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Diversity of viruses in *Cryphonectria parasitica* and *C. nitschkei* in Japan and China, and partial characterization of a new chrysovirus species

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ABSTRACT

We surveyed native populations of the chestnut blight fungus, *Cryphonectria parasitica,* in Japan and China, and *C. nitschkei,* a sympatric species on chestnut trees in Japan, to learn more about the diversity of hypoviruses and other double-stranded (ds) RNA viruses. In a sample of 472 isolates of *C. parasitica* and 45 isolates of *C. nitschkei* from six prefectures in Japan, we found 27 containing one or more dsRNAs. Twelve isolates of *C. parasitica* and two isolates of *C. nitschkei* were infected with *Cryphonectria hypovirus* 1 (CHV-1); four of these 12 *C. parasitica* isolates also contained other dsRNAs that did not hybridize to CHV-1. In China, only one of 85 *C. parasitica* isolates was CHV-1-infected; no dsRNAs were detected in the other isolates from China. No other known hypoviruses were found in this study. However, we found two previously undescribed dsRNAs in Japan approximately 9 kb in size that did not hybridize to each other or to any known dsRNAs from *C. parasitica*. We also found three additional groups of dsRNAs, one of which represents the genome of a new member of the virus family *Chrysoviridae* and was found only in *C. nitschkei*; the other two dsRNAs were found previously in isolates of *C. parasitica* from Japan or China. The most significant result of this survey is the discovery of novel dsRNAs that can be characterized in future research.

Introduction

Fungal viruses have been found in a large number of species, representing all major fungal groups (Buck 1986; Ghabrial 1994). Although the vast majority of fungal viruses have little or no detectable effect on host phenotypes (Ghabrial 1980), a few exceptions have received much attention because of their potential for biological control of fungal plant pathogens (Nuss & Koltin 1990). In particular, the prospects of biological control with viruses have motivated much of the research on the chestnut blight fungus, *Cryphonectria parasitica,* since the 1970s (van Alfen *et al.* 1975; Anagnostakis 1982; Nuss 1992; Nuss 1996; Dawe & Nuss 2001). In southern Europe and in a few locations in North America (e.g. Michigan), chestnut blight has been significantly reduced by hypovirulence, caused by viruses in *C. parasitica*, allowing some degree of recovery of chestnut trees (Fulbright *et al.* 1983; Heiniger & Rigling 1994; Milgroom & Cortesi 2004). Viruses associated

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with hypovirulence are members of the family *Hypoviridae*; these are commonly referred to as hypoviruses (Hillman & Suzuki 2004; Nuss *et al.* 2005). Despite repeated attempts, hypoviruses have not become established or controled chestnut blight in eastern North America (MacDonald & Fulbright 1991; Liu *et al.* 2002; Milgroom & Cortesi 2004). To learn more about hypoviruses for biological control and to look for additional viruses suitable for controlling chestnut blight, we set out to survey the diversity of viruses found in *C. parasitica* in native populations in Asia.

C. parasitica is native to east Asia, and was first found in North America and Europe in 1904 and 1938 respectively, where it caused devastating losses of American and European chestnuts, *Castanea dentata* and *C. sativa*, respectively (Anagnostakis 1987). Based on import records of *Castanea* spp., some of which were presumably infected with *C. parasitica* (Anagnostakis 1992; Heiniger & Rigling 1994), and analysis of restriction fragment length polymorphisms (RFLPs) in *C. parasitica* (Milgroom *et al.* 1996), this fungus appears to have been introduced primarily from Japan. However, there is at least one documented introduction of *C. parasitica* into the US from China (Shear & Stevens 1913), and large numbers of Chinese chestnuts (*Castanea mollissima*) were imported into the US after the epidemic was well underway (Anagnostakis 1992), suggesting that China may be an additional source for the introduction of *C. parasitica* into North America.

Although a good way to understand pests and their antagonists used for biological control is to study them in native, as opposed to introduced, populations, relatively little is known about *C. parasitica* or hypoviruses in *C. parasitica* in Asia compared with Europe and North America. The best-studied hypovirus, *Cryphonectria hypovirus* 1 (CHV-1) has been found in *C. parasitica* in Japan and China (Liang *et al.* 1992; Liang *et al.* 1994; Quan *et al.* 1994; Wang *et al.* 1996; Peever *et al.* 1998; Liu *et al.* 2003), and also in Korea (B. Cha & M. G. M., unpubl. data). CHV-1 was also recently found in a sympatric species of *Cryphonectria* on chestnut trees in Japan (later identified as *C. nitschkei*; Myburg *et al.* 2004), and evidently has been transmitted between the two *Cryphonectria* species (Liu *et al.* 2003). However, the distribution of hypoviruses in Asia, is not known except for CHV-1. CHV-2, which had previously been found only in New Jersey, USA (Hillman *et al.* 1992), was found in three *C. parasitica* isolates from one location in eastern China (Peever *et al.* 1998). This finding suggests that CHV-2 may have been introduced into North America from China, although the direction of migration is uncertain without further study. In contrast, CHV-3 (Smart *et al.* 1999), which is found primarily in Michigan, USA (Fulbright *et al.* 1983; Peever *et al.* 1997), and CHV-4 (Linder-Basso *et al.* 2005), which is common throughout eastern North America (Enebak *et al.* 1994; Peever *et al.* 1997), have not been found in Asia (Peever *et al.* 1998). Finally, a previous survey of hypoviruses in Asia also identified new viral dsRNAs that had not been identified before in *C. parasitica* (Peever *et al.* 1998). More extensive surveys are likely to find additional dsRNAs, extending our knowledge of mycoviruses further (Hillman & Suzuki 2004).

More extensive surveys are needed to determine whether other mycoviruses are found in *C. parasitica* in Asia. The need for further sampling is greatest in Japan because it is the most likely source for the introduction of *C. parasitica* into North America and relatively limited sampling has been done there. Therefore, the objective of this research was to search for hypoviruses (in addition to CHV-1) and other viral dsRNAs in both *Cryphonectria* species known from chestnuts, *C. parasitica* and *C. nitschkei*, in Japan and China, as an extension of previous surveys (Peever *et al.* 1998; Liu *et al.* 2003).

Materials and methods

dsRNA screening

In a previous study (Liu *et al.* 2003), we sampled 472 isolates of *Cryphonectria parasitica* and 45 isolates of *C. nitschkei* collected from Japanese chestnut trees in Japan. Species identifications were based on colony morphology and pigmentation, and confirmed by RFLPs in the ITS region of the nu-rRNA subunit. All of these isolates were screened for dsRNA; however, we reported results only for CHV-1 and concentrated on the interspecies transmission of CHV-1. In this report, we present data for all other dsRNAs found by screening the same sample of isolates. We used a miniprep method described by Morris *et al.* (1983) for detection of dsRNA. Isolate EP102 from Virginia, which contains CHV-4 at a low titre (Peever *et al.* 1997; Liu *et al.* 2002), was used as a positive control for dsRNA in all preps. This miniprep method was compared with an immunoblot method used previously (Peever *et al.* 1997). All 95 isolates from Japan that were previously screened for dsRNAs by immunoblotting (Peever *et al.* 1998) were also screened by the miniprep method described above to compare techniques.

In addition to dsRNA screening of *C. parasitica* and *C. nitschkei* from Japan, we screened 85 Chinese isolates of *C. parasitica* from a 1992 collection (Milgroom *et al.* 1996). These isolates were collected from eight subpopulations in six provinces in eastern China (Table 1). All isolates from China were sampled from Chinese chestnuts growing in orchards.

Northern blotting and hybridizations

Protocols for Northern blotting and hybridizations were as described previously (Peever *et al.* 1997; Peever *et al.* 1998; Liu *et al.* 2003). All dsRNA-containing isolates found by miniprep screening were cultured again to obtain larger quantities of purified dsRNAs. We used CF11 cellulose column chromatography to purify dsRNAs (Morris & Dodds 1979). As reported in Liu *et al.* (2003), we probed all Northern blots with ^{32}P-labelled dsRNA from CHV-1/EP43. The dsRNAs that did not hybridize to CHV-1 were probed with ^{32}P-labelled dsRNAs that had been previously found in *Cryphonectria parasitica* from Asia. Instead of exhaustively probing all dsRNAs, we selectively probed with known dsRNAs of similar size. We probed with two dsRNAs previously found in Asia (Peever *et al.* 1998): (1) 1.8- and 2-kb dsRNAs from isolate 09269 from Fengcheng, Liaoning Province, China; and (2) a 3-kb dsRNA from isolate JA28. These dsRNAs were cut from agarose gels and purified using RNAid according to manufacturer's instructions (Q-BIOgene, Solon, OH). When larger dsRNAs (9–12 kb), similar in size to other hypoviruses, were found that did not hybridize to CHV-1, they were labelled and probed against dsRNA from other known hypoviruses: CHV-2/NB58, CHV-3/GH2, and