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FUNGAL DISEASES

Bchex virulence gene of *Botrytis cinerea*: characterization and functional analysis

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Abstract We previously identified *Bchex* as a highly expressed gene during filamentous growth in *Botrytis cinerea*. The gene encodes the principal protein of the Woronin body and has been shown to seal septal pores in response to cellular damage. In the present study, Southern blot analysis of genomic DNA indicated that the gene exists as a single copy in the *B. cinerea* genome. The gene was differentially expressed during various developmental stages: expression was high in germinating conidia and the mycelial stage and lower in resting conidia and the appressorial stage. For functional analyses, homologous recombination was used to obtain a Δ*Bchex* knockout mutant. Growth of the mutant was strongly reduced growth in complete medium and in defined media with sucrose, fructose or pectin as the carbon source. After detached tomato leaves were inoculated with the *Bchex* mutant, lesion development was markedly reduced compared to the control, suggesting that *Bchex* participates in normal growth, germination and virulence of this fungus.

Keywords *Botrytis cinerea* · Woronin body · Virulence

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Introduction

Botrytis cinerea, also known as gray mold fungus, is an important pathogen of nursery plants, vegetables, ornamental, field and orchard crops including stored and transported agricultural products. Due to the broad host range of *B. cinerea*, considerable efforts are invested in protecting the agricultural produce against *Botrytis* before and after harvest.

Traditional chemical control remains the main way to reduce the incidence of gray mold. However, chemical pesticides are becoming less accepted due to the development of resistance and to negative public perception regarding its safety. Owing to the enormous economical relevance of *B. cinerea*, it is thus necessary to develop new and efficient strategies to prevent infection. Our approach to resolve this problem is to identify virulence genes involved in infection and use this knowledge to generate resistant plants and design new, more specific and efficient fungicides.

The interaction of this fungus with plants begins with conidial attachment to the host surface and continues with conidial germination. The fungus grows through polarized tip extension, resulting in the formation of a tubular cell (hyphae), which is subsequently compartmentalized by the formation of perforated septa. Then, penetration structures are formed, which colonize the plant tissue. In previous work, we described a preliminary analysis of an EST library from *B. cinerea* growing in axenic cultures. From

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this analysis, we identified the hex gene (*Bchex*) as being highly expressed during filamentous growth (Silva et al. 2006). This gene, which encodes the principal protein of the Woronin body, is involved in hyphal growth. When hyphae are damaged, Woronin bodies appear to plug the septal pores within a few minutes (Collinge and Markham 1985).

In addition, *hex*-1 mutants of *Magnaporthe grisea* and *Aspergillus oryzae* also lack visible Woronin bodies and display defects consistent with the loss of damage-induced plugging of the septal pore (Maruyama et al. 2005; Soundararajan et al. 2004). However, Woronin bodies may also be a new type of peroxisomal vesicle (Jedd and Chua 2000). Peroxisomes are a ubiquitous class of subcellular organelle that carry out diverse functions associated with anabolic and catabolic pathways, including peroxide metabolism, the β-oxidation of fatty acids and the biosynthesis of phospholipids (Lazarow and De Duve 1976; Subramani 1998). This information could be important because, as described previously, lipid droplets are abundant in fungal conidia and are mobilized during conidial germination in *M. grisea* (Barbosa et al. 2006; Thines et al. 2000; Weber et al. 2001). Enzymes involved in lipid breakdown via the glyoxylate cycle, a metabolic process carried out in peroxisomes, have been shown to be upregulated in germinating conidia, and disruption of the genes encoding glyoxylate cycle enzymes delays or inhibits conidial germination (Solomon et al. 2004; Wang et al. 2003).

Herein, we report the isolation and characterization of *Bchex* and its expression profile during growth and development. In addition, our phenotypic characterization of knockout mutants suggests that *Bchex* is essential for normal growth, germination and virulence of this fungus.

Materials and methods

Fungal growth

Botrytis cinerea isolates B05.10 and A1 were grown on malt yeast extract agar (MYE) medium (2% [w/v] malt extract, 0.2% [w/v] yeast extract and 1.5% [w/v] agar; complete medium) at 22°C.

Nucleic acid manipulation, expression and Southern blot analysis

Fungal isolates were grown in 50 mL of MYE liquid medium for 96 h at 22°C without shaking. Total DNA from *B. cinerea* was isolated according to Murray and Thompson (1980). RNA was extracted from frozen mycelium from different developmental stages using the Trizol reagent protocol. Southern blot hybridization experiments were performed with genomic DNA digested with three restriction enzymes (*Eco*RI, *Hin*dIII and *Ava*I). DNA probes were labeled with biotin (BioNick Labeling System, Invitrogen, Carlsbad, CA, USA).

Isolation, cloning and sequencing of *Bchex*-coding region from a *B. cinerea* cDNA library

Specific primers H_1 and H_4, which flank the *Bchex*-coding region, were designed from contig 609 obtained from the cDNA library (Silva et al. 2006) (Table 1). The PCR mixture contained one unit of *Taq* polymerase, 10 µM of each primer, 1.5 mM $MgCl_2$; 12.5 mM dNTPs, approximately 20–40 ng of *B. cinerea* genomic DNA and H_2O. This PCR yielded a product of ca. 700 base pairs (bp). Cloning experiments were performed using standard protocols (Sambrook et al. 1989). Primers H_1 and H_4 were also used to amplify *Bchex* from the B05.10 isolate.

Real-time PCR

Primers for real-time PCR assays and amplification efficiencies (E) are shown in Table 1. The real-time PCR reactions were performed in the Mx3000P QPCR System (Stratagene, Santa Clara, CA, USA) using the KAPA SYBR PROBE FAST qPCR Kits (Kapa Biosystems, Woburn, MA, USA). The 20 µL PCR reactions contained 2 µL of a 1:50 diluted cDNA sample, 200 nM of each primer and 1× KAPA SYBR PROBE FAST qPCR Master Mix Universal (Kapa Biosystems). The reference dye ROX was included at a final concentration of 5 nM. The cycling protocol was as follows: initial denaturation for 20 s at 95°C; followed by 40 cycles of 3 s at 95°C and 20 s at 56°C; then a final extension of 3 s at 72°C. Fluorescence was measured after the final extension. Specific amplification was confirmed by a single peak in the melting curve. For each experimental condition, total RNA was extracted from two independent *B. cinerea* cultures. Each RNA sample was retro-transcribed, and the expression of all genes was assessed for the same cDNA sample. The reactions for each target gene were performed in triplicate and in the same PCR run. Expression of the genes was calculated by the method of Livak and Schmittgen (2001). Transcript levels were normalized against the expression levels of the reference genes encoding elongation factor 1α and actin (qPCR using primers BcAct-RT1; BcAct-RT2; BcEF-RT1 and BcEF-RT2 listed in Table 1). Previously, the stability of gene expression of reference gene was evaluated using the Excel-based application geNorm (Vandesompele et al. 2002).

Table 1 Oligonucleotide primers used in this work

Primer (gene)	Sequence (5′–3′)	Restriction site	Efficiency qPCR primers
H_1	ccgtgttcttgatctcca		
H_2	gatacggatgctaccac		
H_3	atcttctttccctctctcca		
H_4	tatgtgttgggcagcggcgt		
H_5	cat**ctcgag**ccgttaccatcccttgccaccac	*Xho*I	
H_6	atgaaaacaaaaaggaaactc		
Hyg-1	gt**gaattc**tgcagctgtggagccgcattc	*Eco*RI	
Hyg-2	ct**gaattc**catgaattgaaggcggcactggc	*Eco*RI	
TuB-F	gttggctgttaacatggttcc		
TuB-R	cttcaccagtgtaccaatgca		
BcAct-RT1	tctgtcttgggtcttgagag		98
BcAct-RT2	ggtgcaagagcagtgatttc		97
BcEF-RT1	atgctatcgaccctccttcc		99
BcEF-RT2	gttgaaaccgacgttgtcac		97
hex-A-RT1	tctacttcaacgaggcttc		94
hex-A-RT2	caccagattgaccgaaaac		95

Disruption of the *Bchex* gene

To construct a *Bchex* knockout vector, we isolated *hex* by PCR using primers H_1/H_2 and H_3/H_4 (Table 1) from genomic DNA of isolate B05.10 (Fig. 3a). The fragment obtained was ligated to a linearized pBluescript vector containing a hygromycin-resistance cassette. The resulting knockout plasmid was verified by PCR (data not shown). *Botrytis cinerea* was transformed with the PCR product according to the protocol of Reis et al. (2005).

Fungal transformation

The haploid *Botrytis cinerea* wild-type strain B05.10 was grown on tomato malt agar (1.5% [w/v] ME with 250 g of homogenized tomato leaves per liter, 1.5% [w/v] agar) and incubated for 5–14 days at 22°C. Transformation was performed as described previously (Reis et al. 2005), and transformants were selected on SH agar (1% [w/v] malt extract, 0.4% [w/v] glucose, 0.4% [w/v] yeast extract, 1.5% [w/v] agar, pH 5.5) supplemented with hygromycin (70 µg/mL each). After single-spore isolation, transformants were cultivated without selection on tomato malt agar.

Fungal growth and germination assays

Botrytis cinerea conidia were collected from dishes and resuspended in MYE medium. Five microliters of conidia suspension (10^5 conidia/mL) placed onto MYE agar. Petri dishes were incubated at 22°C, and colony diameters were measured for 4 days. Germination was tested on glass microscope slides positioned in the wells of a 12-well microtiter plate, with 500 µL of a conidial suspension (2.5×10^4 conidia/mL) either in double-distilled water or in solutions containing various supplements. After 7 h at 22°C, germination rates were determined. Conidia were counted as germinated when the germ tube length exceeded the diameter of the conidium. Subsequent hyphal growth was tested on a minimal medium, consisting of 1.5% (w/v) agar and salt solution containing KH_2PO_4, K_2HPO_4, $MgSO_4{\cdot}7H_2O$, KCl, $FeSO_4{\cdot}7H_2O$, supplemented with either 1% (w/v) sucrose, fructose, pectin or oleic acid as a carbon source (Cotoras et al. 2009).

Infection tests

The ability of *B. cinerea* mutants to colonize tomato leaves was assessed as described by Benito et al. (1998). Conidia were harvested, washed and resuspended in MYE medium. The suspension was incubated for 1 h at room temperature. Detached tomato leaves were surface-sterilized with 10% sodium hypochloride and placed on Petri dishes containing water agar (1.5% [w/v] agar). Five microliters of a conidial suspension (10^5 conidia/mL) was placed on one half of the upper side of a tomato leaf. On the other half, sterile MYE medium was deposited as a control. Petri dishes were incubated at 22°C.

Sequence analysis

The predicted amino acid sequence of *Bchex* was aligned with the sequence of HEX of *Sclerotinia sclerotiorum* (XP_001595438.1), *Neurospora crassa* (XP_963707.1), *Chaetomium globosum* (XP_00122427) and *M. grisea*

(XP_366620.2) using CLUSTAL W (Thompson et al. 1994).

Results

Identification and characterization of *Bchex*

From a *B. cinerea* cDNA library described previously (Silva et al. 2006), we identified a sequence highly homologous to the *hex-1* gene from *N. crassa* (AF001033). Gene-specific primers were designed and used to amplify the *Bchex* gene from genomic DNA of *B. cinerea* strain A1. After subcloning, the amplified fragment of ca. 546 bp was sequenced (not shown). *Bchex* has a coding region interrupted by two introns of 50 and 66 bp. The open reading frame of *Bchex* encodes a predicted (mature) polypeptide of ca. 160 amino acids. This polypeptide sequence has 71% identity with the *N. crassa* Woronin body major protein (AF001033), 65% identity with HEX-1 of *A. nidulans* (AF239659) and 76% identity with HEX-1 of *M. grisea* (AF170544). These levels of identity were sufficiently high to assign a similar putative function to the *hex* gene of *B. cinerea* (Fig. 1). Analysis of the structure of Woronin bodies has led to the suggestion that they are specialized types of peroxisomes (Jedd and Chua 2000). The HEX-1 protein of other fungi has a typical peroxisomal targeting signal, PTS1 at the C-terminus (Tey et al. 2005). Subsequently, using the same primers, we isolated a full-length *Bchex*-coding region from B05.10 DNA. After digestion with restriction enzymes (*Eco*RI, *Hin*dIII and *Ava*I) and under moderate stringency hybridization conditions, it was possible to show that *Bchex* also occurs as a single copy in the genome of B05.10 isolate (See supplementary material). To monitor the expression of the *Bchex* gene, we ran a quantitative RT-PCR analysis with RNA samples from nongerminated conidia on agar dishes after 0, 1, 3, 6 and 12 h of incubation. On the basis of the hex mRNA levels in nongerminated spores, germinating spores and at appressorium formation and vegetative mycelium stages of *B. cinerea*, we conclude that *Bchex* expression is at a maximum at 3 and 12 hpi (Fig. 2). This pattern is similar to that described for *M. grisea*, where HEX transcripts accumulate only in germinating spores and vegetative cells, not at the appressorial stage (Asiegbu et al. 2004).

Generation of Δ*Bchex* mutants

To examine the role of the Woronin body in *B. cinerea*, we made disruption mutants of *Bchex*. After transformation with PCR fragments carrying the disruption construct, hygromycin-resistant colonies were isolated, and their DNA analyzed. We obtained 30 hygromycin-resistant colonies. Preliminary analysis of 20 colonies by PCR led to the identification of four putative knockout mutants (not shown). After single-spore isolation, confirmation of the targeted deletions was obtained for Δ*Bchex* and ectopic

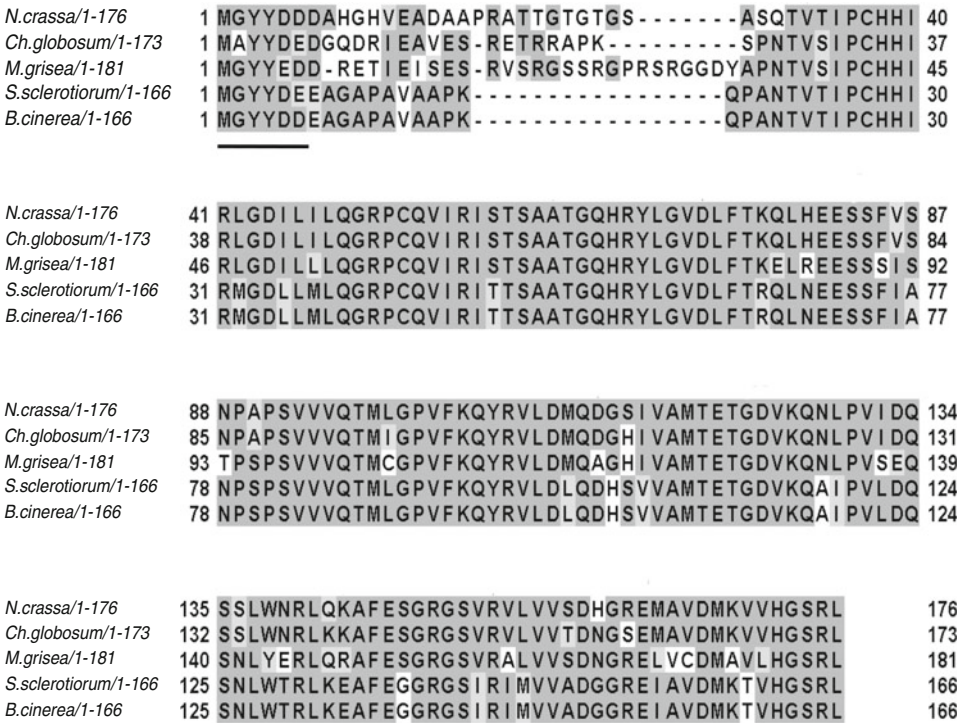


Fig. 1 Sequence alignment of HEX proteins. Alignment of deduced amino acid sequence of the HEX protein from *Botrytis cinerea* with sequences from other ascomycetes available in GenBank database. Residues that are identical in all sequences are marked in *black*. *Underlined* sequences correspond to the destination signal for the peroxisome

mutants by means of PCR using H_5 and H_6 specific primers (Fig. 3b). As expected, Δ*Bchex*-1, Δ*Bchex*-2 and ectopic mutants yielded a band at ca. 2.6 kb due to the introduction of the hygromycin cassette. As expected, the wild-type isolate did not have a PCR product at ca. 2.6 kb. As might be expected, only Δ*Bchex* mutants had a band at 0.6 kb corresponding to correct knockout insertion. Further confirmation of the targeted deletions was obtained for two Δ*Bchex* mutants by means of RT-PCR (Fig. 3c; Table 1).

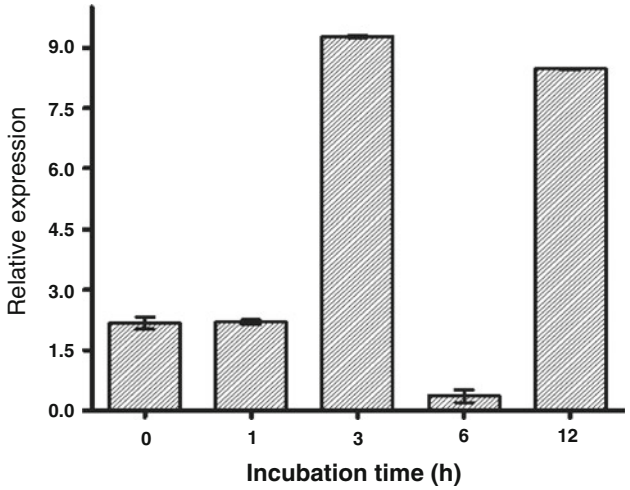


Fig. 2 Expression of *Bchex* at different developmental stages of *Botrytis cinerea*. Relative expression of *Bchex* during germination on agar plates after 0, 1, 3, 6 and 12 h of incubation. Means of three biological replicates, with three replicates each are shown. *Bars* indicate standard errors

Morphology and infection phenotype of Δ*Bchex* mutants

Macroscopic observation of *Bchex* mutants showed differences in colony morphology when compared to the wild-type B05.10. On 2% MYE medium, colonies of the wild-type strain and the ectopic mutants were gray, less dense and larger than the small, compact colonies with thickly packed mycelia observed for the *Bchex* knockout mutants (Fig. 4a). Examination of germination morphology differed markedly between mutants and the wild type at different times. The mutants take longer to germinate, and they are circular and smaller than the wild type (Fig. 4b). When conidia germinated in complete medium, the germination rate of the *Bchex* mutants was strongly reduced. For instance, <10% of mutant conidia germinated after 7 h of incubation, reaching 100% germination at 15 h. In comparison, 100% of the wild type had germinated by 5 h (Fig. 4c). Clearly, germination was delayed for the mutants.

We also studied the effect of the carbon source on the germination of the mutant strains. With pectin, fructose,

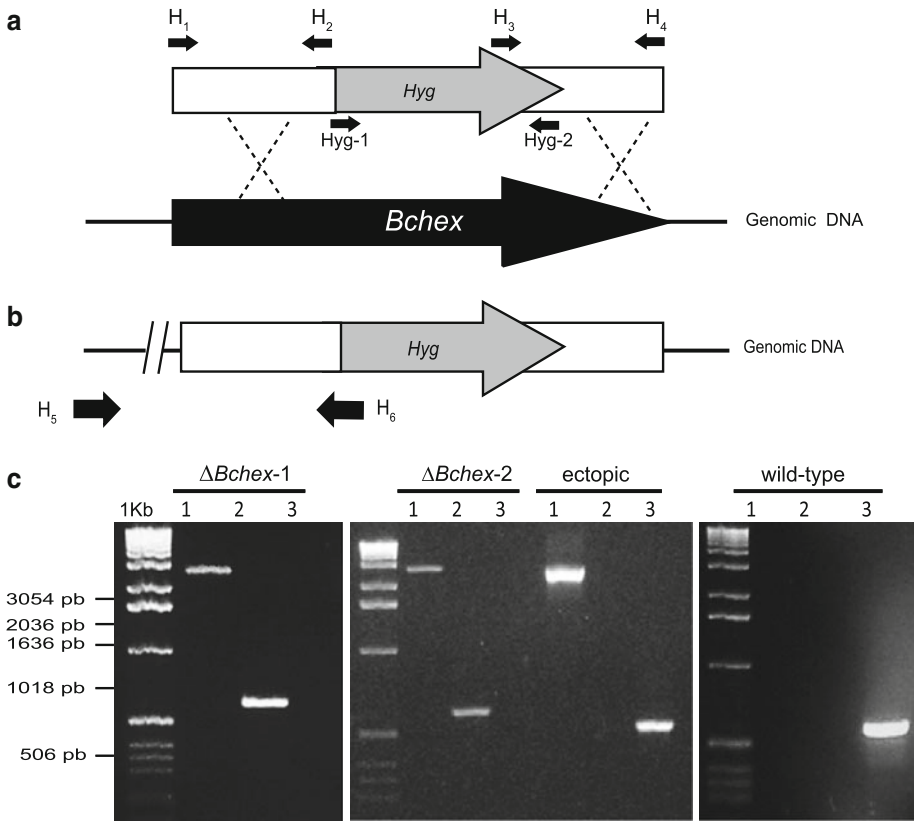


Fig. 3 Schematic diagram of the *Bchex* mutants. **a** Cloning strategy. The region coding for the *Bchex* gene is indicated in *white boxes*. *Gray color* indicates the hygromycin-resistance cassette. A *dashed line* indicates the homologous recombination. **b** Configuration of the expected *Bchex* mutant (Δ*Bchex*). A *black arrow* indicates the sites of the primers H_5 and H_6. **c** Gel of electrophoretic products of expected PCR DNA fragments from Δ*Bchex* gene. *Lane 1* PCR products using primers Hyg-1 and Hyg-2 (Table 1). *Lane 2* PCR products using primers H_5 and H_6 (Table 1). *Lane 3* cDNA obtained from mRNA of *hex* gene from two individual transformants (Δ*Bchex*-1 and Δ*Bchex*-2), ectopic and wild type, respectively

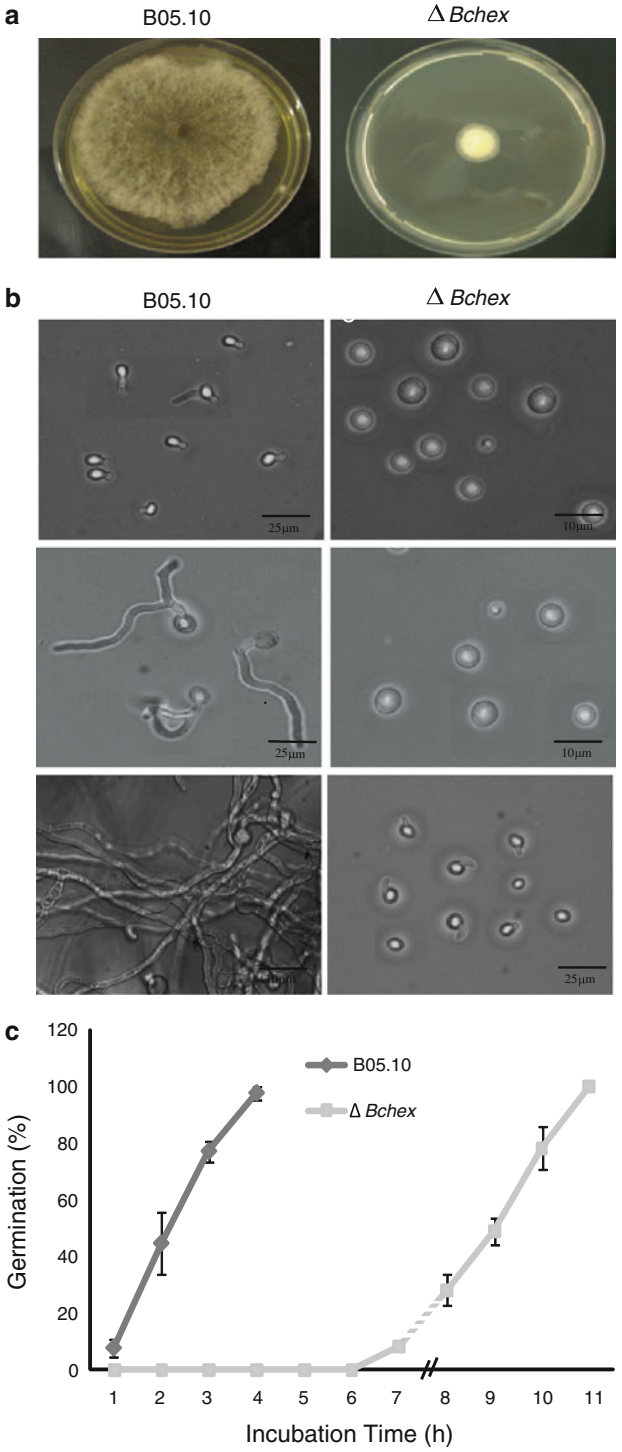


Fig. 4 Morphology and germination of the wild-type and *Bchex* strains. **a** B05.10 wild type (*left*) and Δ*Bchex* mutant (*right*) grown on complete medium for 5 days. **b** Micrographs of B05.10 wild type and Δ*Bchex* mutants incubated on complete medium at 22°C for 3, 7 and 12 h. **c** Germination rate of wild type (*black line*) and Δ*Bchex* mutant (*gray line*) under same conditions. Values are the mean ± SD of three independent experiments with at least two replicates each

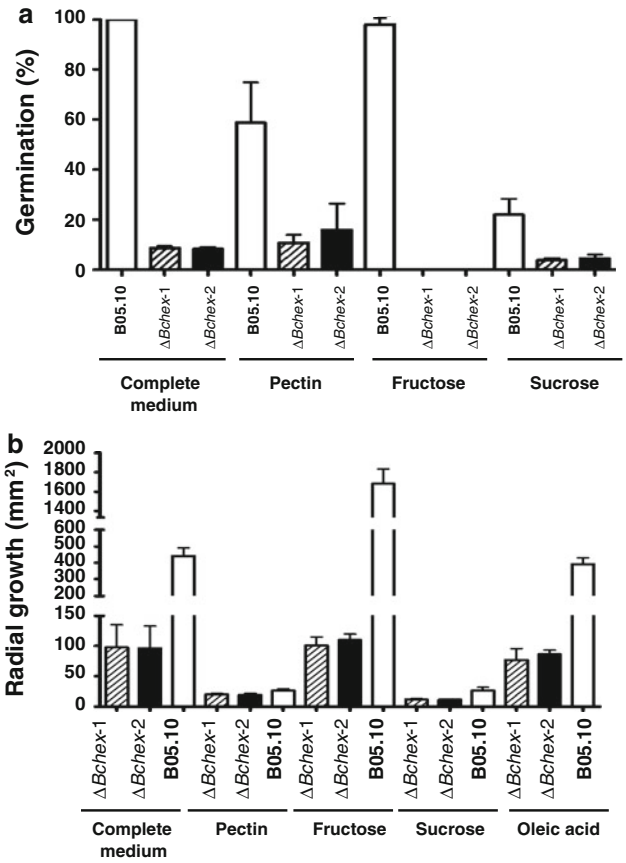
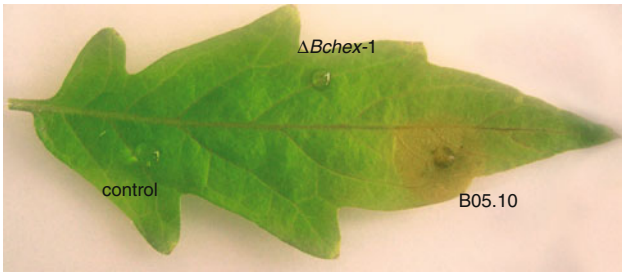


Fig. 5 Germination of conidia (**a**) and growth of hyphae (**b**) of wild type and Δ*Bchex* mutants of *Botrytis cinerea* grown on different carbon sources. Wild type (*white boxes*) and Δ*hex* mutants (*hatched* and *black boxes*) of *B. cinerea* conidia after 7 h. **b** Radial growth after 4 days. Values are the mean ± SD of three independent experiments with at least two replicas each

and sucrose, we observed the same germination pattern in which the wild type had higher levels than did the mutants (Fig. 5a). Interestingly, the Δ*Bchex* was unable to grow when an agar block of mycelium was cut and cultured in liquid medium.

To evaluate the physiological role of *Bchex* gene, radial hyphal growth was studied. Consistent with the observed morphological differences, all *Bchex* mutants revealed a pleiotropic phenotype, with dramatically reduced growth on complete medium. To clarify whether *Bchex* growth is dependent on the carbon source, we evaluated fructose, sucrose and pectin as the carbon source (Fig. 5b). On sucrose and pectin as the carbon source, the mutants and the wild type displayed a marked reduction in hyphal growth in comparison with those grown on complete and fructose media. In the presence of oleic acid, hyphal growth of the mutants was reduced compared to the wild type. Nevertheless, hyphal growth of the wild type was



	day 1	day 2	day 3	day 4	day 5
Δ*Bchex*-1	0.0	0.0	0.1 ± 0.2	4 ± 6.9	4.1 ± 6.8
Δ*Bchex*-2	0.0	0.0	0.0	1.2 ± 1.3	1.3 ± 0.7
B05.10	3.4 ± 0.2	25.2 ± 11.2	120.3 ± 20.2	318.5 ± 160.6	580.4 ± 66.6

Fig. 6 Tomato leaf at 72 h and mean lesion area (mm^2) ± SD after inoculation with *Botrytis cinerea* wild type and Δ*Bchex* mutants. Leaves were inoculated with a droplet of conidia from wild type B05.10 and Δ*Bchex* in three independent experiments with at least two replicates each

always greater in comparison with the mutants. Thus, *Bchex* appears to participate not only in germination but also in hyphal growth.

To check the pathogenic performance of the mutants, we inoculated plant tissues with conidia of the mutants and the wild type (Fig. 6). The Δ*Bchex* conidia failed to cause lesions on the host leaf, but the wild-type strain caused a typical lesion after 72 h. The lesion was small and restricted to a primary lesion accompanied with chlorosis. On the basis of the marked absence of lesions, we conclude that the Δ*Bchex* was greatly reduced in virulence and could not infect and colonize tomato leafs.

Discussion

The *hex* gene from *B. cinerea* was identified and isolated from a cDNA genomic library of this fungus (Silva et al. 2006). The predicted protein HEX-1 from *B. cinerea* shares 71 and 76% identity with the HEX protein from *N. crassa* and *M. grisea*, respectively (Asiegbu et al. 2004; Tenney et al. 2000).

The functional studies carried out here were facilitated by the fact that the gene existed as a single copy in the genome of *B. cinerea*. In this study, the phenotypic effects of disruption of *Bchex* were cleary manifested. Consistent with reports for *M. grisea* (Asiegbu et al. 2004), the *Bchex* mutant displayed a dark-pigmented conidial phenotype compared to the wild type and the ectopic mutants. On the other hand, on complete medium as well as on different carbon sources, growth rate of the *Bchex* mutant was reduced in comparison with the wild type. These results diverge from those reported for other ascomycetes in which mycelia of *hex*-1 mutants grew at a typical rate in complete medium (Asiegbu et al. 2004; Jedd and Chua 2000). On the other hand, the hypothesis that Woronin bodies function as plugs of septal pores has been based entirely on microscopic observations (Collinge and Markham 1985). Our results provide important evidence that this hypothesis appears correct because the *Bchex* mutant was unable to grow when an agar section of the colony was excised and cultured in liquid medium. Therefore, it is tempting to suggest that *Bchex* is strongly involved in hyphal elongation and mycelial growth in *B. cinerea*. These results are concordant with those for *M. grisea* and *A. oryzae* mutants with defects consistent with the loss of damage-induced plugging of the septal pore (Maruyama et al. 2005; Soundararajan et al. 2004).

The characterization of Δ*Bchex* allowed us to conclude that the participation of this gene is not limited to growth and hyphal development; germination was also delayed, permitting us to suggest that the decreased growth may be due to late germination. Therefore, the *Bchex* gene could be important at different developmental stages in *B. cinerea*. In agreement with this hypothesis, analysis of mRNA levels by qPCR in ungerminated spores, germinating spores, and during appressorium formation and vegetative growth of *B. cinerea* showed that *Bchex* transcripts accumulated substantially only during spore germination and hyphal growth. These results are similar to those described by Asiegbu et al. (2004) for *M. grisea.* Perhaps *Bchex* encodes proteins involved in plugging or sealing of the septal pore as well as in the repair of damaged hyphae, indicating that they may play an essential role in the survival of the pathogen in the host tissue.

Thus, how could this gene affect *B. cinerea* germination? Interestingly, a link between Woronin bodies and peroxisomes has been described for *N. crassa* (Managadze et al. 2007). Evidence shows that Woronin bodies may be a new type of peroxisomal vesicle (Jedd and Chua 2000); consequently, these bodies will be related to energy obtained by fatty acid metabolism. Therefore, a deletion of *Bchex* could result in a reduction of cellular respiration, affecting germination. Supporting this hypothesis, many spores can germinate and sustain growth of the germ tube for at least a short time in the absence of exogenous nutrients (Barbosa et al. 2006; Lucas and Knights 1987), indicating that they use endogenous supplies of carbon and nitrogen to support this early growth and development. Lipids and carbohydrates are thought to be the most important sources of energy in fungal and oomycete spores. Consequently, an incorrect assembly of peroxisomes, due to inactivation of *Bchex*, could implicate an impediment in normal germination. In growth assays using oleic acid as carbon source, hyphal growth was remarkably reduced, supporting our hypothesis that Woronin bodies are not limited to a structural role but may also have a role in cellular metabolism associated with β-oxidation.

Regarding the virulence of *Bchex* mutants, the infectious hyphae appear incapable of invading host tissue and remain confined to the site of entry into the host. As a result of this, the virulence of the Δ*Bchex* strain was diminished on tomatoes leaves. However, Δ*Bchex* was able to produce chlorosis in the host. This phenotype can be explained because the hyphae are frequently lysed during growth within the host by the action of host glucanases and chitinases. Deletion of the *Bchex* gene could result in defects in septal pore sealing, reducing the virulence of *B. cinerea*. On the other hand, *B. cinerea* secretes non-specific phytotoxins capable of killing cells from a large spectrum of plants. Among the numerous metabolites isolated from fermentation broth (Collado et al. 2007), the most well known is the sesquiterpene botrydial. Botrydial is produced during plant infection (Deighton et al. 2001), and induces chlorosis and cell collapse, which seems to facilitate both penetration and colonization (Colmenares et al. 2002; Siewers et al. 2005). The presence of chlorosis in lesions produced by the *Bchex* mutants indicates that the pathogen is metabolically active, which could explain why the inactivation of *Bchex* reduces the virulence in the mutant. Simultaneously, due to the reduced hyphal elongation and colonization of Δ*Bchex*, the host could respond to the attack through a rapid production of reactive oxygen species, which is required for defense in order to limit the access of pathogens to water and nutrients, thus preventing the colonization of host tissue (Greenberg and Yao 2004). Therefore, based on these analyses, both phenomena could be acting synergistically to yield the avirulent phenotype.

Finally, the identification of *Bchex* from *B. cinerea* as a virulent gene, demonstrated the importance of Woronin body in pathogenicity of this fungus, leading us to suggest that *Bchex* is a suitable target for the design of *B. cinerea* resistance in plants.

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