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Identification of new pathogenicity related to the gene encoding hypothetical protein in the gray mold fungus (*Botrytis cinerea*)

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Abstract

Botrytis cinerea is an important necrotrophic fungus that causes gray mold disease in various important crop plants, leading to substantial economic losses. Herein, *Agrobacterium tumefaciens*-mediated transformation (ATMT) technique was used to create an insertional mutant library of *B. cinerea* to investigate its pathogenicity-related genes. Among 1,734 transformations only 46 mutants decreased the pathogenicity on tomato leaves. C2M52 is one of the 46 mutants that its pathogenicity reduction was analyzed deeply. The T-DNA integrated into the promoter region of the hypothetical protein (*BcHP*) gene. The gene replacement approach via homologous recombination was used to generate knock-out mutant (Δ*bchp*) and its functional complementary (Δ*bchp-C*) strains. The results warranted that the Δ*bchp* mutant strain displayed a significant difference in the virulence on tomato leaves relative to the wild type. These results were consistent with the attenuated pathogenicity caused by T-DNA integration mutant (C2M52). That subsequently revealed the crucial role of *BcHP* gene in the pathogenicity of *B. cinerea*.

Keywords: *Agrobacterium tumefaciens* -mediated transformant, *Botrytis cinerea*, hypothetical protein, and pathogenicity.

Introduction

Botrytis spp. attack more than 1400 species belong to 596 genera of host plants, many of which are main crops. Around 586 of the 596 genera are attacked by *Botrytis cinerea* (Elad 2016), causing an annually economic loss ranging from $10 to $100 billion worldwide (Romanazzi et al., 2016; Weiberg et al., 2013). This big loss has made this particular pathogen attracting the interest of considerable farmers, advisers, extension staff, agriculture experts, researchers and students in many fields (Sundgren 2014; van Kan 2006; Williamson et al., 2007). Notably, this pathogen is mainly controlled by chemical fungicides which are highly costly and ineffective for long term use due to the development of resistance in pathogen, and non-biodegradable that are destroy the main important sources of the environmental ecosystem. Therefore, it is necessary to develop alternative strategies to control this disease in an effective and sustainable way (Couderchet 2003; Fang et al., 2014; Vos et al., 2015). A molecular approach to the study of *B. cinerea* pathogenicity was facilitated in 1994 by the development of a protoplast-based transformation system of fungus (Hamada et al., 1994). At present, molecular researchers have completed the sequencing of the whole genome and analysed two strains of *B. cinerea* (B05.10 and T4) which lead to practical model by overwhelming the molecular strategy. This might enhance the phenotypic and genotypic variability of this species and the incidence of strain-dependence in the infection process (Tudzynski and Kokkelink 2009; van Kan et al., 2016). Consequently, many virulence factors were identified through molecular tools information such transcriptome, proteome, and secretome investigations (Ish-Shalom et al., 2011). There are many recent successfully attempts in *B. cinerea* to identify the virulence genes by random insertional mutagenesis upon usage of *Agrobacterium-tumefaciens* mediated transformation (ATMT) (Feng et al., 2010; Giesbert et al., 2012; Nakachi et al., 2000). ATMT has been established as an important technique to acquire insertional mutant libraries and to identify pathogenicity-related genes. Furthermore, the high efficiency of targeted gene knock-out also permits quick functional analysis of putative pathogenicity-related genes (Li et al., 2012; Paz et al., 2011; Wang et al., 2016). In this study, the characterization of the C2M52 mutant was carried out to show the insertion of T-DNA in the promoter region of the hypothetical protein (*BcHP*) gene. Different approaches were applied to confirm the relation between T-DNA insertional and the observed phenotype. Moreover, the gene knock-out and the complementary analysis were verified the significant role of *BcHP* in the phenotypic and pathogenicity-related gene in *B. cinerea*. To the best of our knowledge, this is the first study showing that hypothetical protein can influence the pathogenicity of *B. cinerea*.

Results

Generation of a mutant library and analysis of T-DNA insertional sites

The plasmid T-DNA was randomly inserted into the genome of *B. cinerea* using ATMT protocol and improved Rolland et al. (2003), to produce a group of 1,734 hygromycin-resistant

transformants obtained by picking single colony after several screening. PCR was employed to investigate the integration of the hygromycin resistance cassette conveyed by the vector pBHt2. The pathogenicity tests were benchmarked towards a detached tomato leaf categorized according to the severity of the lesions. In total, 1076 transformants were showed wild-type-like infection patterns, 612 transformants were slightly different than WT in the virulence, and 46 transformants significantly reduced the virulence relative to the WT. Then 46 transformants were exposed to at least three independent pathogenicity screening test to prove the significant reduction of the virulence. Based on Southern blot analysis, most of the mutants contained a single copy of random insertion of T-DNA as well as some of the mutants were accounted two copies of T-DNA gene inserted into the genome of *B. cinerea* **(**Figure 1**)**. The thermal asymmetrical interbred (TAIL)-PCR was conducted in these transformants to isolate the DNA sequences nearby the left border or the right border of the T-DNA integration. Among 46 of the tested transformants, 32 mutants were recovered in one border at least, to analyze the T-DNA integration sites followed by the comparison of their sequences with the genomic databases of the recipient strains B05.10 and T4 of *B. cinerea*. 58 border sequences were produced 12% corresponding to T-DNA overlapping sequences with a vector. Overall, 7 T-DNA integrations located intergenic of the promoter region, terminator region or within an ORF. The further characterization of mutant No. 52 in the second class (C2M52) warrants that T-DNA integrations were found to be in the promoter region of (BC1G_01794) gene encoding hypothetical protein. This gene show similarity to genes that code for proteins involved in pathogenicity in other fungi [e.g. (*ssv263*) encoding a hypothetical secreted protein which is required for full pathogenicity in *Sclerotinia sclerotiorum*] (Liang et al., 2013). To confirm whether the identified gene is exactly responsible for the observed phenotypes of the T-DNA integration mutant, the knock-out and the complementary of the identified gene in the wild-type strain could be performed as described by (Michielse et al., 2009).

Identification of BC1G_01794 (BcHP)

The T-DNA insertion of the C2M523 mutant was found in the promoter region 129 bp of the start codon of BC1G_01794 gene encoding hypothetical protein under accession number (NCBI ID: XP_001559638.1) as retrieved from the NCBI database. The BC1G_01794, hereafter named (*BcHP*), is the coding sequence consists of 1467 bp. It is interrupted by the two introns of 74 and 67 bp. The predicted protein involves 441 amino acids with a molecular weight of 46345.8 D and theoretical pI: 4.27. Moreover *BcHP* protein contains 44 negatively charged residues and 24 positively charged residues. The Sequence alignment for the hypothetical protein of *B. cinerea* shared 60%, 58%, 42%, 34% of identity with hypothetical proteins in *Aspergillus carbonarius, Oidiodendron maius, Verruconis gallopava* and *Rachicladosporium antarcticum*, respectively (Figure 2A). The structural model (Figure 2B) of the hypothetical protein of *B. cinerea* contains a six alpha-helices 21 beta strand, with a molecular surface area of 407.168 Å2.

Molecular recognition of BcHP deletion mutants and its complementary strains

The *BcHP* deletion mutants and their corresponding complementation strains were constructed using gene homologous replacement (Figure 3A) as mentioned in "Materials and methods". One of each mutant and complementary strains were chosen to be detected with three pairs of primers (P5+P6, P7+P8, and P9+P10) primers by PCR **(**Table 1). The results clearly demonstrated that the targeted band of the *BcHP* gene is appeared in the WT and complementary strain, while it disappeared in the deletion mutants using gene specific primers (P5+P6). The targeted bands of the inserted hygromycin gene are detected in the mutants and complementary strains but disappeared in the WT strain via using of P7+P8 and P9+P10 primers, respectively (Figure 3B). This result clearly warrants that *BcHP* gene is successfully replaced by hygromycin gene. That is further confirmed by using southern blot in presence of gene-specific primers (P5+P6) as a probe after digestion the genomic DNA of the whole strains with *Xho*1. The WT has a 5.4 kb hybridizing band while the mutant shows no band **(**Figure 3C**)**. Also, the complementation transformant is marked by a random insertion of a *BcHP* gene into the genome of the Δ*bchp* mutant.

Real-time PCR demonstration of the up-regulation of the BcHP during infection

Real-Time RT-PCR analysis was performed to determine expression patterns of *BcHP* gene in *B. cinerea* during fungal interaction with host plants. The sRNA was constructed from *B. cinerea*-inoculated tomato leaves (3-week-old) at 0, 12, 24 and 36 hours post-inoculation (hpi) time points. The housekeeping actin was used as the RT-PCR internal control standard. The relative expression of *BcHP* gene is significantly up-regulated during the infection of tomato leaves in early stages (Figure 4A). Thereafter, the WT and mutant strains were cultured on PDA plates on cellophane overlay to evaluate the expression level of Δ*bchp* mutant. The results showed up-regulation in the response of expression level of *BcHP* gene (Figure 4B). This finding of the gene expression pattern is consistent with our experimental results.

Pathogenicity analysis

To estimate the pathogenic performance of Δ*bchp* mutants, the conidia of WT, Δ*bchp* mutant and Δ*bchp*-C complementary strains were prepared with a concentration of 1×10^5 conidia/ml. An 8µl of the as-prepared conidial suspension was dropped over the unwounded tomato leave and observed daily. The pathogenicity analysis was considered based on measuring the average diameter of the lesions grown on tomato leaves. The lesion diameter on tomato leaves of the mutants was significantly smaller compared to WT and complementary strains (Figure 4A). This difference among the *BcHP* knock-out mutants, complementary and WT strains in pathogenicity were further confirmed by the pathogenicity assays with mycelial plugs (Figure 4B). These findings showed the essential role of *BcADH* in the fungal pathogenicity and consistent with the attenuated pathogenicity caused by C2M52 T-DNA insertion mutant.

Discussion

Reviewing the literature, we found that ATMT has been widely used as a method to identify the virulence factors in different fungal species (Li et al., 2012; Paz et al., 2011; Wang et al., 2016; Zhang et al., 2015). In the present study,

Table 1. The mutagenic primers were used in this study.

No.	Primer name	Sequence from 5' to 3'
1	HPUp-F	CTCGAGTGATTGGGTCTCTTGGCTCT
2	PHUp-R	GAATTCCGGGAATACTTCTTGGTCTG
3	PHDN-F	GGATCCAGCAAAGAAAAGGCGAAGTG
4	PHDN-R	CTGCAGGTGGATAGAAGGGTGGAGCA
5	PHDet-F	TCAGTTTATTTGAGGGAGAGGA
6	PHDet-R	GAGATGGATGTTACGAGGATGG
7	PHOutUP-F	GTTCGGCAAAACTCTCCAC
8	Hptb-RC	ACAGACGTCGCGGTGAGTTCA
9	PHOutDN-R	ACAATACGAGACGGGTGACA
10	Hpta-RC	TGCGCCCAAGCTGCATCAT
11	PHCo-R	GAATTCTGATAAGAACGGATGTCGGA
12	PHCo-F	CTGCAGCTGGTGGATAGAAGGGTGGA
13	PHprobe-F	AGTGCCGTTCCCTCCAGACC
14	PHprobe-R	TACCAGCGTAGAGAATCATA
15	HPq-PCR-F	TCTACACCATCAAAATCCAAGC
16	HPq-PCR-R	GGAACAGCAGTACAAGAAACGA
17	SP RB1	GGCACTGGCCGTCGTTTTACAAC
18	SP RB2	AGGGTTCCTATAGGGTTTCGCTCT
19	SP RB3	AACGTCGTGAACTGGGAAAACCC
20	SP LB1	CATGTGTTGAGCATATAAGAAACC
21	SP LB2	CCCTTCCCAACAGTTGCGCAG
22	SP LB3	GAATTAATTCGGCGTTAATTCAGT
23	AD-1	TGTAAAAGATCGCCCAGCGCAGC

Primer sequence underline is the restriction enzyme site.

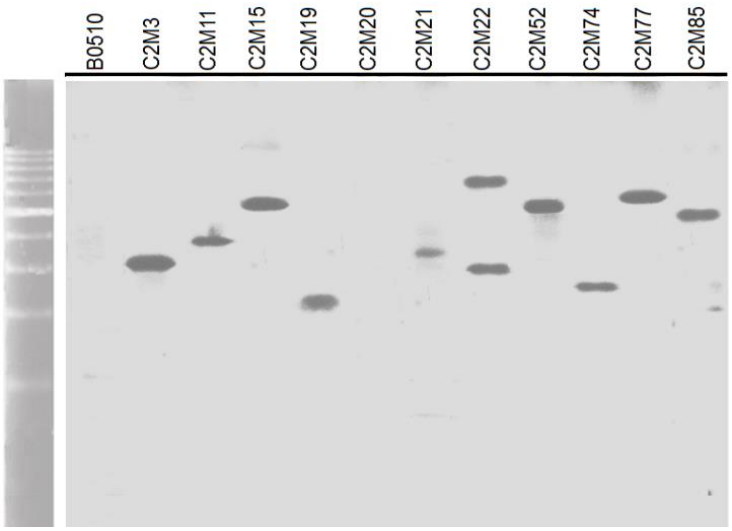

Fig 1. Southern blot analyses, the Genomic DNA of the WT B05.10 and T-DNA integration mutants were hybridized with hygromycin B resistance gene (*hph*) as a probe and the DNA digested with *EcoR*1. Probe labeling and hybridization was performed using DIG DNA labeling and Detection kit (Roche) according to the manufacture's instruction. The result presented mutants contained a single copy of random insertion of the T-DNA. As well as some of the mutants were accounted two copies into the genome of *B. cinerea*

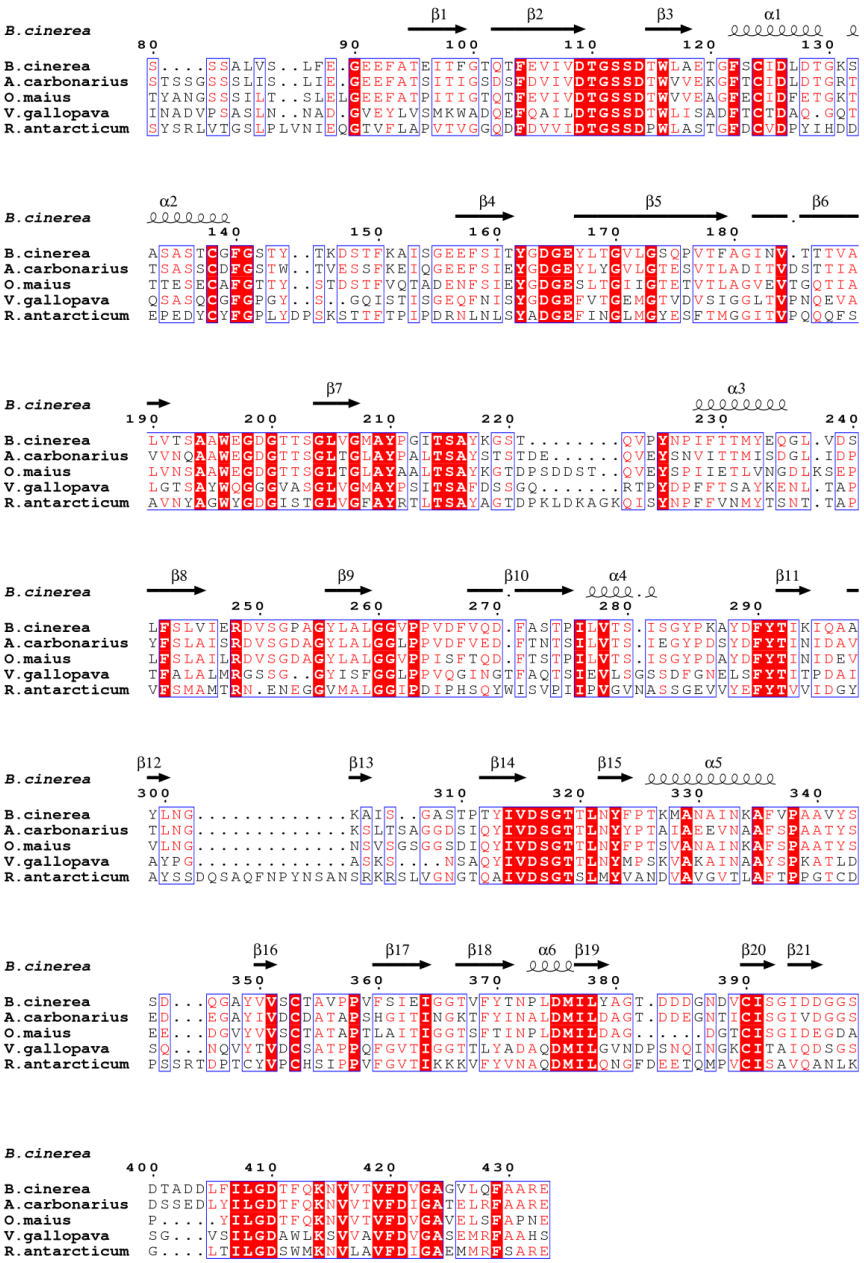

Fig 2A. Sequence alignment for the hypothetical protein of *Botrytis cinerea*, *Aspergillus carbonarius*, *Oidiodendron maius*, *Verruconis gallopava* and *Rachicladosporium antarcticum*. The sequence identity between them and hypothetical protein of *Botrytis cinerea* were 60%, 58%, 42%, 34%, respectively. The alignment was performed using http://espript.ibcp.fr/ESPript/ESPript/index.php. Red boxes refer to high conserved sequence

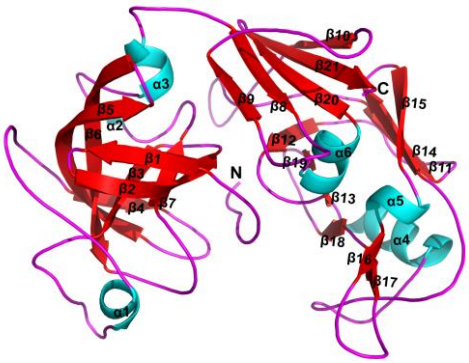

Fig 2B. The schematic drawing of the three-dimensional structures model shows the hypothetical protein of *Botrytis cinerea.* The β-strands are numbered β1-β21 and the α-helices α1-α6. having 6 alpha helix and 21 beta sheet, with a molecular surface area of 407.168 $Å^2$. The N refers to N-terminal, while C refer to C-terminal.

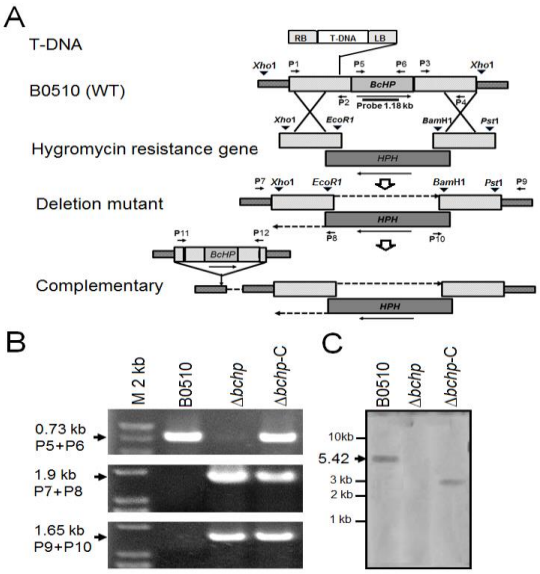

Fig 3. Schematic representation of the T-DNA insertion side and knock-out strategy of the *BcHP* gene. (A) T-DNA insertion in a promoter of the BcHP gene. The target gene of *BcHP* replaced with hygromycin resistance cassette (*HPH*). Primer (codes 1, 2, 3 and 4) PCR primers to amplify upstream and downstream fragments of *BcHP* gene, (5, 6, 7, 8, 9 and 10) PCR primers to detect transformants, (11 and 12) PCR primers to amplify full-length fragment for complementation of *BcHP* including an open reading frame (ORF), promoter regions, and terminator regions (see Table 1). (B) Detection of the WT, Δ*bchp* deletion and complementation with PCR amplification as described above. (C) Southern blot analysis, the genomic DNA of WT, mutants, and complementary were hybridized with a gene-specific fragment of *BcHP* as a probe and digested with *Xho*1.

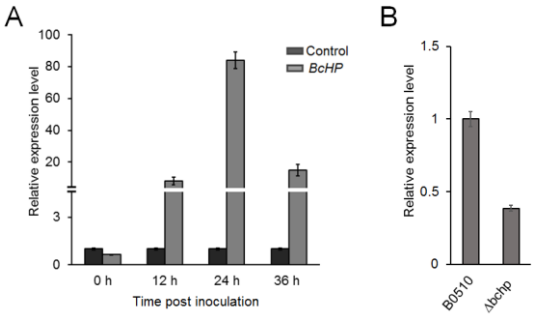

Fig 4. Relative expression level was determined by qRT-PCR (A) the response of *BcHP* gene in *B. cinerea* strain B0510 during infection (B) the expression of the WT (B0510) and Δ*bchp* knock-out during cultural growth on PDA. The bars represent the standard error of the means of three independent experiments.

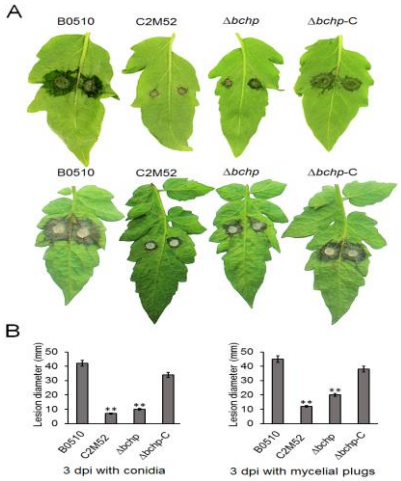

Fig 5. Pathogenicity assays of the indicated *B. cinerea* strains. (A) Infection of tomato leaves with conidia and mycelial plugs of the WT, mutants and their complementary strains. (B) Lesion size was analyzed at 3 dpi. The means were calculated from three independent experiments with three replications. Significant differences in the measurements were compared to the WT. * and ** indicate significance at P<0.05, and 0.01, respectively.

the primary analysis of the newly developed library of 1734 mutants in *B. cinerea* was started and is still in progress. The ATMT-based mutant collection requires the identification of emerging factors that were involved in the induction of virulence in *B. cinerea*. These factors were also examined and validated the studies of Rolland and his coworkers (Rolland et al., 2003). That is owing to the high stability and transformation rate of ATMT approach. Around 46 stable mutants were obtained and showed a substantial decrease in the pathogenicity amongst total mutants found in the library. This was relatively a notable result, due to existence of most heterogenic mutants showing drastic phenotype (Rolland et al., 2003). Notably, the studies of Rolland and his coworkers revealed the successful production of *B. cinerea* mutants contained randomly integrated T-DNA at single sites via efficient transformation of *B. cinerea* using *Agrobacterium tumefaciens*. Herein, in our investigation a high percentage of T-DNA integration is proved to be single ones as displayed by Southern analysis (Figure 1), similar to previously reported in yeasts, many filamentous fungi and plants (Alonso et al., 2003; Maruthachalam et al., 2011; Michielse et al., 2009; Nakamura et al., 2012; Zhang et al., 2015).

The genes which are involved in the production of certain phenotype by ATMT usually generate a wonderful method. However, it is important to investigate each gene in a more descriptive way, because it cannot be considered to prove that the integration of T-DNA is almost associated with the real phenotypic expression, which is also previously manifested by scientist (Blaise 2007; Jeon 2007; Michielse et al., 2009). The alteration of gene function via T-DNA integration led to analysis of phenotypes than the targeted deletions of the relevant genes (Idnurm 2004.). That is noticed after deletion of the *BcHP* gene, which can be determined by partial loss of function. The pathogenicity parameters of a type hypothetical protein and the relationship among T-DNA integration and the observed phenotype are determined by the T-DNA insertion mutant C2M52, which can be proven by targeted gene replacement. In this regard, the *BcHP* gene is knocked out and its complementary strains are produced to examine their pathogenicity compared with the WT strain. The virulence results warrant that the lesion diameter of the Δ*bchp* mutant is substantially lower than WT and Δ*bchp*-C complementary strains implying the particular role of the *BcHP* gene in the host infection. These results are inconsistent with the previously published report by Liang et al. (2013) reporting that the pathogenicity of knock-out mutants of (*ssv263*) gene encoding a hypothetical secreted protein in *S. sclerotiorum* is significantly reduced compared to WT. Similarly, the deletion mutant of extracellular polysaccharide and virulence-related gene (*epv*) is one of six genes encoding a hypothetical protein in *Xanthomonas oryzae* pv. *oryzicola* is dramatically impaired pathogen virulence and extracellular polysaccharide (EPS) production (Qian et al., 2012).

Materials and methods

Fungal, bacterial strains and growth conditions

In this investigation *B. cinerea* strain B05.10 was used as a host for gene integration, gene replacement and a wild type (WT). The WT, mutants, and corresponding complementary strains generated in this study were grown in potato dextrose agar (PDA) (20% (w/v) potato, 2% (w/v) dextrose and 1.5% (w/v) agar) or minimal medium (MM) (0.1% (w/v) K_2HPO_4, 0.1% (w/v) KH_2PO_4, 0.05% (w/v) $(NH_4)_2SO_4$, 0.02% (w/v) NaCl, 0.05% (w/v) $MgSO_4$, 0.002% (w/v) $CaCl_2$, 0.002% (v/v) $FeSO_4$ and 0.5% (v/v) glucose) then the pH adjusted at 6.9, followed by incubation at 20°C.

Escherichia coli (DH5α) was used to propagate all plasmids. Meanwhile, A. *tumefaciens* strains *Agl*-1 was used for the transformation grown in (LB 0.5% (w/v) yeast extract, 1% (w/v) peptone, and 1 % (w/v) NaCl) supplemented with ampicillin or kanamycin (50 μg/ml) when required.

Primer's design and DNA extraction from B. cinerea strain B05. 10

All the primers used in this study were listed in the Table 1. Genomic DNA was extracted from the mycelia of the WT strain B05.10 as reported elsewhere (Cenis 1992; Millar et al., 2000). The fragment flanking *BcHP* gene was generated by PCR system (Roche Diagnostic, Penzberg, Germany). The PCR amplifications were performed by using the following parameters: initial denaturation at 94 °C for 2.5 min, followed by 35 cycles of denaturation at 94 °C for 40 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 6 min. The resultant PCR product was added into a recovery Kit (Clontech Laboratories, California) and then purified, cloned, and sequenced. All mutations were confirmed by DNA sequencing.

The tumefaciens mediated transformation (ATMT) of B. cinerea

The ATMT process was performed and quantified for the transformation of *B. cineraea* as described previously (Islam et al., 2012). Briefly, the *A. tumefaciens* strain *Agrl*-1 were cultured in LB broth medium supplemented with kanamycin (100 μg/ml) and rifampicin (50 μg/ml), then incubated at 28 °C for 2 days. Afterward, the *A. tumefaciens* cells were diluted to an optical density of OD_{600}=0.15 and seeded in an induction medium (IM) containing 200μM acetosyringone (AS) for 6 h before mixing with an equal volume of a fresh *B. cinerea* conidia suspension (1×10^6 conidia/ml^{-1}). This mixture (200 μl) was then sprayed onto a nylon membrane (3×3 cm; Millipore Co., Bedford, MA, USA) and plated on IM amended with 200 μM AS followed by incubation at 28°C for 2 days in the dark. Afterward, the membrane was transferred upside-down on the PDA plates supplemented with hygromycin B (100 μg/ml), streptomycin (100 μg/ml), and cefotaxime (200 μg/ml). The hygromycin-resistant colonies were appeared after about 3-7 days. The colonies of transformants were transferred individually onto the PDA plates amended with hygromycin B (100 μg/ml) and incubated at 28 °C for 2 days. The Single hyphal tip from each transformant was collected with a sterile needle and transferred to a fresh PDA plate containing hygromycin B (100 μg/ml) and kept for further experiments.

Screening of transformants and standard molecular methods

The transformants were initially screened by PCR to identify potential homologous recombination events at the gene locus using the primers designed to amplify the mutated gene locus. Fungal genomic DNA was isolated for the southern blot according to the Chi et al., protocol (Chi et al., 2009). Southern blot analysis of deletion mutants was performed as described by DIG high primer DNA labeling and detection according to the protocol of starter kitII (Roche applied science, Germany). The hybridization was carried out in 2x SSC, 0.1% SDS, washing buffer, maleic acid buffer, detection buffer, blocking solution and antibody solution in

the presence of denaturing DIG-labeled DNA probe (about 25 ng/ml DIG Easy Hyb) by boiling for 5 min. Then the mixture was cooled rapidly and mixed with 4µl DIG high primer followed by incubation at 37 ℃ for 16 to 20 hours. Thereafter, the mixture was filtered and washed for 15 min in 2 x SSC and 0.1% sodium dodecyl sulfate (SDS) at 65-68°C.

Knock-out of BcHP gene and complementation

The knock-out mutant strain of *BcHP* gene was generated by amplification of upstream (567 bp) and downstream (594 bp) flanking sequences fragments being amplified in the presence of *B. cinerea* (B05.10) genomic DNA with pair primers HPUp-F + PHUp-R and PHDN-F + PHDN-R by the PCR respectively (Table 1). Then, the target fragment was recovered by using the recovery Kit protocol (Sangon, Shanghai, China). The upstream fragment was inserted into the *Xho*I/*Eco*RI sites of the deletion pMD^{18}-T vector in order to generate the plasmid of pMD^{18}T-*BcHP*-Up, while the downstream fragment was inserted into the *BamHI*/*Pst*I sites of the deletion pMD^{18}-T vector to generate the plasmid of pMD^{18}T-*BcHP*-DN. The sub-cloned fragments were inserted into two sides of the pGEM hygromycin-B resistance cassette (*HPH*). The obtained plasmid was transformed using ATMT into *B. cinerea* strain (B05.10). The individual transformants were transferred onto PDA plates supplemented with hygromycin B (100 µg/ml). To complement the Δ*bchp* strain, a (2720 bp) full-length *BcHP* gene including an open reading frame (ORF), promoter regions, and terminator regions was amplified from the genomic DNA of B05.10. The PCR product was cloned in front of (p*DUC*) chloramphenicol resistance cassette into the *EcoR*1–*Pst*I sites, leading to a plasmid p*DUC-chip*-C (Silar 1995). The as-obtained plasmid was transformed into the *Agl*-1 to be constructed with Δ*bchp* mutant using ATMT.

Pathogenicity assays

The conidia of 10-day-old fungal strains were cultured on PDA plates. Meanwhile, mycelia were isolated from the fungal strains cultured on PDA plate for three days. Then, tomato leaves were inoculated with conidia and mycelia to investigate the pathogenicity assays. Then a solution of conidia of WT, mutant, and complementary strains was prepared with a concentration of (1×10^5 conidia/ml) for the assay analysis. An 8 µl of the as-prepared solution was dropped on the unwounded tomato leave as previously described (Klimpel et al., 2002). Thereafter, the infected plants were incubated in a plastic spreader box at 20 ℃ under high humidity, and dark condition for 3 days. Following that, the lesion diameters were measured daily to evaluate the virulence. Three independent experiments with three replicates were conducted.

Real-time PCR

Total RNA was extracted using Trizol reagent (TAKARA, Dalian, China) and treated with RNase-free DNase according to the manufacturer's instructions. Briefly, the First-strand cDNA was synthesized by reverse transcription using a Prime Script RT reagent kit (TAKARA, Dalian, China). The obtained cDNAs were used for gene expression analysis with CFX96 real-time PCR system (BioRad, Hercules, CA, USA). Each qPCR reaction conatined 12.5 µL SYBR Premix Ex Taq TM (TAKARA, Dalian, China) and 0.1 µg cDNA in addition to 7.5 pmol of each gene specific primer (Table 1) with a final volume of 25 µL. The gene expression levels were calculated via using 2(ΔΔCT) method (Livak and Schmittgen 2001).

Conclusion

In summary, T-DNA insertion mutagenesis via (ATMT) was used to produce the mutant libraries of *B. cinerea*. In one T-DNA integration analysis, the virulence of *B. cinerea* was identified to be associated with the hypothetical protein (*BcHP*) gene. The knock-out mutant (Δ*bchp*) and its complementary (Δ*bchp-C*) strains were generated via the gene replacement approach. The Δ*bchp* mutant strain revealed a substantial difference in pathogenicity on tomato leaves compared with wild-type. That subsequently revealed the crucial role of *BcHP* gene in the pathogenicity of *B. cinerea*.

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