover, the various levels of CPG-1 expression by transgenic co-suppression correlated with phenotypic changes similar to the viral infection (Fig. 2). However, no changes among virus-containing and their virus-free isogenic strains were detected in the level of G_{α} protein using G_{α}-specific toxin (Fig. 3). In conclusion, G_{α}- and its related proteins are important for fungal processes, such as growth and development, but further studies are required to confirm whether the hypoviruses are directly involved in the regulation of these genes or gene products.

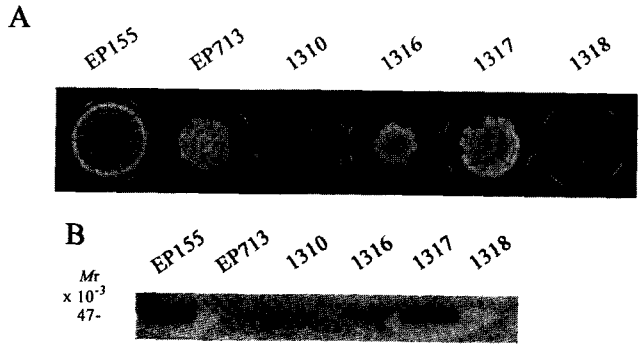

Fig. 2. Colony morphology and Western analysis of selected *C. parasitica* strains transformed with sense copies of *cpg-1*. (**A**) Colony morphology of selected *cpg-2* sense transformants. Eleven of 18 randomly selected *cpg-1* transformants from a single representative transformation exhibited altered phenotypic traits compared with the nontransformed virulent strain EP155. When cultured on PDA, many of the sense transformants grew slowly and had uneven colony margins, sparse aerial hypahe, an intense brown-orange pigmentation rather than the normal orange pigmentation, and absence of concentric orange growth rings, suggesting disruption of response to light/dark cycles. These alterations were stable and qualitatively similar among the transformants but varied in severity between transformants as shown by representative sense transformants 1310, 1316, 1317, and 1318. (**B**) Western analysis of selected sense transformants. Lysates prepared from nontransformed *C. parasitica* virulent (EP155) and hypovirulent (EP713) strains and *cpg-1* sense transformants 1310, 1316, 1317, and 1318 were probed with CPG-1 specific antisera. Migration position of a M_r 47,000 size marker is indicated on the left. Severity of phenotypic alterations exhibited by the four transformants when cultured on PDA correlated with the level of CPG-1 suppression (comparison A and B) (Choi et al., 1995).

Does the hypovirus affect the signal transduction in *C. parasitica*?

Kim et al. (2002) reported the presence of a novel Ser/Thr kinase, *cppk1*, the transcription of which is specifically regulated by the presence of hypovirus. The protein product of *cppk1* had a catalytic activity of protein phosphorylation and its transcriptional accumulation was specifically up-regulated by the presence of hypovirus, which appears to be the first example of the transcriptional up-regulation by hypovirus with a known function. Kinase assay using *E. coli*-expressed CPPK1 exhibited the presence of cellular target protein with a size of 50 kDa and 44 kDa, which strongly indicates that the *cppk1* is one of the components in fungal signaling cascade with many downstream target proteins. The transformation of virus-free strain with a vector containing *cppk1* derived by a constitutive promoter can reproduce some, but not all, viral symptoms, such as reduced pigmentation, asexual sporulation, and female sterility, in virus-free transformants with a concomitant increased accumulation of transcript and its protein product

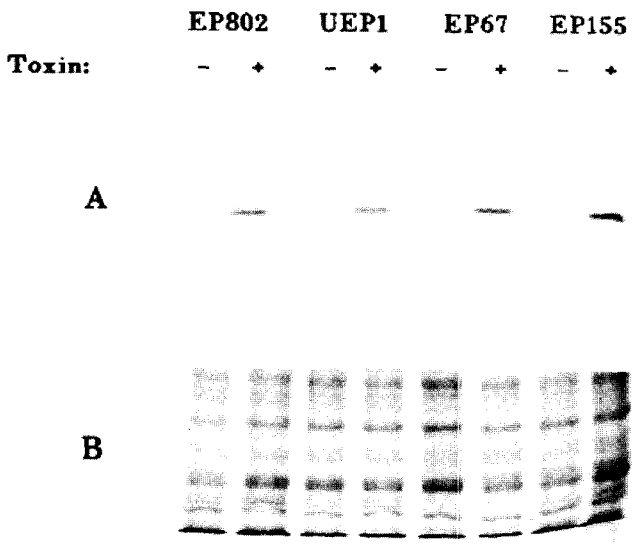

Fig. 3. Detection of the Ga_i sub-unit of *C. parasitica* by pertussis toxin ADP-ribosylation. Crude plasma membranes were extracted from virulent strains EP155 and EP67 and their respective isogenic CHV1-infected strains UEP1 and EP802, and labeled with pertussis toxin by the method of Turner and Borkovich (1993). (**A**) Protein labeled with [32P]NAD in the presence (+) or absence (–) of pertussis toxin. (**B**) Polyacrylamide gel of the same membrane preparation as in panel but stained with Coomassie blue. A total of threefold more protein was loaded in each lane of panel B than in each lane of panel A (Zhang et al., 1998).

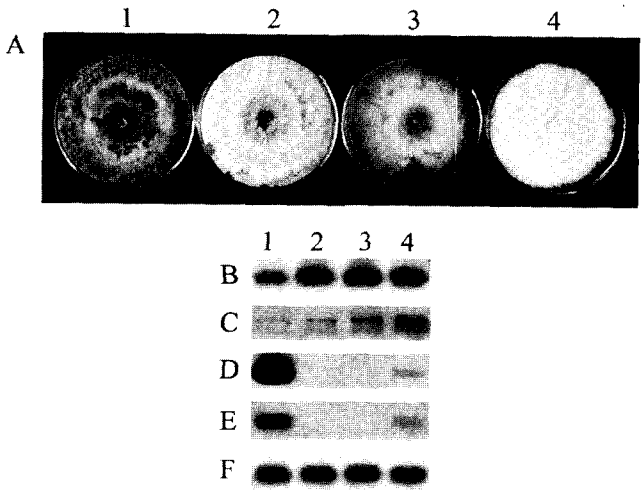

Fig. 4. Phenotypic and molecular characteristics of selected *cppk1* overexpression transformants. (**A**) Colony morphology, when cultured on PDAmb, (**B**) Northern blot analysis of *cppk1* transcript, (**C**) Western blot analysis of CpPK1, (**D**) Northern blot analysis of *Mf2/1* transcript, (**E**) Northern blot analysis of *Mf2/2* transcript. Panels B, C, D, and E contain samples prepared from 5-day old culture. Equal loading amount of protein preparations is normalized by Bradford assay, and Northern blot analyses are normalized with *Gpd* probe (**F**). Numbers 1, 2, 3, and 4 indicate strains of EP155/2, TPK13-1, TPK15-1, and UEP1, respectively (Kim et al., 2002).

(Fig. 4). The *cppk1* study clearly demonstrates that the hypovirus disturbs fungal signaling at least one part by transcriptional up-regulation of *cppk*1, which results in reduced pigmentation and conidiation, and female sterility. Disruption mutant of *cppk1* gene has recently been constructed and it will be of interest to find the relationship between the biological function of gene in signaling and its involvement in symptom development.

As an alternative approach to prove the viral regulation of fungal signal transduction pathway, several genes belonging to representative components of the signal transduction pathway were cloned from *C. parasitica* based on the sequence homology, and the viral regulation of their expression were examined. One of the early examples can be a mitogen-activated protein kinase (MAPK). In this study, high-osmotic glycerol (HOG) homologue from *C. parasitica* was cloned, and it was proven that the induction of this gene product is also affected by the hypovirus infection, which is another example of hypoviral regulation of fungal gene expression (in review).

There are many examples that provide confirmative evidences for the viral regulation of fungal signaling. To understand the mechanism by which mycoviruses perturb fungal gene expression, knowledge of the host genes that are affected, as well as their roles in fungal development and virulence, are important. Assessing the roles of signal transduction pathways in symptom development, it is possible to infer the correlation between a specific signal pathway and its corresponding symptom, which will provide researchers with better strategies for the biological control of fungal pathogen without disturbing other desirable characteristics of a hypovirulence-inducing strain. In addition, it can provide valuable information on how mycovirus coordinates and regulates fungal gene expression, which suggests the general mechanism of viral regulation of host gene expression.

Acknowledgement

This study was supported by the Technology Development Program for Agriculture and Forestry, Ministry of Agriculture and Forestry, Republic of Korea. We thank the Research Center for Industrial Development of BioFood Materials at Chonbuk National University for kindly allowing us the use of their facilities for this research.

References

Chen, B., Choi, G. H. and Nuss, D. L. 1994. Attenuation of fungal virulence by synthetic infectious hypovirus transcripts. *Science* 264:1762-1764.

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

Choi, G. H., 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.

Dickman, M. B. and Yarden, O. 1999. Serine/Threonine protein kinases and phosphatases in filamentous fungi. *Fungal Genet. Biol.* 26:99-117.

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.

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

Kasahara, S., Wang, P. and Nuss, D. L. 2000. Identification of *bdm*-1, a gene involved in G protein β-subunit function and α-subunit accumulation. *Proc. Natl. Acad. Sci. USA* 97:412-417.

Kim, D. H., Rigling, D., Zhang, L. and Van Alfen, N. K. 1995. A new extracellular laccase of *Cryphonectria parasitica* is revealed by deletion of *Lac*1. *Mol. Plant-Microbe Interact.* 8:259-266.

Kim, M. J. Choi, J. W., Park, S. M., Cha, B. J., Yang, M. S. and Kim, D. H. 2002. Characterization of a fungal protein kinase from *Cryphonectria parasitica* and its transcriptional upregulation by hypovirus. *Mol. Microbiol* 45:933-941.

Rigling, D. 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.

Van Alfen, N. K., Jaynes, R. A., Anagnostakis, S. L. and Day, P. R. 1975. Chestnut blight: Biological control by transmissible hypovirulence in *Endothia parasitica*. *Science* 189:890-891.

Varley, D. A., Podila, G. K. and Hiremath, S. T. 1992. Cutinase in *Cryphonectria parasitica*, the chestnut blight fungus: suppression of cutinase gene expression in isogenic hypovirulent strains containing double-stranded RNAs. *Mol. Cell Biol.* 12:4539-4544.

Wang, P., Larson, T. G., Chen, C. H., Pawlyk, D. M., Clark, J. A. and Nuss, D. L. 1998. Cloning and characterization of a general amino acid control transcriptional activator from the chestnut blight fungus *Cryphonectria parasitica*. *Fungal Genet. Biol.* 23:81-94.

Zhang, L., Baasiri, R. A. and Van Alfen, N. K. 1998. Viral regulation of fungal pheromone precursor gene expression. *Mol. Cell. Biol.* 18:953-959.

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.

Zhang, L., Villalon, D., Sun, Y., Kazmierczak, P. and 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.