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RESEARCH ARTICLE

BcDR1, a putative gene, regulates the development and pathogenicity of *Botrytis cinerea*

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Abstract ***Botrytis cinerea*** **is one of the important phytopathogenic fungi. Cloning of the genes related to their development and pathogenicity is fundamental to the pathogen control. A mutant (BCt160), which produces abnormal conidia and no sclerotia, was identified from** ***Botrytis cinerea*** **mutant library generated by** ***Agrobacterium tumefaciens*****-mediated transformation (ATMT). Southern blotting analysis showed that one T-DNA insertion occurred in the genome of the mutant. TAIL-PCR (thermal asymmetric interlaced PCR) and bioinformatic analysis indicated that the exogenous T-DNA insertion occurred in the second exon of a putative gene BC1G_12388.1, named as** ***BcDR1*** **(*****B. cinerea*** **development-related gene 1). The function analysis of** ***BcDR1*** **gene showed that the** ***BcDR1*** **was related to development, morphological differentiation, and pathogenicity of** ***B. cinerea*****, suggesting that** ***BcDR1*** **gene was required for the development and pathogenicity of** ***B. cinerea*****.**

Keywords *Botrytis cinerea*, T-DNA mutagenesis, *BcDR1*, functional analysis

Introduction

Botrytis cinerea, one of the worldwide and important plant-necrotrophic pathogenic fungi, can infect at least 235 dicotyledonous species including a wide range of important crops, fruits, vegetables and ornamental plants, and can cause significant yield losses (Elad et al., 2004). The typical symptoms caused by the pathogen on the leaves and soft fruits include decay, collapse and water soaking of parenchyma tissues and gray conidium groups on the surface of various organs. During different stages of development, *B. cinerea* is a typical necrotroph that kills plant cells which subsequently serve as nutrient sources (Williamson et al., 2007; Rivera et al., 2009).

In recent years, *B. cinerea* has become a model organism for molecular plant pathology and developmental biology. Moreover, *B. cinerea* strain B05.10 (http://www.broad.mit.edu/annotation/genome/botrytis_cinerea/Home.html) and T4 (http://urgi.versailles.inra.fr/index.php/urgi/Species/Botrytis) have been sequenced, which may contribute to understanding of the phenotypic and genotypic variability, the occurrence of virulence and development-related factors of this pathogen. The significant progresses in functional genomics of *B. cinerea* will build important theoretical basis for the control of gray mold diseases caused by this pathogenic fungus. By now, nearly 30 virulent genes have been obtained in *B. cinerea* (Choquer et al., 2007). *B. cinerea* secretes many enzymes and metabolites which are presumed to help kill the host cells subsequently (Vankan, 2006; Tellier et al., 2008). In most cases, *B. cinerea* enzymes are found to degrade plant cuticle and cell wall components such as hemicellulose (Brito et al., 2006), pectin (Kars et al., 2005), cutin (van der Vlugt-Bergmans et al., 1997) and chitin synthase (Cui et al., 2009). The chemical structures of secondary metabolites have been determined, such as the sesquiterpenes botrydial (PubChem compound: CID: 185781), abscissic acid (CID: 5375200) and botcinic acid for the polyketide (CID: 11509607). Some growth development pathways, involving growth (Zheng et al., 2000; Nierman et al., 2005; Rui and Hahn, 2007; Segmuller et al., 2007), sporulation (Takano et al., 2001),

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conidial germination (Fillinger et al., 2002; Liebmann et al., 2003; Yamauchi et al., 2004; Zhao et al., 2006), nutrient sensing (Thevelein et al., 2005; Bahn et al., 2007) and sclerotia formation (Jurick and Rollins, 2007), have been reported in some phytopathogenic fungi. In our preliminary studies, the ATMT mutant library of *B. cinerea* was constructed using *Agrobacterium tumefaciens* AGL-1 carrying the binary vector plasmid pBHtl.

In this study, a novel mutant (BCt160) which cannot produce sclerotia but abnormal conidia was screened from the ATMT mutant library. PCR, Southern blotting and RT-PCR techniques were used to analyze and identify T-DNA mutant insertion sites and the mutant gene. The results will promote the research of development mechanism and molecular pathogenicity in *B. cinerea*.

Materials and methods

Strains and plasmids

The wild-type strain BC22 of *B. cinerea* isolated from diseased tomatoes was used for ATMT mutant library construction. Mutant strain BCt160 was obtained by screening transformation of the BC22 strain previously, using *Agrobacterium tumefaciens* AGL-1 carrying the plasmid pBHt1 (Mullins et al., 2001). The *Agrobacterium tumefaciens* strain AGL-1 was kindly provided by Professor Zonghua Wang at the Department of Plant Pathology, Agricultural and Forestry University of Fujian, China. It carried the hygromycin phosphotransferase gene which can resist hygromycin B (Lazo et al., 1991). The *B. cinerea* strains were cultured on potato dextrose agar (PDA) medium at 20°C in dark.

DNA and RNA manipulations

Genomic DNA of *B. cinerea* was extracted with modified CTAB (cetyl triethyl ammnonium bromide) method (Drenth et al., 1993). Total RNA was isolated from the frozen fungal mycelia using an RNA extraction kit (Cat. No. SK1322, Sangon, China). The quality and quantity of RNA were measured by the nucleonic acid and protein detection instrument (NanoDrop ND-1000, USA). The first-strand cDNA was synthesized according to the manuscript of promega kit (Cat. No. A3500, promega, China). PCR was carried out in 20 μL reaction volume using first-strand cDNA as template under the following conditions: initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, at 66°C for 45 s and at 72°C for 30 s, with final 10 min extension at 72°C.

Identification of mutant BCt160

Using the genomic DNA of wild-type BC22 and mutant BCt160 as templates, respectively, PCR amplifications were performed to identify the T-DNA insertion in mutant BCt160 with specific primers (P1: 5′-CGCCCAAGCTGCATCATCGAA-3′, P2: 5′-CGACAGCGTCTCCGACCTGA-3′) of the hygromycin resistance gene. Southern blotting was used to confirm the presence of exogenous hygromycin resistance gene in the genome of mutant BCt160. Genomic DNA of wild-type BC22 and mutant BCt160 were digested by *Hin*dIII, then electrophoresed with 0.8% agarose gel and transferred onto a nylon membrane. The transferred DNA was hybridized with DIG-labeled DNA probe for 16 h, and thereafter, the immunological detection was run for more than 16 h (DIG DNA Labeling and Detection Kit, Cat. No. 1093657, Roche Applied Science, Germany).

Acquisition of the flanking sequence of T-DNA insertion site in mutant BCt160

TAIL-PCR was used to amplify the flanking sequence of T-DNA insertion site in mutant BCt160. The thermal cycling settings and reaction conditions of TAIL-PCR were performed according to the previous report (Mullins et al., 2001). Taking genomic DNA of mutant BCt160 as template, PCR products were amplified with the primer combination AD4 (5′-TCGTNCGNACNTAGGA-3′) and LB1/LB2/LB3, respectively. The secondary and tertiary PCR products were analyzed by 1% agarose gel electrophoresis. The tertiary PCR products were purified using QIA quick columns (TIANgel Midi Purification Kit, Cat. No. Dp209-03, Qiagen, Germany) and sequenced by Sangon Co. Ltd., China.

Bioinformatics analysis of the mutant

To determine the T-DNA insertion site and mutant gene, the flanking sequences of T-DNA insertion site obtained by TAIL-PCR were jointed into one sequence according to the common sequence between them and aligned with the genome sequences in *B. cinerea* genome database (http://www.broad.mit.edu/annotation/genome/botrytis_cinerea) using the BLAST program. To further know about the function of the mutant gene, bioinformatics analysis of mutant gene proceeded. General BLAST programs of EBI (http://www.ebi.ac.uk/Tools/blast/) were used for the nucleotide acid and amino acid sequences homology alignment. DNASTAR software was used for the local sequences homology alignment and phylogenetics analysis.

Gene identification of the mutant

Taking genomic DNA of wild-type BC22 and mutant BCt160 as templates, *BcDR1* (BC1G_12388.1) gene-specific primers (P3: 5′-CGTAAACACTTCAGCGAG-3′, P4: 5′-TAAGCGTGCCATACCAGAG-3′) and T-DNA specific primer LB3 were used for verifying T-DNA insertion of *BcDR1* gene. Taking the equal aliquots of cDNA of wild-type BC22 and mutant BCt160 as templates, expression levels of *BcDR1* were analyzed by RT-PCR with specific primers of

BcDR1 (P3, P5: 5′-GCACAACGTGTTGAAGTC-3′); at the same time, *Tublin* gene (F: 5′-AVTGGGCTAAGGGTCATT-3′, R: 5′-TCTCCGTAAGATGGGTTG-3′) was used for equal loading control.

Phenotype analysis of the mutant

Wild-type BC22 and mutant BCt160 strains were inoculated in PDA medium and cultured in the darkness at 20°C for 10 d, respectively. Conidial morphogenesis was observed using microscope, and the number of conidia was calculated. Mycelium of wild-type BC22 and mutant BCt160 were inoculated on the surface of mature tomatoes for virulence detection. The lesion diameter of tomatoes was measured 3 days after inoculation. The experiment was repeated three times.

Results

Identification of mutant BCt160

A mutant strain BCt160, which produced abnormal conidia, was screened from ATMT mutant library by microscopic observation. By using specific primers of the hygromycin resistance gene for verifying T-DNA insertion of mutant BCt160, a band of 800 bp was amplified from the mutant strain BCt160, and no PCR product in WT (wild-type BC22-no insertion) (Fig. 1A). The copy number of T-DNA in mutant BCt160 was analyzed by Southern blotting using the hygromycin resistance gene as the probe (Fig. 1B). The result of hybridization with single band in mutant BCt160 indicated that T-DNA only had a single copy *in vivo*.

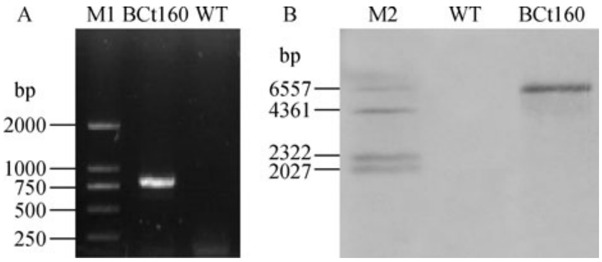

Figure 1 PCR analysis and Southern blotting of hygromycin resistance gene. Note: (A) PCR amplification of hygromycin B resistant gene. (B) Southern blotting of BCt160 mutant with hygromycin B resistant gene. M1 is DNA marker DL2000. M2 is DNA molecular-weight marker DIG labeled, 0.12–23.1 kb.

Bioinformatics analysis of the mutant

The flanking sequence of T-DNA insertion site in mutant BCt160 was obtained by TAIL-PCR technique (date not shown). The comparison of the sequence obtained by TAIL-PCR with *B. cinerea* database indicated that the T-DNA inserted the second exon of the putative gene BC1G_12388.1 (*BcDR1*) (Fig. 2 A). The *BcDR1* gene contained 4 exons and 3 introns and encoded a putative protein of 414 amino acids. By phylogenetics analysis of *BcDR1* from different fungi species retrieved from GenBank database including *Botrytis cinerea*, *Sclerotinia sclerotiorum* and *Aspergillus nidulans*, the phylogenetic tree showed that *BcDR1* shared 48% sequence similarity to putative Zn(II)$_2$Cys$_6$ transcription factor from *Aspergillus nidulans* (Fig. 2 B).

Gene identification of the mutant

To identify the mutant gene further, PCR amplifications were performed with *BcDR1* specific primer P4 and T-DNA specific primer LB3. Fragment of 750 bp PCR product was amplified in mutant BCt160, with no PCR product in the wild-type strain (Fig. 3 A). Taking genomic DNA of mutant BCt160 and WT as templates, *BcDR1* gene-specific primers P3 and P4 were used for PCR amplifications. A band of 700 bp was amplified from WT, with no PCR product in the mutant BCt160 (Fig. 3 B). This result also confirmed that *BcDR1* gene was inserted by a T-DNA in the mutant BCt160.

Analysis of the *BcDR1* gene expression in mutant BCt160

Using *Tublin* gene as control, expression levels of *BcDR1* in mutant BCt160 and wild type were analyzed using RT-PCR technique. The results showed that expression of *BcDR1* gene in mutant BCt160 was significantly lower than that of wild type (Fig. 4). The results further verified that gene *BcDR1* was mutated in the mutant BCt160.

Phenotypic analysis of the mutant BCt160

It was found that strain BCt160 did not produce sclerotia and its sporulation significantly increased, compared to wild type (Fig. 5 A, Table 1) through phenotypic analysis. Conidia changed morphologically; they were rod-like and larger than those of the wild type (Fig. 5 B, Table 1). Pathogenicity tests showed that the pathogenicity of the mutant BCt160 significantly decreased (Fig. 5 C, Table 1), suggesting that the *BcDR1* genes are involved in the development of *B. cinerea* and signal transduction regulation of pathogenicity.

Discussion

Agrobacterium tumefaciens-mediated transformation is an important technology for genetic transformation. Since it was established in yeast (Piers et al., 1996) and other filamentous fungi (de Groot et al., 1998), this technique was successfully applied to over 60 species of filamentous fungi (Michielse et al., 2005). Moreover, fungi are mostly with the genetic features of haploid, and mutants can be expressed in phenotype without the homozygous process. However, the

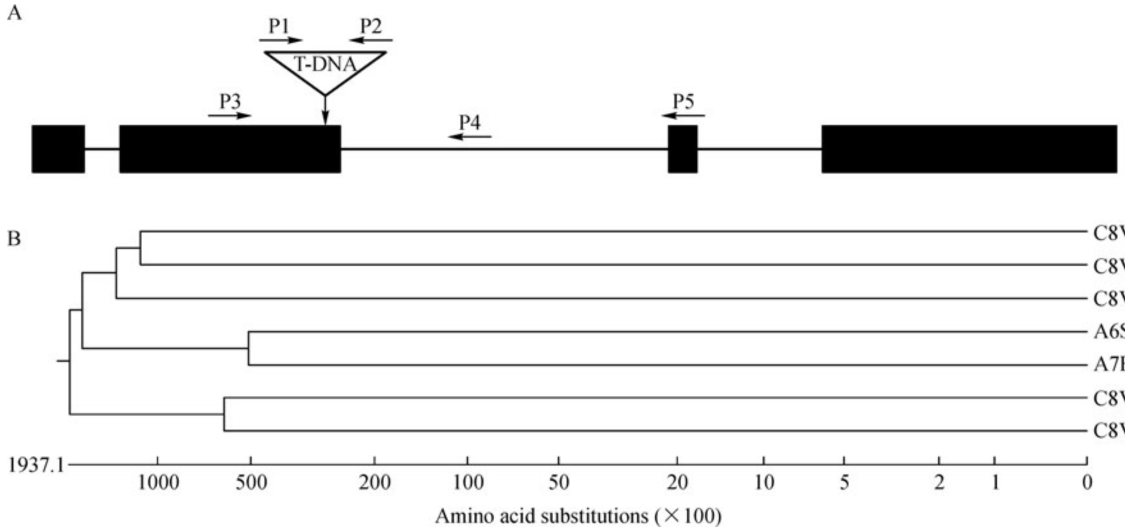

Figure 2 Bioinformatics analysis of *BcDR1*. Note: A means illustration of the BC1G12388.1 locus with the T-DNA insertion in mutant BCt160, and primers used for identifying mutant BCt160 and mutant gene. B means phylogenetics analysis of *BcDR1* from different fungi species retrieved from GenBank database including *Botrytis cinerea* (A6SIW8; BC1G_12388; *BcDR1*), *Sclerotinia sclerotiorum* (A7ERY9; SS1G_08094) and *Aspergillus nidulans* (C8VQP5, C8V280, C8V4S7, C8VRR4, C8VTY5; Putative Zn(II)$_2$Cys$_6$ transcription factor).

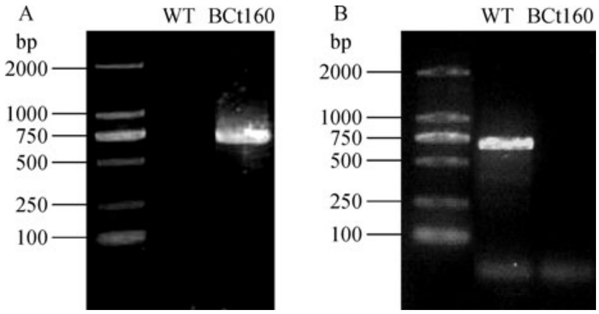

Figure 3 PCR analysis results of *BcDR1* gene. Note: A represents amplification using LB3 and P4. B represents amplification using *BcDR1* gene specific primers.

classical REMI technique successfully applied in several phytopathogens did not perform well in *B. cinerea* (Balhadère et al., 1999; Tudzynski and Siewers, 2004). Therefore, more results of mutant gene function obtained by ATMT analysis could be expected.

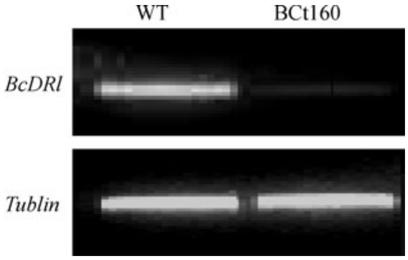

Figure 4 RT-PCR analysis results of *BcDR1*.

Pathogen *B. cinerea* as model organisms of developmental biology and molecular plant pathology, *Agrobacterium tumefaciens*-mediated transformation was established for *B. cinerea* in Bruel's laboratory (Rolland et al., 2003), and also an ATMT mutant library of *B. cinerea* was built in our early study. In this study, the inserted vector with antibiotic selected marker led to gene deactivation. By screening mutant library of *B. cinerea*, a spore-morphogenesis-variant, no-sclerotia,

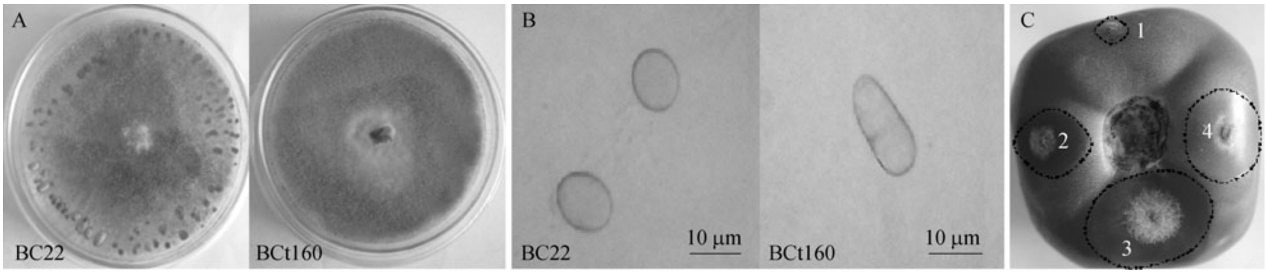

Figure 5 Phenotypic analysis of BCt160 mutant. Note: A is phenotypic characteristics of BCt160 mutant cultured in PDA medium. B is conidium characteristics of BCt160 mutant. C is pathogenicity assay of BCt160 mutant (1, BCt160^{3dpi}; 2, BC22^{3dpi}; 3, needle inoculation BC22^{3dpi}; 4, needle inoculation BCt160^{3dpi}).

Table 1 Phenotypic analysis between mutant strain BCt160 and wild-type BC22

Strains	The area of sclerotias (mm^2)	The quantity of sporulation (10/cm^2)	The size of conidia	Lesion diameter with inoculation no wound (mm)	Lesion diameter with wound inoculation (mm)
BC22	> 2000 ^{a}	12.1±0.3 ^{a}	(8.5±0.3) μm × (9.7±0.3) μm ^{a}	15.4±1.3 ^{a}	31.7±2.1^{a}
BCt160	0 ^{b}	30.0±0.4 ^{b}	(8.7±0.4) μm × (19.2±0.2) μm ^{b}	7.2±0.9 ^{b}	23.7±1.7 ^{b}

virulence-reduced mutant BCt160 was obtained. We assumed that *BcDR1* gene was involved in the development and pathogenicity of *B. cinerea*. BLASTP analysis showed that the protein of BcDR1 displayed high homology to SS1G_08094 of *Sclerotinia sclerotiorum* and the putative Zn(II)$_2$Cys$_6$ transcription factor of *Aspergillus nidulans*. The putative Zn(II)$_2$Cys$_6$ transcription factor of *Aspergillus nidulans* represses sexual development upon integration of several environmental signals (Vienken and Fischer, 2006), which coincides with our experimental hypothesis. This paper is the first report of the functional analysis of putative gene *BcDR1*. In-depth study of the gene's function will clearly provide basis for researching its pathogenic molecular mechanism and controlling *B. cinerea*. The specific mechanisms of *BcDR1* gene regulation, development and pathogenicity, especially the cause of significant virulence reduction by the mutant conidia, remain unclear and will be our further research emphases.

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