FEMS Microbiology Ecology 51 (2005) 293–301

FEMS MICROBIOLOGY Ecology

www.fems-microbiology.org

Dynamics of double-stranded RNA segments in a *Helicobasidium mompa* clone from a tulip tree plantation

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Received 20 April 2004; received in revised form 23 June 2004; accepted 11 September 2004

First published online 29 October 2004

Abstract

Eighty-three isolates of the violet root rot fungus, *Helicobasidium mompa*, were collected in a tulip tree plantation and analyzed for the dynamics of double-stranded (ds) RNA for five years. They were divided into eight mycelial compatibility groups (MCGs). Prevalent MCGs 60 and 68 included 61 and 11 isolates, respectively. Electrophoretic profiles of dsRNA in the first year collection of MCG 60 contained no or a single large dsRNA (more than 10 kb) with or without small dsRNAs (ca. 2.0–2.5 kb). Additional dsRNA fragments, i.e., a middle dsRNA (ca. 8.0 kb) or another type of small dsRNAs, became evident within MCG 60 isolates with time. Northern hybridization revealed the relatedness of all large and middle dsRNA fragments within MCG 60 but small fragments of dsRNA were variable. Large dsRNA fragment differed from that in other MCGs even in the same field. Correlation between specific dsRNA fragments and hypovirulence was not observed. Possible explanations for the accumulation of dsRNA fragments during the growth of disease patch by MCG 60 are discussed in terms of their internal changes such as evolution of novel dsRNA fragments from pre-existing viruses or fungal genomic DNA and horizontal transmissions.

Keywords: Double-stranded (ds) RNA; *Helicobasidium mompa*; Mycelial compatibility group (MCG); Transmission; Mycovirus

1. Introduction

Helicobasidium mompa Tanaka is a basidiomycetous fungus that colonizes rhizosphere with the mycelial strand and produces flat, resupinate fruiting body annually on the trunk base, unlike the typical "mushroom" fungi that raise fruiting body above the substrate. The fungus causes violet root rot of many plant species, e.g., apple trees, mulberry, asparagus, and forest trees [1]. Many trials on biological control of violet root rot have been made, but few experiments have been successful under field conditions. Long-term biological control may be hampered by the perennial growth of the pathogen as well as hosts and by the difficulty in the establishment of antagonists in the rhizosphere. Matsumoto [2] proposed the use of mycoviruses to control the disease, because they exist within the cytoplasm of the host fungus and are free from microbial interactions in soil. The following evidence is the basis for the exploitation of mycoviruses: field isolates of *H. mompa* spreads

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0168-6496/$22.00
doi:10.1016/j.femsec.2004.09.011

clonally by the extension of mycelial strands in soil rather than propagation by basidiospores [3] as reported in *Armillaria* spp. [4–6]. Clonal growth of the fungus should facilitate the spread of mycovirus through the mycelial network. To apply mycoviruses as biocontrol agents against violet root rot in the field, it is prerequisite to recognize how they spread through the mycelial network and to predict if interference occurs when plural mycoviruses occur in the same strains.

Viruses commonly occur in fungi and have been detected from all fungal taxa [7]. Most of the characterized mycoviruses contain segmented, double-stranded (ds) RNA genome. Some dsRNAs are known to function as the hypovirulence factor and regarded as a potential biocontrol agent [8], e.g., CHV1 in *Cryphonectria parasitica* [9], D-factor in *Ophiostoma ulmi* [10], and *Reovirus*-like elements in *Rosellinia necatrix* [11] and in *C. parasitica* [12]. In *H. mompa*, only a dsRNA fragment was reported as a hypovirulence factor, though most dsRNAs were neutral [13].

The origin of dsRNA is controversial. Most dsRNAs are considered to have co-evolved with host cells through vertical transmission [14,15]. Laboratory experiments implied that horizontal transmission by hyphal anastomosis [16–18] and spontaneous emergence of dsRNA from fungal genome DNA [19] are the alternative acquisition mechanisms of dsRNA. Moreover, similar mycovirus species were detected from distantly separate fungal taxa, e.g., dsRNA elements belonging to the genus *Partitivirus* from ascomycetes fungi, *Fusarium poae* [20] and basidiomycetes fungi, *H. mompa* [21]. Therefore, mycoviruses seems to be actively disseminated across fungal taxa; however, it is very difficult to prove the way of acquisition of novel dsRNAs in the field observation.

Bissegger et al. [22] studied the population structure of *C. parasitica* in European chestnut forests by repeated isolation of the fungus from the same cankers for four years. Colonies of isolates that had produced orange pigmentation became unable to produce it (white colony) during the 4-year collection, suggesting that natural infection of hypovirus occurred in the field. However, they also pointed out that about 30% of the cankers were taken over by isolates differing in vegetative compatibility groups (VCGs) during the study [22]. The transition in VCGs makes the analysis of viral dynamics in the *C. parasitica* populations difficult due presumably to the airborne epidemics of chestnut blight.

Our previous studies [23,21] revealed that *H. mompa* isolates frequently contained dsRNAs (65%: 363/559) in Japan and that various dsRNA species were detected by dsRNA banding profiles and nucleotide sequences implying that horizontal transmission of mycoviruses frequently occurs in nature. In this article, we annually collected *H. mompa* isolates from a tulip tree plantation for 5 years and examined their clonality and the dynamics of dsRNA banding profiles in the same clone.

2. Materials and methods

2.1. Study site and fungal isolates

A 34-year-old tulip tree (*Liriodendron tulipifera*) plantation in Iwate was selected as a study site of dsRNA in *H. mompa*. The plantation was approximately 45×45 m, containing 200 tulip trees and a few shrubs. Trees with fruiting bodies were marked, and three spots each of a fruiting body were collected for five consecutive years from 1999 to 2003 for fungal isolation. All isolates were originated from vegetative hyphae newly grown from fruiting bodies in moist Petri dishes [24]. They were maintained on oatmeal agar (BD Difco, Sparks) slants at 4 °C. Three isolates were obtained from each fruiting body in each tree, and one isolate represented the three isolates from the same trees, and isolate number was assigned (Table 1). When isolates from the same trees contained different dsRNA banding patterns, they were treated separately with different isolate numbers (Table 1, e.g., trees 1, 2, 3, and 4).

Mycelial compatibility groups (MCGs) were distinguished by the presence of a demarcation line along the colony junction in paired cultures on oatmeal agar plates. Small agar blocks with mycelia were cut from the colony margin of oatmeal agar plate cultures. Six blocks were inoculated at equal distance around the edge of oatmeal agar plates and one in the center. Plates were incubated at 25 °C for 2 weeks. When the dark demarcation line was observed between colonies, the two isolates involved were regarded as belonging to different MCGs.

2.2. Extraction of dsRNA

Isolates were grown on cellulose membrane spread on potato-dextrose agar plates for one week. Cellulose membrane attached with mycelia was stripped from the plates and ground in liquid nitrogen to a fine powder. Fungal dsRNA was extracted according to the method of Arakawa et al. [25]. Total nucleic acids were extracted by phenol–chloroform treatment and ethanol precipitation, and subsequently incubated overnight at 37 °C in nuclease buffer (50 units of DNase I [Takara, Ohtsu], 20 units of S1 nuclease [Takara, Ohtsu], 30 mM sodium acetate [pH 4.6], 5 mM $MgCl_2$, 100 mM NaCl, 1 mM $ZnSO_4$). dsRNA fraction was then obtained after phenol–chloroform treatment and ethanol precipitation. Samples were separated by electrophoresis on 1.0% agarose gel in TAE buffer (40 mM Tris–acetate, 1 mM EDTA [pH 8.0]) and on 5% polyacrylamide gel in TBE buffer (89 mM Tris–borate, 2 mM EDTA [pH 8.0]), stained with ethidium bromide (100 ng/ml) and visualized under UV irradiation.