DEVELOPMENT OF A SIMPLIFIED DETECTION METHOD OF DSRNA FROM THE HYPOVIRULENT STRAINS OF THE CHESTNUT BLIGHT FUNGUS (*CRYPHONECTRIA PARASITICA)*

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Abstract
Cryphonectria parasitica, the casual pathogen of chestnut blight disease. Double-stranded RNA viruses cause reduces virulence (hypovirulence) in the host fungus and disturbs the developmental process of the fungus. In this study, we present a modified procedure which further improves the dsRNA isolation efficiency from C. parasitica. The new protocol is less time-consuming and it requires smaller amount of fungal materials as well as reagents reducing the financial costs and isolation time significantly.

INTRODUCTION

The causal agent of chestnut blight disease is a heterothallic fungus, *Cryphonectria parasitica (Murr.) Barr* (Syn. *Endothia parasitica* [Murr.] And.) is one of the most important fungal pathogens of chestnut in Europe and North America. *Cryphonectria parasitica*, is classified as a quarantine organism, list A2 by the EPPO and is also quarantine significance for NAPPO and IASPC. Chestnut blight is a most important and widespread disease of chestnut in Hungary today.

Fungicide treatments are not efficient in managing the disease and only biological control by virus mediated attenuation provides acceptable and effective results. *C. parasitica* is a natural host of at least five hypovirus species, some of which attenuate the virulence of the host fungus (Hillman and Suzuki 2004). The fungal strains infected by the viruses have been studied intensively to develop a biological method for control of chestnut blight disease.

Hypovirulence causes a reduction in fungal virulence and perturbs host developmental processes such as sporulation and virulence (MacDonald and Fulbright 1991; Mccabe et al. 1999). Virus infected strains express symptoms of reduced virulence (hypovirulence), reduced asexual and sexual sporulation, and reduced pigmentation.

Mycoviruses are unencapsulated double-stranded RNA viruses located in the cytoplasm of the fungus (Choi and Nuss 1992). The sizes of the dsRNA genomes range between 10 and 13 kbp. Mycovirus does not have extracellular phase and are transmitted from infected to non-infected fungal strains via hyphal anastomosis converting. They are also mitted into asexual conidia but not into sexual ascospores (Anagnostakis 1988). This character has an important role to the successful control of chestnut blight disease by artificially and naturally disseminating hypovirulent strains as a biological control agent (Heiniger and Rigling 1994; Milgroom and Cortesi 2004).

The first fast and simple method to isolate and screen of dsRNA mycoviruses from virus infected fungal tissue and plant was reported by Morris and Dodds (1979). The original procedure have been subjected to several modifications by different authors (Allemann et al. 1999; Allen et al. 2003; Hillman et al. 1990; Peever et al. 1997; Rigling et al. 1989).

In this study, we present a modified procedure which further improves the dsRNA isolation efficiency from C. parasitica. The new protocol is less time-consuming and it requires smaller amount of fungal materials as well as reagents reducing the financial costs and isolation time appreciably. The basis of this method was previously applied on an other ascomycetous fungus, Rosellinia necatrix, by Osaki et al. (2002).

MATERIALS AND METHODS

The hypovirulent strains isolated from Hungary were cultured on PDA plates covered by cellulose based cellophane sheet at 25°C for 8 days in the dark until the culture diameter reaches at least 6 cm. The entire mycelium was collected from the cellophane using a sterile spatula.

One gram of mycelial tissue was grinded to a fine powder in liquid nitrogen using a mortar and a pestle, and transferred into a clean 2ml centrifuge tube, and then it was suspended in 1.8ml of 2xSTE buffer (0.1M NaCl, 50mM Tris, 1mM Na_2EDTA, pH 7.0), 0.2ml of 10% SDS, 2µl of mercaptoethanol, 1ml of chloroform and 1ml water-saturated phenol containing 0.2% 8-hydroxyquinoline. The mixture was homogenized by a Vortex then the homogenate was centrifuged at 8000rpm for 10min. The aqueous phase was transferred into a 2ml centrifuge tubes, and ethanol was added to make a final concentration of 16%. For each 10ml of the 16% ethanol solution, 0.5g CF-11 (Whatman) cellulose powder was added then the mixture was transferred into a Sigma chromatography column. The column was washed four additional times with 1ml 1xSTE/15% ethanol pH 7.0 buffer. The dsRNA was then eluted from CF-11 cellulose with 2ml 1xSTE pH 7.0 buffer and collected in a fresh 2ml centrifuge tube. To precipitate dsRNA, 2.5 volumes of cold ethanol were added to the elution. The precipitate was collected by centrifugation, 12000G for 8 min and was dissolved in 50µl nuclease-free water. The remaining DNA was digested by RNase-free DNase (Promega) according to the manufacturer's instructions. The dsRNA was visualized under UV light in 0.7% agarose gel stained with ethidium bromide (0.5µg/ml) at 70 V for 60 min (Fig. 1).

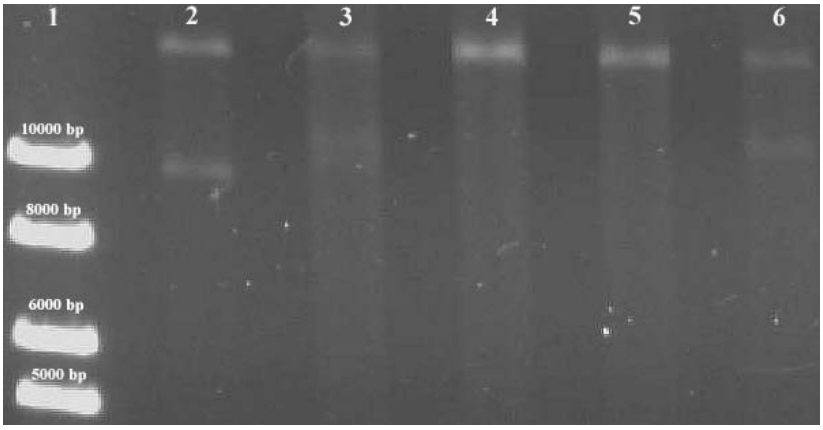

Fig. 1: Agarose gel electrophoresis of dsRNA isolated from *C. parasitica* hypovirulent strains. Lane 1: 1 kb DNA Ladder Fermentas; Lane 2-6: dsRNAs extracted from hypovirulent strains.

CONCLUSION

The new modified protocol allows rapid and efficient isolation from dsRNA *C. parasitica*. The new method is less time-consuming and it requires smaller amount of fungal materials as well as reagents reducing the financial costs and isolation time significantly. The described protocol was successfully applied for dsRNA detection in *C. parasitica* originated from cankers of chestnut trees in Hungary. The relative high concentration of extracted dsRNA makes the modified protocol suitable and for the rapid detection of hypovirus in *C. parasitica*.

REFERENCES

1. Allemann, C., Hoegger, P., Heiniger U., Rigling, D. (1999). Genetic variation of Cryphonectria hypoviruses (CHV1) in Europe, assessed using restriction fragment length polymorphism (RFLP) markers. *Molecular Evolution 8, 843-854.*
2. 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, 1263-1265.*
3. Anagnostakis, S.L. 1988. *Cryphonectria parasitica*, cause of chestnut blight. *Advanced Plant Pathology 6, 123-136.*
4. Choi, G. H., Nuss, D. L. (1992). Hypovirulence of chestnut blight fungus conferred by an infectious viral cDNA. *Science 257, 800-803.*
5. Heiniger, U., Rigling, D. (1994). Biological control of chestnut blight in Europe. Annual *Review of Phytopathology 32, 581-599.*
6. Hillman, B.I., Shapira, R., Nuss, D.L. (1990). Hypovirulence-associated suppression of host functions in *Cryphonectria parasitica* canbe partially relieved by high light intensity. *Phytopathology 80, 950-956.*
7. Hillman, B.I., Suzuki, N. (2004). Viruses in the chestnut blight fungus. *Advances in Virus Research 63, 423-472.*
8. MacDonald, W.L., Fulbright, D.W. (1991). Biological control of chestnut blight: use, limitations of transmissible hypovirulence. *Plant Disease 75, 656-661.*
9. McCabe, P.M., Pfeiffer, P., van Alfen, N.K. (1999). The influence of dsRNA viruses on the biology of plant pathogenic fungi. *Trends in Microbiology 7, 377-381.*
10. Milgroom, M.G., Cortesi, P. (2004). Biological control of chestnut blight with hypovirulence. A critical analysis. *Annual Review of Phytopathology 42, 311-338.*
11. Morris, T.J., Dodds, J.A. (1979). Isolation and analyses of double-stranded RNA from virus-infected plant and fungal tissue. *Phytopathology 87, 1026-1033.*
12. Osaki, H., Wei, C.Z., Arakawa, M., Iwanami, T., Nomura, K., Matsumoto, N., Ohtsu, Y. (2002). Nucleotide sequences of double-stranded RNA segments from a hypovirulent strain of the white root rot fungus *Rosellinia necatrix*: Possibility of the first member of the Reoviridae from Fungus. Virus genes 25, 101-107.
13. Peever, T.L., Liu, Y-CH., Milgroom, M.G. (1997). Diversity of Hypoviruses and other double-stranded RNAs in *Cryphonectria parasitica* in North America. Phytopathology 87, 1026-1033.
14. Rigling, D., Heiniger, U., Hohl, H.R. (1989). Reduction of laccase activity in dsRNA-containing hypovirulent strains of *Cryphonectria (Endothia) parasitica*. Phytopathology 79, 219-223.