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ORIGINAL PAPER

Cold induced *Botrytis cinerea* enolase (*BcEnol-1*) functions as a transcriptional regulator and is controlled by cAMP

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Abstract *Botrytis cinerea* is a necrotrophic fungal plant pathogen that can survive, grow and infect crops under cold stress. In an attempt to understand the molecular mechanisms leading to cold tolerance of this phytopathogen, we identified an enolase, *BcEnol-1*. *BcEnol-1* encodes a 48 kDa protein that shows high identity to yeast, *Arabidopsis* and human enolases (72, 63 and 63%, respectively). Northern analysis confirms that an increase in transcript abundance of *BcEnol-1* was observed when *B. cinerea* mycelium was shifted from 22 to 4°C. In order to understand its regulation during cold stress, *BcEnol-1* expression was studied in *B. cinerea* mutants viz *Δbcg1* (mutant of *B. cinerea* for *bcg1)*, *Δbcg3* (mutant of *B. cinerea* for *bcg3)* and *Δbac* (mutant of *B. cinerea* for adenylate cyclase). A decrease in enolase expression in these mutants was observed during cold stress suggesting enolase activation by a cAMP mediated cascade. Expression of enolase was restored with the exogenous addition of cAMP to the *Δbac* mutant. Recombinant enolase protein was also found to bind to the promoter elements of transcripts belonging to the Zinc-C_6 protein family and calpain like proteases. Based on these results we conclude that enolase from *Botrytis* is cold responsive, influenced by cAMP and acts putatively as a transcriptional regulator.

Keywords *Botrytis cinerea* · Enolase · Cold stress · DNA-binding activity · cAMP regulated

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Introduction

Low temperature is an important environmental factor that greatly influences the growth, development and survival of many organisms. Numerous physiological and molecular changes occur during cold acclimation. Among them, the transcriptional activation and repression of genes by low temperature are of central importance. The reprogramming of gene expression results in the accumulation of protective proteins and hundreds of metabolites, some of which are known to have intracellular protective effects.

In bacterial *systems* many small acidic shock proteins are reported to be responsible for adaptation towards cold stress (Beckering et al. 2002). Recently, it was shown in *Escherichia coli*, that families of cold shock proteins (CSPs) also function as transcription antiterminators or translational enhancers at low temperature by destabilizing RNA secondary structure (Bae et al. 2000). There are reports about the role of fatty acid desaturase activity in *Bacillus subtilis* that provides membrane adaptation during cold treatment (Weber et al. 2001). *Arabidopsis*, when exposed to low temperatures rapidly induces a small family of genes encoding transcriptional activators known as C-repeat/dehydration responsive element (DRE) and its DNA binding protein/factor (CREB/CBF) (Yamaguchi-Shinozaki and Shinozaki 1994, 2005; Lee et al. 2002). Also in *Arabidopsis*, an increase in freezing tolerance involves the

production of cryoprotective polypeptides such as COR15a and synthesis of low molecular-weight cryoprotectants, including proline and raffinose (Steponkus et al. 1998).

Although the cold-shock response has been widely studied in bacteria and plants, relatively little attention has been paid to fungal systems. In yeast, cold adaptation leads to the accumulation of protectants such as trehalose, glycerol and heat-shock proteins (Kandror et al. 2004; Piper et al. 1998). Recently, it has also been shown that changes in membrane fluidity are the primary signals triggering the cold-shock response. Notably, this signal is transduced and regulated through cAMP mediated stress pathways using transcriptional factors such as Msn2/4p, the high-osmolarity of glycerol, and mitogen-activated protein kinases (Martinez-Pastor et al. 1996; Kandror et al. 2004; Izawa et al. 2006; Panadero et al. 2006; Aguilera et al. 2007).

B. cinerea infects a wide range of harvested crops during storage, shipment and marketing, due to conditions favorable for its development and infection. Given the right conditions, it may out-compete other fungi on senescing tissue or harvested plant organs (Droby and Lichter 2004). The fungus gains a significant advantage over other pathogens due to its ability to grow at low temperature whereas; its pathogenicity is reduced at warmer temperatures and at low humidity. *Botrytis* is also able to survive in soil during chilling conditions as melanized mycelium or as sclerotia in the decaying plant debris, thus making it an efficient and devastating post harvest pathogen (Staats et al. 2005). Cold storage is an important post harvest treatment to slow plant senescence; however, its lower chilling limit is the freezing point of the tissue and the sensitivity to the chilling injury. Natural hosts like grapes and strawberries can tolerate near freezing storage temperatures but these conditions do not prevent *B. cinerea* from causing disease during cold storage (Droby and Lichter 2004). Integrated pest management strategies for strawberries and grapes are limited approaches to solve this problem. The promise of alternative methods, gleaned from a better insight into the biology of the organism, is that effective treatments can be targeted directly to the pathogen with reduced environmental, health or quality side effects. To accomplish this goal, we are currently studying the factors and regulatory mechanisms of cold induced transcripts in *B. cinerea*.

From our initial studies of the shot-gun proteomic analysis of *B. cinerea* secretions collected from the mock infection of plant tissues, an enolase protein was identified (personal communication). Previous studies have also shown that enolase has been secreted *in planta* from the pathogenic fungus *Fusarium gramienarum* or extracellularly using a non-conventional secretory pathway from yeast (Nombela et al. 2006; Paper et al. 2007). Enolase is a glycolytic enzyme that converts 2-phospho-D-glycerate to phosphoenolpyruvate. Recently, using a reverse genetics approach it has been shown that cold-responsive gene transcription in *Arabidopsis* is also controlled through a bifunctional enolase, which acts as a transcriptional repressor (Lee et al. 2002). In this paper, we describe the cloning and characterization of a cold-induced enolase from *B. cinerea* (*BcEnol-1*). *BcEnol-1* was upregulated during cold conditions and cAMP influences the transcript accumulation. We have further shown that enolase from *B. cinerea* has a DNA binding activity which suggests that it might also act as a transcriptional regulator during cold stress.

Materials and methods

Media and culture conditions

Botrytis cinerea Pers. Fr. (teleomorph *Botryotinica fuckeliana* de Bary Whetz.) strain BO5.10 was grown on potato dextrose agar at 22°C for maintenance and spore collection. For cold stress 10^6 spores of *B. cinerea*, *Δbcg1* mutant (mutant of *B. cinerea* for *bcg1*, Schade et al. 2004), *Δbcg3* (mutant of *B. cinerea* for *bcg3*, Doehlemann et al. 2006) and *Δbac* mutant (mutant of *B. cinerea* for adenylate cyclase, Klimpel et al. 2002) were spread on cellophane membranes overlaid on Gamborg's B5 Medium (Sigma, St Louis, MO, USA) supplemented with 4% grape pulp (GP). All fungi were grown for 5 days then exposed to cold (4°C) (and with 2 mM of cAMP in case of *Δbac* mutant) for 1, 4, 10 and 24 h. To study the effect of cAMP on the expression of enolase at ambient temperatures, the fungal hyphae grown on Gamborg's B5 and GP at 22°C were floated on a fresh liquid Gamborg's B5 Medium supplemented with 1 mM of the cAMP analogue (adenosine 3′, 5′-cyclic monophosphate, 8-bromo-sodium salt, Calbiochem, CA, USA) at room temperature. Fungal mycelia were harvested after 30 min, 1 and 3 h. Similarly, for heat stress the plates were shifted to 40°C at given time points. Growth rate of the cultures were calculated based on the ratio of colony size at 4 and 22°C. Experiments were carried out twice by using five to six replicates for each time points and the representative values were plotted as bar graphs.

Cloning and expression of purified enolase

Two micrograms of cold induced DNA-free RNA from *B. cinerea* was used for the first-strand cDNA synthesis using the Transcriptor First Strand cDNA Synthesis Kit RT-PCR (Roche) with oligo-dT primers following the manufacturer's guidelines. A 1.3 kb fragment of the *B. cinerea* enolase cDNA (XM_001561215) was PCR amplified with the forward and reverse primers, 5′ ATGGCTATCACTAA GATTCACGC 3′ and 5′ TTACAAGTTGACGGCAGTT CTG 3′, respectively, then cloned into the pGEMT Easy

vector (Promega Inc., Madison, WI, USA) for sequence verification. For protein expression, the fragment was subcloned into the *Hind*III and *Xho*1 cloning sites of the pET28b vector (Novagen Inc., San Diego, CA, USA). The final construct was transformed into the Rosetta *E. coli* strain (Novagen Inc.) and grown to an OD of 0.6–0.7 at A_{600} in LB medium containing kanamycin (50 μg/ml) and chloramphenicol (25 μg/ml). The T7 promoter was induced with the addition of 1.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 22°C for 4 h. Bacterial cells were harvested, suspended in the Bug Buster Reagent (Novagen Inc.) and sonicated. The expressed His-enolase fusion protein was purified using a Ni-NTA column as described by the manufacturer (Novagen Inc.), fractionated in a 12% denaturing SDS-PAGE and visualized with Coomassie Blue staining.

Enzyme assay, immunoblot and gel shift analysis

Activity measurements for enolase were performed in 50 mM Tris–HC1 (pH 7.4), containing 1 mM $MgC1_2$, 0.01 mM EDTA and 2.5 mM of 2-phospho-D-glycerate as modified slightly from a previously published procedure (Westhead 1996). For total protein extraction, *B. cinerea* mycelium was grounded and the extraction was performed using the above mentioned buffer omitting the substrate. The reaction was incubated at 25°C for 10 min and the absorbance at 230 nm was measured against a blank, without added substrate. Immunoblot analysis was performed as described (Van der Straeten et al. 1997). His-Enolase pure protein (30 μg) was subjected to Western blot analysis. After nonspecific binding was blocked using 5% non-fat dried milk, the membrane was briefly incubated with a rabbit anti-human α-enolase antibody (product 6880-0410; Biogenesis, Kingston, NH, USA) at a 1:1,000 dilution for 1 hour at room temperature. Bound primary antibody was visualized by incubating the membrane with a secondary antibody (HRP goat anti-rabbit; Vector Laboratories, Burlingame, CA, USA) at a 1:100 dilution for 1 h. Luminescence detection was performed with the SuperSignal WestPico Kit (Pierce Inc., Rockford, IL, USA) according to the manufacturer's instructions.

Electrophoretic Mobility Shift Assays (EMSA) was performed as described by (Subramanian and Miller 2000) with some modification. The following double-stranded DNA oligonucleotides were used for probes in EMSAs; a Zn/C6 promoter element (5′-TATAAATATTGTTAA CTTTATATAAAATCCTTAGAATATTTCA-3′) and CAL promoter element (5′-TCCCTTTCAAGCCCTATATAAA ATCCTTATGACACCCTATGT-3′). Briefly, 5 μg of poly (dI–dC) (nonspecific competitor) was added to 1× binding buffer (Hao et al. 1998) with 8 μg of purified His-enolase fusion protein. The reaction mixture was incubated 15 min on ice for any non-specific binding. The doubled stranded oligonucleotides for Zinc-C_6 and CAL promoter elements were 5′ end labeled with [γ-P^{32}] ATP in the presence of T4 polynucleotide kinase and further purified with a QIAquick nucleotide removal kit (QIAGENE, MD, USA). After the addition of a 5' end-labeled oligonucleotide (5 pmol), the reaction was incubated for 25 min at room temperature. For specific competition assays, unlabeled cold-competitor oligonucleotides were added at varying concentrations to the reaction mixture. The resulting complexes were then separated on a native 6% polyacrylamide gel at room temperature in 0.5× Tris borate-EDTA at 10 V/cm. After electrophoresis, the gel was dried at 80°C for 1 h and further visualized by autoradiography (Sambrook et al. 1989).

cDNA synthesis and quantitative real-time PCR

Total RNA was isolated from *B. cinerea* cultures grown on cellophane and overlaid on Gamborg's B5 Medium supplemented with 4% grape pulp (GP) for 5 days then shifted to 4°C for 1, 4 10 and 24 h. All RNA samples were treated with RNase-free DNase at 37°C for 30 min using the DNA-free kit (Ambion Inc., Austin, TX, USA) in order to ensure the presence of only RNA amplicons prior to cDNA synthesis. Two micrograms of cold induced DNA-free RNA was used for the first-strand cDNA synthesis using the Transcriptor First Strand cDNA Synthesis Kit RT-PCR (Roche) with random hexamer primers following the manufacturer's guidelines. qRT-PCR, with gene-specific primers and probes from the Roche (Indianapolis, IN, USA) Universal Probe Library Assay Design Center for mouse probe library, were used to detect and quantify specific gene expression. 18S-ribosomal RNA (18S rRNA, BC1G_15191.1; BROAD accession number) was the internal control/housekeeping gene with the forward 5′-CGTTATCGCAATT AAGCAGACA-3′ and reverse 5′-TCTTGATTTTGTGG GTGGTG-3′ primers. All values obtained were normalized against a *B. cinerea* 18S rRNA as an internal control. Reactions were performed in triplicate on pools of cDNAs synthesized from each of two independent preparations of RNA. All cDNA samples of each treatment were amplified simultaneously in one PCR assay. All quantitative PCR analyses were performed using an ABI PRISM 7900 HT (Applied Biosystems).

Southern blot hybridization and Northern analysis

Ten micrograms of genomic DNA from *B. cinerea* were digested with either *BamH*1 (which cuts once inside the gene) or *EcoR*1 (no internal cut sites), respectively. Fragments were fractionated on 0.8% agarose gel and blotted onto Hybond-N membranes (Amersham Pharmacia

Biotech, Piscataway, NJ, USA), using 20× SSC. Membranes were hybridized with a full length *BcEnol-1* cDNA as a probe. For Northern analysis, *B. cinerea* was exposed to cold for 1, 4, 10 and 24 h and total RNA isolated. RNA at 10 µg per lane was fractionated on formaldehyde denaturing agarose gels and transferred to Hybond-N membranes, as described (Sambrook et al. 1989). A full length enolase cDNA of 1.3 kbp was randomly labeled with [α^{32}P] dCTP using the Rediprime DNA labeling kit (Amersham Pharmacia Biotech). The labeled cDNA was used as a probe to detect enolase transcripts in the hybridization of membrane-bound nucleic acids as described previously (Sambrook et al. 1989). The membrane was washed twice (with 2 and 0.1× SSC, 0.1% SDS) before being exposed to autoradiographic film. All Northerns were carried out in duplicate with two independent culture experiments. The Northern picture shown is a representation of these blots. The blots were stripped using 0.1% boiling SDS, rinsed with 2× SSC and were reprobed with [α-^{32}P] dCTP labeled 18S rRNA as an expression control.

Results

BcEnol-1 is induced by cold and cAMP but not by heat stress

Induction of the *Arabidopsis* enolase was shown by Lee et al. (2002) to be influenced by cold. To check the specificity of enolase from *B. cinerea* toward cold treatments, Northern blot analysis was performed. Our data shows that transcript accumulation of enolase starts within the first hour after cold treatment and continues to increase until 10 hours in wild-type *B. cinerea*. Enolase expression further decreases at 24 h post cold induction. The size of the transcript detected was 1.3 kbp (Fig. 1a). This suggests that fungal enolase is induced during cold stress. To correlate the enhanced transcriptional activity of enolase with its enzymatic activity we performed enolase assays under in vivo conditions. Our results show an increase in the enzymatic activity when compared to the control, but this activity was not further enhanced with time (Fig. 1a; lower panel). There is a positive correlation of the increase in level of enolase activity to the transcriptional accumulation of *BcEnol-1* when compared to the control. Since the *B. cinerea* (BO5.10) genome sequence has only 5.4× coverage and contained some gaps, we performed Southern analysis to verify the copy number of enolase in the genome. Autoradiographic imaging of the probed blot confirms that enolase exists as a single copy in the *B. cinerea* genome (Fig. 1b). Furthermore, in order to understand the regulatory mechanism of enolase, we performed a motif search of the 2 kbp sequence upstream of the enolase "ATG" start sequence. The scan was performed on the DNA sequence by using the Motif Search site at the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://motif.genome.jp/). Our analysis predicted that the promoter region consists of as many as 12 heat shock factor responsive elements (HSF; AGAAN consensus sequence) and 5 CREB, cAMP-responsive element binding protein with a TGACGTYA consensus sequence (data not shown). To test whether the enolase is a heat shock responsive gene, we performed a Northern analysis of heat treated (40°C) versus room temperature (22°C) grown fungus. Our results indicated that enolase expression is independent of heat stress (Fig. 1c). A positive control *hsp70* was expressed at all time points during heat stress compared to the control.

In order to examine whether cAMP influences the expression of enolase, wild-type and *Δbac* mutants of *B. cinerea*, cultures were supplemented exogenously with a cAMP analogue. When wild-type *B. cinerea* was supplemented with cAMP in Gamborg's medium (G5) the expression of enolase was enhanced, compared to that of G5 media alone (Fig. 1d). In wild-type and *Δbac* mutants of *B. cinerea*, enolase is induced at the earliest time point, i.e. 30 min; and that the level of induction is maintained for the entire 3 hours of incubation (Fig. 1d). This data shows that cAMP influences the expression of enolase in wild-type as well as in *Δbac* mutants of *B. cinerea*.

Expression of *BcEnol-1* in *Δbac*, *Δbcg1* and *Δbcg3* mutants during cold stress

To further dissect the signaling cascade which influences the expression of enolase from *B. cinerea* during cold stress, we used the deletion mutants for the Gα subunits *BCG1* (*Δbcg1*) and *BCG3* (*Δbcg3*), and adenylate cyclase (*Δbac*). Since cAMP levels influence the accumulation of enolase in *B. cinerea*, these mutants were subjected to cold stress and Northern analysis was performed. The expression level of enolase differed in all the three mutants. In the *Δbac* mutants, no induction of enolase was observed until 10 h of cold treatment (Fig. 2a). The expression of enolase transcripts was significantly reduced in the mutant compared to the wild-type and only a slight induction was observed at 24 h post treatment. This strong reduction in enolase expression in the *Δbac* mutants supports a possible role for adenylate cyclase in regulating gene expression. To elucidate the role of cAMP on the expression of enolase under cold conditions, *Δbac* mutants was supplemented with the membrane permeable cAMP analogue. Induction of enolase expression was observed after 4 h of treatment and was maintained until 24 h. This restoration in enolase expression under cold conditions suggests that transcript abundance of enolase is controlled by cAMP. Furthermore, to study the role of the Gα proteins on enolase expression

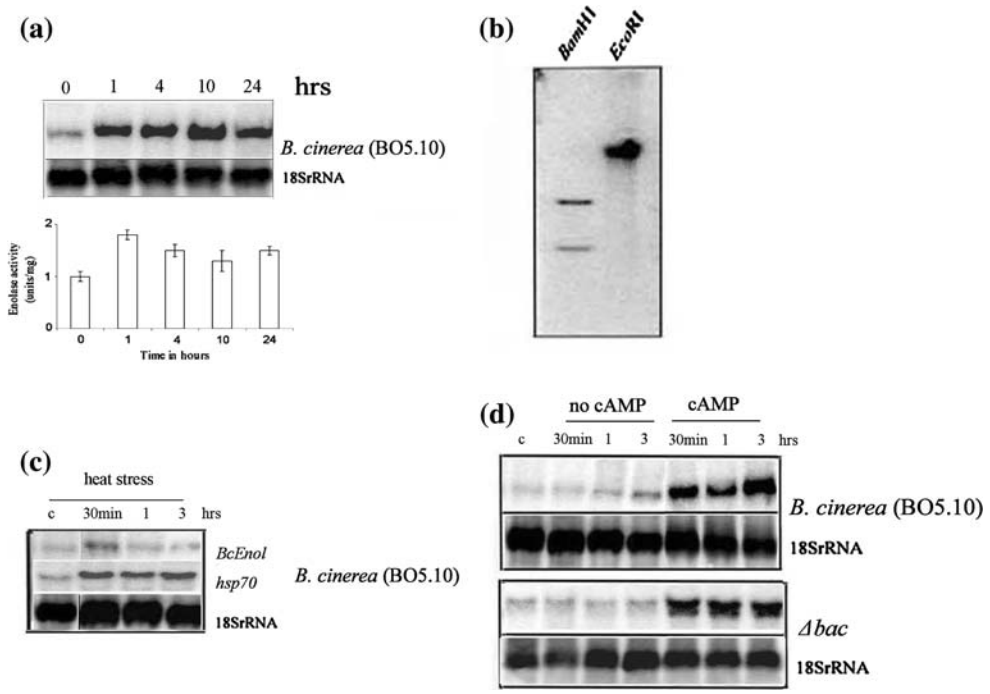

Fig. 1 Induction of *BcEnol-1* under different stresses. **a** Effect of cold stress (4°C) on the expression of *BcEnol-1*. As a loading control, RNA was hybridized to an 18S rRNA probe (BC1G_15191.1) from *B. cinerea*. *Lower panel* indicates the enolase activity at different time points expressed as units/mg. *Error bars* represent standard error ($n = 4$). **b** Southern blot analysis of *BcEnol-1*. *B. cinerea* genomic DNA was digested with *Bam*H1 or *Eco*RI, and subjected to Southern hybridization with a full-length *BcEnol-1* probe under low-stringency conditions as described in the "Materials and methods". The X-ray film exposure times were varied to confirm probe hybridization to single bands. **c** Effect of heat shock treatment on transcript accumulation of *BcEnol-1*. Northern blot analysis was performed from 5-day-old cultures of *B. cinerea* grown at 22°C on G5 + GP agar and then shifted to 40°C. As a loading control, RNA was hybridized with an 18S rRNA probe from *B. cinerea*. Heat stress control *hsp70* was blotted on the same membrane after stripping the blot. **d** Effect of cAMP on *BcEnol-1* expression. *B. cinerea* cultures were supplemented with or without an analogue of 1 mM cAMP and were harvested after 30 min, 1 and 3 h of incubation. Fractionated total RNA (10 μg) was blotted and hybridized with the *BcEnol-1* gene-specific probe under high-stringency conditions as described in the "Materials and methods". As a loading control, RNA was hybridized with an 18S rRNA probe from *B. cinerea*

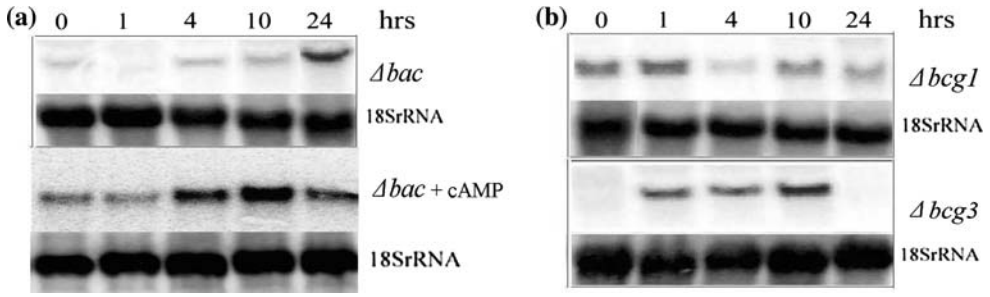

Fig. 2 Gene expression patterns of *BcEnol-1* under cold stress in *Δbac*, *Δbcg1* and *Δbcg3* mutants of *B. cinerea*. Effect of cold stress (4°C) on *BcEnol-1* expression in *Δbac* (**a**), *Δbcg1* and *Δbcg3* mutants (**b**) of *B. cinerea*. As a loading control, RNA was hybridized to an 18S rRNA probe (BC1G_15191.1) from *B. cinerea*

during cold stress we used two Gα mutants of *B. cinerea, Δbcg1* and *Δbcg3*. A decrease in the accumulation of enolase expression was observed in *Δbcg3*, when compared to wild-type *B. cinerea* (Fig. 2b). Surprisingly, the expression was down-regulated during cold-treatment in the *Δbcg1* mutants in contrast to the wild-type (Fig. 2b) which suggests that *bcg1* might be involved in regulating the expression of enolase during cold stress.

Phenotypic colony morphology of *B. cinerea* during cold conditions

The cAMP-mediated cascade is important for cold tolerance in yeast (Aguilera et al. 2007). The decrease in the level of enolase expression in the *Δbac* mutants of *B. cinerea* suggests that the cAMP pathway could also play a role in cold tolerance for *B. cinerea*. To address this possibility,

we studied the radial growth rate of the *Δbac* mutants at 4°C and compared it to that of the wild-type. *Δbac* mutants and wild-type *B. cinerea* were subjected to cold treatment after 1 day incubation at room temperature. Colony diameters were measured after each day and bar graphs were plotted based on the relative growth rate under cold conditions compared with growth at 22°C (Fig. 3a, b). Our results show a decreased growth rate of the *Δbac* mutants when compared to the wild-type *B. cinerea* under cold stress. A sharp decline in the growth rate was observed for the *Δbac* mutants (from 0.5 to 0.22, arbitrary units) while the wild-type growth rate showed no significant change (0.6, arbitrary units; Fig. 3a, b). These results support the *Δbac* as a cold sensitive mutant of *B. cinerea*.

Protein sequence analysis of BcEnol-1

Multiple sequence alignment analysis (Corpet 1988) and comparison of the protein sequence indicates a high identity with yeast, human and *Arabidopsis* enolases at 72, 63 and 63%, respectively (Fig. 4). The DNA-binding and repression domains of enolase were also highly conserved in human, *Arabidopsis* and *B. cinerea*. This conservation in sequence supports the ability of BcEnol-1 to also bind specific DNA sequence(s) from this fungus. Sequence alignment of a putative plasminogen binding domain of *B. cinerea* with other bacterial, fungi, *Arabidopsis* and human enolases; further suggests conservation of this activity. In the crystal structure of enolase from *Streptococcus pneumoniae* the peptide sequences, $_{248}$FYDKERKVY$_{256,}$ D$_{250}$, E$_{252}$, K$_{251}$ and K$_{254}$ were found to be critical for plasmin(ogen)-binding by systematic permutation (Bergmann et al. 2003; Ehinger et al. 2004). The *B. cinerea* enolase has three out of the four aforementioned amino acids conserved in its putative plasmin(ogen)-binding site (PPBS; Fig. 4) suggesting a further interacting role.

B. cinerea enolase possesses DNA binding activity

A comparison of the *Arabidopsis LOS2*, human *ENO1* and *Botrytis* enolase sequences revealed that the putative DNA-binding and repression domains are mostly conserved. Perhaps, the *Botrytis* enolase may also have the ability to bind certain DNA elements, sequences similar to the human c-*myc* or *Arabidopsis ZAT10* promoter elements (Ray and Miller 1991; Lee et al. 2002). To check the DNA binding activity of the fungal enolase we cloned the cDNA (1,317 bp) into a pET28b vector for protein expression. The IPTG induced His-Enolase fusion protein migrated as an approximate 54 kDa (6 kDa Histidine + 48 kDa enolase protein) protein in an SDS-PAGE gel (Fig. 5a; Lane 3). *B. cinerea* enolase is predicted to be a 48 kDa protein with a calculated pI of 5.3. Enolase enzyme activity assays were performed for the purified recombinant protein by using 2-phospho-D-glycerate as a substrate (data not shown). The linear increase in OD was observed at 230 nm with an increasing time of substrate incubation. Western analysis was performed using a human enolase antibody (Fig. 5a; Lane 4) in order to detect a 54 kDa fungal His-enolase fusion protein.

To identify the possible targets for enolase, a 1 kb upstream promoter region of the *B. cinerea* genome was searched to identify any conserved c-*myc* and *ZAT10* promoter elements in genes shown to have expressed ESTs. The query sequences used were "XXXTTGACGCGTTA TATAAAATCCGTATTX" for *ZAT10* promoter elements and "XXXXTCGCGCTXXXTATAAAAGCCGXXTTX" for *c-myc* promoter elements. In this comprehensive search with our criteria, we found ten putative hits (data not shown). Hypothetical and predicted proteins were not used in the current study. Figure 5b lists some of the selected putative *Botrytis* enolase targets based on the high percentage homology of the conserved promoter sequence matches with *c-myc* and *ZAT10* promoter elements. XM_001545248 encodes for a protein belonging to a zinc cluster protein family (Zinc-C$_6$) and XM_001546816 for a calpain cysteine proteases (CAL). Remaining transcripts encode for either hypothetical or other predicted proteins. To address the possibility of enolase having DNA binding activity, we performed an electrophoretic mobility shift assay (EMSA) with the *B. cinerea* promoter fragment of XM_001545248 (−728 to −690 upstream of the translation initiation site, Zinc-C$_6$) and XM_001546816 (−152 to −115 upstream of the translation initiation site, CAL). Our gel-shift analysis showed a strong shift in the DNA-protein complex with promoter elements of XM_001545248. To determine the specificity of the gel shift assays, a competition assay was performed by adding ×5, ×50, ×100, ×200 molar excess of the cold-oligonucleotide in the binding reaction mixture. A decrease in the intensity of the labeled DNA-protein complex was shown to occur with an increase in the molar ratio of the cold-competitor (Fig. 5c). A sequence alignment of XP_001545298 with other functionally known Zinc-C$_6$ fungal-type regulatory proteins indicates conserved patterns for a DNA-binding domain signature (CX$_2$CX$_6$CX$_{5-12}$CX$_2$CX$_{6-8}$C; Fig. 6a). Furthermore, enolase binds weakly to the XM_001546816 promoter element. Competition experiments showed no observed shifts with an increase in the addition of cold competitors. The intensities in the gel-shift for the CAL promoter element were considerably reduced when compared with the binding to the Zinc-C$_6$ promoter elements (Fig. 5d). For each shift assay, cold poly(dI–dC) competitors were included in each reaction mixture to provide nonspecific targets for the protein, this supports a specificity for enolase proteins towards each promoter element.

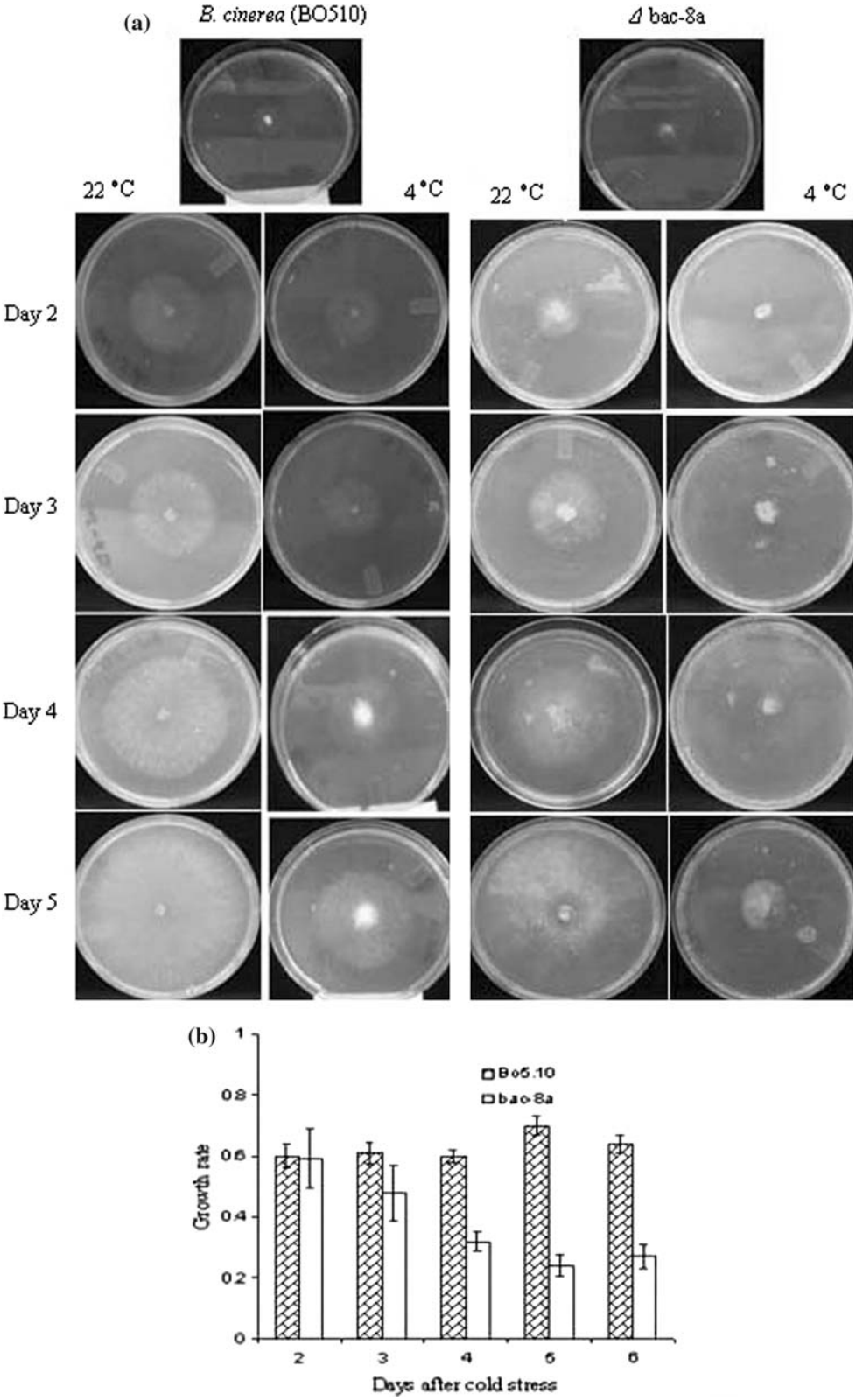

Fig. 3 Effect of cold stress on the growth rate of *Δbac* mutants and wild-type *B. cinerea*. **a** Growth of *Δbac* mutants and wild-type *B. cinerea* after indicated days. **b** *Bar* graphs for the calculated growth rate (ratios of radii of culture at 4 and 22°C). Two independent experiments were performed by using five replicates for each time points. *Error bars* represent standard error ($n = 5$)

To study EST expression for the selected promoter elements, we performed qRT-PCR to verify the regulation of these genes under cold conditions. The Ct values obtained were normalized against 18S rRNA expression, since 18S rRNA expression was shown to be consistent in each treatment (data not shown). For example, the Ct values for 18S rRNA varied between 27.62 and 28.65, while expression for target genes ranged from 35.45 to 45.52. Our results

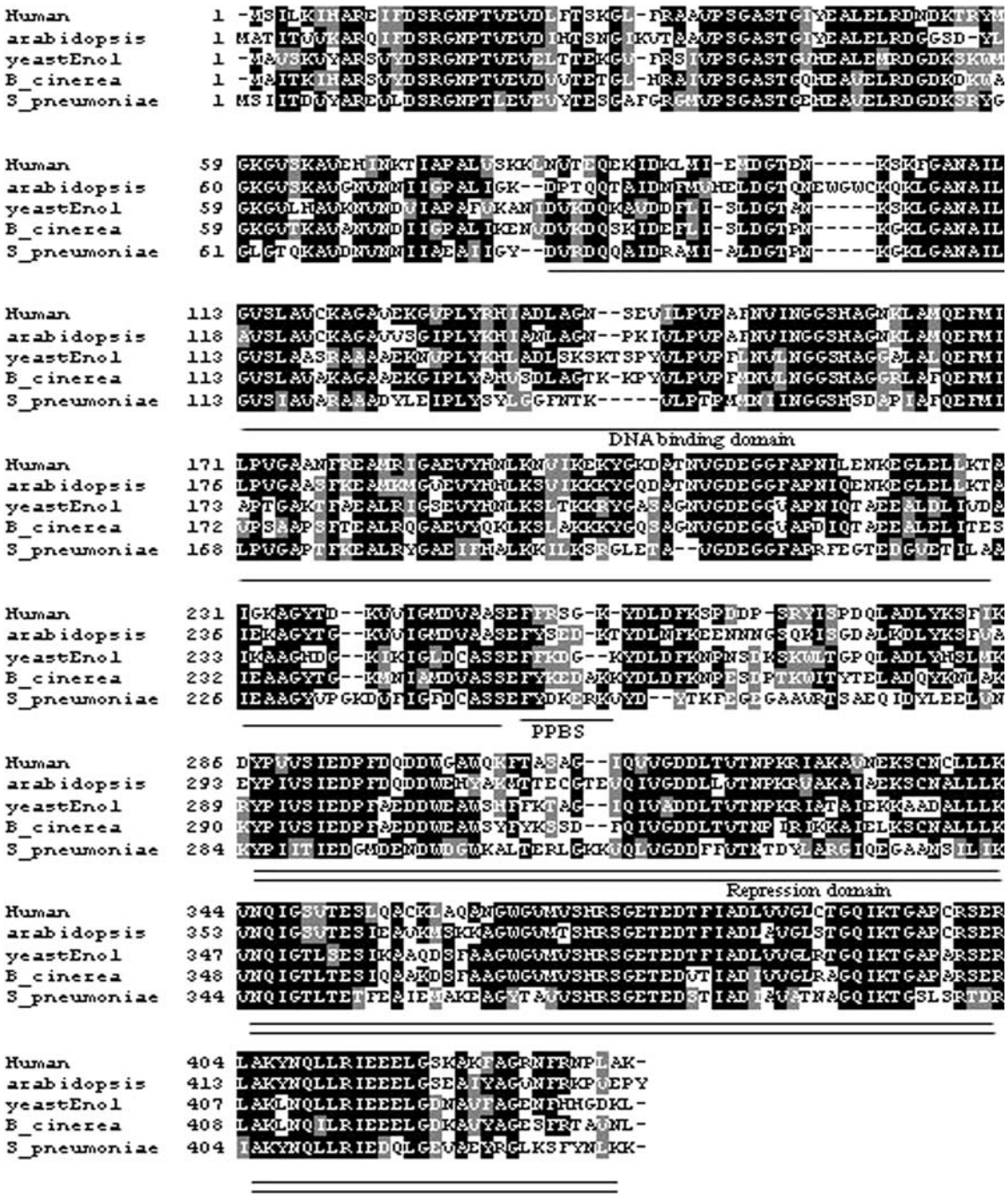

Fig. 4 Amino acid sequence alignments of enolases from human (AAP36132), *Arabidopsis* (NP_181192), yeast (NP_011770), *Botrytis* (XP_001561265) and *Streptococcus pneumoniae* (NP_345598). The DNA-binding domain is *underlined* and the transcriptional repression domain is *double-underlined*. Putative plasmin(ogen)-binding site (PPBS) is shown

indicate that within the first hour XM_001545248 was down-regulated from its basal expression level in wild-type *B. cinerea*. This repression was greatest at 10 h. At this time, transcript accumulation was repressed two to three-fold over that of the control (Fig. 6b; left panel). This suggests that some of the transcripts might be negatively regulated by BcEnol-1. Alternately, XM_001546816 did not undergo any significant changes in the level of transcript accumulation when compared with the control in wild-type *B. cinerea* (Fig. 6b; left panel). Kinetic profiles for these two transcripts were also studied in *Δbac* mutants of *B. cinerea* under cold condition. Our results showed a cumulative increase in the induction of XM_001545248 until the fourth hour of cold treatment along with a further decrease with the increasing time period. Similarly, XM_001546816 was also highly induced at 10 hours post

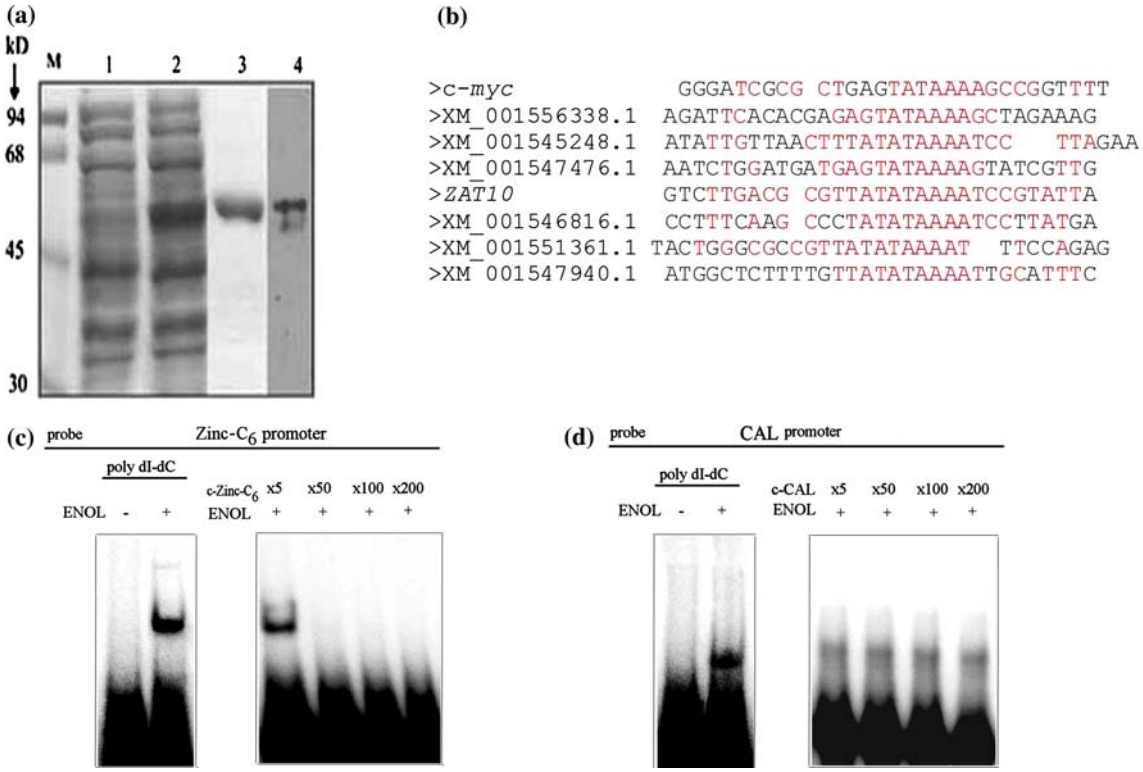

Fig. 5 Protein expression and DNA binding activity of BcEnol-1 by using EMSA. a Expression and immunoblot analysis of recombinant His-enolase fusion protein induced in a Rosetta *E. coli* strain. *Lane 1* cells transformed with pET28b without IPTG; *lane 2* after IPTG induction; *lane 3* purified *B. cinerea* His-enolase extracts. *Lane 4* immunoblot analysis of *B. cinerea* enolase with 30 µg of His-Enolase purified protein. b Sequence alignment for c-*myc*, *ZAT10* and predicted DNA binding promoter elements of *B. cinerea* enolase. The red color indicates the conserved nucleotides of these elements. Promoter elements were identified by Blastn searches for c-*myc* and *ZAT10*-like elements in the *B. cinerea* genome (http://www.broad.mit.edu/annotation/genome/botrytis_cinerea/Home.html). c EMSA with the *B. cinerea* Zinc-C_6 promoter element as DNA probe. Cold-competitors, poly[dI-dC] in the left panel and the varying molar excess of c-Zinc-C_6 in the *right panel*. d EMSA with the *B. cinerea* CAL promoter element as a DNA probe. Cold-competitors, poly[dI-dC] in the left panel and the varying excess molar of c-CAL in the *right panel*

cold stress treatment and was further decreased at 24 h (Fig. 6b; right panel). This increase in the expression of the transcript in *Δbac* mutants when compared to wild-type *B. cinerea* suggests a partial removal of repression by enolase due to absence in production/accumulation of cAMP.

Discussion

Botrytis cinerea is a necrotrophic fungal plant pathogen that can survive, grow and infect crops under cold stress. In an attempt to study the regulation of cold induced genes in *B. cinerea*, we identified *BcEnol-1* that encodes for a 48 kDa enolase protein. *BcEnol-1* is highly induced when subjected to cold and possesses DNA binding activity. Protein sequence analysis revealed that enolases from *B. cinerea*, human and *Arabidopsis* are well conserved. Enolase, 2-phospho-D-glycerate hydrolase (EC 4.2.1.11), is an abundant intracellular enzyme that catalyzes the conversion of 2-phospho-D-glycerate to phosphoenolpyruvate, the only dehydration reaction in the glycolytic pathway. To our knowledge, this is the first report of an enolase as a cold-responsive gene from fungi.

Enolase, one of the most abundant enzymes in the cytosol, was found in the cell wall of *S. cerevisiae*, lacking the typical signal peptide sequence (Edwards et al. 1999). Although no classical signal peptide was predicted in the BcEnol-1 protein sequence by SignalP computer analysis (Bendtsen et al. 2004), Secretome-P analysis (Dyrløv Bendtsen et al. 2004) predicts a possible non-classical secretion mechanism with an NN score of 0.505 that is at the lower threshold for the prediction. Multiple alignments with enolases from various other organisms support the presence of a plasminogen-like binding site in the *B. cinerea* enolase that supports some level of membrane activity. Enolase from *Leishmania mexicana*, *Streptococcus pneumoniae* and *Candida albicans* all display plasminogen binding activity and participate in plasmin activation and penetration inside host cells (Vanegas et al. 2007; Bergmann et al. 2001, Jong et al. 2003). The use of α-enolase for tissue invasion has also been demonstrated for several eukaryotic cell lines (Redlitz et al. 1995; Lopez-Alemany

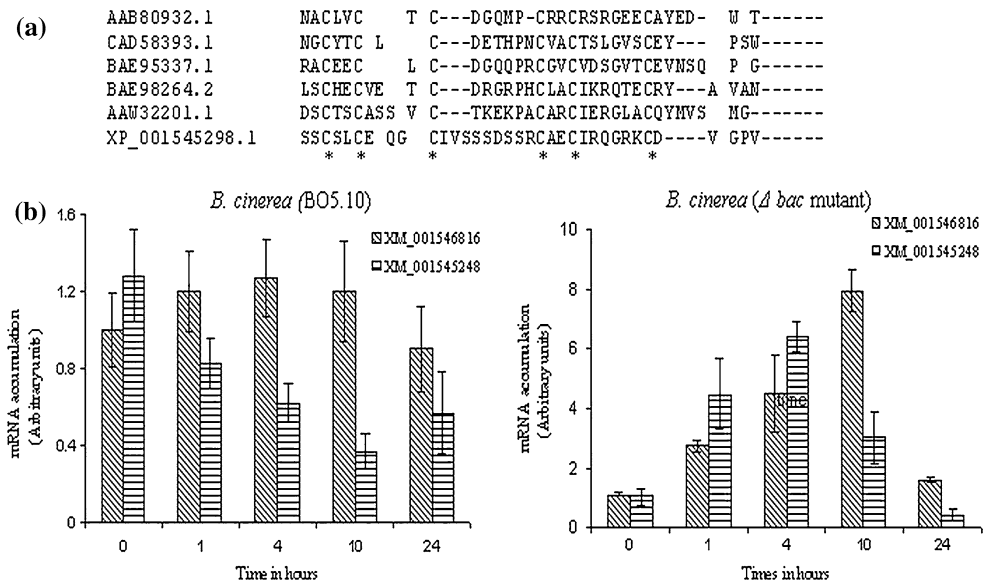

Fig. 6 Sequence comparison of Zinc-C_6 finger like proteins and qRT-PCR analysis of the Zinc-C_6 and CAL gene transcripts in *Δbac* mutants and wild-type *B. cinerea*. **a** Amino acid sequence comparisons of the DNA binding domain of Zinc-C_6 finger proteins from *Neurospora crassa*, AAB80932; *Emericella nidulans*, CAD58393; *Monascus purpureus*, BAE95337; *Fusarium oxysporum*, BAE98264; *Aspergillus oryzae* var. *effusus*, AAW32201 and *B. cinerea*, XP_001545298. The consensus sequence of Zinc-C_6 finger proteins determined by Schjerling and Holmberg 1996 is shown (27). b qRT-PCR analysis of selected gene transcripts, XM_001545248 and XM_001546816 under cold stress in *Δbac* mutants and wild-type *B. cinerea*. For controls, 18S rRNA (BC1G_15191.1) expression was also monitored in each sample. *Error bars* represent standard error ($n = 4$)

et al. 1994). As a possible evolutionarily conserved site, an alignment of peptide sequences present evidence for a putative binding motifs for yet unidentified host proteins uniquely interacting with the enolase from this organism.

In an attempt to characterize *BcEnol-1*, *B. cinerea* was grown on Gamborg's B5 medium supplemented with 4% grape-pulp to mimic infection conditions while exposing to various stress conditions. *BcEnol-1* expression was induced by cold but not by the presence of grape pulp. In yeast upon cold induction, an increase in accumulation of metabolic enzymes particularly in glycolysis was observed (Schade et al. 2004). Correspondingly, *Arabidopsis* enolase, *LOS2*, is highly induced during cold stress (Lee et al. 2002). An increase in activity of α-glucuronidase reporter gene (GUS) was observed when the promoter element of *LOS2* was fused and subjected to cold treatment (Lee et al. 2002). Wild-type *B. cinerea* and *Δbac* mutant, when supplemented with exogenous cAMP, showed an increase in enolase transcripts which supports a regulatory role of cAMP on enolase. Although, under cold stress, the expression of enolase in the *Δbac* mutant undergoes a significant decrease, it was restored with the addition of cAMP. This further reinforces that enolase is induced via a cAMP mediated cascade at 22°C as well as during exposure to cold. A slight accumulation of enolase transcript was still observed in the *Δbac* mutant with cold treatment after 24 h (Fig. 2d), this might be due to the presence of a constant basal level of cAMP of an "unknown origin" despite the deletion of the coding region (Klimpel et al. 2002). In yeast, cAMP mediated cold tolerance has been widely reported and it occurs via phosphorylation of transcription factors like Msn2p and Msn4p (Aguilera et al. 2007). We also propose that the cAMP mediated cascade plays an important role in the regulation of fungal enolase. As reported previously, *Δbac* mutants supplemented with 2 mM cAMP completely restored the process of sporulation and partially restored the colony growth rate (Klimpel et al. 2002). Altogether these observations support that, in *B. cinerea*, cold signals are transduced through the cAMP-PKA pathway and that adenylate cyclase is essential in determining the genetic response to cold.

Since, cAMP levels are easily influenced by the concentration of glucose; we measured the glucose levels in our 4% grape-pulp by using glucose oxidase assays. No measurable amounts of glucose were detected in the 4% pulp. Therefore glucose levels would have an insignificant effect on any further repression of the cAMP profile. As Gα subunits of heterotrimeric G proteins are often upstream components of cAMP-mediated signaling pathways, *B. cinerea Δbcg1* and *Δbcg3* were also tested for regulation of enolase. The *B. cinerea Δbcg1* mutant shows an opposite regulation of enolase expression. The transcript levels decrease during cold treatment. *BCG1* was shown to only partially control the adenylate cyclase activity (Schulze Gronover et al. 2001). Supplemented cAMP fully restored colony morphology and sporulation in *Δbcg1* mutant (Schulze

Gronover et al. 2001), while other features, e.g. protease secretion and toxin production were not affected (Schulze Gronover et al. 2004). However, in *Δbcg3* a reduced expression of enolase was also observed. Nevertheless, results of these experiments support a possible mechanism for *BCG1* in the regulation of enolase through cAMP signaling pathways. Further studies are required to define the complete cAMP signaling mechanism in *B. cinerea* during cold stress. Heat shock induction was also reported for other yeast glycolytic proteins, such as phosphoglycerate kinase and glyceraldehyde phosphate dehydrogenase, *GAPDH* (Piper et al. 1998). Yeast enolase is identical to the yeast heat shock protein, *HSP28*, and is involved in thermotolerance and growth control of yeast (Iida and Yahara 1985). *BcEnol-1* is not a heat inducible transcript as in the case of enolase from tomato which is similar in sequence to the yeast enolase (Van der Straeten et al. 1997). These results show the varying role of enolases from organism to organism.

Sequence comparisons of enolase from *B. cinerea* have allowed us to speculate a possible DNA binding activity. Multiple sequence alignments show that enolases from *Botrytis*, *Arabidopsis* (LOS2) and human (α-enolase) are highly conserved in their C-terminal regions which is necessary for active repression (Ghosh et al. 1999). Previous studies have revealed that the LOS2 is capable of binding to the promoter of human c-*myc* and promoter of *STZ/ZAT10* in *Arabidopsis*, which encodes a putative transcriptional repressor (Lee et al. 2002). Our initial experiments support the binding of enolase from *B. cinerea* to the *c-myc* and *ZAT10* promoter elements (data not shown). Furthermore, we used a bioinformatics approach to search the *B. cinerea* genome for c-*myc* and *ZAT10*-like promoter elements. Our searches have identified six putatively conserved promoter sequences. EMSA results confirm that enolase from *B. cinerea* binds to the promoter element of XM_001545248 and XM_001546816. Similarly, human α-enolase cDNA encodes the Myc-binding protein-1 (MBP-1), which binds to the c-*myc* P2 promoter and down-regulates c-*myc* expression (Subramanian and Miller et al. 2000). XM_001545248 codes for predicted protein belonging to the Zinc-C_6 family with a conserved domain consensus sequence of $CX_2CX_6CX_{5-12}CX_2CX_{6-8}C$. The binding intensities show that fungal enolase binds preferentially to the promoter of this Zinc-C_6 protein over that of CAL. Zinc-C_6 like finger proteins contain a DNA-binding domain consisting of six cysteine residues bound to two zinc atoms that commonly recognize CGG triplets (MacPherson et al. 2006). They are known to regulate genes involved in variety of stress, biosynthesis or metabolic responses (MacPherson et al. 2006). These experimental results led us to propose a possible additional role for enolase as a transcription regulator. The increase in the expression of XM_001545248 and XM_001546816 in *Δbac* mutants when compared to wild-type *B. cinerea* suggests that cAMP influences the expression of these targets genes by regulating the expression of *BcEnol-1*. Interestingly, *ZAT10* was also down-regulated in wild-type *Arabidopsis* under cold stress which represses the transcription of *COR/KIN/RD/LT1* (Lee et al. 2002). Therefore, we propose that *Botrytis* enolase might have a similar function as a transcriptional repressor.

Based on these results, we conclude that enolase can act as an important regulating molecule in *Botrytis* and possibly controlled by the cAMP-mediated signaling cascade after *B. cinerea* undergoes cold treatment at 4°C. Fungal enolases are multifunctional proteins that can either act in the cytoplasm as a key enzyme in the glycolytic pathway, in the nucleus as a transcription factor/repressor, or possibly at the cell membrane. Additional experiments are in progress to validate the various cellular functions served by this enzyme and its potential role in fungal pathogenicity under cold conditions.

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