Characterization of the Carbohydrate Binding Module 18 gene family in the amphibian pathogen *Batrachochytrium dendrobatidis*

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A R T I C L E   I N F O

A B S T R A C T

Batrachochytrium dendrobatidis (*Bd*) is the causative agent of chytridiomycosis responsible for worldwide decline in amphibian populations. Previous analysis of the *Bd* genome revealed a unique expansion of the carbohydrate-binding module family 18 (CBM18) predicted to be a sub-class of chitin recognition domains. CBM expansions have been linked to the evolution of pathogenicity in a variety of fungal species by protecting the fungus from the host. Based on phylogenetic analysis and presence of additional protein domains, the gene family can be classified into 3 classes: Tyrosinase-, Deacetylase-, and Lectin-like. Examination of the mRNA expression levels from sporangia and zoospores of nine of the *cbm18* genes found that the Lectin-like genes had the highest expression while the Tyrosinase-like genes showed little expression, especially in zoospores. Heterologous expression of GFP-tagged copies of four CBM18 genes in *Saccharomyces cerevisiae* demonstrated that two copies containing secretion signal peptides are trafficked to the cell boundary. The Lectin-like genes *cbm18-ll1* and *cbm18-ll2* co-localized with the chitinous cell boundaries visualized by staining with calcofluor white. *In vitro* assays of the full length and single domain copies from CBM18-LL1 demonstrated chitin binding and no binding to cellulose or xylan. Expressed CBM18 domain proteins were demonstrated to protect the fungus, *Trichoderma reeseii*, *in vitro* against hydrolysis from exogenously added chitinase, likely by binding and limiting exposure of fungal chitin. These results demonstrate that *cbm18* genes can play a role in fungal defense and expansion of their copy number may be an important pathogenicity factor of this emerging infectious disease of amphibians.

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1. Introduction

Batrachochytrium dendrobatidis (*Bd*) is an aquatic, flagellated chytrid fungus and is the causative agent of chytridiomycosis, one of the major contributors of worldwide decline in amphibian populations. *Bd* is a member of the early diverging Chytridiomycota Phylum, and is an emerging infectious disease of amphibians. A second chytrid pathogen, *Batrachochytrium salamandrivorans sp. nov.*, was reported to cause lethal skin infections in salamanders, resulting in steep declines in salamander populations in northwestern Europe (Cheng et al., 2011; Martel et al., 2013). Chytridiomycosis not only causes individual

mortality, but also devastates entire amphibian populations, causing declines leading to eventual extinction (Cheng et al., 2011).

In 1998, *Bd* was first identified as a cause of amphibian disease and isolated from the epidermis of an infected frog (Longcore et al., 1999). *Bd* colonizes the stratum corneum of amphibian skin or larval mouthparts (Marantelli et al., 2004). Extensive colonization causes a series of physiological effects such as disruption of the osmoregulatory function of the skin, resulting in dehydration, osmotic imbalance, and eventual asystolic cardiac arrest (Marcum et al., 2010; Voyles et al., 2007, 2011, 2012, 2009). From the host side, amphibians such as *Xenopus laevis*, employ both innate and adaptive components of the immune system to resist *Bd* infection (Ramsey et al., 2010). Some amphibians produce antimicrobial peptides (Rollins-Smith, 2009; Rollins-Smith and Conlon, 2005) and antifungal metabolites (Becker et al., 2009), which provide non-specific protection against the pathogen, and the secreted antibodies in the mucus of *X. laevis* exposed to *Bd* can provide specific anti-*Bd* protection (Ramsey et al., 2010). The three species of amphibians have demonstrated acquired

immunity to *Bd* that overcomes pathogen-induced immunosuppression (McMahon et al., 2014).

Previous studies have focused on aspects of *Bd* biology including ecology, evolution, and pathogenesis but few results have linked specific molecules in the fungus to virulence progression. The availability of whole genome sequences and analysis has enabled computational searches for genes that have contributed to the evolutionary transition to pathogenicity. During the initial analysis of *Bd* genome from two isolates, JEL423 and JAM81, the expansion of CBM18 gene family was identified, which implicated expansions as an important recent adaption in the transition to pathogenicity (Abramyan and Stajich, 2012; Fisher et al., 2012; Joneson et al., 2011; Rosenblum et al., 2013).

CBM18 is a subclass of chitin binding domains that have convergently evolved in fungi, plants, and arthropods (Suetake et al., 2000). The CBM18 domain is highly conserved across the eukaryotes that contain it. The founding member of the described family is the hevein domain in plants, first discovered in the latex of rubber tree (*Hevea brasiliensis*) (Archer, 1960). The *Bd* CBM18 domains are comprised of ∼44 residues organized around a homologous (cysteine) pattern of $X_3CGX_7CX_4CCSX_4CX_6CX_3C$ and are identifiable with the Pfam domain PF00187 (Chitin_bind_1). The *Bd* CBM18 genes were categorized into three groups: lectin-like (LL) group, tyrosinase-like (TL) group and deacetylase-like (DL) group, according to the secondary domain in the gene itself (Abramyan and Stajich, 2012). There are 18 predicted genes in the JEL423 genome with CBM18 domains and the domain copy number ranges from one to eleven in the genes. One of the largest observed number of CBM18 domains in any one gene in fungi or other species is the 11 noted in the LL gene BDEG_01757 (Abramyan and Stajich, 2012). The similarity and relationships between the domains was previously described and led to ascribing a letter name to groups of domains that were phylogenetically most similar (e.g. A, B, C, D) (Abramyan and Stajich, 2012). Copy numbers of the CBM18-containing genes in other species of fungi range from 1 to 4 copies in *Aspergillus* fungi, and only one copy in *Neurospora crassa* (http://pfam.xfam.org/) (Finn et al., 2014).

Chitin is the major component of chytridiomycete cell walls (Bartnicki-Garcia, 1968; Kroh et al., 1977) and likely important in the rigidity and shape of the sporangia. Examination of gene expression in *Bd* has indicated that genes for chitin synthases and chitin-binding proteins vary in expression between zoospore and sporangium life stages (Rosenblum et al., 2008). Components released by *Bd* cell walls also have an inhibitory effect on the proliferation of amphibian lymphocytes (Fites et al., 2013). The treatment of Nikkomycin Z, a chitin synthase inhibitor, on *Bd* cells dramatically alters the cell wall stability and completely inhibits growth of *Bd* at 250 μM (Holden et al., 2014).

Previous work has identified the significant expansion and evidence for positive selection in the domain copies of CBM18s in the *Bd* genome, suggesting a potential role they may have in protecting the pathogen from recognition or degradation by the amphibian host (Abramyan and Stajich, 2012). Here, we further investigate the function of CBM18s *in vitro* by testing for expression. This family was chosen because of the observed recent dramatic expansion of copy number and that CBMs have been implicated in fungal protection from host defenses such as *Avr4* (CBM14), which prevents plant factors from degrading fungal chitin (van den Burg et al., 2006). As currently few efficient transformation systems have been developed for early diverging fungi, and none so far for Chytridiomycota fungi including *Bd*, we limited our experiments to evaluation of protein functions using a heterologous expression system. Characterizing whether *Bd* CBM18s can serve as protective factors will provide support for whether the recent expansion in copy number of this family is important for *B. dendrobatidis* pathology. This is explored by testing if any of the CBM18 proteins are

secreted and localized to the exposed fungal wall, can protect the fungus from plant chitinases, and can bind chitin to potentially avoid recognition or degradation by host defenses.

2. Materials and methods

2.1. Strains and culture condition

Bd inoculations were carried out with the virulent *Bd* diploid strain JEL423 obtained from the collection of Joyce Longcore (University of Maine, U.S.A). Cultures were maintained on medium containing 1% Tryptone, 3.2% Glucose and 1% Agar at room temperature. *Trichoderma reeseii* strain RUT C30 was obtained from Fungal Genetics Stock Center (Kansas City, Missouri, USA) (McCluskey et al., 2010). The culture was maintained on potato dextrose agar medium at room temperature.

2.2. RNA isolation, RT-PCR and quantitative PCR

Bd RNA was extracted with an RNeasy Mini Kit (Qiagen, Hilden, Germany) with the standard protocol and a DNase digestion. First-strand cDNAs were synthesized by using the SuperScript cDNA Synthesis kit (Invitrogen, USA). cDNA samples were used as template for RT-PCR with gene-specific primer sets (listed in Table S1). Real-time PCR was performed in triplicate using the iQ SYBR Green Supermix (Bio-Rad, USA) as recommended by the manufacturer. Gene expression changes were determined with a protocol for relative quantification in real-time (Pfaffl, 2001), using β-Tubulin (BDEG_03462) as the internal control for comparison. Analysis of variance (ANOVA) was performed to evaluate significance of the qPCR expression values.

2.3. Genomic DNA isolation and fusion PCR to obtain full-length DNA

Genomic DNA from *Bd* was extracted by standard methods (Qiagen DNA extraction kit; Qiagen, Hilden, Germany). The *cbm18* genes were identified as corresponding loci in the *Bd* JEL423 genome sequence (https://www.broadinstitute.org/annotation/genome/batrachochytrium_dendrobatidis) and the naming scheme adopted from previous work (Abramyan and Stajich, 2012). The full-length transcripts from these genes were obtained by a fusion PCR strategy using primers shown in Table S2. All of the four selected *cbm18* genes contain two exons. In the first step, two separated exons for each gene were amplified using it own primer set: P1and P3, P4 and P6, listed in Table S2, and synthesized based on the *Bd* genomic DNA. The fusion PCRs were performed in a 20 μl final volume containing 100 ng of *Bd* genomic DNA, 300 nM final concentration of each primers, 0.2 mM dNTPs, 1× phusion PCR buffer and 1.25 U of phusion enzyme. The PCR cycling conditions were 98 °C for 30 s, and then 30 cycles of 98 °C for 20 s, 55 °C for 20 s, and 68 °C for 1 min, followed by a final extension at 68 °C for 5 min. In the second step, using gene specific primers (P2 and P5 primers, listed in Table S2) fuses the two fragments purified from first fusion PCR reactions to the full-length genes with all sequences in the correct open-reading frame. The PCR reaction was performed in a 50 μl final volume including 1 μl of each template, 400 nM final concentration of each primer, 0.2 mM dNTPs, 1× phusion PCR buffer and 1.25 U of phusion enzyme. The PCR cycling conditions were 94 °C for 2 min, and then 30 cycles of 94 °C for 20 s, 55 °C for 20 s, and 68 °C for 2 min, followed by a final extension at 68 °C for 10 min. The final product was gel purified by PCR purification kit (Qiagen, Hilden, Germany). The purified PCR fragment was cloned into PGEM-T Vector (Promega, Medison, WI, USA) and then transformed into *E. coli* JM109 competent cells by heat shock. The positive clone was selected based on colony PCR

verification with gene specific primers. Plasmid DNA was isolated from this clone using Plasmid Miniprep kit (Qiagen, Hilden, Germany) and further subjected for sequencing.

2.4. Localization of the CBM18-GFP fusion proteins

The CBM18 coding sequences were amplified from sequenced plasmid DNA by Fusion PCR using gene specific primer pairs as follows:

For CBM18-LL1, 5′-CTGCAGATGGTGGTGATTATACATGC-3′ and 5′-CTCGAGCTACAACTCGGCTTTTGAC-3′. For CBM18-LL2, 5′-CTGC AGATGGTGGCGATTATACATGC-3′ and 5′-CTCGAGCTACAACTCGCCT TTTAACC-3′. For CBM18-LL3, 5′-CTGCAGATGCCGCCCACAAT TAAACC-3′ and 5′-CTCGAGCTACAACTCGCCTCTTAACC-3′. For CB M18-TL1, 5′-ATCGATATGACTCTGGTTGCTAC-3′ and 5′-CTCGAGTTA TTTGTCTGTGGTCCATA-3′. The products were cloned into the XhoI and BamHI sites of the vector pBS1303. The gene was driven by galactose promoter, and fused to the 5' end of GFP to generate pBS1303-CBM18s-GFP constructs. The construct was transformed into yeast strain 834 using a modified Lithium Chloride transformation method. The GFP fluorescence was visualized under fluorescence microscope, after induction by 1% galactose for overnight. Localization of the GFP-tagged CBM18-LL1 and CBM18-LL2 proteins was performed using Confocal microscopy on a Leica SP5 following the manufacturer's protocol. FM4-64 (Cat# T13320, Invitrogen) staining to visualize yeast vacuolar membrane was performed following previously described methods (Vida and Emr, 1995). Calcofluor white (Cat#6726, Eng Scientific Inc) staining was conducted according to the manufacturer's protocol.

2.5. Production of recombinant CBM18 and truncated version proteins

To express full-length and truncated versions of CBM18-LL1 as a fusion with the His tag in *E. coli*, DNA fragments encoding the full length (amino acid 1-467), the three repeats of A domain (MA, amino acid 20-208), the four repeats of D domain (MD, amino acid 213-467), the single A domain (amino acid 88-131) and the single D domain (amino acid 213-251) were amplified from the original CBM18-LL1 plasmid using the primer sets listed in Table S3. The PCR products of CBM18-LL1, MA and MD were digested with EcoR1 and Xho1, cloned into the vector pET-28a (Novagen) and confirmed by sequencing. The PCR products of A and D domains were digested with EcoR1 and Xho1, cloned into the pET-Mal vector (Sweeney et al., 2005) with maltose binding protein (MBP) in the N-terminus and 8xHis in the C-terminus and confirmed by sequencing. The recombinant plasmids were transformed into *E. coli* BL21 stain and positive colonies were identified based on kanamycin resistance marker. Growing the cells in LB medium containing 1 mM IPTG induced expression of His fusion protein.

The expression, purification and western blotting were performed according to the manufacturer's protocol (Novagen).

2.6. Polysaccharide affinity precipitation assay

The affinity of the CBM18-LL1, MA, MD, A domain, D Domain, and Avr4 protein for different polysaccharides was determined by incubating each of these proteins (at a concentration of 5 μg/ ml) with the following insoluble polysaccharides (5 mg): chitin beads (cat# E8036S, New England Biolabs, Beverly, MA, U.S.A.), crab shell chitin, cellulose, and xylan (cat#, C3641, C6288, X4252, Sigma, St. Louis, U.S.A.). The incubations were performed in 500 μl of buffer containing 50 mM Tris/HCl, pH 8.0, and 150 mM NaCl. After 2 h of gentle rocking at cold room, the insoluble fraction was collected by centrifugation (1 min, 10,000 rpm) and the supernatant was collected. The insoluble fraction was washed three times with incubation buffer and subsequently boiled in 120 μl of 1% SDS solution. Presence of proteins in both supernatant and pellet was examined by SDS–PAGE gel electrophoresis followed by Coomassie staining (Avr4) or western blot to the 8-His tag (*Bd* CBM18 domains).

2.7. Fungal growth assay in vitro

Tobacco basic chitinase (ChiI) was over-expressed in *E. coli* and purified according to Jongedijk and associates (Jongedijk et al., 1995). Subsequently, the purified protein was screened for antifungal activity by challenging 50 μl of an overnight liquid culture of 100 mycelia/ml of *T. reesei* with 40 μl of the individual fractions. Approximately 10^3 conidia and germlings of *T. reesei* were incubated overnight at room temperature in 50 μl of potato dextrose broth in 96-well plates. Subsequently, the A or D domain of CBM18-LL1, protein, or Avr4 protein was added to the mycelia suspensions at a final concentration of 10 or 100 μM. After a 2 h incubation period, 40 μl of extract containing tobacco chitinase was added. Fungal growth was assessed microscopically after 24 h of incubation at 22 °C.

3. Results

3.1. Cloning and gene structure of the cbm18 genes

Expression of nine *cbm18* genes (Table 1), from members of the LL and TL groups previously classified (Abramyan and Stajich, 2012), was evaluated by RT-PCR (Fig. 1A) with gene-specific primers (Table S1). The mRNA expression levels, which were also confirmed by real-time RT-PCR (Fig. 1B), showed differences across the nine genes from *Bd* cells that were grown in standard rich media conditions in two life stages, zoospore and sporangium. LL group genes showed the higher expression level, while TL group genes

Table 1
CBM18 genes used in the study.

Scheme name	Group	Name	SignalP score[a]	FungiDB link
BDEG_00287	Lectin-like	CBM18-LL1	0.914	http://fungidb.org/gene/BDEG_00287
BDEG_01757	Lectin-like	CBM18-LL2	0.871	http://fungidb.org/gene/BDEG_07157
BDEG_00269	Lectin-like	CBM18-LL3	0.112	http://fungidb.org/gene/BDEG_00269
BDEG_00257	Lectin-like		0.915	http://fungidb.org/gene/BDEG_00257
BDEG_00285	Lectin-like		0.112	http://fungidb.org/gene/BDEG_00285
BDEG_00262	Lectin-like		0.116	http://fungidb.org/gene/BDEG_00262
BDEG_06106	Tyrosinase-like	CBM18-TL1	0.486	http://fungidb.org/gene/BDEG_06106
BDEG_06105	Tyrosinase-like		0.484	http://fungidb.org/gene/BDEG_06105
BDEG_06104	Tyrosinase-like		0.853	http://fungidb.org/gene/BDEG_06104

[a] Signal peptide predication was based on the SignalP (4.1 Server). The cutoff is 0.450.
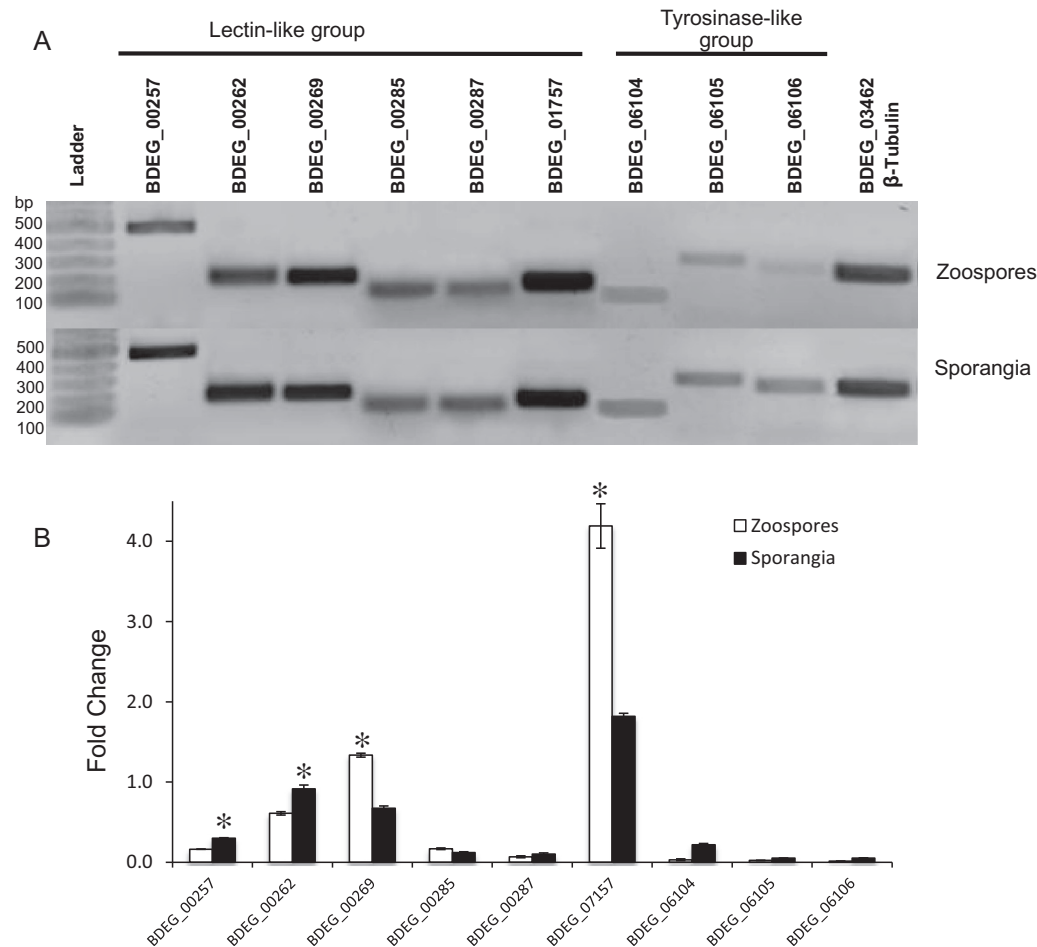
Fig. 1. qPCR measuring expression levels of nine *cbm18* genes in *Bd* zoospores and sporangia life stages. (A) Expression of nine *cbm18* genes using RT-PCR with gene-specific primers. A *Bd* β-Tubulin gene, BDEG_03462, served as an internal control. (B) Expression of nine *cbm18* genes using quantitative PCR. Each column represents three replicates and bars indicate the SEs. *Indicates significant differences between the two life stages at $p < 0.05$.

had a lower level, especially in zoospores; their expressions were rarely detected (Fig. 1A and B). BDEG_00269 and BDEG_01757 genes were highly expressed in zoospores compared to sporangia (Fig. 1B). The expressions of BDEG_00257 and BDEG_00262 genes had a dramatic increase in sporangia compared to zoospores. BDEG_03462, identified as housekeeping gene β-Tubulin, served as an internal control.

The LL group is the largest group of identified CBM18s, containing genes with the most number of copies of the domain (11 in gene BDEG_01757) and the largest number of genes. LL domains also showed evidence for positive selection (Abramyan and Stajich, 2012). In accord with our evidence from gene expression levels and the importance of LL group, three genes from the LL group (Table 1), *cbm18-ll1* (BDEG_00287), *cbm18-ll2* (BDEG_01757) and *cbm18-ll3* (BDEG_00269), and one gene from the TL group, *cbm18-tl1* (BDEG_06106) were chosen for functional analysis.

The predicted gene sequences of the four *cbm18* genes were obtained based on the JEL423 strain annotation. Primers were designed from the exon boundaries and amplified from *Bd* genomic DNA by fusion PCR. The amplified sequences of three genes were the same as those in the predicted gene model. However, *cbm18-ll1* shows a longer transcript product than was predicted by the genomic sequence. The genome annotation predicted that the *cbm18-ll1* gene encodes a 334 amino acid protein, containing three copies of the A domain, and two copies of the D domain. However, the amplified *cbm18-ll1* length was 1398 bp and is predicted to encode a protein of 465 amino acids, including two more copies

of D domain at C-terminal end, compared with the predicted one. The corrected sequence model is deposited in GenBank as KM099424.

The CBM18-LL2 protein, encoding 1033 amino acids, consists of 11 copies of CBM18 motifs, representing the largest expansion of the domain within any of the genes. The CBM18-LL3 protein, encoding 577 amino acids, contains six copies of the motif. The CBM18-TL1 protein, encoding 634 amino acids, contains only one copy of the motif and an additional tyrosinase domain.

3.2. Subcellular localization of CBM18s

To investigate CBM18 intracellular localization, constructs containing the CBM18-GFP fusion genes in the plasmid pPS1303 were generated. The fusion genes and GFP control in the pPS1303 vector driven by the galactose-inducible promoter were transformed into *S. cerevisiae*. Protein expression was visualized by green fluorescence of the fused GFP protein in an inverted fluorescence microscope. The control GFP protein can also be observed in the cytoplasm (Fig. 2A and e).

The CBM18-LL1 and CBM18-LL2 proteins localized to cell boundaries (Fig. 2A, a and b) and displayed intracellular aggregates. The localization of CBM18-LL1 and CBM18-LL2 GFP was further verified using confocal Leica SP5 microscope, showing cell surface localization of these two fusion proteins (Fig. 2B, a and b). Analysis of the protein sequences with SignalP (Petersen et al., 2011) predicts a secretion signal peptide in the
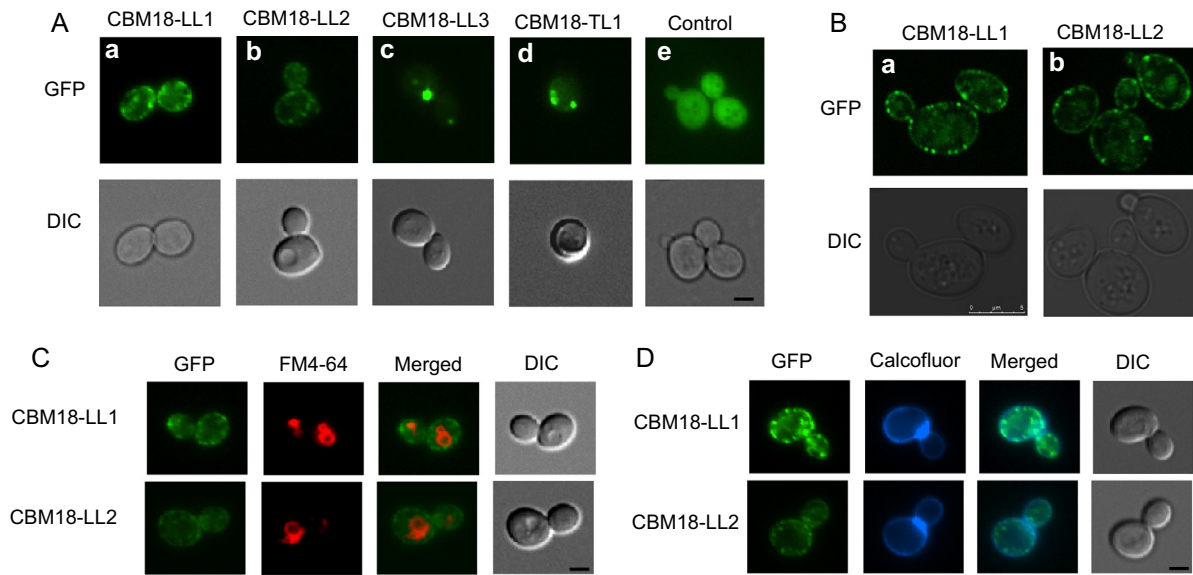
Fig. 2. Subcellular localization of CBM18s. (A) Subcellular localization of CBM18-GFP proteins in *S. cerevisiae* as revealed by fluorescence microscope. For each panel, the photographs were taken in dark field for green fluorescence (upper) and bright field for the morphology of the cells (lower). a, CBM18-LL1; b, CBM18-LL2; c, CBM18-LL3 and d, CBM18-TL1. Bar = 1 μm. (B) The CBM18-LL1-GFP (a) and CBM18-LL2-GFP (b) cells were examined via Leica SP5 confocal microscopy. Bar = 5 μm. (C) The CBM18-LL1-GFP and CBM18-LL2-GFP cells stained with vital dye FM4-64 for 20 min at 30 °C. Bar = 1 μm. (D) The CBM18-LL1-GFP and CBM18-LL2-GFP cells were stained with Calcofluor white for 5 min at room temperature. Bar = 1 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

CMB18-LL1 and CMB18-LL2, while none are present in the CBM18-LL3 and CBM18-TL1 (Table 1). These results indicate the CBM18-LL1 and CBM18-LL2 proteins are trafficked to the outside of the cells through the secretion pathway, and may function at the cell surface.

The CBM18-LL3 GFP fusion protein, which lacks a signal peptide (Table 1), formed intracellular agglomerations (Fig. 2A and c), and showed a distinctly different pattern of localization from CBM18-LL1 and CBM18-LL2. Although phylogenetic methods indicate it is a member of the LL group, this difference in localization is likely due to a lack of a signal peptide sequence. The CBM18-TL1 GFP fusion protein, which has a low SignalP score (Table 1) indicating it does not encode a typical secretion signal peptide, also formed intracellular bodies (Fig. 2A and d). The results suggest CBM18 proteins have separate cellular roles as not all localize to the cell surface.

To further investigate the details of subcellular localization of the LL proteins CBM18-LL1 and CBM18-LL2, we performed co-staining assays with dyes to label the organelles of *S. cerevisiae*. When co-stained with FM4-64, a dye to label vacuole membranes, the FM4-64 and the LL protein signals were mutually exclusive (Fig. 2C), indicating that these two proteins were not destined to vacuole. When co-stained with calcofluor white, a dye to selectively bind to chitin or cellulose in the cell wall of fungi, each LL protein was observed to co-localize with the calcofluor white signal at the cell boundary (Fig. 2D), indicating that the proteins were distributed to the cell surface and could be binding to chitin present and enriched in the daughter bud neck.

3.3. Expression of CBM18-ll1 in E. coli

To address the biochemical properties of the CBM18-LL1 protein, a His-tagged version of the protein was constructed and expressed in the *E. coli* protein expression system. However, insufficient expression was observed with the long form of the protein, which contains a signal peptide, three copies of the A domain (named MA), and four copies of the D domain (named MD).

To further investigate the functions of these domains, five constructs containing truncated versions of the CBM18-LL1 protein were made (Fig. 3A). The vector pET-28a was constructed with either the full-length *cbm18-ll1* gene, MA (three A domains), or MD domain (four D domains), and preceded by a His tag sequence to provide an immunoreactive epitope. The pET-MAL vector, which contains maltose-binding protein (MBP) at N-terminus, was used to express an A domain or D domain alone, which is a single copy of the 44 amino acid domain. The N-terminal MBP tag increases the efficiency of expression and ability to detect these single short domains, which were not well expressed in pET-28a. The recombinant CBM18-LL1 protein and truncated versions were induced by 1 mM IPTG, purified using a nickel column and confirmed by western blot using anti-His monoclonal antibody (Fig. 3B).

3.4. CBM18-LL1 binds specifically to chitin

To investigate whether the CBM18-LL1 protein or the CBM18 domain has affinity for insoluble polysaccharides, we performed an affinity precipitation assay *in vitro*. This assay tested for binding of the CBM18-LL1 protein or individual copies of the domain to polysaccharides chitin, cellulose or xylan. The gene *Avr4*, which is a chitin-binding lectin found in *Cladosporium fulvum* (van den Burg et al., 2006) (now named *Passalora fulva* (Crous and Braun, 2003)) was used as a positive control in this affinity precipitation assay. Previously *Avr4* had been successfully reported to have chitin-specific binding properties and we confirmed Avr4 protein readily binds chitin in our assay by affinity precipitation (Fig. 4F). The full length CBM18-LL1 protein was shown to have specific but weak affinity when precipitated in the presence of chitin (by both magnetic chitin beads and crab shell chitin), but not with the other polysaccharides cellulose and xylan (Fig. 4A). The MA and MD domain constructed proteins showed very strong chitin binding affinity (Fig. 4B and C). A single copy of CBM18 domain, either the A domain or D domain, also has a similarly strong capability to bind chitin (Fig. 4D and E). MBP alone did not bind any of the polysaccharides (data not shown). These results
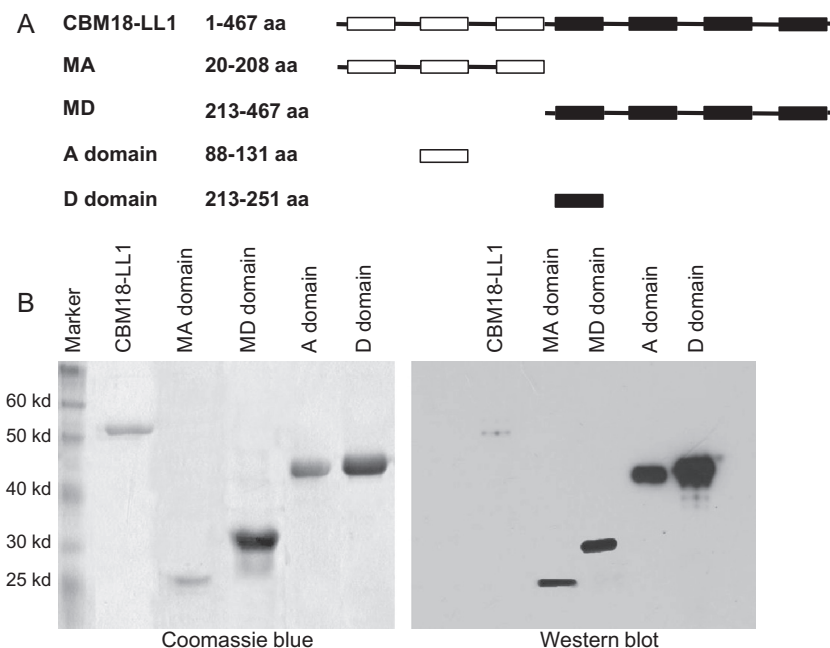
Fig. 3. Protein expression of full length and truncated versions of the CBM18-LL1 protein. (A) CBM18-LL1 full length and truncated constructs used in bacterial expression. The CBM18-LL1 protein contains three copies of A domain (white) and four copies of D domain (black). (B) Expression and purification of the various truncated versions of CBM18-LL1 from *E. coli* by affinity resin. The His-fusion protein was stained with Coomassie blue (left) and confirmed by western blot analysis using an anti-His monoclonal antibody (right).
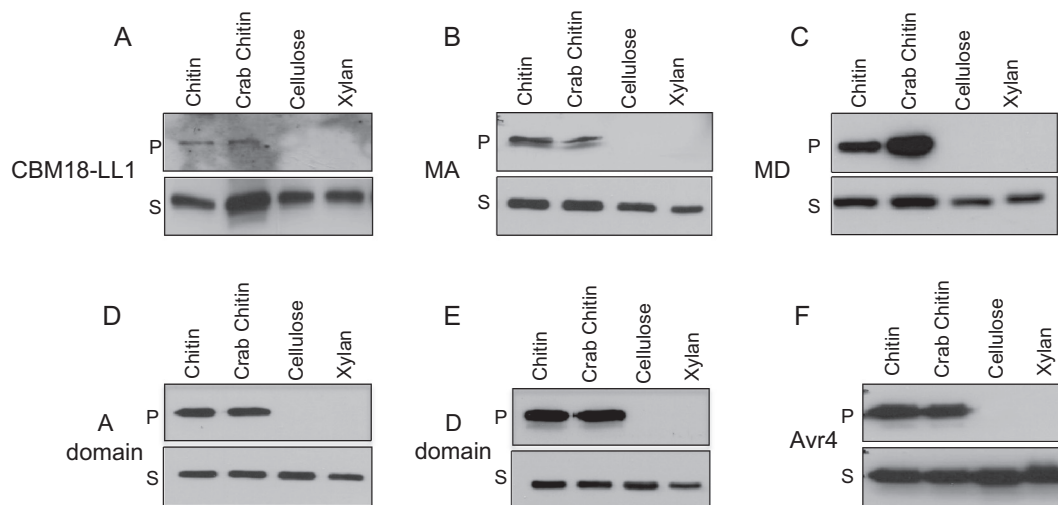

Fig. 4. Affinity precipitation showing Chitin-specific binding activity of the CBM18-LL1 protein. Western blots for the entire protein (A), individual CBM18 domains (D or E), or a partial combination of the CBM18 domains (B or C). The Avr4 protein (F) served as a positive control and is shown by Coomassie blue staining. S: protein remaining in the concentrated supernatant fraction. P: protein bound to insoluble polysaccharide fraction.

demonstrated chitin-specific binding activity of the CBM18-LL1 protein: whether the entire protein, individual CBM18 domains, or a partial combination of the CBM18 domains.

3.5. CBM18 domain protects fungi in vitro against hydrolysis by tobacco chitinase

The growth of the fungi *T. reesei* is inhibited when treated with plant chitinase (Roberts and Selitrennikoff, 1988). Taking advantage of this system we examined whether CBM18 domains can protect *T. reesei* against hydrolysis by basic tobacco chitinase (ChiI), which is well-characterized (Sela-Buurlage et al., 1993).

Here we tested the protein constructs with only the single CBM18 domains, because they showed high protein expression and strong chitin binding affinity.

The growth of *T. reesei* is inhibited by tobacco ChiI alone (Fig. 5B), compared to non-treatment (Fig. 5A). In the presence of 0.3 μM ChiI, nearly all *T. reesei* mycelia were lysed within 24 h. However, when the A or D domain proteins were added to the medium and incubated for 2 h, the addition of chitinase did not affect growth of *T. reesei* mycelia (Fig. 5D and E). This indicates that the *T. reesei* mycelia were protected against ChiI by either the A or D domain protein. The Avr4 protein was also tested as a positive control (Fig. 5C) and showed similar properties to the CBM18
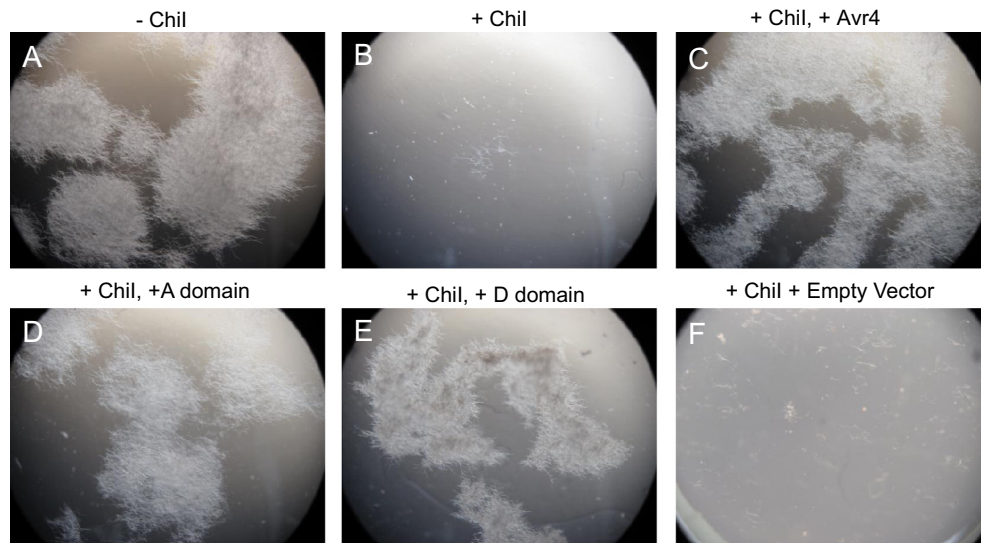
Fig. 5. Growth inhibition of the fungus *T. reesei* treated with tobacco basic chitinase (ChiI) in the presence of CBM18 domain. (A) Growth of *T. reesei* mycelia for 24 h. (B) Mycelia incubated with 0.3 μM ChiI for 24 h. (C) Similar as in B, except that the mycelia were pre-incubated with Avr4. (D) Similar as in C, except pre-incubated with A domain. (E) Similar as in D, except pre-incubated with D domain. (F) Pre-incubated with fraction from empty vector stain, then treated with ChiI.

domains. A negative control expressing an empty vector and the extracted fraction showed no protection (Fig. 5F). These results indicate the CBM18 domain can protect fungi *in vitro* against hydrolysis by tobacco chitinase.

4. Discussion

Previous findings identified the species-specific CBM18 expansion and positive selection in the amphibian pathogen *Bd* (Abramyan and Stajich, 2012). In the *Bd* genome 67 CBM18 copies were identified, while only 10 CBM18 copies could be found in the genome of *Homolaphlyctis polyrhiza,* one of the closest known relatives to *Bd*, and non-pathogenic to amphibians (Joneson et al., 2011). One explanation of this observation is that recent adaptation by gene family expansion is important to *Bd* biology and perhaps its pathogenic lifestyle. This phenomenon has been observed for peptidase and keratinase gene families in the *Onygenales* fungi and the evolution of the mammal pathogen *Coccioidies* (Muszewska et al., 2011; Sharpton et al., 2009). More evidence from the analysis across nine pezizomycete and two basidiomycete species support that the pattern of gene duplication enrichment, gene family expansion and contraction reflect adaptation within pathogenic life histories (Powell et al., 2008).

The expression pattern of nine genes from the two examined groups indicated LL genes had higher transcript levels than the TL genes in both life stages. Although zoospores lack cell walls, the chitin-binding *cbm-18* genes were still expressed differentially in zoospore stage. Interestingly, the BDEG_01757 gene, which contains 11 copies of the CBM18 domain, and BDEG_00269 gene showed an elevated transcriptional level in zoospores as compared to the sporangia. Previous work has shown that zoospore life stage of the chytrid, *Blastocladiella emersonii*, contains stored, but not newly transcribed mRNA (Johnson and Lovett, 1984). The elevated expression could indicate importance of these genes in early sporangia development. The BDEG_00262 gene showed a significant increase of expression in sporangia compared with zoospores. However, this protein does not have a signal peptide (Table 1) and was not further tested in this study. The BDEG_00287 gene is the shortest gene in the LL group and has a constant expression level in the two life stages. Overall the RT-PCR results indicate that

all of the *cbm18* genes are expressed in at least one life stage tested, suggesting they may play a role in the cell biology of zoospores or sporangia.

Localization of CBM18 containing proteins reveals that some members of the gene family are trafficked to the cell surface. The LL proteins CBM18-LL1 and CBM18-LL2, when fused with GFP, localized to the cell boundary surface. Both proteins encode a secretion signal peptide (Table 1) and have similar pattern of localization in *S. cerevisiae*. The CBM18-LL3 protein (Table 1), which is phylogenetically classified with the other LL genes, but lacks a secretion signal peptide and failed to show a similar localization at the cell surface. The CBM18-TL1 GFP-fused protein, which is a non-SP, formed intracellular aggregates. According to these observations, it is likely the secretion signal is necessary for trafficking to the cell surface.

The heterologous expression in *S. cerevisiae* also shows that *Bd* proteins with predicted signal peptides could be successfully trafficked for secretion in a cross-species heterologous expression. Previous work on a related family of genes has demonstrated that a *S. cerevisiae* chitin-binding module with GFP fused to the N or C terminus, directed by the *tglA* secretion signal peptide from *Aspergillus oryzae*, was found to localize to the cell surface in *A. oryzae* (Tabuchi et al., 2010). To further define the location of intracellular aggregates in the cells, FM4-64, was used to label vacuole membranes to test for co-localization with the LL GFP-fused protein. The intracellular LL signals did not overlap with vacuole in the co-stain assay. Calcofluor white, a dye to selectively bind to chitin in the cell wall, serves as a good indicator to outline the cell wall. The surface signal of the LL GFP-fused proteins overlaid calcofluor white, confirming that the LL proteins were delivered to the cell wall. Some of the CBM18 proteins, which do not localize to the cell surface, may play other roles in the *Bd* lifestyle.

The *in vitro* experiments confirm that the protein is able to recognize and bind chitin. The CBM18 domains are highly conserved in sequence with the hevein domain found in plants and has been shown to be able to bind chitin (Archer, 1960). The avirulence factor Avr4 in leaf mold fungus *C. fuvum* is a chitin-binding lectin containing a chitin-binding domain (CBM14) and can bind chitin specifically (van den Burg et al., 2006). When the residues Trp541 and Trp542 located on the surface of chitin-binding domain (CBM5) of chitinase J from alkaliphilic *Bacillus sp.* J813 were

mutated, the mutated protein had a significant decrease in binding capacity to chitin (Uni et al., 2012). Here we find that the CBM18-LL1 protein *in vitro* is able to specifically bind chitin, and not cellulose and xylan. The full length CBM18-LL1 protein showed a weaker affinity to chitin binding, compared with the MA, MD, A and D domain. This may be due to a limitation of the *E. coli* protein expression system where the full-length protein may not be folding properly. The strong binding affinities of the individual domains or partial copy of the proteins indicate that a single domain is able to perform the binding alone. To further examine if CBM18-LL1 domains specifically bound chitin, an assay containing a mixture of chitin beads, xylan and cellulose, was used to perform the affinity precipitation. The results (Fig. S1A and B) indicate the amount of CBM18 domain protein precipitated by chitin beads, in the presence of xylan and cellulose, was not affected when compared to the precipitate of chitin beads alone. When the chitin concentration was increased, up to 50 mg, with a fixed amount of CBM18 domain protein, the detected amount of precipitated also protein did not change (Fig. S1C and D). Furthermore when small amounts of chitin beads were used a similar amount of protein was seen in the blots suggesting the binding substrate is saturated even at the lowest chitin concentration we could assay.

Protection of *T. reeseii* from plant chitinases indicates the domain can serve the same role and general binding to available and exposed chitin. Pre-incubated *T. reeseii* with the A or D domains were protected when exposed to plant chitinase while untreated mycelia were susceptible and failed to grow. The mechanism for this protection may be that the single CBM18 domains bind the available chitin in the cell wall of *T. reeseii* to serve as a shield to prevent the chitinases from attacking their chitin substrate. The Ecp6 protein in *C. fuvum*, possessing three Lysin (LysM) domains also known as CBM50, was shown to bind chitin (de Jonge et al., 2010). However, the Ecp6 protein failed to protect the fungus *Trichoderma viride* against hydrolysis by crude extras of tomato leaves containing intracellular basic chitinases (de Jonge et al., 2010).

We propose the model that in *Bd*, the CBM18 proteins are secreted into the cell surface where the protein can bind chitin present in the cell wall. *Bd* cell wall is likely to have more chitin content than other close relatives (Sain, Mélida, Bulone and Stajich, unpublished data). It is possible to enhance the protection with multiple copies of CBM18 in *Bd*. We do not know *in vivo* which form of CBM18 protein, the full-length, a single copy of a domain, or a partial combination, is primarily binding chitin. Previous studies showed the primary AVR4 protein product, which is 135 amino acids long, is processed by plant and/or fungal proteases at both the N and C terminus, and the most abundant form of functional AVR4 was found to correspond to an internal sequence 86 amino acids in length (Joosten et al., 1997). This suggests that CBM18 protein may also go through posttranslational processing before having a functional form.

We hypothesize that CBM18 protein binds chitin in the *Bd* cell wall and thereby protects it from chitinases that may be part of the animal antifungal defenses. CBM18 may also function as an integral cell wall protein imbedded in the extracellular matrix to prevent detection by amphibian host cells. CBM18 proteins could bind chitin in the *Bd* cell wall to prevent chitinase hydrolysis from the host. Little work on frog chitinases has been done to demonstrate any role they play in innate immunity to invading fungi, but the *X. laevis* genome contains 21 genes that are similar to chitinase and could play a role in host defense. Future work should investigate the impact of frog chitinases on the *Bd* cell wall, whether invading *Bd* sporangia have increased CBM18 expression, and whether these or other fungal genes play a defensive role that is important for its success as a pathogen. Finally, it also will be important to have a better understanding of the substrate of the

CBM18 deacetylase copies and the role of the tyrosinase domain-containing copies in the vegetative or pathogenic life stages of *Bd*. Additional roles of the DL group are could be in converting chitin to chitosan (Kafetzopoulos et al., 1993) to further prevent detection by the host immune system that is likely tuned for chitin recognition (Baker et al., 2011). Understanding the potentially complex role that CBM18 family plays in protection or immune evasion and what evolutionary pressures drove the expansion of the gene family could help link ecological context in understanding the history of this emerging pathogen.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2015.03.003.

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