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EglD, a putative endoglucanase, with an expansin like domain is localized in the conidial cell wall of *Aspergillus nidulans*

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ABSTRACT

Although the process of conidial germination in filamentous fungi has been extensively studied, many aspects remain to be elucidated since the asexual spore or conidium is vital in their life cycle. Breakage and reformation of cell wall polymer bonds along with the maintenance of cell wall plasticity during conidia germination depend upon a range of hydrolytic enzymes whose activity is analogous to that of expansins, a highly conserved group of plant cell wall proteins with characteristic wall loosening activity. In the current study, we identified and characterized the *eglD* gene in *Aspergillus nidulans*, an expansin-like gene the product of which shows strong similarities with bacterial and fungal endo-β1,4-glucanases. However, we failed to show such activity *in vitro*. The *eglD* gene is constitutively expressed in all developmental stages and compartments of *A. nidulans* asexual life cycle. However, the EglD protein is exclusively present in conidial cell walls. The role of the EglD protein in morphogenesis, growth and germination rate of conidia was investigated. Our results show that EglD is a conidial cell wall localized expansin-like protein, which could be involved in cell wall remodeling during germination.

1. Introduction

Aspergillus nidulans asexual reproduction leads eventually to cell differentiation. A germinating conidiospore forms tubular hyphae that will develop into the mycelium, a network of interconnected cells that differentiate into conidiophores (for a review see Adams et al., 1989). The formation and maintenance of the morphologically specialized cellular forms prerequisites cell wall remodeling that typically involves alterations in its composition and organisation. Concisely, the cell wall is a plastic and dynamic structure that is constantly changing in response to environmental signals and the different stages of the fungal cell cycle. A broad number of cell wall modifying enzymes that permit cell wall plasticity, reorganization and adaptation is necessary in this process.

The *A. nidulans* cell wall is a complex structure consisting of polysaccharides such as glucans and chitin, and a minority component of proteins and lipids together with galactose and mannose (Bull, 1970; Zonnenveld, 1971). Cell wall polysaccharides can be classified as alkali-soluble (a-1,3-glucan) and alkali insoluble, consisting of chitin, a-1,4-glucan, β-1,4-glucan and b-1,3-glucan (Zonnenveld, 1971). There is evidence of extensive cross-linking among chitin, a β-1,4-linked homopolymer of *N*-acetylglucosamine (GlcNAc), glucans and other wall components (Cabib et al., 2001; Klis et al., 2002).

Conidial germination in *A. nidulans* is triggered by nutrients in a process whose molecular details are mostly unknown. It is characterized by conidial swelling, adhesion, nuclear reorganization and hyphal growth. At the molecular level, the activation of the *rasA* and the cAMP signaling pathways are involved in the regulation of initial steps of germination (Fillinger et al., 2002; Lafon et al., 2005). Based on genetic and biochemical evidence, it has been suggested that dormant conidia of *A. nidulans* contain a pre-existing pool of mRNA molecules and ribosomes primed for rapid activation and translation during early germination (Loo, 1976; Aramayo et al., 1989; Osherov and May, 2000). The presence of a carbon source activates the translation machinery to initiate loading of pre-existing ribosomal subunits onto mRNA molecules, producing a rapidly growing pool of newly synthesized proteins (Mirkes, 1970). Two such proteins encoded by the *cetA* (AN3079.2) and the *calA* (AN7619.2) genes, members of a novel family of fungal genes of unknown function, showed high homology to plant thaumatin-like defense proteins (Greenstein et al., 2006; Balaish et al., 2007). Deletion of both genes caused profound defects in germination and their role as cell wall-softening agents is under investigation (Balaish et al., 2007).

Cell wall degradation is required during conidia germination since the rigid cell walls of conidia must first be loosened to form germ tubes. Breakage and reforming of cell wall polymer bonds and maintenance of cell wall plasticity during morphogenesis,

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may depend upon the activities of a range of hydrolytic enzymes found intimately associated with the fungal cell wall. Gene annotation programmes predict that *A. nidulans* enzymes with chitinase or glucanase activity are more than the already characterised ones (Takaya et al., 1998; Wei et al., 2001).

The activity of these cell wall polymer modifying enzymes is analogous to that of expansins, a highly conserved group of plant cell wall proteins. Expansins exhibit wall loosening activity, are involved in plant cell expansion and other developmental events during which cell wall modification occurs (for reviews see Cosgrove, 2000; Cosgrove et al., 2002).

In the present work, we identified and characterized an expansin-like gene in *A. nidulans*, named *eglD*. This gene is similar to genes encoding plant expansins as well as bacterial and fungal endo-β1,4-glucanases. The *eglD* gene is constitutively expressed in all developmental stages and compartments of *A. nidulans* asexual life cycle. The temporal expression pattern and cellular localization of the EglD protein and its role in morphogenesis, growth and germination rate of conidia were investigated. According to our results EglD is a cell wall localized protein with putative endonuclease activity, which is exclusively synthesized in conidia and could be involved in cell wall remodeling during germination.

2. Materials and methods

2.1. Media and strains

Minimal (MM), Complete (CM) media and growth conditions for *A. nidulans* have been previously described (Cove, 1966). Supplements were added when necessary. Urea was used as nitrogen source at a final concentration of 5 or 10 mM as indicated. Glucose and fructose were used as carbon sources at final concentrations of 1% w/v and 0.1% w/v, respectively. The inducers of the *alcA* promoter, 2-butanone and ethanol, were both used at a final concentration of 50 mM.

The *A. nidulans* strains and plasmids used in this study are listed in Tables 1 and 2, respectively. *yA*$^{+}$, *pabaA1* used as a wild-type (wt) strain, *eglD::sgfp*, *alcA*$_{(p)}$*::eglD::sgfp*, *5′eglD::sgfp::eglD3′* and *eglD::argB* (wt1) were isolated after transformation of protoplasts of an *argB2* strain with plasmids pGEM:EglD-sGFP-argB, pGEM:alcA-EglD-sGFP-argB, pGEM:5′EglD-sGFP-EglD-3′-argB and pGEM:EglD-argB, respectively. For plasmid details see below and Table 3. Strains *pyr4* (wt2) and *ΔeglD* were isolated after transformation of a *pyrG89* strain with plasmid pGEM:*pyr4* and an 8 kb D-J PCR product, respectively. Finally, strains *ΔeglD::eglD* and *ΔeglD::EglD::sgfp* were isolated after co-transformation of a *ΔeglD* strain with plasmids pGEM:EglD or pGEM:EglD-sGFP-argB, respectively, and plasmid pJVF085 (kindly provided by Prof. C. Scazzocchio; Cultrone et al., 2007). This plasmid derived by introducing the *pantoB* gene into the multi-cloning site of the pGEM T Easy vector (PROMEGA).

pantoB100, *pabaA1*, *pyrG89*, and *argB2* indicate auxotrophies for D-pantothenic acid, *p*-aminobenzoic acid, uracil/uridine and L-arginine, respectively. *yA*$^{+}$ indicates green conidia, while *yA2* results in yellow conidia. These markers do not affect the regulation of gene products involved in cell wall morphogenesis. For more details see http://www.gla.ac.uk/Acad/IBLS/molgen/aspergillus/. *A. nidulans* protoplast transformation was carried out as described in Tilburn et al., 1983.

2.2. Standard plasmid and nucleic acid manipulations

Plasmid isolation from *Escherichia coli* DH5α strain and standard DNA manipulations were performed as previously described (Sambrook et al., 1989). Total genomic DNA isolation from *A. nidulans* has been described by Hoffman and Winston, 1987, whereas total RNA extraction was carried out with the TRIzol® Reagent (GIBCO-BRL) according to the manufacturer instructions. Polymerase Chain Reaction (PCR) was carried out using *Taq* DNA polymerase (New England Biolabs). DNA sequencing of plasmid constructs was carried out using the ABI 310 Genetic Analyzer at the Institute of Biology, NCSRD, Athens, Greece. Reverse transcription (RT-PCR) for the isolation of the *eglD* cDNA clone was carried out using the PowerScript™ reverse transcriptase (Clontech), oligo-dT as reverse primer and total RNA extracted from a wild-type strain, grown on minimal medium supplemented with 5 mM urea and 0.1% fructose for 4 h at 37 °C. The oligonuleotide primers used for amplification, cloning and sequencing of the *eglD* gene and the *cDNA* clone named as EGLD-BamHI and EGLD-EcoRI as well as all the primers used in the present study are listed in Table 3.

Plasmid pGEM:EglD was derived by cloning the *eglD* gene to the multi-cloning site of pGEM T Easy vector (PROMEGA). The *eglD* gene was amplified from total genomic DNA of a wild-type strain using the EGLD-BamHI and EGLD-EcoRI primers. Plasmid pGEM:EglD-argB was constructed by cloning the *argB* sequence, amplified from plasmid pAN335:argB (Argyrou et al., 2001), to the PstI site of plasmid pGEM:EglD using appropriate primers (ARGB-PstI and ARGB-L-PstI). The *argB* gene encodes an enzyme that participates in arginine catabolism (Berse et al., 1983; Johnstone et al., 1985) and is used as a selection marker.

An in-frame fusion of the *sgfp* gene to the 3′-end of the *eglD* gene with a linker of four (DIGG) amino acids (Tavoularis et al., 2001), driven by native *eglD* promoter sequences was cloned into vector pGEM:EglD-argB to generate plasmid pGEM:EglD-sGFP-argB. The *sgfp* sequence was amplified from a pBluescript vector containing the EcoRV–HindIII fragment of plasmid pA4 (Tavoularis et al., 2001; Erpapazoglou et al., 2006) using primers GFP-KpnI-F and TrpC-KpnI-R. This fragment, containing also the *trpC* transcriptional terminator (*trpC*$_{Term}$; Pokorska et al., 2000) downstream the

Table 1
Aspergillus nidulans strains used in this study

Strains	Genotypes	Reference
wt	*yA*$^{+}$, *pabaA1*	CS2498 (Fungal Genetics Stock Center)
argB2	*yA2; argB; pantoB100*	Berse et al. (1983)
pyrG89	*yA*$^{+}$, *pyrG89, pabaA1; pantoB100*	Palmer and Cove (1975); Oakley et al. (1987)
creA^{d}*1*	*creA*^{d}*1, pabaA1*	Bailey and Arst (1975); Shroff et al. (1997)
eglD::sgfp	*yA2; argB; eglD::sgfp; pantoB100::argB*	This study
alcA$_{(p)}$*::eglD::sgfp*	*yA2; argB2; alcA*$_{(p)}$*::eglD::sgf; pantoB100::argB*	This study
5′eglD::sgfp::eglD3′	*yA2; argB2; pantoB100,5′eglD:sgfp:eglD3′::argB*	This study
wt1 (*eglD::argB*)	*yA2; argB2; eglD; pantoB100::argB*	This study
wt2 (*pyr4*)	*yA, pyrG89*$^{+}$*, pabaA1; pantoB100::pyr4*	This study
ΔeglD	*yA*$^{+}$*, pyrG89, pabaA1; eglDΔ; pantoB100::pyr4*	This study
ΔeglD::eglD	*yA*$^{+}$*, pabaA1; ΔeglD::eglD; pantoB100::pantoB*	This study
ΔeglD::EglD::sgfp	*yA*$^{+}$*, pabaA1; ΔeglD::EglD::sgfp; pantoB100:: pantoB*	This study

Table 2
Plasmids used in this work

Cloning vector	Description	Reference
pGEM T Easy vector	T/A cloning vector	Promega Biosciences, San Luis Obispo, USA
pGEM:PYR4	pGEM:*pyr4*	This study
pGEM:EglD	pGEM:*eglD*	This study
pGEM:EglD-argB	pGEM:*eglD:trpC$_{Term}$:argB*	This study
pGEM:EglD-sGFP-argB	pGEM:*eglD:sgfp:trpC$_{Term}$:argB*	This study
pGEM:alcA-EglD-sGFP-argB	pGEM:*alcA$_{Prom}$:eglD:sgfp:trpC$_{Term}$:argB*	This study
pGEM:5'EglD-sGFP-EglD3'-argB	pGEM:*5'eglD:sgfp eglD3':trpC$_{Term}$:argB*	This study
pGEM:EglD:BAD-Y-tail	pGEM:*eglD:BAD:Y-tail*	This study
pPYR4	pBS KS(+):*pyr4* (*BglII-HindIII* fragment of pFB6)	Buxton and Radford (1983)
pJVF085	pGEM:*pantoB*	Cultrone et al. (2007)
pRG3	pGEM:*18SrRNA*	Delcasso-Themousaygue et al. (1988)
pA4	pBS:*prnB:DIGG:sgfp:trpC$_{Term}$*	Tavoularis et al. (2001)

Table 3
Oligonucleotides used in this study

Name	Sequence
EGLD-BamHI	5′-CGGGATCCCATGAAGTACCAGCATTTCTC-3′
EGLD-EcoRI	5′-CGGAATTCTCAGAAGTTTCCATCAGC-3′
ARGB-PstI	5′-AAAACTGCAGGGGGTAGTCATCTAATG-3′
ARGB-L-PstI	5′-AAAACTGCAGGTCGACCTACAGCCATTG-3′
GFP-KpnI-F	5′-CGGGGTACCGGATCCATGGTGAGCAAGGGC-3′
TrpC-KpnI-R	5′-CGGGGTACCTTCTCGAGTGGAGATGTGGAG-3′
EglD-GFP-F	5′-GTGACGGCTGATGGTACCTTCTGATCTCCA-3′
EglD-GFP-R	5′-TGGAGATCAGAAGGTACCATCAGCCGTCAC-3′
alcA-EglD-NcoI	5′-CATGCCATGGGGAAGTACCAGCATTTC-3′
alcA-EglD-NcoI-R	5′-CATGCCATGGGGAGCTAGTCTACTGTGC-3′
UPEGLD-F	5′-CCTTTTCCAAGGTAGCCTCATTATAC-3′
UPEGLD-R	5′-GGAAAAGCGATTATAAATGAAAGGACG-3′
DOWNEGLD-F	5′-TGGATTATCCTCTGAGTTCTCTATTCG-3′
DOWNEGLD-R	5′-ATTTGAATGTAACTTCTCGCAACCCC-3′
PYR4EGLD-F	5′-CGTCCTTTCATTTATAATCGCTTTTCCTAAGCTTAGACTTCAACAACCCCACCATC-3′
PYR4EGLD-R	5′-CGAATAGAGAAACTCAGAGGATAATCCATGGATCTTCATCATTCGTCGCTTTCGGG-3′
NEGLD-F	5′-AGCCTGCCTTTTCAGCAAAGC-3′
NEGLD-R	5′-TACTCTAGTGTGAGGCGGTAC-3′
EGLD-RTPCR-F	5′-CCCCACTCATCCTGCGTAAC-3′
EGLD-RTPCR-R	5′-GAAGTTTCCATCAGCCGTCAC-3′
18SrRNA-RTPCR-F	5′-CCGTTCTTAGTTGGTGGAGTGA-3′
18SrRNA-RTPCR-R	5′-GCTCTATCCCCAGCACGACA-3′
EglD-BAD-F-KpnI	5′-CGGGGTACCACGGCTGCTGCTCCT-3′
EglD-BAD-R- KpnI	5′-CGGGGTACCTTAAGCGACTTCATTCACC-3′

sgfp sequence, was cloned to the KpnI site of plasmid pGEM:EglD-argB. The KpnI site was introduced in plasmid pGEM:EglD-argB by site-directed mutagenesis using primers EglD-GFP-F and EglD-GFP-R 5′ according to Kunkel et al., 1987. Plasmid pGEM:EglD-sGFP-argB was introduced by transformation in an *argB2* train, which is unable to grow in media lacking arginine. Plasmid pGEM:alcA-EglD-sGFP-argB was constructed as follows: the *eglD* open reading frame fused with a linker of four amino acids (DIGG) to the *sgfp* gene, was amplified from plasmid pGEM:EglD-sGFP-argB, using oligonucleotides alcA-EglD-NcoI and alcA-EglD-NcoI-R, and cloned in the NcoI restriction site downstream the *alcA* promoter of vector pGEM:*alcA* (Felenbok, 1991). The *argB* sequence was cloned to the PstI site of this plasmid to give pGEM:alcA-EglD-sGFP-argB. Finally plasmid pGEM: 5′eglD-sGFP-eglD3′-argB was constructed as follows: a second KpnI site was introduced by site directed mutagenesis in pGEM:EglD-sGFP-argB, immediately downstream the start codon of the *eglD* gene. The derived plasmid was digested with KpnI, in order to extract the EglD-sGFP sequence and clone the *sgfp* gene as a KpnI fragment in the remaining plasmid sequence. Finally, plasmid pGEM:EglD:BAD was constructed as follows: a fragment containing the Biotin Acceptor Domain (BAD) of oxaloacetate decarboxylase of *Klebsiella pneumoniae* (Consler et al., 1993; Frillingos and Kaback, 1996) followed by Y-tail, an epitope corresponding to the C-terminal dodecapeptide residues of *Escherichia coli* lactose permease (LacY; Carrasco et al., 1984), was amplified from plasmid pA-tag (Kafasla et al., 2007) using oligonucleotides EglD-BAD-F-KpnI and EglD-BAD-R-KpnI and cloned in the KpnI restriction site of plasmid pGEM:EglD-argB.

Inactivation of the *eglD* gene was achieved through the replacement of its coding sequence by the *pyr4* gene of *Neurospora crassa* (Buxton and Radford, 1983), via homologous double-crossover. A *pyrG89* strain, unable to grow in the absence of uracil and uridine (Balance and Turner, 1985; Oakley et al., 1987) was transformed with a linear tripartite construct composed of the upstream and downstream regions of the *eglD* locus flanking the *pyr4* gene. The *pyr4* sequence was used for the complementation of the *pyrG89* mutation, in order to avoid homologous recombination at the marker genetic locus (*pyrG*). The tripartite fragment was constructed by the Double-Joint PCR (D-J PCR) technique as described in Yu et al., 2004, with the exception that overlapping sequences were generated at the ends of both the *eglD* flanking regions and the *pyr4* gene for the assembly of the three molecules. Regions upstream and downstream the *eglD* gene were amplified using primers UPEGLD-F UPEGLD-R and DOWNEGLD-F DOWNEGLD-R, respectively. Marker gene *pyr4* was amplified from plasmid pPYR4 using primers PYR4EGLD-F and PYR4EGLD-R. It was cloned to the multi-cloning site of pGEM T Easy vector (PROMEGA) to produce plasmid pGEM:*pyr4*. The Expand High Fidelity PCR system (Roche Molecular Biochemicals, Mannheim, Germany) and nested primers NEGLD-F and NEGLD-

R were used for the construction and amplification of the 8 kb disruption cassette.

The primers used to amplify the *eglD* cDNA from genomic DNA of a wild-type strain and the radish 18 S ribosomal RNA gene from pRG3 (Delcasso-Themousaygue et al., 1988) were EGLD-RTPCR-F, EGLD-RTPCR-R and 18SrRNA-RTPCR-F and 18SrRNA-RTPCR-R, respectively.

Southern blot analysis was carried out according to Lockington et al., 1985 and Northern blot analysis using the glyoxal method as described by Tazebay et al., 1995.

An *eglD*-specific fragment, amplified from genomic DNA of a wild-type strain using primers EGLD-*BamHI* and EGLD-*EcoRI* was used as a probe in northern blots, whereas a ~1.5 kb EcoRI restriction fragment of plasmid pRG3 was used as 18 S *rRNA*-specific probe.

2.3. Fluorescence microscopy-laser scanning confocal microscopy

5×10^5 conidia per ml inoculated on sterile cover slips embedded in appropriate liquid culture media were incubated for different time intervals as indicated, at 25 °C (Tavoularis et al., 2001, 2003). Cover slips were washed with phosphate-buffered saline (PBS) and observed by epifluorescence microscopy (EFM) and laser scanning confocal microscopy (LSCM).

Samples were observed and photographed with an AXIOPLAN ZEISS phase-contrast fluorescent microscope and appropriate filters. LSCM was carried out on a BIO-RAD MRC 1024 CONFOCAL SYSTEM (Laser Sharp Version 3.2 Bio-Rad software, zoom 2×, excitation: 488 nm/Blue, samples at Laser Power 30%, Kalman filter $N = 5–6$, 0.3 μm cut, iris: 7–8, crypton/argon laser, Nikon DIAPHOT 300 Microscope, 60× (oil immersion) lens, emission filter 522/DF35. lens reference: Plan Apo 60/1.40 oil DM, Nikon Japan 160175, 60 DM/ Ph4, 160/0.17).

For microscopic observation of asexual compartments of *A. nidulans*, strains were grown between a square cube (5 × 5 mm) of solid agar minimal medium, positioned on a Petri dish with the same growth medium and a glass cover slip. Strains were allowed to grow in the dark for 4 days at 25 °C. The cover slip was then removed and placed on a slide with a drop of minimal medium. Samples were sealed with silicone and immediately observed microscopically. For the examination of asexual compartments in three-dimensional, dry samples, EFM gave, by far, better results than LSCM (Pantazopoulou et al., 2007). Thus, results shown are those from EFM.

2.4. Reverse transcriptase-PCR

RNAs were prepared from freshly harvested *A. nidulans* strains and conidia germinated in MM at 25 or 37 °C as indicated, using the TRIzol® Reagent (GIBCO-BRL) method and were further purified according to the RNeasy Mini Protocol for RNA Cleanup in the RNeasy Mini Kit (Qiagen). RNA was extracted from conidiating mycelia as described by Timberlake, 1980. To avoid contamination with genomic DNA, ~10 μg of each RNA sample were treated and cleaned up with TURBO DNA-*free*™ kit (Ambion). The absence of DNA contamination was verified with conventional PCRs in which using specific-*eglD* and 18S *rRNA* set of primers at least 2 μg of each RNA sample as template were amplified for 40 cycles (the absence of products indicates the absence of a detectable DNA contamination under the experimental conditions used). The quality of RNA was confirmed in a conventional 2% agarose gel stained with ethidium bromide (1 mg/ml). The concentration of each RNA sample was calculated using the nanodrop equipment (ND-1000 Spectrophotometer) according to the instructions of the manufacturer.

Similar two micrograms of each RNA sample were used for reverse transcription using the SuperScript™ II RNase H-reverse transcriptase (Invitrogen) according to the instructions of the manufacturer. Briefly, ~2 μg of RNA template were heated for 10 min at 70 °C with 250 ngr of Random Hexamer Primers and chilled immediately on ice for 3 min. Buffer and DDT were added at the appropriate concentrations and the mix was annealed at 25 °C for 5 min. Reverse transcriptase was added and a second incubation at 25 °C for 10 min was followed. Finally, an extension step at 42 °C for 1 h and a heat inactivation step at 70 °C for 15 min were performed. One microliter of each RT-PCR reaction was used as template for the amplification of the *eglD* transcript (278 bp) using primers EglD-RT PCR-F and EglD-RT PCR-R and the control 280 bp fragment of the 18 S *rRNA* transcript using primers 18SrRNA-RTPCR-F and 18SrRNA-RTPCR-R (Kato et al., 2003). For semi-quantitative analysis of transcript levels, cDNA was diluted 10-, 100-, 1000-fold. The number of cycles required to produce detectable difference in band intensity between dilutions (to avoid saturation) was determined with Et-Br staining. More precisely, PCR reactions were conducted for number of cycles ranging from 9 to 41, with an interval of three cycles. Results indicated 15 cycles for 18*S rRNA* and 30 cycles for *eglD*, after repeating the experiment three times.

2.5. Membrane protein extracts preparation and Western blotting

Conidiospore suspensions of *A. nidulans* strains were grown in MM containing 5 mM urea and 0.1% fructose as sole nitrogen and carbon sources, respectively, and the appropriate auxotrophies at 37 °C. For detection via the BAD epitope, 10 mg D-biotin/100 ml of minimal media was added. Mycelia (10 h) or swollen germinated conidiospores (4.5 h) were collected, washed with minimal media and ground in liquid nitrogen. The powder was weighed and suspended in 2 ml cold extraction buffer (10 mM Tris, pH 7.5, 5 mM $MgCl_2$, 100 mM NaCl, 0.3 M Sorbitol, 1 mM PMSF, 1 mM DTT, Protease inhibitors cocktail (Sigma, 1:500)) per gram. Suspensions were centrifuged at 2000*g* for 10 min at 4 °C followed by centrifugation of the supernatant at 42,000 rpm (Beckman OPTIMA MAX ultracentrifuge, MLA130 rotor) for 60 min at 4 °C. The gelatinous pellet was resuspended in 100 μl of buffer containing 20 mM sodium-phosphate buffer pH 7.5, 250 mM sucrose, 100 mM NaCl, 0.15% NP-40, 1:1000 protease inhibitors cocktail, 1 mM PMSF, 1 mM DTT, and 0.7 M β-mercaptoethanol, and separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 7.5% gel. Proteins were transferred to nitrocellulose membrane, probed with avidin-HRP antibody (Amersham) that recognizes the *in vivo* biotinylated BAD-epitope (Kafasla et al., 2007), and visualized by ECL (enhanced chemiluminescence; Jackson ImmunoResearch, USA). Protein concentration was determined using a modified Bradford assay (Bradford, 1976).

For the zymogram, cell wall/membrane protein extracts, after centrifugation at 13,000 rpm (~16,000 RCF) for 5–10 min, were incubated for 60 min at 4 °C with immobilized monomeric avidin–Sepharose beads (PIERCE) pre-equilibrated with equilibration buffer. The resin was washed twice with 20 volumes of equilibration buffer and biotinylated proteins were eluted in SDS sample buffer (10% glycerol, 100 mM DTT, 2% SDS, 0.1% bromophenol blue). A sample of the eluted proteins were separated by SDS–PAGE on a 7.5% gel, transferred to nitrocellulose membrane and probed with the avidin-HRP antibody. The rest was incubated at 37 °C for 20 min and analysed by electrophoresis on 12% polyacrylamide gel containing 0.2% CMC. Zymogram was carried out according to Beguin et al., 1983.

3. Results

3.1. Cloning of an expansin-like gene in A. nidulans

In silico analysis of the *A. nidulans* genome database (http://www.broad.mit.edu/annotation/fungi/aspergillus/faq.html) using

a representative expansin protein sequence from *Cucumis sativus* as a probe (Shcherban et al., 1995), revealed an ORF of 1.152 bp in supercontig 1.131 (scaffold 10), coding for a polypeptide chain of 366 amino acid residues with 30% identity and 40% similarity to plant expansins. This protein sequence with a calculated molecular mass of 36.7 kDa (http://au.expasy.org/tools/protparam.html; Gasteiger et al., 2005) has many putative homologues in fungal and bacterial species as shown by TBLASTN programmes (http://www.ncbi.nlm.nih.gov/blast/). The most similar (more than 50% sequence identity) are fungal hypothetical or characterized cellulases from *Neosartorya fischeri* (XP001259883), *Aspergillus oryzae* (BAE62388), *A. fumigatus* (XP753833) and *Magnaporthe grisea* (XP367645), bacterial endo-β1,4-glucanases that belong to glycoside hydrolase family 5 from *Clavibacter michiganensis* subsp. *Sepedonicus* (AAK16222), *Xanthomonas campestris* (AAM42805) and *X. oryza* (YP452835), plant expansins from *Oryza sativa* (XP642827) and expansin-like proteins from *Dictyostelium discoideum* (NP001049005) (Fig. 1). On the other hand, *A. nidulans* characterized endo-β1,4-glucanase *eglB* (AAM54071), β-1,3-endoglucanase *eglC* (AAT90341), 1,4-beta-D-glucan-cellobiohydrolyases *chbA* (AAM54071) and *chbB* (AAM54069) show low similarity with the product of the above gene. In accordance with the *A. nidulans* nomenclature rules (Martinelli and Kinghorn, 1994) and based on its similarity to endoglucanases, the gene was named *eglD* (AN7735.2) and the corresponding protein EglD (for Endoglucanase D).

The amino terminal domain (+45 to +192 from the +1 Met) of EglD is rich in serine and threonine residues that could be glycosylated by O linked glycosyl chains, as previously reported for a chitinase protein (Cts1p) of *Saccharomyces cerevisiae* (Kuranda and Robbins, 1991) (Fig. 1A). The carboxyl terminal domain (+183 to +273) shows an extensive similarity to expansin family-45 endoglucanase-like domain (PS50842 or Pfam HFDL domain; HLDL in EglD), a single family domain conserved in bacterial endo-β1,4-glucanases (Marchler-Bauer et al., 2005). It also shows an extensive similarity to a rare lipoprotein A (RlpA)-like double-psi beta-barrel domain (+210 to +263), a conserved region that has the double-psi beta-barrel (DPBB) fold, often indicating enzymatic activity (Marchler-Bauer et al., 2005) (Fig. 1A). Both domains are well conserved among EglD, bacterial and fungal endoglucanases as well as plant expansins as supported by phylogenetic trees created for each of these domains separately, using the Neighbour Tree Builder method (Kimara 2 parameter, data not shown).

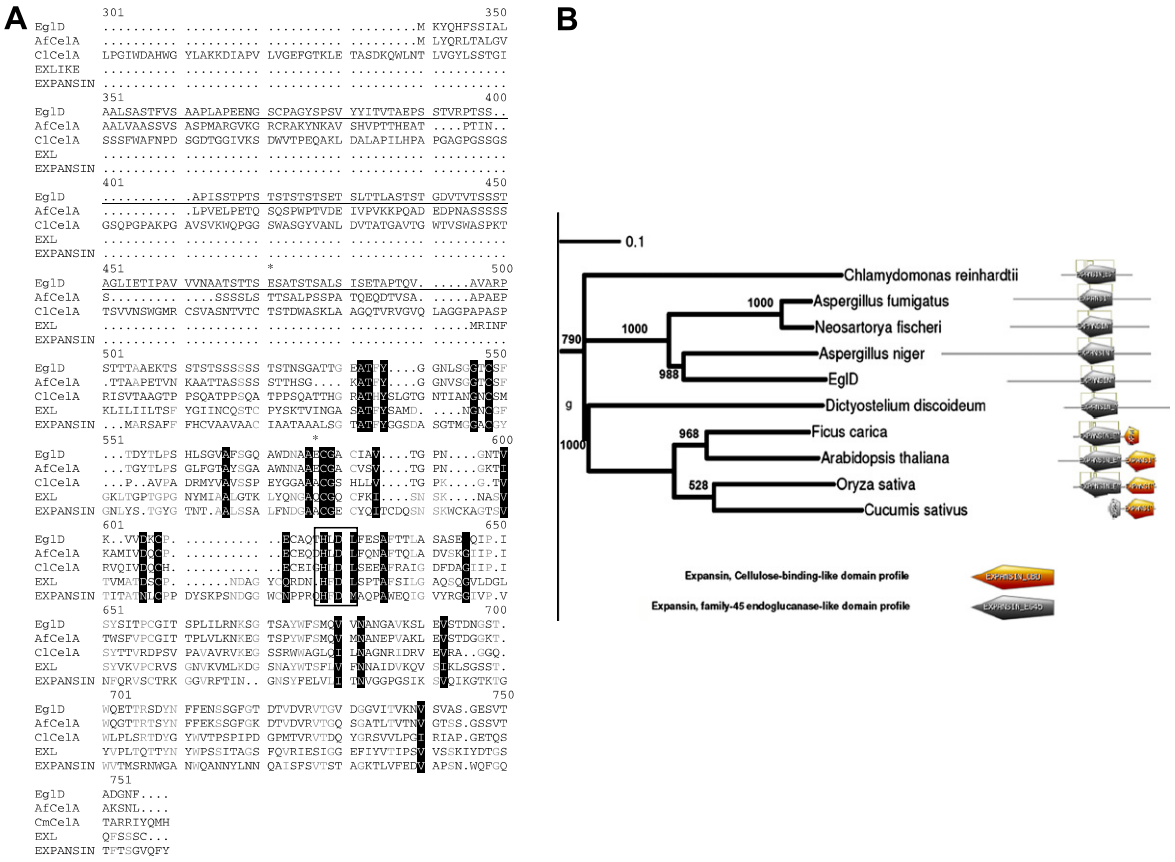

Fig. 1. (A) Multiple alignment of the EglD sequence against endo-β1,4-glucanases from *Aspergillus fumigatus* (AfCelA) and *Clavibacter michiganiensis* (ClCelA), an expansin-like protein from *Dictyostelium discoideum* (EXL) and an alpha expansin protein from *Oryza sativa* (EXPANSIN), using the Multalin version 5.4.1 of INRA (Corpet, 1988). The gap and gap length weight parameters used were 12 and 2, respectively. Letters in black boxes represent conserved amino acid residues in all proteins aligned, whereas gray letters represent conserved amino acid residues in two or three proteins. The amino terminal region, rich in serine/threonine residues that could be glycosylated by O-linked glycosyl chains, is underlined. The conserved HFDL (HLDL in EglD) expansin family-45 endoglucanase-like domain (Pfam:PS50842) is within the black square. Phylogenetic relationships between EglD and its putative homologues from *Aspergillus niger* (Accession No. XP_001390300), *A. fumigatus* (Accession No. XP_753833), *Neosartorya fischeri* (Accession No. XP_001259883), *Chlamydomonas reinhardtii* (Accession No. XP_001703263), *Dictyostelium discoideum* (Accession No. XP_642827), *Ficus carica* (Accession No. AAR27066), *Arabidopsis thaliana* (Accession No. NP_172717), *Oryza sativa* (Accession No. AAL24497) and *Cucumis sativus* (Accession No. AAB37746). (B) The phylogenetic tree was computed with the ClustalW reconstruction program (www.ebi.ac.uk/Tools/clustalw2), using as input the multiple sequence alignment of the above members corresponding to the EglD endoglucanase-like domain (+183 to +273). The filtering algorithms "Correct Distance" and "Ignore Gaps" were set on and Neighbor Joining was selected for tree type and clustering method. Bootstrap values per 1000 bootstrap are depicted on the nodes, suggesting that EglD can be safely classified alongside other fungal endoglucanases.

Moreover, all expansin proteins retrieved from the Refseq dataset and the top BLAST hits were analyzed for their domain architecture in Prosite. Our results revealed that all of them contain the Pfam Expansin-45-endoglucanase domain shown in Fig. 1A, which has three disulfide bridges. The Neighbor-joining phylogenetic dendrogram obtained from a multiple alignment using ClustalW (Fig. 1B) shows that EglD is clustered in a fungal branch as expected. All the fungal proteins in this branch share a common architecture with the Pfam Expansin-45-endoglucanase domain (two of the three disulfide bridges are conserved), being the only functional domain identified in Prosite. The other members of this tree (*Chlamydomonas reinhardti*, *Ficus carica*, *Arabidopsis thaliana*, *Oryza sativa, Cucumis sativus*, *Dictyostelium discoideum*) diverge from the fungal branch mainly due to the presence of the third disulfide bridge inside the expansin domain. Finally, all higher plant species carry an additional domain, the expansin cellulose-binding domain (PS50843), which distinguishes them from fungal proteins.

Southern blots of total genomic DNA extracted from a wild-type strain and hybridized with a probe from the *eglD* region (see Section 2) confirmed that there is only one copy of the *eglD* gene in *A. nidulans* genome (data not shown). The *eglD* gene is found in supercontig 1.131 which corresponds to chromosome IV and has an intron of 51 bp (between nucleotides +674 and +725, with respect to the adenine base (+1) of the ATG initiation codon). The presence of the intron was verified by sequencing the complete *eglD* cDNA clone obtained by RT-PCR using mRNA isolated from conidiospores of a wild-type strain germinated for 4 h at 25 °C in minimal media supplemented with urea and glucose as nitrogen and carbon sources, respectively. The cDNA clone was amplified using oligonucleotides EGLD-BamHI and EGLD-EcoRI designed from supercontig 1.131 (data not shown).

3.2. eglD is constitutively expressed during asexual developmental stages

RT-PCR in total RNA extracted from germinated conidia (8 h) of a wild-type strain cultured in media containing urea and glucose as sole nitrogen and carbon sources, respectively, revealed that *eglD* transcripts are accumulated at relatively low levels (Fig. 2A lane 1 and data not shown). Sequence analysis of the promoter region of the *eglD* gene identified one conserved putative *creA* (glucose repressor) binding site (SYGGRG) (Felenbok et al., 2001), suggesting that *eglD* mRNA expression may be repressed by glucose, i.e., is under Carbon Catabolite Repression (CCR). To test this possibility we performed semi-quantitative RT-PCRs in total RNAs extracted from germinated conidia (8 h) of a wild-type strain cultured in media containing fructose instead of glucose as sole carbon source and from a *creA* derepressed (*creA*^{d}1) mutant strain exhibiting failure of CCR in response to repressed carbon sources (Shroff et al., 1996, 1997). Both strains were cultured in media containing urea as sole nitrogen source and glucose or fructose as alternative carbon sources. The results presented in Fig. 2A showed that *eglD* transcript accumulation is similar in all cases, indicating that *eglD* gene expression is not affected by CCR. The primers used for RT-PCR were expected to produce amplicons of 278 bp (*eglD* cDNA) and 280 bp (18 S *ribosomal RNA* gene), respectively (Kato et al., 2003).

We then examined by RT-PCR using total RNA whether the expression of the *eglD* gene is transcriptionally regulated at different developmental stages and/or compartments of *A. nidulans* asexual life cycle. RNA was extracted from a wild-type strain grown for 0–20 h: rested (ungerminated) conidia (0 h), swollen germinated conidia (2, 4, 6, 8 and 10 h), germlings (12 h), young mycelia (14, 16 and 18 h), old mycelia (20 h) and conidiating mycelia. Minimal media supplemented with fructose (Fig. 2C) or glucose (data not shown) as alternative carbon sources and urea as sole nitrogen source, were inoculated with conidiospores of a wild-type strain. Samples were taken after 0, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 h intervals of growth and from conidiating mycelia (Fig. 2B). The results presented in Fig. 2 (B and C) show that *eglD* mRNA transcripts are detected in all stages of *A. nidulans* asexual life cycle, from the stage of rested to the stage of swollen germinated conidia, during the onset of germ tube emergency, in young and old mycelia as well as in conidiating mycelia, indicating that *eglD* gene is constitutively expressed during development.

3.3. EglD protein is expressed during the onset of the asexual life cycle

In order to investigate the localization of the EglD protein we have studied the expression of chimeric EglD-sGFP molecules in a wild-type strain grown in minimal media with urea and fructose as nitrogen and carbon sources, respectively, in all developmental stages and compartments of *A. nidulans* asexual life (for sGFP: Chiu et al., 1996; Suelmann et al., 1997; Fernández-Álbaros et al., 1998; Valdez-Taubas et al., 2000; Tavoularis et al., 2001, 2003).

An integrative plasmid expressing chimeric EglD-sGFP molecules and carrying the *argB* gene of *A. nidulans* (see Section 2) was introduced by transformation in an *argB2* strain, which is unable to grow in media lacking arginine. We searched for single plasmid integration events at the resident locus the transformants isolated by Southern blots. Even though we obtained single copy integrations these were ectopic. The corresponding strains showed a radial growth, density of the colony and conidiation comparable to a wild-type strain carrying the untagged *eglD* gene and one of them was selected for further analysis (data not shown). Conidiospores of this transformant (EglD-sGFP) were allowed to germinate for 4 h (swollen germinated conidia) at 25 °C in minimal media containing urea as sole nitrogen source and fructose or glucose as alternative carbon sources and were observed by Laser scanning confocal microscopy (LSCM) and/or Epifluorescence Microscopy (EFM). As shown in Fig. 3A and B EglD specific GFP fluorescence as patches is associated only with the periphery of swollen germinated conidia and fluorescence intensity is lower in the presence of glucose compared to fructose. This visual estimation of fluorescence intensity was constantly observed in all experiments performed. Additionally, conidiospores of EglD::sGFP and AlcA$_{(p)}$::EglD::sGFP (see below) strains were allowed to germinate for 20 h (old mycelia) in urea and fructose as sole nitrogen and carbon sources, respectively, and were observed by EFM and/or LSCM. As shown in Fig. 3C-left, patches of *5′eglD*-EglD and *alcA*-EglD specific GFP fluorescence are associated only with conidial cell wall while no fluorescence was detected in the germtubes. Finally, we took advantage of the strain expressing EglD-sGFP molecules to study EglD expression in conidiophores by EFM. This strain was grown for 4 days at 25 °C in dark, with urea and fructose as sole nitrogen and carbon sources, respectively. Samples for microscopy were prepared as described in Section 2. As shown in Fig. 3D, EglD-sGFP molecules are clearly fluorescing only in the periphery of conidiospores, while they are absent from metulae and phialides.

3.4. Expression of the eglD gene is regulated post-transcriptionally

A plasmid expressing in-frame fusions of the *sgfp* coding sequence to the ATG initiation codon of the *eglD* gene and containing the *argB* gene was used to transform protoplasts of an *argB2* strain. The expression of the *sgfp* gene in this construct is under the control of the *eglD*-5′ and -3′ flanking sequences (see Section 2). *argB*$^{+}$ transformants were selected on minimal media lacking arginine. All transformants showed growth phenotypes similar to that of a wild-type strain in all media tested, and one of them with ectopic single copy integration revealed by Southern blots, was used for

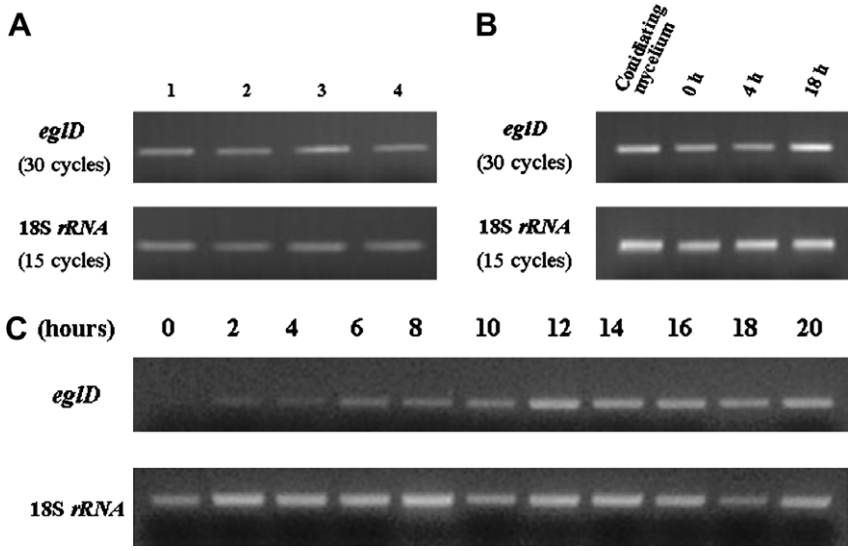

Fig. 2. Expression of the *eglD* gene. *eglD* transcript levels in (A) germinated conidia of a wild-type and a *creA^{d}1* strains grown under different nutritional conditions, (B) conidiating mycelia, rested conidia, germinated conidia and young mycelia of a wild-type strain and (C) at various asexual developmental stages of *A. nidulans* life cycle of a wild-type strain. (A) Expression pattern of the *eglD* gene in a wild-type and a *creA^{d}1* strains using semi-quantitative RT-PCRs. Similar ten micrograms of total RNAs were extracted from mycelia of wild-type (lanes 1 and 2) and *creA^{d}1* strains (lanes 3 and 4), grown in liquid minimal media supplemented with urea as sole nitrogen source and 0.1% fructose (lanes 2 and 4) or 1% glucose (lanes 1 and 3) as alternative carbon sources, for 10 h at 37 °C. (B) Expression pattern of the *eglD* gene by semi-quantitative RT-PCRs using RNAs extracted from conidiating mycelia, rested conidia (0 h), germinated conidia (4 h), and young mycelia (18 h) of a wild-type strain. Similar ten micrograms of total RNAs were extracted from conidiospores of a wild-type strain grown for the time indicated in liquid minimal media supplemented with 10 mM urea and 0.1% fructose as sole nitrogen and carbon sources, respectively, at 25 °C. (C) Detection of *eglD* expression using RT-PCRs in dormant conidiospores (0 h, lane 0), germinated conidia (2, 4, 6, 8 and 10 h, lanes: 2–10), young mycelia (12, 14, 16 and 18 h, lanes: 12–18) and old mycelia (20 h, lane: 20) of a wild-type strain grown for the indicated time at 25 °C. 18 S *rRNA* steady-state levels are used to monitor the amount of RNA loading in each lane.

further analysis (data not shown). Rested conidiospores of this strain (0 h), swollen germinated conidia (4 h), young mycelia (16 h) and old mycelia (24 h) at 25 °C grown in the presence of urea and fructose as sole nitrogen and carbon sources, respectively, were analyzed by EFM and LSCM. As shown in Fig. 4 expressed 5′EglD-sGFP-EglD3′ molecules were homogenously distributed all over rested conidiospores, swollen germinated conidia, young and old mycelia.

The expression profile of *eglD* (Fig. 2B and C) together with the cellular distribution of the EglD-sGFP and 5′EglD-sGFP-EglD3′ chimeric molecules (Figs. 3 and 4) indicate that although *eglD* mRNA transcripts are expressed in all developmental stages of the asexual life cycle of *A. nidulans,* EglD-sGFP molecules are exclusively restricted to the periphery of germinating conidiospores (apart from the developing germination tubes). On the contrary, sGFP molecules expressed under the control of the *eglD*-5′ and 3′ regulatory sequences are homogenously distributed all over the conidiospores as well as along the entire length of the germtubes. These results clearly indicated that expression of the *eglD* gene is constitutive while localization of the EglD protein is highly regulated.

3.5. Function of the eglD gene

3.5.1. Overexpression of the eglD gene

In order to overexpress the *eglD* gene we replaced the *eglD*-5′ flanking sequences with the strong, ethanol-inducible, glucose-repressible, *alcA* promoter (Adams and Timberlake, 1990; Felenbok, 1991; Felenbok and Kelly, 1996; Felenbok et al., 2001) as described in Section 2. The plasmid overexpressing the *eglD* gene (pGEM:alcA-EglD-sGFP-argB) was introduced by transformation in an *argB2* strain. All 8 transformants isolated showed growth similar to wild-type strains carrying either the untagged *eglD* gene or a tagged variant expressed from its native promoter, in media containing urea and fructose as nitrogen and carbon sources, respectively. Ectopic single copy plasmid integration events occurred in two of the transformants as revealed by Southern blots, and one was selected for further analysis (data not shown). In this transformant the functionality of the *alcA* promoter driving the expression of the *eglD-sgfp* sequence was subsequently confirmed by Northern blots. Under induced (ethanol or butanone) repressed (glucose) conditions *eglD* mRNA steady state levels were very low, while under derepressed (fructose) induced (ethanol or butanone) conditions a dramatic over-expression of *eglD* transcript was observed (data not shown). This corresponds to approximately 4–5 times the expression of the *eglD* gene from its native promoter under non-induced derepressed conditions and about 50 and more than 90 times under derepressed conditions induced with ethanol or butanone, respectively.

In the strain overexpressing the *eglD* gene localization of chimeric EglD-sGFP molecules under derepressed (fructose), repressed (glucose), induced (+butanone) and non-induced (−butanone) conditions was examined by LSCM. Our results presented in Fig. 3A–C showed that EglD-sGFP molecules expressed from the *alcA* promoter have a distribution similar to that of the EglD-sGFP molecules expressed from the *eglD* native promoter, both in germinated conidia (4 h and 30 min) and old mycelia (16 h). In other words, the *alcA*-driven *eglD* expression does not interfere with conidial specific cell wall localization of EglD-GFP molecules. Moreover, EglD specific GFP fluorescence is more intense in a strain expressing *alcA*-EglD-GFP molecules grown under induced (+butanone), derepressed (fructose) conditions than in a strain where *eglD* expression is driven by its native promoter grown under fructose and/or glucose (Fig. 3A and B). These results clearly indicated that when *eglD-sgfp* expression is driven by the *alcA* promoter EglD levels are increased, in accordance to Adams and Timberlake, 1990.

3.5.2. Inactivation of the eglD gene

Inactivation of the *eglD* coding sequence by the introduction of the *pyr4* gene of *N. crassa* at its locus due to homologous double-crossover events occurred in ~30% of the transformed strains (as

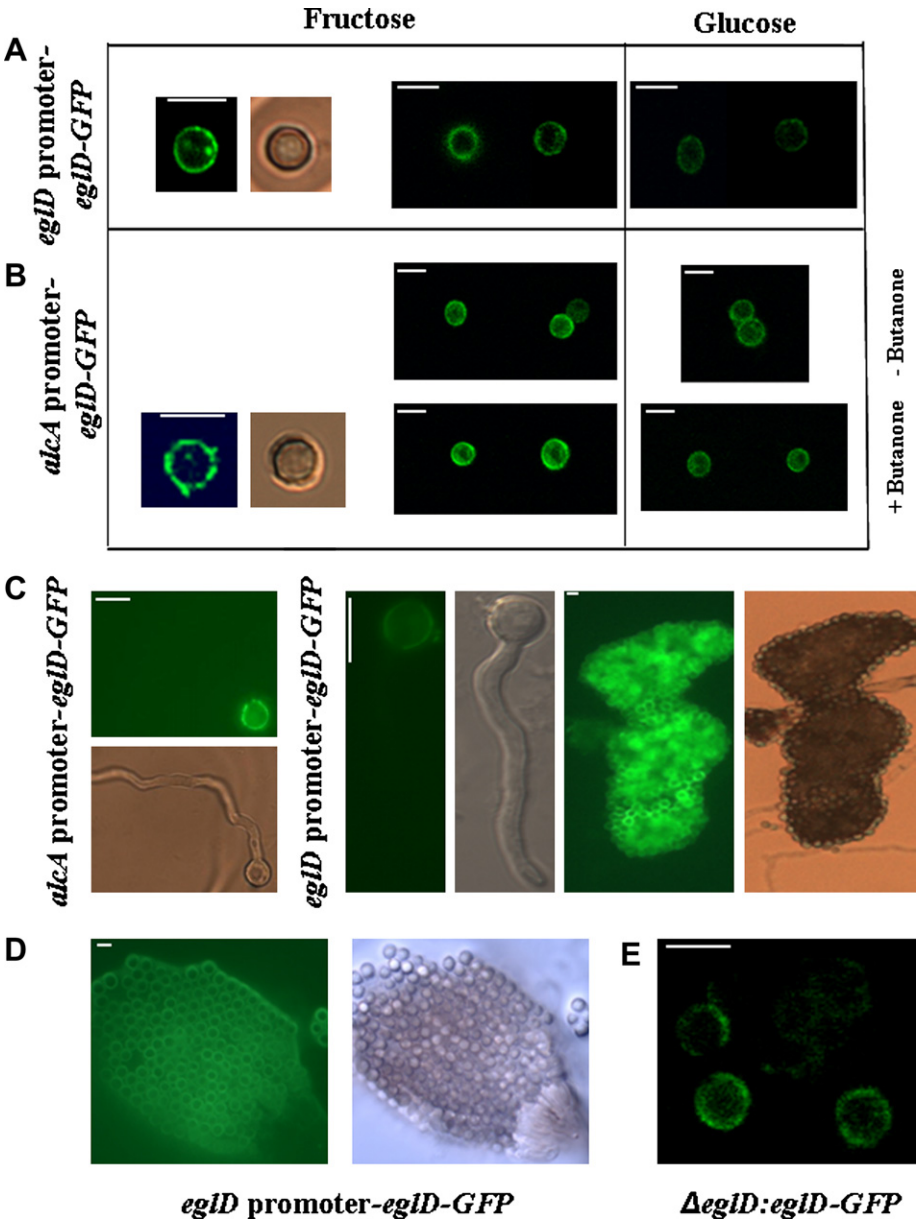

Fig. 3. Subcellular localization of the EglD protein. Representative pictures from laser scanning confocal microscopy (A, B, C-left, E) and epifluorescence microscopy (C-right, D) of wild-type strains expressing chimeric EglD-sGFP (A, C-left, D) and alcA-EglD-sGFP (B, C-right) molecules, or *ΔeglD:eglD-sgfp* strain expressing chimeric EglD-sGFP (E). The strains were grown in the presence of 5 mM urea as sole nitrogen source and 0.1% w/v fructose or 1% w/v glucose as alternative carbon sources, at 25 °C for 4 h (swollen germinated conidiospores) A, B, C-right, E, 16 h (young mycelia) C-left, and 4 days (conidiophores) D. Strains expressing $alcA_{(p)}$::EglD::sGFP molecules were induced with 50 mM 2-butanone for the last 2 h of their growth. GFP fluorescence is shown on the left panels while Nomarski pictures of the same samples on the right and down panels. Bar 5 μm.

revealed by Southern blot analysis, data not shown), indicating that *eglD* gene is not essential for growth. All inactivated strains showed identical phenotypes and grew similarly to a wild-type strain on minimal media supplemented with urea and fructose or glucose as sole nitrogen and carbon sources, respectively, and one was used for further study (data not shown).

In order to examine the role of the *eglD* gene product during asexual development, the phenotypes of *ΔeglD, $alcA_{(p)}$::eglD::sGFP* and wild-type strains were compared regarding specific characteristics of *A. nidulans* asexual life cycle (conidiation, timing of conidial germination, shape, size and number of conidiospores) as well as in a series of morphological tests (CMC (carboxymethylcellulose) plate assay described in Lockington et al., 2002, growth tests on intact plant material, temperature and osmotic stress effects). In every case all strains showed similar phenotypic profiles (data not shown).

Recently, EglC, a putative β1,3-endoglucanase was implicated in digestion of sugar polymers during sexual development in *A. nidulans*. Deletion of the *eglC* gene resulted in hyphae more resistant to cell wall lyzing enzymes, implying that the cell wall structure of an *eglC*-null mutant is different from that of a wild-type strain (Choi et al., 2005). Thus, conidiospores of *ΔeglD, $alcA_{(p)}$::eglD::sgfp* and wild-type strains were digested with glucanex, a mixture of cell wall lyzing enzymes, and the number of protoplasts was counted in each strain separately. Our results presented in Table 4 showed that at 1 h or more of glucanex treatment, *ΔeglD* strain generates less protoplasts than a wild-type strain expressing the *eglD* gene from its native promoter, suggesting that conidiospore cell wall structure is altered in the absence of EglD. Moreover, Table 4 shows that *alcA-eglD-sgfp* strain overexpressing the *eglD* gene generates a number of protoplasts similar to that of a wild-type strain, imply-

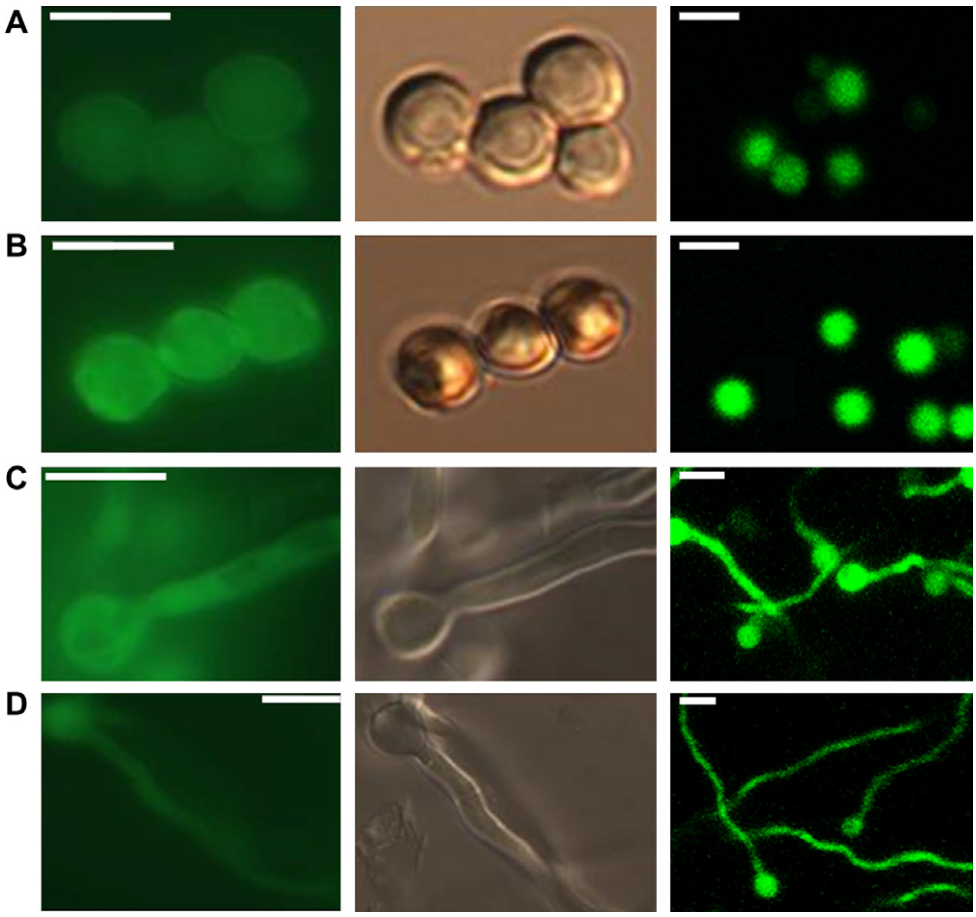

Fig. 4. Subcellular localization of sGFP molecules expressed from the *eglD* promoter. Representative pictures from epifluorescence microscopy (left panels), and laser scanning confocal microscopy (right panels) of a wild-type strain expressing sGFP proteins under the control of native *eglD* 5′ and 3′ regulatory sequences. The strain was grown at 25 °C in the presence of 20 mM urea and 0.1% w/v fructose as sole nitrogen and carbon sources, respectively: (A), rested conidiospores; (B), swollen germinated conidiospores (4 h); (C), young mycelia (16 h); and (D), old mycelia (24 h). GFP fluorescence by EFM is shown on the left panels while Nomarski pictures of the same samples on the middle panels. Bar 5 μm.

ing that conidiospore cell wall structure is not significantly altered when EglD protein levels are increased.

3.5.3. Functionality of the EglD-sGFP protein molecules

In order to test the functionality of the EglD-sGFP molecules, an integrative plasmid expressing an in-frame fusion of the *sgfp* coding sequence to the carboxyl terminus of the *eglD* gene was introduced by transformation to the Δ*eglD* strain. The expression of the *sgfp* gene in this construct is under the control of the *eglD*-5′ and -3′ flanking sequences. Ectopic single copy plasmid integration events occurred in three of the 18 transformants analyzed, as revealed by Southern blots (data not shown). Conidiospores of these three transformants were grown in minimal media with urea and fructose as sole nitrogen and carbon sources, respectively, for 4 h and 30 min at 25 °C, and analyzed by LSCM. Our results presented in Fig. 3E showed that distribution of EglD-sGFP molecules in all three

Table 4
Protoplastation assay

Strain	No. of protoplasts × 10^{-5}/ml [a](average ± standard deviation)			
	30 min	1 h	1 h 30 min	2 h 30 min
eglD^{+} *(wt1)*	2.5 ± 0.5	16 ± 3.0	15 ± 4	15.6 ± 2.4
eglD^{+} *(wt2)*	2.4 ± 0.8	15 ± 3.6	14 ± 2.2	15.3 ± 2.2
ΔeglD	5.6 ± 2.3	3.7 ± 1.8	3.17 ± 0.6	3.6 ± 0.5
alcA-eglD (butanone)	4.6 ± 3.7	16.1 ± 2.2	16.9 ± 3.5	14.7 ± 1.5
ΔeglD:EglD	5.2 ± 0.8	12 ± 2.5	13 ± 2.0	14.0 ± 2.5
ΔeglD:EglD-sgfp	4.5 ± 2.3	10.2 ± 1.8	12.3 ± 1.7	13.5 ± 1.0

Number of protoplasts formed by different strains after treatment with Glucanex.
An equal number (~6.5 × 10^{6}) of conidia of strains expressing a wild-type *eglD* gene (wt1, wt2), having the *eglD* gene deleted (Δ*eglD*) or overexpressed (*alcA*$_{(p)}$*::eglD::sgfp*), as well as of a *ΔeglD::EglD::sgfp* strain expressing EglD-sGFP molecules and a *ΔeglD::EglD* strain, were allowed to germinate for 4 h and 30 min in MM at 37 °C with appropriate supplements in the presence of urea as sole nitrogen source and fructose or glucose (data not shown) as alternative carbon sources, respectively. In strains expressing alcA-EglD-sGFP molecules 0.1% v/v 2-butanone was added for 2 h before harvesting germinated conidia to induce the expression of the *eglD* gene driven by the *alcA* promoter (Adams and Timberlake, 1990). Germinated conidia were transferred to an isosmotic solution and treated with 500 mg of Glucanex® (Novozymes) to generate protoplasts according to Yelton et al., 1984. After 30 min, 1 h, 1 h and 30 min and 2 h and 30 min, samples were plated on isosmotic (viable protoplasts) and non-isosmotic (conidia) media. The number of protoplasts in each sample was counted using a Haematometer. The results showed are the mean value of at least five independent experiments.

strains is similar to that of a wild-type strain (Fig. 3E), and one was used for further analysis. In this strain the functionality of the EglD-sGFP molecules was analyzed by counting the number of protoplasts produced after digestion of its conidiospores with glucanex (see above). Our results presented in Table 4 showed that *ΔeglD::EglD::sGFP* strain generates a number of protoplasts similar to that of a wild-type strain.

3.5.4. Immunoblot analysis of the EglD protein

To investigate the temporal expression pattern of the EglD protein, a tagged version of the *eglD* gene was constructed in an integrative plasmid as described in Section 2. This plasmid expresses EglD-tagged molecules having the Biotin Acceptor Domain (BAD) (Consler et al., 1993; Frillingos and Kaback, 1996) fused in frame to the carboxyl terminus of the protein, followed by a Y-tail epitope (Carrasco et al., 1984; Kafasla et al., 2007). This EglD-tagged construct was introduced by transformation in an *argB2* strain. Southern blots of *argB*$^+$ transformed strains selected on minimal media in the absence of arginine, showed single copy plasmid sequences ectopically integrated in their genomes. All transformants showed identical phenotypes and grew similarly to a wild-type strain carrying the untagged *eglD* gene on all media tested and one of them was used for further study. As shown in Fig. 5 the BAD-Y-tail tag allowed specific detection of EglD in cell/wall membrane proteins extracted from swollen germinated conidia (4 h/25 °C) and young mycelia (14 h/25 °C) of a strain expressing EglD-tagged molecules by blotting with avidin-HRP that recognizes the *in vivo* biotinylated BAD-epitope. No EglD was detected in cell/wall membrane proteins of a wild-type strain expressing untagged EglD molecules or in protein fractions excreted to the medium from swollen germinated conidia (5 h), young mycelia (12 h) and old mycelia (24 h) of either of the two strains, indicating that EglD is not a secreted protein (data not shown). The approximate molecular weight of EglD molecules was found to be ~66 kDa. Considering the size of the epitope used, the protein has slower electrophoretic mobility compared to the predicted ~50 kDa, most likely due to post-translational modifications.

Our attempts to detect a glucanase activity of EglD were unsuccessful. Specifically, *in vivo* biotynilated EglD-BAD-Y-tail molecules extracted from cell/wall membrane preparations of germinated conidia (4 h and 30 min/37 °C) and young mycelia (12 h/37 °C) were immobilized on avidin-coated beads, through their BAD domain according to Kafasla et al., 2007. EglD-BAD-Y-tail molecules were detected by immunoblotting analysis with avidin HRP antibody to be the only eluted biotinylated proteins. They were examined in a zymogram and a plate assay for their activity to digest carboxymethylcellulose (CMC), a representative substrate for cellulase activity (Lockington et al., 2002). No clear zone or spot corresponding to the EglD-BAD-Y-tail protein was detected following visualization with 0.3% (w/v) Congo red (Beguin et al., 1983; data not shown) in both assays.

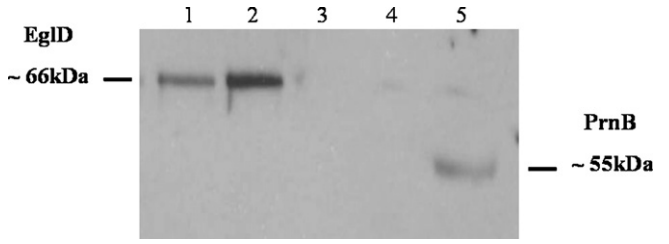

Fig. 5. Western blot analysis of EglD-BAD-Y-tail protein molecules. Thirty micrograms of protein molecules extracted from cell wall/membrane fractions of swollen germinated conidia (4.5 h/25 °C) (lanes 1 and 3) and young mycelia (14 h/25 °C) (lanes 2 and 4) of strains expressing tagged-EglD (EglD-BAD-Y-tail) (lanes 1 and 2) or untagged EglD (*eglD*$^+$) (lanes 3 and 4) molecules. Proteins were analyzed by SDS/7.5%PAGE followed by electroblotting onto nitrocellulose membrane and probing with avidin HRP antibody. Tagged molecules of the major proline transporter of *A. nidulans* (PrnB-BAD-Y-tail) extracted from cell wall/membrane fractions of young mycelia (14 h/25 °C) of a strain expressing tagged-PrnB molecules (Kafasla et al., 2007) were used as control (lane 5).

4. Discussion

In the present work, we report the cloning and functional characterization of a unique expansin-like gene *eglD* in *A. nidulans*. EglD is a spore specific cell wall protein as revealed by the subcellular localization of chimeric EglD-GFP molecules, that shows significant sequence similarity to fungal cellulases and bacterial endo-β1,4-glucanases. All these proteins together with plant expansins share a common functional domain, the Pfam: 45-endoglucanase family domain. Despite the lack of direct evidence of an *in vitro* enzymatic activity of EglD, the inactivation of the *eglD* gene drastically increased resistance of the fungal cell wall against lyzing enzymes. Considering that synergy is often necessary for the activity of cell wall associated enzymes (Vries et al., 2001 and references therein), our results suggest that EglD is a significant component of an enzymatic mixture implicated in *A. nidulans* cell wall remodelling function during germination.

A number of distinct, non-catalytic regions have been identified in fungal cell wall associated enzymes. The serine/threonine-rich domain located towards the C-terminus of EglD is a common non catalytic region of many yeast cell wall proteins, which undergoes heavy O-linked mannosylation of serine/threonine residues (Willer et al., 2005 and references therein). This pattern of glycosylation is thought to confer an extended, rod-like configuration of proteins and, in the case of cell wall hydrolases, it may ensure that the active site of the enzyme is projected towards the surface of the cell or elsewhere within the wall structure (Popolo and Vai, 1999). This type of modification could explain the result of the immunoblot analysis of the EglD protein, showing a higher molecular weight than predicted from the peptide backbone.

Genes of fungal cell wall hydrolytic enzymes are usually repressed by the presence of carbon sources such as glucose which can be easily metabolised, while they are induced in the presence of their substrate polymers. This conditional expression results in glucose accumulation from hydrolysis of fungal or plant cell wall polysaccharides when glucose is not directly available (Aro et al., 2005 and references therein). However, expression of the *eglD* gene is not subjected to glucose mediated carbon catabolite repression (CCR) as shown by RT-PCR analyses. In other words, the regulation pattern of the *eglD* gene differs from that of the majority of genes encoding enzymes that participate in degradation of plant cell wall polysaccharides, such as xylanolytic and cellulolytic enzymes (deVries and Visser, 2001). This in combination with the lack of EglD endoglucanase activity according to zymogram analysis and CMC plate assays may exclude a direct polysaccharide hydrolytic activity of this protein, and further experiments should be conducted to access its biological function.

In summary, our *in silico* data and protoplastation assay suggest that EglD may have an endoglucanase activity. On the other hand, the *eglD* gene shows a different pattern of regulation than the majority of genes encoding cell wall-modifying enzymes. Specifically, *eglD* gene expression is not under carbon catabolite repression (CCR) as the expression of the majority of genes encoding cell wall-modifying enzymes, which is both glucose repressed and inducer dependent (Prade et al., 2001 and references therein).

Moreover, although *eglD* mRNA transcripts are constitutively present in all developmental stages of *A. nidulans* asexual life, EglD protein is present only in conidia, being absent from hyphae, metulae and phialides of conidiophores. Developmental regulators such as wetA (Marshall and Timberlake, 1991) may be directly or

indirectly involved in conidial specific translation of *eglD* mRNA transcripts. However, further studies are needed to identify *cis* sequences and/or *trans* acting factors that are implicated in EglD developmental regulation.

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