British Mycological Society promoting fungal science

journal homepage: www.elsevier.com/locate/funbio

DsRNA-free transmissible hypovirulence associated with formation of intra-hyphal hyphae in *Botrytis cinerea*

Jian Bo CAO[a,b], *Yu ZHOU*[a], *Lei ZHANG*[a], *Jing ZHANG*[a], *Long YANG*[a], *Li Hong QIN*[b], *Dao Hong JIANG*[a], *Guo Qing LI*[a,*], *Hung-Chang HUANG*[c]

[a]*The State Key Laboratory of Agricultural Microbiology and Key Laboratory of Plant Pathology of Hubei Province, Huazhong Agricultural University, Wuhan 430070, Hubei, China*
[b]*Electron microscopes Laboratory, Huazhong Agricultural University, Wuhan 430070, China*
[c]*Agriculture and Agri-Food Canada, Research Centre, Lethbridge, Alberta T1J 4B1, Canada*

ARTICLE INFO

Article history:
Received 9 November 2010
Accepted 21 April 2011
Available online 7 May 2011
Corresponding Editor:
Geoffrey Michael Gadd

Keywords:
Botrytis cinerea
Intra-hyphal hyphae
Oilseed rape
Transmissible hypovirulence

ABSTRACT

A spontaneous mutant CanBc-3HV and its parental strain CanBc-3 of *Botrytis cinerea* were investigated in terms of pathogenicity, colony morphology, hypovirulence transmissibility, presence of double-stranded RNA (dsRNA), and formation of intra-hyphal hyphae (IH). Results showed that inoculation of CanBc-3HV on detached leaves of *Brassica napus* did not produce any visible necrotic lesions (20 °C, 72 h), whereas inoculation of CanBc-3 caused necrotic leaf lesions. Compared to CanBc-3, CanBc-3HV grew slowly, formed numerous mycelial sectors, sporulated sporadically and failed to produce sclerotia on potato dextrose agar (PDA) (20 °C, 15 d). Hypovirulence and the abnormal cultural characteristics of CanBc-3HV were transmissible from CanBc-3HV to CanBc-3 in pair cultures on PDA. However, the transmission was unsuccessful from CanBc-3HV to another virulent strain CanBc-2 of *B. cinerea*. These results suggest that transmission of the hypovirulence and the abnormal cultural characteristics of CanBc-3HV are strain-specific. No dsRNA was detected in mycelia of either CanBc-3HV or CanBc-3, implying that the hypovirulence of CanBc-3HV is caused by a transmissible element (TE) of non-RNA mycoviral origin. Formation of IH through self-infection was observed in CanBc-3HV, CanBc-3T1 (a hypovirulent derivative of CanBc-3 trans-infected by TE in CanBc-3HV), but was not observed in CanBc-3, suggesting that IH formation is associated with the hypovirulence of CanBc-3HV. To our knowledge, this is the first report of dsRNA-free transmissible hypovirulence associated with IH formation in *B. cinerea*.

Introduction

Hypovirulence usually refers to debilitated ability of selected fungal strains to cause disease on susceptible host plants (Anagnostakis 1982; Nuss 2005). This phenomenon has been reported to exist in numerous plant pathogenic fungi (Ghabrial & Suzuki 2009; Pearson *et al.* 2009) including *Botrytis cinerea* Pers.: Fr. (Castro *et al.* 2003; Wu *et al.* 2007), the causal agent of plant gray mold disease. Fungal hypovirulence can be caused by mycovirus infection (Ghabrial & Suzuki 2009; Pearson *et al.* 2009), mutation of mitochondrial genes (Monteriro-Vitorello *et al.* 1995; Baidyaroy *et al.* 2000) and/or spontaneous generation of DNA plasmids (Charter *et al.* 1993; Monteiro-Vitorello *et al.* 2000). Hypovirulent fungal strains are potential biocontrol

* *Corresponding author*. Tel.: 86 27 87286880.
E-mail address: guoqingli@mail.hzau.edu.cn
1878-6146/$ – see front matter
doi:10.1016/j.funbio.2011.04.008

agents for management of fungal diseases by attenuating pathogenicity/virulence of wild fungal strains through transmission of 'hypovirulence element (HE)' or mycoviruses (Anagnostakis 1982; Nuss 2005). Meanwhile, understanding of the interaction between fungal hosts and 'HE' or mycoviruses, and between hypovirulent fungal strains and host plants at molecular level might help to unveil the underlying mechanisms of fungal pathogenesis (Nuss 2005; Zhang *et al.* 2010).

Botrytis cinerea [teleomorph: *Botryotinia fuckeliana* (de Bary) Whetzel] is a ubiquitous plant pathogen causing gray mold disease on more than 200 plant species including oilseed rape (*Brassica napus* L.) in temperate and subtropical climates (Williamson *et al.* 2007). *Botrytis cinerea* is a necrotrophic pathogen (van Kan 2006) and no fully resistant crop cultivars to this pathogen have ever been identified (Williamson *et al.* 2007). Therefore, control of plant gray mold disease depends greatly on application of fungicides. However, public concerns over the potential risk of fungicide residues in plant produces and formation of fungicide resistance in populations of *B. cinerea* due to the use of fungicides suggest a need to explore alternative measures including biological control for management of plant gray mold disease.

Discovery and characterization of the transmissible hypovirulence of *B. cinerea* provide a novel strategy for control of *B. cinerea*. Previous studies indicated that RNA mycoviruses could infect *B. cinerea*. Some RNA mycoviruses such as the 6.8-kb-dsRNA mycovirus (Castro *et al.* 2003) and *B. cinerea* mitovirus 1 (BcMV1), an ssRNA mycovirus (Wu *et al.* 2010), can cause hypovirulence and hypovirulence-associated phenotypes, including abnormal colony morphology, reduced sporulation and suppressed sclerotial formation, in *B. cinerea*. However, information about the hypovirulence not caused by RNA mycoviruses in *B. cinerea* is not available in the literature.

A strain of *B. cinerea*, designated as CanBc-3, was isolated from oilseed rape in our previous study (Zhou 2009). The strain CanBc-3 had the normal colony morphology of *B. cinerea* on potato dextrose agar (PDA) and was highly virulent on oilseed rape. During successive mycelial sub-culturing of CanBc-3 on PDA, a cultural variant, designated as CanBc-3HV, was obtained. In the present study, strains CanBc-3 and CanBc-3HV of *B. cinerea* were investigated for fulfilling the following objectives: (i) to characterize cultural features on PDA and pathogenicity on oilseed rape; (ii) to determine transmissibility of the hypovirulence and the abnormal cultural characteristics of strain CanBc-3HV; (iii) to detect the presence of dsRNA; and (iv) to observe formation of intra-hyphal hyphae (IH) in strain CanBc-3HV and to establish the relationship between IH formation and hypovirulence in *B. cinerea*.

Materials and methods

Strains of Botrytis cinerea

Five strains including CanBc-1, CanBc-1c-66, CanBc-2, CanBC-3, and CanBc-3HV were used in this study. Strains of CanBc-1, CanBc-2, and CanBC-3 were isolated from field samples of oilseed rape grown in Wuhan of China in 2004 (Wu *et al.* 2007; Zhou 2009). Strain CanBc-1 was a hypovirulent strain infected with BcMV1 (Wu *et al.* 2010). A double-stranded RNA (dsRNA) of about 3.0 kb in size was consistently detected in mycelia of CanBc-1 (Wu *et al.* 2007). Strain CanBc-1c-66 is a single-conidium derivative of CanBc-1. It became virulent on oilseed rape and lost the 3.0-kb dsRNA (Wu *et al.* 2007). Strain CanBC-3HV is a cultural variant of CanBC-3 through successive sub-culturings on PDA. Stock cultures of these strains were maintained on PDA at 4 °C. Working cultures were established by transferring stock agar plugs containing mycelial mats onto PDA in Petri dishes, which were then incubated at 20 °C for 4–7 d.

Hyphal growth rate and colony morphology

Strains CanBc-3 and CanBc-3HV were compared for hyphal growth rate and colony morphology on PDA. Mycelial agar plugs (MAPs) (6 mm diameter) were removed from the margin area of 2-d-old PDA cultures using a sterilized cork borer and transferred to the centre of Petri dishes (9 cm diameter) each containing 20 ml PDA, one MAP per dish and three dishes per strain. The dishes were incubated at 20 °C in dark for 2–15 d. Colony diameters were measured at day 2 and day 3 after incubation and the radial growth rate (RGR) was calculated with the formula: RGR (cm/day) = $(D_3-D_2)/2$, where D_2 and D_3 represent colony diameters at day 2 and day 3, respectively. The experiment was repeated once.

Pathogenicity assay

Pathogenicity of strains CanBc-3 and CanBc-3HV was compared in a detached leaf pathogenicity assay. Seeds of oilseed rape (*Brassica napus* cultivar Zhongyou Za No. 4) were sown in a field in early Oct. of 2009, and the plants were watered as required. Leaves were excised from the central part of each seedling of oilseed rape in early Dec. of 2009. Eight leaves with the average size of 10 × 8 cm (length × width) were placed in two lines on moist towels in an enamel tray (45 × 30 × 2.5 cm, length × width × height). MAPs (6 mm diameter) were removed from the margin of 3-d-old PDA cultures of each strain and placed on leaves of oilseed rape with the mycelial side down facing the leaf surface, two MAPs per leaf and four leaves per strain. Plain PDA agar plugs without mycelia were used in the control treatment. The trays were individually sealed with transparent plastic films (100 μm thick) (Gold Mine Plastic Industry Ltd., Jiangmen, China) to maintain high humidity and incubated in a growth chamber at 20 °C under fluorescent light with the regime of 12-h light/12-h dark. Leaf lesion diameter around each MAP was measured after incubation for 72 h. The experiment was performed twice.

Detection of dsRNA

MAPs of strains CanBc-1, CanBc-1c-66, CanBc-2, CanBc-3 or CanBc-3HV were inoculated on sterilized cellophane films placed on PDA in Petri dishes, four MAPs per dish. The dishes were incubated at 20 °C in dark for 3 d for strains CanBc-1c-66, CanBc-2, and CanBc-3, and for 6 d for strains CanBc-1 and CanBc-3HV. Mycelial mats in each dish were harvested using a sterilized spatula. Extraction and purification of dsRNA from three mycelial samples (three replicates) for each strain were carried out using the procedures described by Morris & Dodds (1979) and Wu *et al.* (2007, 2010). Presence of dsRNA in the