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Isolation and identification of *Sclerotinia* stem rot causal pathogen in *Arabidopsis thaliana**

Ai-rong WANG[1,2], Wen-wei LIN[2], Xiao-ting CHEN[2], Guo-dong LU[2], Jie ZHOU[†‡2], Zong-hua WANG[†‡2]

([1]*Computer and Information College, Fujian Agriculture and Forestry University, Fuzhou 350002, China*)

([2]*Ministry of Education Key Laboratory of Biopesticide and Chemical Biology, Fujian Agriculture and Forestry University, Fuzhou 350002, China*)

[†]E-mail: yxxc19204@sina.com; zonghuaw@163.com

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Abstract: A new stem rot disease is found to occur naturally on *Arabidopsis* plants in greenhouses of Fuzhou, China. In order to identify its pathogen, we conducted a series of fungal isolation and purification, plant reinoculation, and ascus and ascospore induction from the sclerotia. The isolate caused typical water-soaked lesions after reinoculation and produced sclerotia both on *Arabidopsis* plants and culture medium plates, and the sclerotia could be induced to produce discal apothecia and 8 binucleate ascospores per ascus. These disease symptom and fungal morphology data revealed that the fungus *Sclerotinia sclerotiorum* (Lib.) de Bary was the pathogen for *Arabidopsis* stem rot. To confirm this, we further amplified its large subunit ribosomal DNA (LSU rDNA) by polymerase chain reaction (PCR), and compared the sequence with the known LSU rDNA sequences in GenBank. The results show that the sequence shares the highest identities with the LSU rDNAs of different *S. sclerotiorum* strains. Taking all these data together, we concluded that the fungus that caused the *Arabidopsis* stem rot is *S. sclerotiorum* (Lib.) de Bary. This is the first report that *Arabidopsis* is naturally infected by *S. sclerotiorum*.

Key words: *Sclerotinia sclerotiorum*, *Arabidopsis thaliana*, Large subunit ribosomal DNA (LSU rDNA), Systematic classification
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INTRODUCTION

Sclerotinia sclerotiorum (Lib.) de Bary is a facultative parasitic Ascomycete fungus (Kirk *et al*., 2001), and can grow well even in an unfavorable environment and survive for up to 8 years in soil in the sclerotia form (Adams and Ayers, 1979). It can infect as many as 408 plant species including many important crops, such as rapeseeds, sunflower and soybean, and many vegetables (Boland *et al*., 1994; Bolton *et al*., 2005). It causes water-soaked lesions on the leaves or stem rot in stems of some infected plants. The most obvious symptoms of plants infected by *S. sclerotiorum* are necrotic tissues covered with patches of fluffy white mycelia, and sclerotia are produced after mycelial growth when the nutrition is not sufficient or other conditions are favourable for sclerotial development (Christias and Lockwood, 1973).

Sclerotia play an important role in disease cycles as they are the primary structures for their long-term survival and produce inocula for further infection (Willetts and Wong, 1980). Sizes of sclerotia are dramatically different depending on their host. Sclerotia germinate either carpogenically or myceliogenically, resulting in two distinct categories of diseases under different environmental conditions. Hyphae developed when sclerotia germinate myceliogenically and can directly attack plant tissues under soil. However, apothecia are produced when sclerotia germinate carpogenically and ascospores can be projected to the air and infect aboveground portions

‡ Corresponding authors

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of host plants (Bardin and Huang, 2001; Le Tourneau, 1979; Bolton *et al*., 2005).

The large subunit ribosomal DNA (LSU rDNA) has been widely used as a potential marker for fungal species identification in recent years. The LSU is a part of the rDNA gene sequence of the nuclear genome, which is arranged in ribosome clustered at tandem repeat manner (Long and Dawid, 1980; Sonnenberg *et al*., 2007). Ribosomal genes are highly conserved in different forms of living organisms (Woese *et al*., 1990), but are actually composed of a mixture of conserved and divergent regions. It is considered that LSU rDNA is an imprint of evolutionary history for different organisms (Lydeard *et al*., 2000).

The new disease, with similar symptoms to sclerotia stem rot, was observed when we grew *Arabidopsis thaliana* in a greenhouse. In order to identify the causal pathogen, we conducted pure isolation and observed the morphology and cytology of the isolate, further sequenced the LSU rDNA and compared the identity with the known *S. sclerotiorum* LSU rDNA sequences. Our results clarify that the *Arabidopsis* sclerotia stem rot disease was caused by an isolate of *S. sclerotiorum*, which will open a new window to study plant-*S. sclerotiorum* interaction by using the model *Arabidopsis* plant system.

MATERIALS AND METHODS

Fungal isolation and purification

We followed a standard procedure by Fang (1998) for fungal isolation and purification. The infected tissues of *Arabidopsis* were collected from local greenhouses, cut into small pieces, and then rinsed 3~4 times with diluted water after treated with 70% (v/v) ethanol for 2~3 s and with 0.1% (w/v) mercuric chloride solution for 3~5 min. The treated tissues were transferred to potato dextrose agar (PDA) medium and cultured at 25 °C. To confirm pathogenicity of the isolate, the pure culture was reinoculated onto *Arabidopsis* plants. When actively growing on PDA plates, mycelium-agar plugs were excised from the margin of the fungal colony and used to inoculate *Arabidopsis* leaves. Inoculated leaves were then incubated in a growth chamber at 23~25 °C with 100% relative humidity until observation.

Treatment of sclerotia at low temperature to induce apothecium development

The sclerotia produced on PDA medium were collected, placed in moist sands evenly, and then cultured in 4 °C for 6~8 weeks and left in place at 15~20 °C under scattered light until the apothecia developed (Smith and Boland, 1989).

Microscopic observation of ascospore morphology

Apothecia were cut into thin slices and stained with trypan blue solution over night to observe the morphology of asci and ascospores, or stained with 0.5 μg/L ethidium bromide (EB) for 30 min to observe nuclei, or stained with 10 μg/ml Calcofluor white solution for 5 min to observe cell walls and septa between cells, then rinsed with sterile water, and photographed with visible, ultraviolet light and fluorescence under an Olympus microscope, respectively.

Polymerase chain reaction (PCR) amplification of LSU rDNA

The fungus was cultured by shaking in liquid PDA medium at 25 °C for 3~4 d. Mycelia were collected by centrifugation and its genomic DNA was extracted using a cetyl trimethyl ammonium bromide (CTAB) procedure as described in Talbot *et al*.(1993) and used as a template for PCR amplification of LSU rDNA.

LSU rDNA was amplified with the universal primers NL1 (5′-GCATATCAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) (O′Donnell, 1993). The PCR conditions were: 94 °C, 2 min for denature; 33 cycles of 94 °C, 30 s; 55 °C, 30 s; 72 °C, 1 min; and 72 °C, 7 min for extension. The PCR product was separated with 1.5% (w/v) agarose gel and sequenced at Shanghai Ding'an Company, China.

Sequence and phylogenetic analysis of LSU rDNA

The LSU rDNA sequence of the fungus was sent to National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) and analyzed using the basic local alignment search tool (BLAST) sequence comparison algorithms. The high homologous sequences were downloaded and analyzed with program Clustal X. The ∗.aln files were opened by Mega software and the phylogenetic tree was generated.

RESULTS

Fungal infection and symptoms

The fungal infection process was observed under natural and manual infection. The fungus infected old leaves firstly and expanded rapidly through the petiole into the stem, causing water-soaked lesions. The upper leaves were subsequently infected until the whole plant finally died off. The disease was frequently observed to trans-infect other healthy plants if the infected leaves made contact with uninfected plants (Figs.1a and 1b). The hyphae grew luxuriantly and adjacent plants could also become infected with the disease through the hyphal growth when the atmosphere humidity reached 90% (Fig.1b). Sclerotia formed on the surface of plants after plants had died (Fig.1c).

To conduct pathogen identification and analysis, the fungus was isolated from infected tissues of *Arabidopsis* and cultured on PDA medium. The fungus started to produce white masses when growing to the edge of the Petri dish (Fig.1d). The size of mycelium masses became bigger and their colors became darker as time proceeded. Finally, many black sclerotia formed (Fig.1e). When the *Arabidopsis* leaves were inoculated with the isolated fungus, the symptoms were the same as those under natural infection (data not shown).

Germination of sclerotia and formation of apothecia

To further observe the biology of sclerotia germination and apothecia formation, sclerotia were first incubated in moist sandy soil for 6~8 weeks at 4 °C and then placed in the condition at 15~20 °C with scattered light. Sclerotia geminated quickly after 20 d. Stipes and receptacles of apothecia were formed at first (Fig.2a), then their tops grew swollen and discal apothecia with cupped centers were formed (Fig.2b). Hollowness in the center became flatter with augmentation of the apothecia and the hymenial layer spread fully until asci became mature and ascospores emanated (Fig.2c). The numbers of apothecia produced per sclerotium were not equal, ranging from only 1 to as many as 8~9 apothecia (Fig.2d).

Morphology of asci and ascospores

Apothecia were cut into thin slices, then stained with trypan blue and observed with a microscope. The hymenial layers of the apothecia were found to be full of asci, each ascus containing 8 ascospores. Mature ascospores were released from the top of the asci (Figs.3a and 3b). Many paraphyses could be seen among the asci (Fig.3c). Each ascospore had two nuclei when stained by EB (Figs.3d and 3e), but no septum was observed when stained with Calcoflour white (Fig.3f). The results suggest that ascospores were single binuclear cells, coinciding with the description in (Kohn, 1979).

LSU rDNA sequence and fungal phylogenetics revealed by the sequence analysis

To study phylogenetics of the isolate, we used a pair of universal primers to amplify the LSU rDNA by PCR (O′Donnell, 1993). A product with a size of roughly 600 bp was obtained, sequenced and deposited in GenBank (accession No. EU926159). The sequence was analyzed using the BLAST sequence comparison algorithms from the NCBI website (http://www.ncbi.nlm.nih.gov/). The analysis revealed that LSU rDNA of our isolate (*S. sclerotiorum* strain FZ001) has close evolutionary relationship with fungal species in *Discomycetes*, including *S. sclerotiorum*, *Botryotinia fuckeliana*, *Scleromitrula shiraiana*, *Sclerotinia veratri* and *Mitrula borealis* (Fig.4). It was closely clustered in the same group with Sclerotiniaceae members, including *S. sclerotiorum*, 28S LSU rDNA of *S. sclerotiorum* strain AFTOL-ID 928, 28S rDNA of *S. sclerotiorum* strain CBS 499.50, 25S LSU rDNA of *S. sclerotiorum* strain WZ0067, LSU rDNA of *B. fuckeliana* and 28S rDNA of *B. fuckeliana* isolate AFTOL-ID 59 (Fig.4). All of these sequences shared more than 99% identities, indicating that they have a notablely tight relationship. *B. fuckeliana*, the teleomorph of *Botrytis cinerea*, produces highly resistant sclerotia, but also produces abundant hyaline conidia (asexual spores) borne on grey, branching tree-like conidiophores which is different from *S. sclerotiorum* (Kirk *et al*., 2001). Other fungi we selected such as *S. shiraiana* strain Hirayama062001, *S. veratri* 96CFEBD7, and two *M. borealis* strains were divergent from this group. These results suggest that our isolate is a strain of *S. sclerotiorum*. We here named it as *S. sclerotiorum* strain FZ001.

(a) (b) (c) (d) (e)

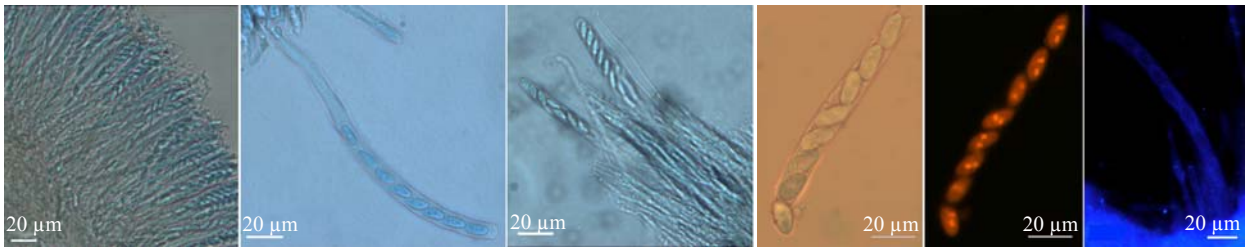
Fig.1 Progress of infected *Arabidopsis* plants, and sclerotia on plants and PDA plates

(a) A primarily infected *Arabidopsis* plant; (b) Heavily infected *Arabidopsis* plants; (c) Sclerotia on dead infected plants; (d) Sclerotia starting to develop on PDA plate; (e) Well developed sclerotia on PDA plates. Arrows indicate sclerotia

(a) (b) (c) (d)

Fig.2 The time course of sclerotia germination and apothecia formation

(a) Primary stage of sclerotia germination; (b) Apothecia being formed; (c) Mature apothecia; (d) Geminated sclerotia with several apothecia formed



(a) (b) (c) (d) (e) (f)

Fig.3 The morphology of asci and ascospores

(a), (b) and (c) Slices of apothecia stained with trypan blue: (a) the hymenial layer with rows of asci which are cylindrical sac-like zygote cells, (b) ascus with eight ascospores, (c) paraphysis among asci; (d) and (e) Ascospores stained with EB: (d) observed under visible light, (e) observed under ultraviolet light; (f) Ascospores stained with Calcoflour white observed under florescence

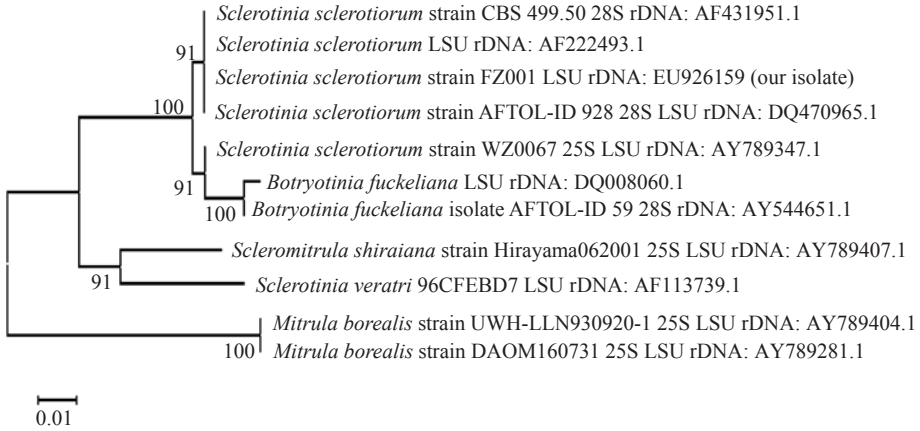


Fig.4 Tree view of phylogenetic relationship of *Sclerotinia sclerotiorum* strain FZ001

The LSU rDNA and other related sequences were retrieved from GenBank, and calculated with neighbor-joining method. Bootstrap values (%) are indicated at the nodes

DISCUSSION

The fungus we isolated could infect *Arabidopsis*, causing water-soaked lesions in leaves, developing sclerotia on the surface of infected tissues and spreading across leaves in contact with each other at high moist conditions. These disease symptoms are the same as that caused by *S. sclerotiorum* (Lib.) de Bary in other plants. Although some reports indicated that *S. sclerotiorum* could infect *Arabidopsis* in manual inoculation, there is no report suggesting that *Arabidopsis* can be infected naturally.

Mycelia of *S. sclerotiorum* (Lib.) de Bary in host plants or in culture look hyaline, septate, branched and multinucleate, and their colors changed from white to dark as melanin accumulates. It cannot produce conidiophore during the asexual period. Hyphae tend to form sclerotia (Kirk *et al*., 2001). These are also the characteristics of our isolate. Our further observation on sclerotia germination and apothecia production confirms that there are three stages during the course of sclerotial development (Chet and Henis, 1975; Bolton *et al*., 2005). At initiation stage, hyphae aggregate to form a white mass, then further aggregate to increase the size of sclerotia; finally, surfaces of sclerotia are delimited, with melanin deposited in peripheral rind cells, and interiors of sclerotia become consolidating. Each sclerotium can produce one or more apothecia consisting of a stipe and a receptacle with a flat to convave hymenial layer (2~10 mm in diameter). Asci are cylindrical sac-like zygote cells and are rowed in the hymenial layer. Each ascus contains eight hyaline, ellipsoid binucleate ascospores [(4~6) μm×(9~14) μm]. The morphology and development of our isolate were similar to those of *S. sclerotiorum* as described by Kohn (1979). Moreover, results of LSU rDNA sequence and systematic analysis show that the fungus is a member of *S. sclerotiorum*.

Taking all morphological, developmental and molecular data together, we confirmed that the fungus isolated from naturally infected *Arabidopsis* plants is a strain of *S. sclerotiorum* (Lib.) de Bary. It is known that *S. sclerotiorum* is a necrotrophic fungal pathogen, both economically and biologically important. Despite decades of dedicated efforts, the pathogenesis mechanism is not well understood yet, and economically important crops still lack the resistant germplasm (Bolton *et al*., 2005). We believe that our findings will provide a base to further study the interaction mechanism of *Arabidopsis* and *Sclerotinia* fungi.

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