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Cloning and characteristics of *Brn1* gene in *Curvularia lunata* causing leaf spot in maize

Tong Liu · Shufa Xu · Lixing Liu · Feihong Zhou · Jumei Hou · Jie Chen

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Abstract The full length cDNA of the *Brn1* was first cloned, and then expression of the *Brn1* was analyzed and the function was identified by silencing technology. Results show that the full length cDNA of the *C. lunata Brn1* gene contains 1001 base pairs and an 801 bp open reading frame encoding 267 amino acids. Semi-quantitative PCR analysis shows that the expression of *Brn1* at 96 h is significantly higher than at 24 and 72 h ($p<0.05$) in both the highly virulent isolate CX-3 and the weakly virulent isolate DD60. *Brn1*-silenced transformants were light brown in culture filtrate, and have significantly reduced toxin production relative to the wild-type. These results imply that *Brn1* gene in *C. lunata* is not only involved in 1,8-dihydroxynaphthalene melanin synthesis, but is also relatively associated with toxin biosynthesis of the pathogen.

Keywords *Curvularia lunata* · Gene silencing · 1,8-dihydroxynaphthalene · Melanin · Toxin

Tong Liu and Shufa Xu contributed equally to this work.

T. Liu · S. Xu · L. Liu · F. Zhou · J. Chen (✉)
School of Agriculture and Biology and Key laboratory of Urban Agriculture (South) of Ministry of Agriculture,
Shanghai Jiaotong University,
800 Dongchuan Road,
Shanghai 200240, China
e-mail: jiechen59@sjtu.edu.cn

Introduction

Curvularia lunata is an important fungal pathogen that causes leaf spot in maize worldwide. In the past decade, the disease spread widely in maize growing areas in China (Dai et al. 1995). To uncover the mechanisms of pathogen infection, most work have thus far focused on the identification of virulence factors, such as cell wall-degrading enzymes, melanin, and toxins (Liu et al. 2009; Xu et al. 2007; Feng et al. 2002). However, we have less understanding of the genes involved in these virulence factors, particularly on the association of the genes related to the production of melanin and toxin synthesis. In a previous proteome study, we found that expression of Brn1 protein has a positive correlation with virulence of the pathogen. The gene encoding Brn1 protein is involved in melanin synthesis in other pathogens, including *Magnaporthe oryzae* (Andersson et al. 1996), *Bipolaris oryzae* and *Colletotrichum lagenarium* (Takano et al. 1997). However, until the current study, no study has revealed the direct correlation of this gene expression with melanin production in *C. lunata* and its involvement in pathogen infection. This is because extensive studies on other phytopathogens have documented that melanin can improve the mechanical strength of appressoria when penetrating host cell walls (Hamilton and Gomez 2002). Also, based on our previous findings, *Brn1* in *C. lunata* might be

involved in the production of a toxin, methyl 5-(hydroxymethyl) furan-2-carboxylate, which has been demonstrated as a crucial virulence factor to maize. In this study, we aim to clone the full length of *Brn1* from the pathogen, and subsequently to investigate the role of *Brn1* in the formation of melanin, as well as its implications in toxin production.

Materials and methods

Fungal isolates, culture conditions, and host plants

The highly virulent isolate of *C. lunata* CX-3 and the weakly virulent isolate DD-60 were selected from more than 300 isolates obtained from diseased maize leaves from six different provinces in China. The eight differential host plants including Shen135, 7899–1, Mo17, 477, C8605, E28, 7922 and Huangzao4 (Chen et al. 2003) were used to identify the virulence types which all strains belong to. The isolates were stored at Prof Jie Chen's laboratory (Shanghai Jiaotong University, China) by growing mycelium on silica granule. For DNA or RNA extraction, the single silica gel granule with the pathogen was cultured on potato dextrose agar (PDA) for a week at 28°C in the dark, then mycelial plugs were taken from the edge of the culture and transferred into potato dextrose broth (PDB) for 5 days at 28°C in the condition of continuous shaking (120 rpm).

HUANGZAO-4, a maize inbred line susceptible to *C. lunata*, was used as the host plant. HUANGZAO-4 has been used extensively in commercial hybrid production in China, and has been well characterized genetically. All seedlings were grown in pots in a growth chamber at 25°C and with a 14 h photoperiod and 60% relative humidity.

Bacterial strain and plasmid

Escherichia coli JM109 was used for cloning and propagation of the plasmids, and was maintained on Luria-Bertani medium. Plasmid pMD-18T (TaKaRa, Japan) was used for cDNA cloning. Fungal RNA silencing vector pSilent-1(Nakayashiki et al. 2005) was obtained from the Fungal Genetics Stock Center (Kansas, USA). Plasmid DNA was isolated from *E. coli* cells using the Plasmid DNA Extracting Kit (Sangon Company, China), following the protocols recommended by the manufacturer.

RNA extraction and cDNA synthesis

Total cellular RNA was extracted from the powdered mycelia of *C. lunata* isolates using a TRIzol Kit (Invitrogen, USA), according to the protocols recommended by the manufacturer. Total RNA was treated with DNase, and was measured by ultraviolet absorbance at $A_{260/280}$ (Eppendorff AG 22331, Germany). The yield of RNA was calculated as follows: $A_{260} \times 40 \times$ dilution factor $= \mu$g RNA/ml. The first-strand cDNA was synthesized using 5 μg of total RNA, Oligo(dT) primer, Random primer and M-MLV Reverse Transcriptase according to the manufacturer's instructions from the M-MLV RTase cDNA Synthesis Kit (Takara, Japan).

Full-length cDNA clone of *Brn1* and sequence analysis

For cloning the full-length cDNA, a pair of primers, A and B (Table 1), for RT-PCR was designed based on the sequence of amino acid identified by mass spectrometry (Xu et al. 2007). The 5′ end of the *Brn1* cDNA sequence was obtained via SMART™ RACE cDNA Amplification Kit (Clontech, CA, USA). For 5′ RACE PCR amplification, the reaction buffer consisted of 0.5 μl Extensor Hi-Fidelity PCR Enzyme Mix (5 U/l), 5 μl of (10 mM) Universal Primer A Mix, 5 μl of $MgCl_2$ (22.5 mmol/l), 1 μl of dNTP mixture (10 mM), 2 μl of specific primer (20 μM), 5 μl of the template (5′ RACE cDNA), and 31.5 μl ddH_2O. The amplification profile was as follows: 1 cycle at 95°C for 1 min, and 29 cycles at 94°C for 30 s, 55°C to 68°C for 30 s, and 72°C for 3 min. After a final extension of 10 min at 72°C, the samples were stored at 4°C. A 3′-Full RACE Core Set (TaKaRa, Japan) was used for the 3′-Full PCR amplification. The reaction solution consisted of 10 μl of the template (3′ RACE cDNA), 0.5 μl of the specific primer (20 μM), 0.5 μl 3 site Adaptor Primer (20 μM), 5 μl 10 × Ex Taq Buffer (Mg^+ plus), 0.25 μl TaKaRa Ex Taq (5 U/μl), and 33.75 μl ddH_2O. The amplification profile was as follows: 1 cycle at 95°C for 1 min, and 30 cycles at 94°C for 30 s, 47 to 68°C for 30 s, and 72°C for 3 min, followed by a final extension of 10 min at 72°C. The amplification was conducted in a Peltier Thermal

Table 1 PCR primers used in this study

Primer name	Sequence (5′-3′)
Primer A	GTCAACTACGCCAACGCCG
Primer B	GTT GTGA GGAGACCATGTG
Primer C	GACGGCAACAACCTGACT
Primer D	CAGTGCTGCTGGGAATGA
Brn1si-Xhol	CCGCTCGAGCAAAGGTCGCCGTCAACTAC
Brn1si-HindIII	CCCAAGCTTCTTGTCACCCCCAGGAATG
Brn1si-kpnI	CGGGGTACCCAAAGGTCGCCGTCAACTAC
Brn1si-BglII	GGAAGATCTGACCTTTCCGTTGACCCACT
Primer E	ATGGCCAACATTGAGCAGACATGCA
Primer F	TTACATGCAAGCAGCACCGTCAATG

Cycler Chromo 4 PTC 200 (Bio-Rad, USA). The resulting 5′ and 3′ RACE PCR amplification products were gel-purified and cloned into pMD 18T-Vector, and sequenced at Beijing AuGCT Biotechnology in Shanghai. The full-length cDNA of *Brn1* was obtained through sequence assembly by searching for the sequence overlap region. Alignment of the full-length cDNA with other homologous sequences was conducted with DNAMAN software (Lynnon Biosoft, Canada) and through GenBank searches (http://www.ncbi.nih.gov/).

Semi-quantitative RT-PCR analyses for *Brn1* expression of *C. lunata* isolates with different virulence and silenced mutants

Total RNA was extracted from the mycelia of CX-3 and DD-60 strains grown in PD medium for 24, 72, 96, and 144 h. Afterwards, to study the expression of the *Brn1*-silenced mutant, total RNA of the mutant T5 was also extracted at 96 h. Based on the cDNA clone of *Brn1*, Primers A and B were employed for *Brn1* amplification. Another pair of primers, C and D, for *GAPDH* nucleotide fragments was designed using PRIMER Premier 5.0 (Premier Biosoft, CA, US) as internal positive control (Xu et al. 2007). The PCR protocol and PCR reaction conditions used in this experiment were optimized to determine the comparative level of gene expression. To reach the plateau phase of PCR amplification, different numbers of amplification cycles (24, 28, 29, 30, and 31 cycles) and different concentrations of Mg^{2+} (15.0, 22.5, and 35.0 mmol/l) were used. PCR solution included 2 μl of $MgCl_2$ (25 mmol/l), 2 μl of dNTP mixture (5 mmol/l), 0.2 μl of Taq (5 U/μl), 1 μl of forward primer A (10 p mol/μl), 1 μl of reverse primer B (10 p mol/μl), and 2 μl of the template (RT product). The mixture was topped-up by ddH_2O to 25 μl. The PCR reaction was conducted with Peltier Thermal Cycler Chromo 4 PTC 200 (Bio-Rad, USA). Approximately 5 μl of the target PCR product was mixed with 1 μl 10×loading buffer; then, the mixture was separated by gel electrophoresis, as previous reported (Ausubel 2004; Jensen and Hahn 2000; Meng et al. 2004). The gene expression levels were analyzed by SynGene GeneTools Analysis Software (Version 3.02.00, Gene Company, USA) after the gel was imaged with Quantity One imaging software by VosaDoc 3000 (Bio-Rad, USA). The statistical analysis of gene expression was conducted with SAS 8.2 ANOVA. The results of the experiment were verified at least three times.

Silencing vector construction and transformation

The *Brn1* silencing vector, pSilentBrn1-2, was constructed by using fungal transformation vector pSilent-1 for use in constructing inverted repeats of the target *Brn1* gene cDNA fragment (533 bp). The sense Brn1-A cDNA fragment (533 bp) was first amplified using primers Brn1si-Xhol and Brn1 si-HindIII, with the *C. lunata* total cDNA as template. The amplified fragment was then digested with *Xho* I and *Hin*dIII, and cloned into the *Xho* I- *Hin*dIII site of pSilent-1, resulting in pSilentBrn1-1. Next, the antisense Brn1-B cDNA fragment (663 bp) was amplified using primers Brn1 si-kpnI and Brn1 si-BglII, with the *C. lunata* total cDNA as template. This amplified fragment was digested with *Kpn*I and *Bgl*II, and cloned into the *Kpn*I-*Bgl*II site of pSilentBrn1-1, resulting in pSilentBrn1-2.

The wild-type strain CX-3 was transformed with pSilentBrn1-2 by the PEG-$CaCl_2$ method. Flasks containing 100 ml of PDB inoculated with spore suspension (approximately 10^4 spores) were incubated at 28°C for 18 h at 120 rpm. The mycelia were collected, centrifuged for 3 min at 3,000×*g*, and washed twice with 0.7 M KCl. Approximately 1 g of mycelia was resuspended in 10 ml of 0.7 M KCl phosphate buffer (pH 5.6) containing 1% lywallzyme (Guangzhou Microbial Culture Collection Center, China) and 1% cellulose (Sigma, America). After incubation at 30°C for 4 h at 100 rpm, the protoplasts were separated from cell debris by filtration through cheesecloth, washed twice in STC (1.2 M sorbitol, 10 mM Tris-HCl at pH 7.5, 50 mM $CaCl_2$) buffer, and then resuspended in STC buffer to a concentration of approximately 10^7/ml.

An aliquot of 100 μl of the protoplasts was gently mixed with 10 μg of plasmid pSilentBrn1-2. A total of 300 μl polyethylene glycol (PEG) solution (40% PEG 4000 in STC buffer) was then added slowly into the mixture, 100 μl each time, at 20 min intervals. The solution was incubated on ice for 20 min, and then at room temperature for another 20 min. Approximately 200 μl of this mixture was overlaid onto a plate containing regeneration medium (PDA+ 0.7 M KCl) with 250 μg/ml hygromycin B. The plates were incubated at 28°C. After incubation for 5 d, the transformants were selected.

Southern blot

Southern blot was performed as previously described (Liu et al. 2010) to assay *Brn1* copy number in genome. Genomic DNA from CX-3 was digested with *Eco*RI, *Pst*I, *Xba*l, and *Hin*dIII for 24 h at 37°C. The *Brn1* DNA fragment amplified with primers E and F was labeled with digoxigenin as probe.

To analyze transgenic *C. lunata*, genomic DNA from the putative silencing transformants was digested with *Eco*RI and *Xba*l for 24 h at 37°C. The double strand *Brn1* cDNA fragment (533 bp) was used as probe and was hybridized with the DNA fragments attached to the filter.

Inoculation and toxin assay

Inoculation assay was carried out with the CX-3 and mutant T5, as described by Liu et al. (2009). Briefly, the fourth leaves of the susceptible maize seedlings at the 7-leaf stage were cut into 2 cm sections. Punctures were made on the leaf surface along the vein. Then, 10 μl of conidia suspension (10^6 spore ml^{-1}) was applied on the wounds, and the leaves were placed on moistened filter paper in Petri plates at 25°C. The symptoms were recorded after 3 d of incubation at 25° C using a standard method (Liu et al. 2010).

To determine whether the mutant retained the ability to produce virulence-related toxins, T5 was cultured in Fries medium, as described by Liu et al. (2010), and 2 μl of crude extract was spotted onto silica gel plates. The production of the toxin methyl 5-(hydroxymethyl) furan-2-carboxylate produced by the mutant was determined as described by Liu et al. (2009). Extracts from the wild-type strain CX-3 were used as positive control. These experiments were conducted in triplicate.

Data analyses

Data were analyzed using standard analysis of variance technique and means were separated using least significant difference (L.S.D) comparisons using the SAS statistical package (SAS Institute 1989).The gene expression data from sampling at 4 time points (24, 72, 96 and 144 h) of each strain were analyzed separately for standard error calculation.

Results

Full length *Brn1*cDNA cloning and its sequence

RACE strategy was used to obtain the full-length cDNA sequence of *Brn1* gene. Subsequently, a 1001 bp cDNA was cloned from the *C. lunata* mycelia, including an open reading frame of 801 bp (GenBank accession no DQ358052). Further analysis suggests that the *C. lunata* Brn1 protein is composed of 267 amino acids with a molecular mass of approximately 43 kDa based on gel electrophoresis (Xu et al. 2007). The amino acid sequence prediction was deduced from NCBI. The BLAST analysis indicates that the deduced amino acid sequence of Brn1 protein is highly homologous with proteins from *Cochliobolus lunatus*, *Cochliobolus heterostrophus*, *Bipolaris oryzae*, and *Alternaria alternata* (Fig. 1).

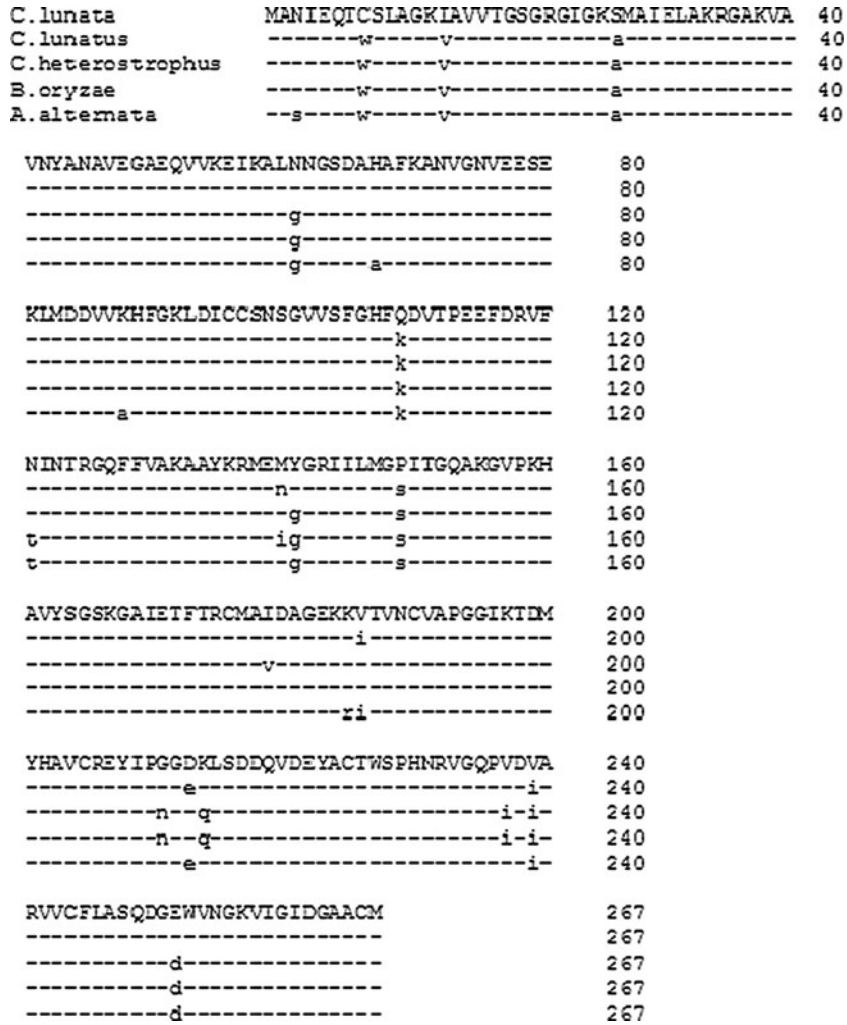

Fig. 1 Alignment of the deduced amino acid sequence of *C. lunata* Brn1 protein with proteins of other microorganisms. Note: Identity of the deduced amino acid sequence of Brn1 protein with protein from *Cochliobolus lunatus* (hydroxynaphthalene reductase) is 96% (AF419330) (Rizner and Wheeler 2003), *Cochliobolus heterostrophus* (Brn1) is 95% (BAA24255), *Bipolaris oryzae* (reductase) is 94% (BAC20956), and *Alternaria alternata* (1, 3, 8-naphthalenetriol reductase) is 94% (BAA36503)

In vitro assay of *Brn1* expression in *C. lunata* isolates with different virulence

PCR amplification conditions were optimized (data not shown), and the changes of *Brn1* expression during the cultural time (24, 72, 96, and 144 h after incubation) were investigated. *Brn1* expression increased with time and peaked at 96 h ($p<0.05$) in both the highly virulent and weakly virulent isolates (Fig. 2a–c). The expression of *Brn1* in the highly virulent CX-3 was higher than the expression in the weakly virulent DD60 at 72, 96, and 144 h. In addition, the colour of the mycelia of both isolates became darker along the time course (Fig. 2d and e).

Role of *Brn1* in melanin biosynthesis

9 hygromycin resistant transformants were obtained with pSilentBrn1-2 by PEG-$CaCl_2$ method, and among which 3 transformants (T2, T4, and T5) showed light brown colonies compared with the wild-type (Fig. 4a). To determine whether the transcriptional unit was integrated in the genome of these 3 transformants, genomic DNA was digested with *EcoR*I and *Xba*I, then probed with the sense *Brn1* cDNA fragment (533 bp). A 3.4 kb fragment representing the entire transcriptional unit of *Brn1* hairpin RNA was found in all 3 transformants, indicating that an intact *Brn1*-inverted repeat element was integrated in the genome (Fig. 4b). Semi-

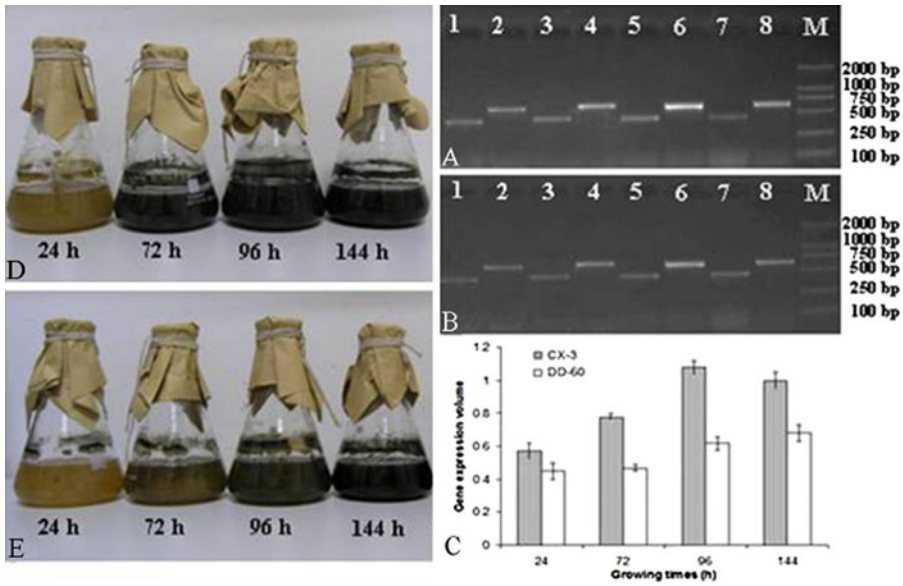

Fig. 2 Mycelial growth and *Brn1* expressions of *C. lunata* isolates after 24, 72, 96 and 144 h at 28°C in PD. **a** PCR products of CX-3 by gel electrophoresis **b** PCR products of DD-60 by gel electrophoresis. For A and B, Lane 1 to 8 represent gene expressions at 24, 72, 96 and 144 h, respectively. Lanes with odd number represent PCR products of the *GAPDH* gene, and lanes with even number represented PCR products of *Brn1* of the isolates. Lane M represents DNA standard (DL2000, TaKaRa, Japan). **c** a diagram comparing gene expressions of the isolates. **d** mycelia of highly virulent isolate CX-3. **e** mycelia of low virulent isolate DD-60

quantitative RT-PCR analysis shows that *Brn1* mRNA expression in the albino phenotype mutant T5 was inhibited significantly compared with that of the wild-type (Fig. 4c), confirming that *Brn1* is involved in melanin biosynthesis.

Association of *Brn1* in toxin biosynthesis

To examine the association of Brn1 expression with toxin biosynthesis, detached maize leaves from 7-leaf stage seedling were used to measure the ability of the mutant to infect host plants. The results revealed that the Brn1-silenced mutant T5 caused a slight tissue maceration surrounding the inoculation site in contrast to a massive, chlorotic tissue maceration associated with the wild type CX-3 inoculation site (Fig. 5a). To further understand the relevance of Brn1 with toxin production, mutant T5 was grown in Fries mediums for 15 d at 28°C; the toxin in cultures was then detected by thin-layer chromatography. The results indicate that T5 lost the capacity to produce toxin. Another assay was conducted by adding of 5 μl of toxin (10^{-4}M) into mutant T5 conidia suspensions, and then using the mixture to treat maize leaves. We found that the typical leaf necrotic spots reappeared (Fig. 5a, b). Therefore, *Brn1* is closely linked to toxin production.

Discussion

This study represents the first report of cloning and characterization of the Brn1 gene from *C. lunata*, a causative agent of maize leaf spot. The Brn1 gene shows over 96% sequence similarities with Brm2 from *Alternaria alternata* and Brn1 from *Cochliobolus heterostrophus*. The latter two genes were functionally characterized to encode a 1,3,8-THN

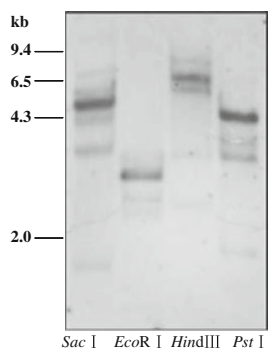

Fig. 3 *Brn1* copy number in genome of *C. lunata* isolate CX-3

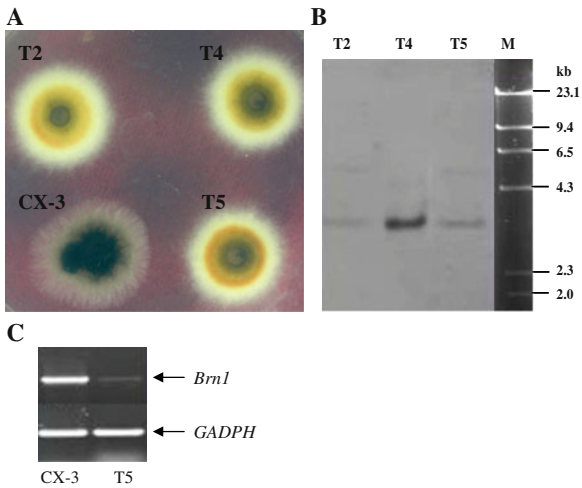

Fig. 4 Characterization of *Brn1* silenced transformants. **a** Colony morphology of the wild-type (CX-3) and hygromycin-resistant transformants with a light brown phenotype (T2, T4, T5) on PDA. **b** Southern blot analysis of the hygromycin-resistant transformants with a light brown phenotype (T2, T4, T5), M: Lambda-DNA-*Hin*dIII DNA marker. **c** Semi-quantitative RT–PCR analysis of *Brn1* mRNA expression in CX-3 and hygromycin-resistant transformants with a light brown phenotype (T5)

reductase that converts 1,3,8-trihydroxynaphthalene to vermelone or 1,8-dihydroxynaphthalene (DHN) in the DHN melanin synthesis pathway (Kawamura et al. 1999; Rizner and Wheeler 2003). Our study showed that the Brn1-silenced mutants of *C. lunata* with decreased Brn1 expression displayed an albino phenotype on colony growth, suggesting that Brn1 gene of *C. lunata* is an orthologue of Brm2 from *A. alternata* and Brn1 from *C. heterostrophus*.

Melanin is known to consolidate the mechanical penetration structures of phytopathogens, such as appressoria and infection pegs, required for effective penetration. Melanin has been recognized as an important virulence determinant in *Wangiella dermatitidis* (Schnitzler et al. 1999; Feng et al. 2001), *Sporothrix schenckii* (Hamilton and Gomez 2002), *C. laenarium* (Kubo et al. 1996), *M. grisea* (Money 1997; Howard and Ferrari 1989) and *Verticillium dahliae* (Wheeler et al. 1978). Previous studies have also indicated that melanin substances, such as DHN, are able to increase the mechanical strength of the germ tube cell, which in turn improves the efficiency of pathogen penetration (Bell et al. 1976; Bell and Wheeler 1986; Idnurm and Howlett 2001; Vidal-Cros et al. 1994). We also found melanin accumulation in the mycelia and germ tube cells of *C. lunata* through immunofluorescence marking (data not shown). The result is consistent with our previous finding that there is a certain percentage of albino mutants present in wild-type strains collected from naturally infected leaves. These albino mutants are weakly virulent, based on artificial leaf inoculation test (Yan et al. 2005). These findings further support our conclusion

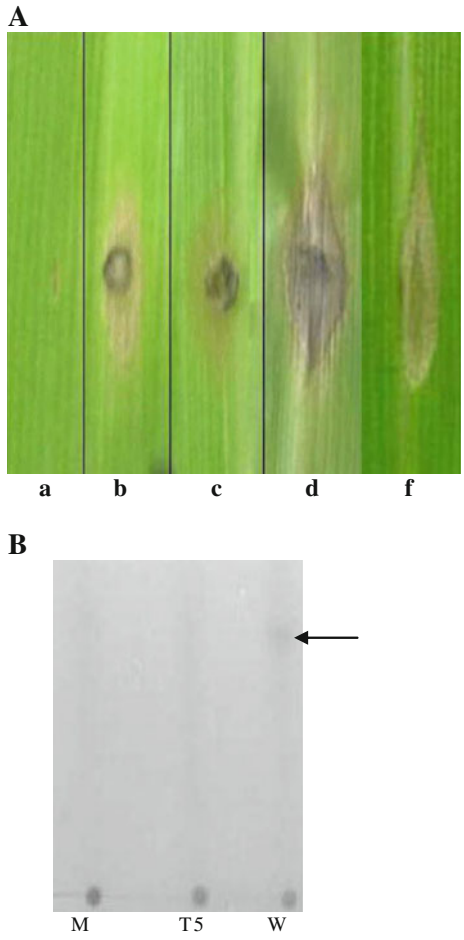

Fig. 5 Disease symptoms and toxin production of the Brn1-silenced mutant. **a** Disease symptoms on the wounded leaf tissues of maize inoculated with droplets of H_2O (**a**), conidia suspension of the wild-type CX-3 (**b**), conidia suspension of the Brn1-silenced mutant T5 (**c**), conidia suspension of the Brn1-silenced mutant T5 in the presence of *C. lunata* toxin (**d**), and toxin (10^{-4} M) (f) , respectively. The representative symptoms on inoculated leaf tissues were photographed 3 days after inoculation. **b** Thin-layer chromatography analysis of toxin productivity in culture extracts of the Brn1-silenced mutant (T5), the wild-type strain (W), and non-inoculated medium (M)

that melanin is a crucial virulence factor for *C. lunata* in susceptible maize leaves.

In addition, the copy number of the gene in *C. lunata* genome was determined by Southern blot analysis prior to the *Brn1* function study. Three to four bands hybridized with the *Brn1* probe with different enzymes (Fig. 3), which implies that the gene might be present either as a single copy or as multiple copies, or that the bands are other closely related genes in the *C. lunata* genome. Thus, RNAi technique was applied with the *Brn1* function analysis. The RNAi mutant indicates that *Brn1* is not only responsible for melanin synthesis but also contributes, to some extent, to toxin production. The reduced toxin production from the *Brn1* RNAi mutant T5 illustrates that the gene might also be a regulator of toxin production. From the result, we hypothesize that a mechanism is present in *C. lunata* that synergistically regulates both virulence factors. In a separate study, we have cloned a toxin production-related gene *Clt*-1 from a *C. lunata* ATMT library and showed that the Brn1-silenced mutant T5 was very low in *Clt*-1 expression (data not shown). Current data further revealed that the Brn1-silenced mutant T5 was deficient in toxin production and in melanin biosynthesis. We proposed that the Brn1 gene may serve as a common regulator controlling two separate metabolic pathways for toxin and melanin biosynthesis, respectively. Furthermore, other virulence factors might also be involved during infection of maize leaves, such as cell wall-degrading enzymes, because silenced *Brn1* mutants are still capable of slight infection in maize leaves (Feng et al. 2002) (Figs. 4 and 5).

Melanins are complex substances; thus, many different enzymes are involved in the synthesis process, which may lead to the production of an array of intermediate metabolites (Butler and Day 1998). Therefore, the role of melanin in the consolidation of pathogen penetration structures might not simply depend on a single melanin, but rather on a mixture of various melanin compounds. Taken together, the real role of the melanin complex in pathogen penetration into host plants should be further investigated.

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