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RESEARCH ARTICLE

Screening of conidium development mutant of *Botrytis cinerea* and functional analysis of the related gene

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Abstract **A novel conidium development mutant was obtained by screening the transformants of *Botrytis cinerea* produced by *Agrobacterium tumefaciens* mediated method, which lost the ability of producing conidia. The flanking sequence of T-DNA insertion site was acquired by TAIL-PCR technology, and then, the T-DNA insertion in the second exon of *BC1G_02800.1* confirmed by BLAST between the flanking sequence and the known sequence in the *B. cinerea* gene database. The mutant gene was identified as *BC1G_02799.1* located in the upstream of *BC1G_02800.1* gene by RT-PCR. The DNA full-length sequence of *BC1G_02799.1* was 1951 bp and contained 1848 bp coding region, which encoded a 615 amino acids putative protein similar to ABC-transporter, and the function of *BC1G_02799.1* gene was unknown to date. Phenotype analysis of the mutant found that the mutant strain colony was white, grew slowly, and did not produce conidium and sclerotia on PDA medium but showed a stronger pathogenicity to tomato leaves and successfully increased the enzyme activity related to pathogenicity compared to the wild type strain. The results suggested that the *BC1G_02799.1* gene was involved in the conidium development, the sclerotia formation, and pathogenicity in *B. cinerea.* Our research will facilitate in understanding the molecular mechanism of conidium development, sclerotia formation, and pathogenic in *B. cinerea*.**

Keywords *Botrytis cinerea*, T-DNA insertional mutant, TAIL-PCR, conidium development

Introduction

Botrytis cinerea, which belongs to subphylum imperfect fungi, is an extremely serious plant pathogen with a wide host range including tomato, strawberry, cucumber, grape, apple, and a variety of vegetables. *B. cinerea* results in damping off, defoliation, blossom blight, and rotting of seedling or fruit of plant, which caused enomous economic loss to agricultural production (Kauffman et al., 1987; Williamson et al., 2007). Gray mold in tomato is seriously spread in the district of Yellow River and Huaihe River, North-east and North-west of China where glasshouses are concentrated. Field production loss rate is often 30%, even reaching 50%–70% in serious situations.

B. cinerea is a typical necrotrophic nonobligate parasite pathogenic fungi. Its main pathogenic factor includes production of both enzymes related to pathogenicity, like cutinase, pectin methylesterase, endopolygalacturonase, and toxin, such as botrydial and botcinolides, which help hypha or conidiophore invade and kill host cells and acquire nutrients from host cells (Stahmann et al., 1992; Klimpel et al., 2002; Choquer et al., 2007). However, the research found that the same pathogenic factor or the same gene related to pathogenicity may play different roles in different strains of *B. cinerea* (Valette-Collet et al., 2003; Viaud et al., 2006). Conidiophore of the pathogen plays a crucial role in the cycle and prevalence of the disease. The disease can be effectively controlled or reduced by inhibiting or lessening conidiophore production. The mutation of adenylate cyclase gene (Klimpel et al., 2002), heterotrimeric G protein α subunit gene, or MAP kinase gene influenced the formation of conidiophore or pathogenicity of *B. cinerea*. Up to now, more than 30 genes involved in the production of secondary metabolites (Siewers et al., 2005), growth of pathogen, germination of conidiophore (Turrion-Gomez et al., 2010), and other aspects were obtained in *B. cinerea*.

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Screening the transformants of *B. cinerea* produced by *Agrobacterium tumefaciens*-mediated method, the strain BMH179 fully lost its ability of conidia production. A novel gene encoding a putative protein similar to ABC-transporter was confirmed to be involved in conidium development, sclerotia formation, and pathogenicity in *B. cinerea*, which will facilitate to understand the molecular mechanism of conidium development, sclerotia formation, and pathogenic in *B. cinerea*.

Materials and methods

Strains and growth conditions

The wild-type strain BC22 and ATMT mutant library of *B. cinerea* were provided by the Molecular Plant Pathology Laboratory of Hebei Agricultural University. BC22 and transformants of *B. cinerea* grew on PDA plates at 20°C.

Screening of conidium development mutant

Strain BC22 and transformants of *B. cinerea* were grown on PDA and cultured in darkness at 20°C for 2 d, respectively. Mycelia of BC22 and transformants were inoculated on the surface of mature tomatoes by placing a piece of mycelial agar of 6.0 mm diameter. Inoculated with *B. cinerea* for 3 days, the mycelia of BC22 and transformants on tomatoes were transferred to new PDA and cultured in darkness at 20°C. The conidia of BC22 and transformants were collected from 7-day-old and 10-day-old cultures by placing mycelial agar slices in distilled water. The conidial production of BC22 and transformants were calculated by using blood counting chamber, respectively. The experiment was repeated three times. The transformant BMH179 fully lost its ability of conidial production.

Isolation of DNA and RNA

Cultured on PDA for 7 d, BC22 and BMH179 were transferred to PD media and cultured in darkness at 20°C for preparing isolation of DNA and RNA. DNA of BC22 and BMH179 were isolated using CTAB method. Total RNA was isolated using Trizol kit and then used to synthesize single-stranded cDNA according to the manuscript of promega kit.

PCR analysis of BMH179

Taking genomic DNA of BC22 and BMH179 as template, PCR was performed to identify the T-DNA insertion in BMH179 using the hygromycin resistance gene specific primers of T-DNA (hph-S: 5'-CGACAGCGTCTCCGACCTGA-3' and hph-AS: 5'-CGCCCAAGCTGCATCATCGAA-3').

TAIL-PCR analysis of BMH179

Flanking sequence of T-DNA insert in BMH179 was obtained by TAIL-PCR. The nested primers (RB1: 5'-GGCACTGGCCGTCGTTTTACAAC-3', RB2: 5'-AACGTCGTGACTGGGAAAACCCT-3', and RB3: 5'-CCCTTCCCAACAGTTGCGCA-3') of T-DNA inserted fragment, random primer (AD3: 5'-CATCGNCNGANACGAA-3') of *B. cinerea* DNA, and PCR procedures were performed as described by Mullins et al. (2001). The secondary and tertiary PCR products were tested by 1.0% agarose gel and sequenced. Alignment of the sequence obtained by TAIL-PCR with the *B. cinerea* B05.10 genome database was performed to analyze the T-DNA insertion site and gene by using the BLAST program.

PCR identification of T-DNA insertion site in BMH179

Specific primers of gene inserted by T-DNA were designed according the upstream and downstream information of T-DNA insertion site, and the primer pairs were the following: LX-1: 5'-CAAAACATCCACCATTACCACGATAG-3', RX-1: 5'-TACTCCATTCTCATCAACCAGCCT-3'). The T-DNA insertion site of BMH179 was identified by PCR using LX-1, RX-1, and T-DNA specific primer LB3 (5'-GAATTAATTCGGCGTTAATTCAGT-3').

Identification of mutant gene in BMH179

For identification of mutant gene in BMH179, semiquantitative RT-PCR was used to determine the expression levels of three specific genes including T-DNA inserted gene, an upstream gene and a downstream gene of T-DNA insertion gene. Using the equal aliquots of cDNA of BC22 and BMH179 as template, RT-PCR was performed with gene specific primers; at the same time, *Tubulin* was used for equal loading (Table 1).

Table 1 Primer pairs for semiquantitative RT-PCR analysis

Gene name	Sequence of primers
Upstream gene	5'-GTAAAGTCTCCGAGTTATGACCGAT-3' 5'-GTGATGAAGCCAAAGGTATCCAAAC-3'
T-DNA insertion gene	5'-AGGGTGTCTTGTTTCTCCAACTCAT-3' 5'-GAGGCTGGTTGATGAGAATGGAGTA-3'
Downstream gene	5'-TATCGGAAGAGTTGGTTTCGTATGA-3' 5'-GTCATAAGGGAAAGAGAGTCTGGCA-3'
Tubulin	5'-ACTGGGCTAAGGGTCATT-3' 5'-TCTCCGTAAGATGGGTTG-3'

Sequencing analysis of mutant gene

To understand the possible functional of mutant gene, bioinformatic analysis of mutant gene was proceeded. Amino acid sequences of mutant gene were obtained from the *B. cinerea* gene database and were aligned by BLAST

programs. The conserved domain of mutant gene was analyzed by ScanPro site. DNASTAR software was used for the local sequences homology alignment and phylogenetic analysis.

Phenotype and virulence analysis of BMH179

After growth on PDA for 2 d, the mycelia of BC22 and BMH179 were placed on mycelial agar of 6.0 mm diameter and transferred to new PDA for the observation of colonial morphology and the measurement of growth rate of BMH179. Mycelia of BC22 and BMH179 were inoculated on tomato leaves for virulence detection (Li et al., 2008). Fungal progression and infection symptoms were monitored for 1, 2, 3, 4, and 5 d after inoculation.

Enzymatic activity of cellulase (Cx), polygalac-tuconase (PG), pectin methylgalactuionase (PMG), and Polygalacturonic acid trans-eliminase (PGTE)

The mycelium plugs of BC22 and BMH179 were inoculated into 50 mL liquid pectinase medium, shaken, and cultured in darkness at 20°C for 12 d, respectively. Cx, PG, PMG, and PGTE of BC22 and BMH179 were extracted, and the enzymatic activity was determined based on description of Wu (2007).

Results and analysis

PCR identification of *Botrytis cinerea* transformant BMH179

Genomic DNAs extracted from wild-type BC22 and BMH179 were amplified, respectively, with hph-S and hph-AS primers for identifying hygromycin gene fragments of T-DNA. About 800 bp expected fragment amplified from BMH179 genomic DNA was shown in gel electrophoresis, and no fragment amplified from BC22 strain genomic DNA was shown in gel electrophoresis (Fig. 1).

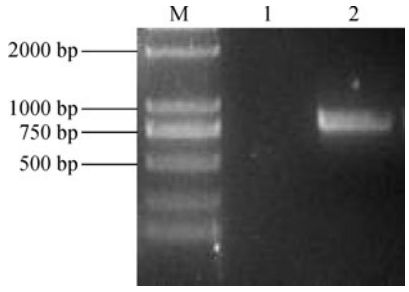

Figure 1 Identification of transformant BMH179 by PCR. M is Trans2K DNA marker; 1 is DNA of wild-type BC22 strain; 2 is DNA of transformant BMH179.

Identification of T-DNA insertion site in BMH179

Taking genomic DNA of BMH179 as template, PCR products were amplified with nested primer pairs (RB1, RB2, and RB3) and random primer AD3. An approximately 700 bp band was amplified by the second PCR amplification, and about 500-bp PCR products were obtained by the third PCR (Fig. 2A). The PCR products were cloned, sequenced, and then analyzed by BLAST between the sequence obtained by TAIL-PCR and the *B. cinerea* gene database. The result indicated that the sequence was homology with *BC1G_02800.1* gene, and the T-DNA insertion site located in the second exon of *BC1G_02800.1* gene.

To further identify the T-DNA insertion site locating in *BC1G_02800.1* gene, PCR was performed with LX-1, RX-1, and T-DNA specific primer LB3. An approximately 1100 bp band was amplified from wild-type using LX-1 and RX-1 primers, which was the full-length *BC1G_02800.1* gene sequence, and no PCR product (from LX-1 to RX-1) was amplified from BMH179. A PCR product of about 250 bp (from LB3 to RX-1) was amplified from BMH179, and no product (from LX-1 to RX-1) was obtained (Fig. 2B). These results showed that *BC1G_02800.1* was inserted by a T-DNA in BMH179.

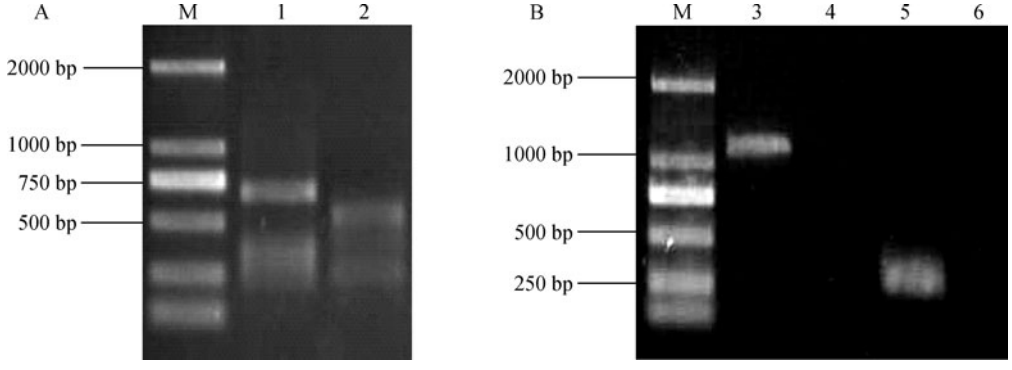

Figure 2 Results of TAIL-PCR (A) and identification of T-DNA insertion site in BMH179 by PCR (B). M is Trans2K DNA Marker; 1 is the secondary PCR of TAIL-PCR; 2 is the tertiary PCR of TAIL-PCR; 3–4 are amplification with primers LX-1 and RX-1; and 5–6 are amplification with primers LB3 and RX-1. 3 and 6 are wild-type BC22. 4 and 5 are BMH179.

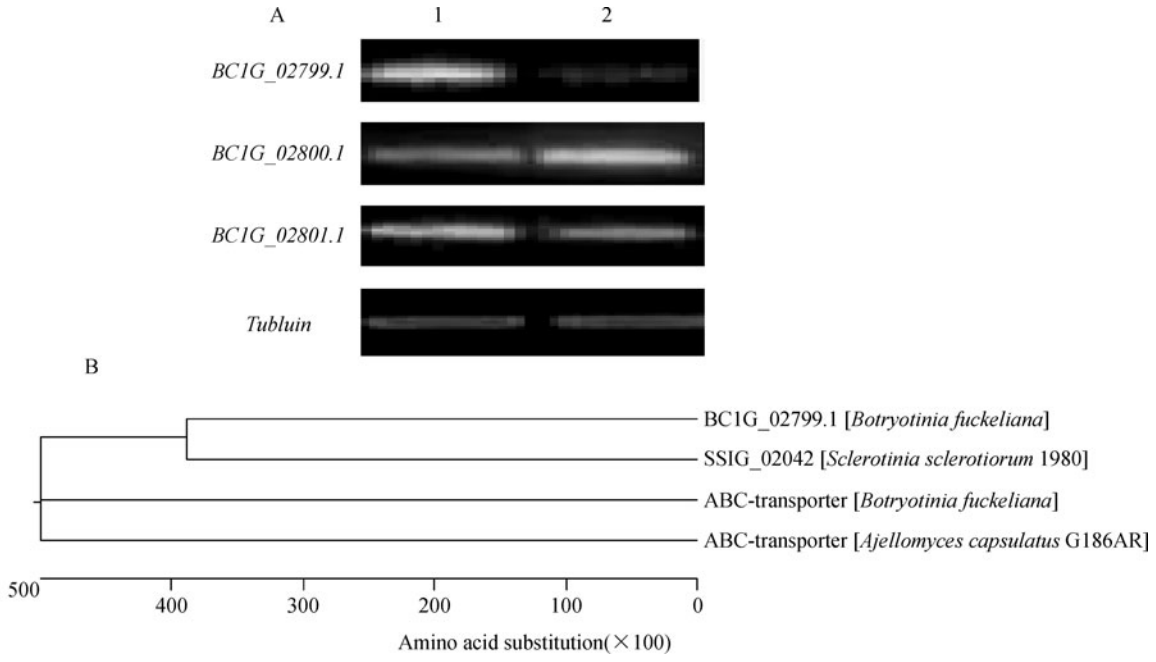

Figure 3 RT-PCR results of the gene *BC1G_02799.1*, *BC1G_02800.1*, and *BC1G_02801.1* in BMH179 (A) and phylogenetic analysis of *BC1G_02799.1* (B). 1 is wide-type BC22, and 2 is BMH179.

Identification of mutant gene in BMH179

Expression levels of T-DNA inserted gene and its upstream and downstream genes were identified by semiquantitative RT-PCR. Compared with wild type BC22, the expression level of *BC1G_02799.1* appeared to be slightly lower in BMH179 (Fig. 3A). The results showed that *BC1G_02799.1* gene was mutated in BMH179.

Sequence searches in databases revealed that the DNA full-length sequence of *BC1G_02799.1* was 1951 bp with a 1848 bp coding region, and a 615 amino acids putative protein was encoded. However, the function of *BC1G_02799.1* gene was unknown to date. Phylogenetic analysis showed that *BC1G_02799.1* shared 98% high identities with *SS1G_02042*, which is similar to ABC-transporter from *Sclerotinia sclerotiorum* (Fig. 3B).

Phenotype analysis of BMH179

The colony of wild-type BC22 in the PDA medium in the early growth stage was gray and then gradually became brown. 7 d after growing in PDA medium, the conidia and sclerotia appeared. The dense colony of BMH179 cultured in the same conditions was always white and did not produce sclerotia (Fig. 4A). The conidial suspension, collected from the culture on 7 d and 14 d in PDA, respectively, was observed by microscope. No conidia were found in the spore suspension collected from BMH179. The conidial suspension concentration, collected from the culture on 7 d and 14 d in PDA, was 6.9×10^6 mL and 7.12×10^6 mL, respectively (Fig. 4B). The growth rate of

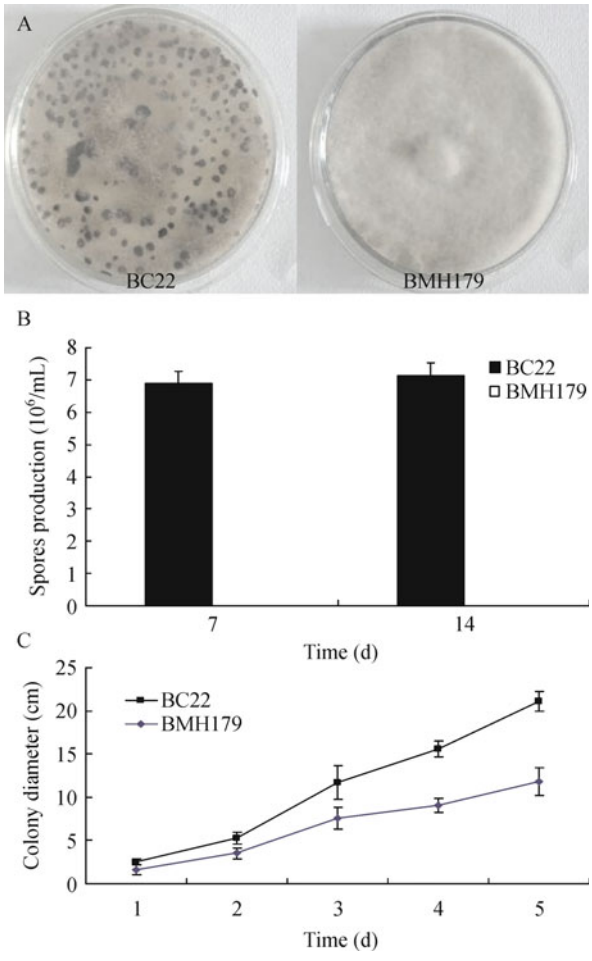

Figure 4 Colony (A), conidial production (B), and growth rate (C) of wild-type BC22 and BMH179.

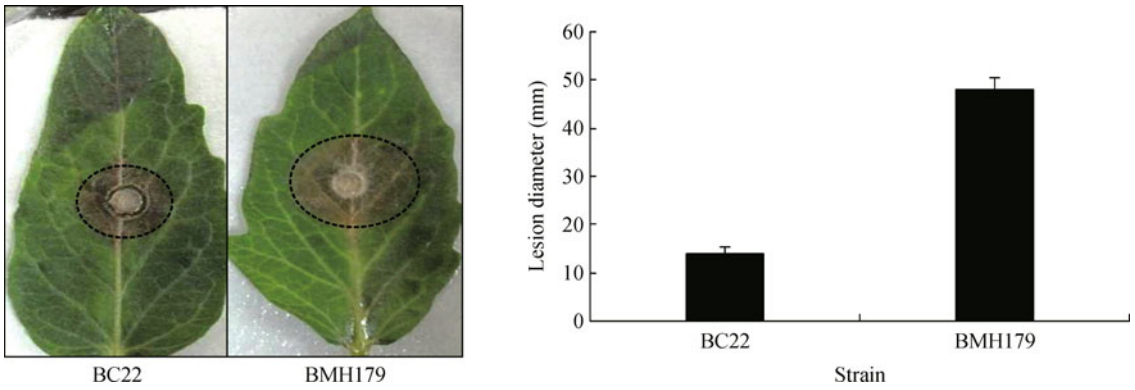

Figure 5 The pathogenicity in the tomato leaf blade comparison between wild-type BC22 and BMH179. * means significant difference at 0.05 probability level.

BMH179 was significantly lower than that of wild-type BC22 (Fig. 4C).

Virulence determination of BMH179

Tomato leaves were inoculated with the mycelial disks from colonies of the wild-type and mutant BMH179, respectively. At 24 h after inoculation, small lesions appeared on the back of all inoculated leaves, but the number of lesions on the back of the leaves inoculated with mutant BMH179 was significantly more than that of wild-type BC22. At 48 h after inoculation, the lesion area on the back of leaves inoculated with mutant strain was larger than that of wild-type BC22 (Fig. 5).

Analysis of Cx, PG, PMG, and PGTE activity

The results showed that Cx activity of mutant BMH179 was significantly higher than that of wild-type BC22. PG and PGTE activities of mutant BMH179 were slightly higher than wild type strain, with lower PMG activity of mutant strain than wild-type BC22 (Fig. 6).

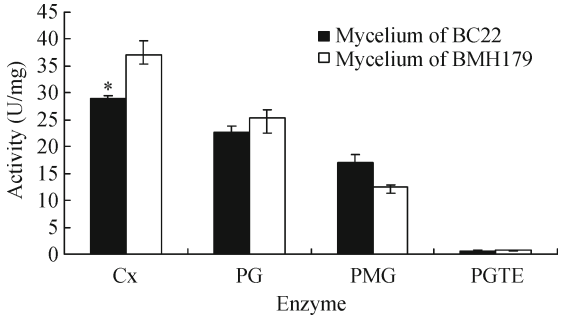

Figure 6 Enzyme activity analysis of wild-type BC22 and BMH179. * means significant difference at 0.05 probability level.

Discussion

Agrobacterium tumefaciens-mediated method is one of the most effective ways to mark and gain the functional genes in phytopathogenic fungi. Segmuller et al. have constructed more than 2800 *B. cinerea* T-DNA insertion mutants, of which more than 30 mutants change their abilities to cause disease (Segmüller et al., 2008). Most genes related to pathogenicity of *B. cinerea* are some genes involved in MAPK-signaling pathway and cAMP-signaling pathway. The *B. cinerea* mutant *Δbcg3* (gene encoding G subunits of heterotrimeric G-proteins named *bcg*) showed conidial production, and the germination rate of conidiospore were reduced compared with wild-type (Doehlemann et al., 2006). In contrast to the *Δbcg3* mutant, the *B. cinerea* adenylate cyclase gene mutant *Δbac* was unable to sporulate in planta, while *in vitro* conidiation was unaffected (Williamson et al., 2007). The *B. cinerea* mutant *Δbcpka1* (gene for catalytic subunit of the cAMP-dependent protein kinase; PKA) grew slowly and produced only small colony on the PDA but was able to sporulate in planta (Segmüller et al., 2007). A strain mutated in the *Δbcpka2* gene (encoding the second catalytic subunit of the cAMP-dependent protein kinase) showed wild-type growth, conidiation, germination, and infection (Williamson et al., 2007). It is thus clear that the mutation of different genes in *B. cinerea* in the same cAMP-signaling pathway affect conidial production differently. Why does adenylate cyclase gene mutant *Δbac* affect conidial production of *B. cinerea* in planta but does not affect the ability to sporulate *in vitro*, and why does not PKA catalytic subunit gene mutant *Δbcpka1* affect conidial production of *B. cinerea* in plant? These problems need to be further studied. The *B. cinerea* MAP kinase gene mutant *ΔBcSAK1* totally loses the ability to sporulate, and its hypha cannot penetrate uninjured plant tissues. However, the production of sclerotium increases (Rui and Hahn, 2007; Liu et al., 2008; Schamber et al., 2010), and the other MAP Kinase gene mutant *Δbmp3* in *B. cinerea* impaired conidiation fails to form sclerotium (Rui and Hahn, 2007). Obviously, both MAPK-signaling pathway and cAMP-signaling pathway take part in the regulation of conidial production of *B. cinerea*. Unfortunately, how much have the gene expressions been regulated by

MAPK-signaling pathway and cAMP-signaling pathway, and what is the outcome of those gene expressions and how have they influenced conidial production of *B. cinerea* in plant or *in vitro*? These problems still need to be further studied. In addition, two small G protein gene mutants *Δbcras1* and *Δbcrac* also completely lose the ability to sporulate; meanwhile, they lose the pathogenicity (Segmüller et al., 2007).

In our study, a novel conidium development mutant was found by screening the transformants of *B. cinerea* produced by *Agrobacterium tumefaciens*-mediated method, which lost the ability of producing conidia and forming sclerotia in vitro and increased pathogenicity to tomato leaves. Its putative protein was similar to ABC-transporter, which is an ATP-driven enzyme on membrane participating in the substance transportation across cell membrane, such as lipid (Panikashvili and Aharoni, 2008; Stefanato et al., 2009). The ten gene fragments (BcatrA-BcatrN) of ABC-transporter from *B. cinerea* have been cloned. Most gene expressions can be induced by germicides, such as Resveratrol, Fenpiclonil, and Fludioxonil, and the mutant of *B. cinerea* accumulating Fludioxonil is more sensitive to germicides than wild type (Del Sorbo et al., 2008). The influence of ABC-transporter on the ability of producing conidia and forming sclerotia and pathogenicity needs to be further studied.

The research discovered that *B. cinerea* at first kills the host cell by secreting poisonous metabolite and exoenzyme before entering plant tissues and then absorbs nutrients from the host cell (Choquer et al., 2007). These enzymes include lipase (cutin-degrading enzyme), pectinase (polygalacturonase, pectin methyl galacturonic acid enzyme, the galacturonic acid trans elimination enzyme, transeliminase), cellulose, xylanase, and so on. Nevertheless, the roles of these enzymes in the pathogenesis of *B. cinerea* are in controversy. *B. cinerea* lipase mutant *ΔBcpls1* is unable to invade unhurt plant tissues but form normal appressorium (Gourgues et al., 2004). The mutant deleted cutinase gene and lipase gene at the same time have no influence on penetrating host cells (Reis et al., 2005). The mutant of *B. cinerea* containing six poly-galacturonic acid genes, when lacking the two genes, descend pathogenicity to several kinds of host plants remarkably, while lacking other four genes, showed no pathogenicity change evidently (Kars et al., 2005). The pathogenicity of single and double mutants in two *Bcpme* genes (encoding pectin methyl galacturonic acid enzyme) in *B. cinerea* B05.10 was not different from wild type, and the pathogenicity of mutant in the gene encoding cellulase had no change evidently (Kars et al., 2005). The mutant deleting *β*-1,4-xylanase gene reduced its pathogenicity and lesion expansion obviously (Espino et al., 2005). The fact was that most of the abovementioned enzymes are encoded by multiple genes (Broome et al., 1995). There probably exists complementation in function between genes. Therefore, making *B. cinerea* mutant lacking all genes encoding certain enzyme at the same time is the effective way of investigating the functions of the enzyme in the pathogenicity.

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