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Molecular characterization of the *Aspergillus nidulans fbxA* encoding an F-box protein involved in xylanase induction

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ABSTRACT

The filamentous fungus *Aspergillus nidulans* has been used as a fungal model system to study the regulation of xylanase production. These genes are activated at transcriptional level by the master regulator the transcriptional factor XlnR and repressed by carbon catabolite repression (CCR) mediated by the wide-domain repressor CreA. Here, we screened a collection of 42 *A. nidulans* F-box deletion mutants grown either in xylose or xylan as the single carbon source in the presence of the glucose analog 2-deoxy-D-glucose, aiming to identify mutants that have deregulated xylanase induction. We were able to recognize a null mutant in a gene (*fbxA*) that has decreased xylanase activity and reduced *xlnA* and *xlnD* mRNA accumulation. The *ΔfbxA* mutant interacts genetically with *creAd-30*, *creB*15, and *creC27* mutants. FbxA is a novel protein containing a functional F-box domain that binds to Skp1 from the SCF-type ligase. Blastp analysis suggested that FbxA is a protein exclusive from fungi, without any apparent homologs in higher eukaryotes. Our work emphasizes the importance of the ubiquitination in the *A. nidulans* xylanase induction and CCR. The identification of FbxA provides another layer of complexity to xylanase induction and CCR phenomena in filamentous fungi.

1. Introduction

Xylan degradation occurs through the action of endo-β-(1,4)-xylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37) (Subramaniyan and Prema, 2002; Polizeli et al., 2005). The filamentous fungus *Aspergillus nidulans* has been used as a model system to study the regulation of xylanase production (MacCabe and Ramón, 2001; Tamayo et al., 2008). When grown on xylose or xylan as single carbon sources, *A. nidulans* produces three xylanases (Fernández-Espinar et al., 1992, 1993, 1994, 1996; Piñaga et al., 1994) and one β-xylosidase (Orejas et al., 1999), encoded by the *xlnA*, *xlnB*, *xlnC* and *xlnD* genes, respectively (MacCabe et al., 1996; Pérez-González et al., 1996, 1998). These genes are activated at the transcriptional level by the master regulator the transcriptional factor XlnR (for a review, see Stricker et al., 2008) and repressed by carbon catabolite repression (CCR) mediated by the wide-domain repressor CreA (Pérez-González et al., 1998; MacCabe et al., 1998, 2001; Orejas et al., 1999, 2001). *A. nidulans creA* encodes a protein of 415 amino acids containing several features characteristic for DNA binding proteins, such as zinc fingers, an alanine-rich region and frequently appearing SPXX and TPXX motifs (Ronne, 1995; Ruijter and Visser, 1997). The CreA has two zinc finger structures of the Cys2His2 type very similar to the zinc fingers of MIG1, a repressor protein in the main glucose repression pathway of *Saccharomyces cerevisiae* (Nehlin and Ronne, 1990). Expression of *xlnR* is not sufficient for induction of genes encoding the xylanolytic complex because the presence of xylose is absolutely required (Tamayo et al., 2008). It has been established previously that CreA indirectly represses *xlnA* [encodes X(22)] and *xlnB* [encodes X(24)] genes as well as exerting direct repression on *xlnA* (Orejas et al., 1999, 2001). Recently, Tamayo et al. (2008) provided the following evidence that CreA-mediated indirect repression occurs through repression of *A. nidulans xlnR*: (i) the *xlnR* gene promoter is repressed by glucose and this repression is abolished in *creAd-30* mutant strain and (ii) deregulated expression of *xlnR* completely relieves glucose repression of *xlnA* and *xlnB*. Thus, CreA and XlnR form a transcriptional cascade regulating *A. nidulans* xylanolytic genes.

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Proteasomal protein turnover is an essential biological process in eukaryotes. Proteins destined for degradation in this system are first covalently linked to a chain of ubiquitin molecules. The critical enzymes responsible for attaching ubiquitin to protein substrates are the E3 ligases (for a review, see Hershko and Ciechanover, 1998). The SCF1s (Skp1, Cullin1, F-box proteins) are the largest family of E3 ligases. In the SCF1 complexes, F-box proteins interact with Skp1 and are exchangeable subunits that help E3 ligase to recognize and target its protein substrates (Skowyra et al., 1997). Eukaryotes have large F-box protein families and the fungal F-box proteins are responsible for several biological function, such as cell cycle, circadian clocks, transcription, development, signal transduction, and nutrient sensing (Jonkers and Rep, 2009a). *S. cerevisiae* has about 20 proteins containing an F-box domain and several *Schizosaccharomyces pombe* F-box proteins have been investigated (Jonkers and Rep, 2009a). In other fungi, there are several reports about F-box proteins, such as GrrA in *A. nidulans* (Krappmann et al., 2006), Fwd1 and Scon2 in *Neurospora crassa* (Kumar and Paietta, 1998; He et al., 2003; He and Liu, 2005), Grr1 and Cdc4 in *Candida albicans* (Atir-Lande et al., 2005; Butler et al., 2006; Li et al., 2006), Pth1 in *Magnaporthe grisea* (Sweigard et al., 1998), Fbp1 (F-box protein 1) in *Fusarium graminearum* (Han et al., 2007), Frp1 in *Fusarium oxysporum* (Duyvesteijn et al., 2005), and Fbp1 in *Cryptococcus neoformans* (Liu et al., 2011).

Here, we screened a collection of 42 *A. nidulans* F-box deletion mutants grown either in xylose or xylan as the single carbon source in the presence of the glucose analog 2-deoxy-D-glucose, aiming to identify mutants that have deregulated xylanase induction. We were able to recognize a null mutant in the gene *fbxA* that has decreased xylanase activity and reduced *xlnA* and *xlnD* mRNA accumulation. The *ΔfbxA* mutant interacts genetically with *creAd-30*, *creB*, and *creC* mutants. FbxA is a novel protein that has a functional F-box domain and a putative cyclic nucleotide-binding domain. This domain is found in proteins that bind cyclic nucleotides (cAMP or cGMP) and the best studied of these proteins is the prokaryotic catabolite gene activator (also known as the cAMP receptor protein) (gene crp; http://www.expasy.org/cgi-bin/nicedoc.pl?PS50042). Blastp analysis suggested that FbxA is a protein exclusive from fungi, without any apparent homologs in higher eukaryotes.

2. Material and methods

2.1. Strains, media and culture methods

A. nidulans strains used are described in Table 1. The media used were of two basic types, i.e., complete and minimal. The complete media comprised the following three variants: YAG (2% w/v glucose, 0.5% w/v yeast extract, 2% w/v agar, trace elements), YUU (YAG supplemented with 1.2 g/L [each] of uracil and uridine), and liquid YG or YG + UU medium with the same composition (but without agar). The minimal media were a modified minimal medium (MM; 1% w/v glucose, fructose or xylose, original high-nitrate salts, trace elements, 2% w/v agar, pH 6.5). Trace elements, vitamins, and nitrate salts were included as described by Kafer (1977). Strains were grown at 37 °C unless indicated otherwise.

2.2. Enzymatic activities

Xylanase (endo-1,4-β-xylanase) assay was performed using Azo-xylan from birchwood as substrate, according to manufacturer's protocol. Briefly, supernatant containing enzymes from xylan-induced *A. nidulans* was mixed with 100 mM sodium acetate buffer (pH 4.5) in an appropriate volume. Reaction mixtures consisted of 0.5 mL of buffered enzyme preparation and 0.5 mL of substrate solution (1% w/v Azo-Xylan birchwood or Azo-CM-cellulose). The samples were incubated at 40 °C for 10 min and the reactions were interrupted by adding 2.5 mL of ethanol (95% v/v) with vigorous stirring. Non-degraded substrate precipitated by ethanol was removed by centrifugation at 1000*g* for 10 min, and the A_{590} was monitored in reaction supernatants. Enzyme activity was determined using MegaCalc™ software (Megazyme, available at http://www.megazyme.com).

2.3. Molecular techniques

Standard genetic techniques for *A. nidulans* were used for all strain constructions and transformations (Kafer, 1977). DNA manipulations were according to Sambrook and Russell (2001). All PCR reactions were performed using Platinum Taq DNA Polimerase High Fidelity (Invitrogen). The primers used in this work are listed on Supplementary Table S1.

The *fbox* deletion cassettes were generated by using *A. fumigatus pyrG* as a prototrophic marker. This marker was PCR-amplified from the pCDA21 plasmid (Chaveroche et al., 2000), and is referred as *zeo-pyrG* cassette (2417 bp) because the amplified fragment contains the zeocin-resistance gene and the *A. fumigatus pyrG* gene. A 2000 bp 5′- and 3′-UTR fragments from *fbox* gene were PCR amplified using *A. nidulans* TNO2a3 (Nayak et al., 2006) as a template. The fusion PCR was done according Szewczyk et al. (2006). Briefly, the PCR primers were synthesized with "20 base pairs tails" such the gene flanking region fragments anneal to the marker during fusion PCR. Fusion PCR has created a cassette containing the gene flanking sequences surrounding the marker and transformation with this fragment has led to replacement of the gene with the marker. The fusion products generated by PCR were gel-puri-

Table 1
Strains used at this work.

Strains	Genotypes	References
Δfbox19::pyro	*Δfbox19::pyroA; Δku70::argB2; argB; pyrG89; pyroA4; cho1*	This work
GFP::Fbox19	*gfp::fbox19::pyrG; Δku70::argB2; argB; pyrG89; pyroA4; cho1*	This work
creAd30	creA30; biA1; wA3	Arst et al. (1990)
SA15B12	creB15; biA1; wA3	Kelly and Hynes (1977)
SA27D8	creC27; niiA4; biA1; wA3	Kelly and Hynes (1977)
Δfbox19 creAd30	Δfbox19; creAd30; cho1	This work
Δfbox19 creB15	Δfbox19; creB15; cho1	This work
Δfbox19 creC27	Δfbox19; creC27; cho1	This work
xlnR AMAI	prG3AMAI::XlnR-stag; pyrG89; pyroA4; wA3	This work
creA AMAI	prG3AMAI::CreA-stag; pyrG89; pyroA4; wA3	This work
Δfbox19 xlnR AMAI	Δfbox19::pyroA; pRG3AMAI::xlnR::stag; Δku70::argB2; argB; pyrG89; pyroA4; cho1	This work
Δfbox19 creA AMAI	Δfbox19::pyroA; pRG3AMAI::creA::stag; Δku70::argB2; argB; pyrG89; pyroA4; cho1	This work
TNO2a3	*Δku70::argB2; argB; pyrG89; pyroA4; cho1*	Nayak et al. (2006)
GR5	pyrG89; pyroA1; wA3	Nayak et al. (2006)

fied and transformed in the *A. nidulans* strains TNO2A3 (*nkuA::argB*; Nayak et al., 2006). The transformation was according to the procedure of Osmani et al. (1987) using approximately 5 μg of linear DNA fragments. *pyrG*+ transformants were selected on YAG medium. Southern analysis demonstrated that the deletion cassettes had integrated at the desired gene locus and a single integration event had occurred.

The construction of the FboxA::GFP fusion cassette was performed according to Colot et al. (2006). Briefly, 1.5 kb regions on either side of the *fbox19* ORF was selected for primer design. The primers amplified the 5′UTR flanking region of the *fboxA* ORF and the *fbox19* ORF, and the 3′UTR flanking region. These fragments were PCR amplified from genomic DNA of the TNO2a3 strain. The *pyrG*, used as a selectable marker for prototrophy was amplified from pCDA21 plasmid, and GFP gene was amplified from pMCB17apx plasmid. The external primers presented cohesive ends with the vector pRS426 used for *in vivo* recombination in yeast. This vector was double digested with *Eco*RI and *Bam*HI for linearization and *S. cerevisiae* strain SC9721 was transformed by the lithium acetate method with these four fragments. The DNA of the yeast transformants was extracted by the method described by Goldman et al. (2003), dialyzed and transformed by electroporation in *Escherichia coli* strain DH10B to rescue the pRS426 plasmid harboring the cassettes. The cassette was PCR-amplified and the fusion products generated by PCR were gel-purified and transformed in the *A. nidulans* strain TNO2A3. The transformation was according to the procedure of Osmani et al. (1987) using approximately 5 μg of linear DNA fragments. *pyrG*+ transformants were selected on YAG medium. Southern analysis demonstrated that the cassette had integrated at the desired gene locus and a single integration event had occurred.

For the construction of XlnR overexpression cassette, about 1 kb of each 5′- and 3′-flanking regions and the ORF were selected for primer design and amplified from *A. nidulans* A4 wild-type strain. The cassette was constructed by PCR-mediated technique. Then, it was cut with *Kpn*I and ligated to the autonomous plasmid pRG3-AMAI (*N. crassa pyr4* marker; Ballance and Turner, 1985). For the experiments to verify the interaction between FbxA and SkpA, DNA sequence corresponding to the *fbxA* first exon was amplified from genomic DNA of the *A. nidulans* using primers F-f and F-r. Complementary DNA products of PCRs were first subcloned into pGEX-4T-1 (GE Healthcare, London, UK) bacterial expression vector in-frame with the N-terminal gst. Then, the *gst-fbxA* (F) and *gst-fbxA* (ΔF) (lacking the coding-sequence for the F-box domain) sequences were amplified using primers gst-f and F-r or gst-f and Δf-r, respectively. The PCR products were cloned into the mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA). The DNA sequence corresponding to the gene encoding SkpA was amplified from from genomic DNA of the *A. nidulans* using the primers skp-f and skp-r and subcloned direct into pCS2 + MT mammalian expression vector (kindly provided by Dr. David L. Turner, University of Michigan, USA) in-frame with N-terminal 6XMyc epitope tag.

2.4. Pull-down assay

For expression of the plasmids, HEK293T cells were grown in DMEM in 10 cm culture dishes supplemented with 10% fetal bovine serum. GST-FbxA$^{1-809}$ (F) or GST-FbxA$^{1-538}$ (ΔF) in pCDNA3.1 (12 μg) was cotransfected with 4 μg of 6XMyc-SkpA. The plasmid constructs were transfected into cells at 70–90% confluence using FuGENE® 6 reagent (Roche Diagnostics, GmbH Mannheim, Germany) following the manufacturer's protocol. The cells were lysed with buffer 25 mM Tris–HCl pH 7.5, 225 mM KCl and 1% NP40 and cleared by centrifugation at 20,000×*g* for 20 min, at 4 °C. For the pull-down assay, the supernatants were incubated with 25 μL of glutathione-Sepharose beads (GE Healthcare, London, UK) for 3 h at 4 °C under agitation. The beads were washed and boiled with sample buffer. The input and pull-down extracts were resolved by SDS–PAGE 12% gel. The proteins were transferred to nitrocellulose membranes and subjected to immunoblot using the anti-GST (Sigma, St. Louis, MO) or anti-Myc (Invitrogen, Carlsbad, CA) antibodies.

2.5. Staining and microscopy

For germling nuclear staining, conidia were inoculated on coverslips. After incubation at the appropriate conditions for each experiment, coverslips with adherent germlings were transferred to fixative solution (3.7% formaldehyde, 50 mM sodium phosphate buffer pH 7.0, 0.2% Triton X-100) for 30 min at room temperature. Then, they were briefly rinsed with PBS buffer (140 mM NaCl, 2 mM KCl, 10 mM $NaHPO_4$, 1.8 mM KH_2PO_4, pH 7.4) and incubated for 5 min in a solution with 100 ng/ml 4′,6-diamidino-2-phenylindole (DAPI-Sigma Chemical, St. Louis). After incubation with the dyes, they were washed with PBS buffer for 10 min at room temperature and then rinsed in distilled water, mounted.

For cell imaging of FbxA protein fused to GFP, conidiospores were grown in glass-bottom dishes (Mattek, Ashland, MA) at 30 °C. Germlings were fixed for 10 min in a fixative solution containing 1× PBS, 5% DMSO, 3.7% formaldehyde, and 10% methanol. The nucleus was DAPI stained as described above. Slides were viewed with a Carl Zeiss (Jena, Germany) microscope using 100× magnification oil immersion objective lens (EC Plan-Neofluar, NA 1.3) equipped with a 100 W HBO mercury lamp epifluorescence module. Phase contrast for the brightfield images and fluorescent images were captured with an AxioCam camera (Carl Zeiss), processed using the AxioVision software version 3.1 and saved as TIFF files. Further processing was performed using Adobe Photoshop 7.0 (Adobe Systems Incorporated, CA).

2.6. RNA isolation and real-time PCR reactions

After harvesting, mycelia was disrupted by grinding, and total RNA was extracted with Trizol (Invitrogen, USA). RNA (10 μg) from each treatment was fractionated in 2.2 M formaldehyde, 1.2% agarose gel, stained with ethidium bromide, and visualized with UV-light in order to check RNA integrity. The samples were submitted to RNAse-free DNAse treatment as previously described (Semighini et al., 2002), purified with RNeasy® Mini Kit (Qiagen), and then quantified in the NanoDrop® 2000 Thermo Scientific (Uniscience).

All PCR reactions were performed using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, USA) and Taq-Man™ Universal PCR Master Mix kit (Applied Biosystems, USA). The reactions and calculations were performed according to Semighini et al. (2002). The primers and Lux™ fluorescent probes (Invitrogen, USA) used in this work are described in Supplementary Table S2.

3. Results

3.1. Screening for A. nidulans fbx deletion strains resistant to 2-deoxy-D-glucose

The *A. nidulans* genome contains approximately 70 genes encoding F-box proteins which are normally bridged by SkpA (Skp1-homolog) to cullin-1 (CulA) (Galagan et al., 2005). We have deleted 42 of them (Supplementary Table S3), and as a first step to investigate which F-box encoding genes could be involved in catabolite repression, we have grown these 42 *fbx* deletion and wild-type strains on either MM plus 1% xylose or 1% xylan and different concentrations of 2-deoxy-D-glucose (2-DG). We recognized

two null mutants as more resistant to 2-DG, *fbx19* (AN4510.4; Supplementary Fig. S1) and *fbx22* (AN5517.4), here named *fbxA* and *fbxB*, respectively (Fig. 1A). FbxA encodes a novel 923 amino acid protein that has a putative F-box domain at amino acid positions 538–585 and a cyclic nucleotide-binding domain signatures and profile (Fig. 1B; cNMP binding motif, PDOC00691, http://www.expasy.org), a domain found in proteins that bind cyclic nucleotides (cAMP or cGMP) sharing a structural domain of about 120 residues; the best studied of these proteins is the prokaryotic catabolite gene activator (also known as the cAMP receptor protein) (gene crp; http://www.expasy.org/cgi-bin/nicedoc.pl?PS50042). In addition, at amino acid positions 650–890 there is an AMN1 superfamily motif, an effector domain of the CAP family of transcription factors; binding of the effector leads to conformational changes and the ability to activate transcription (Fig. 1B; AMN1 superfamily, cd00038; http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi?SEQUENCE=40741663&FULL). Blastp analysis suggested that FbxA is a protein exclusive from fungi, without any apparent

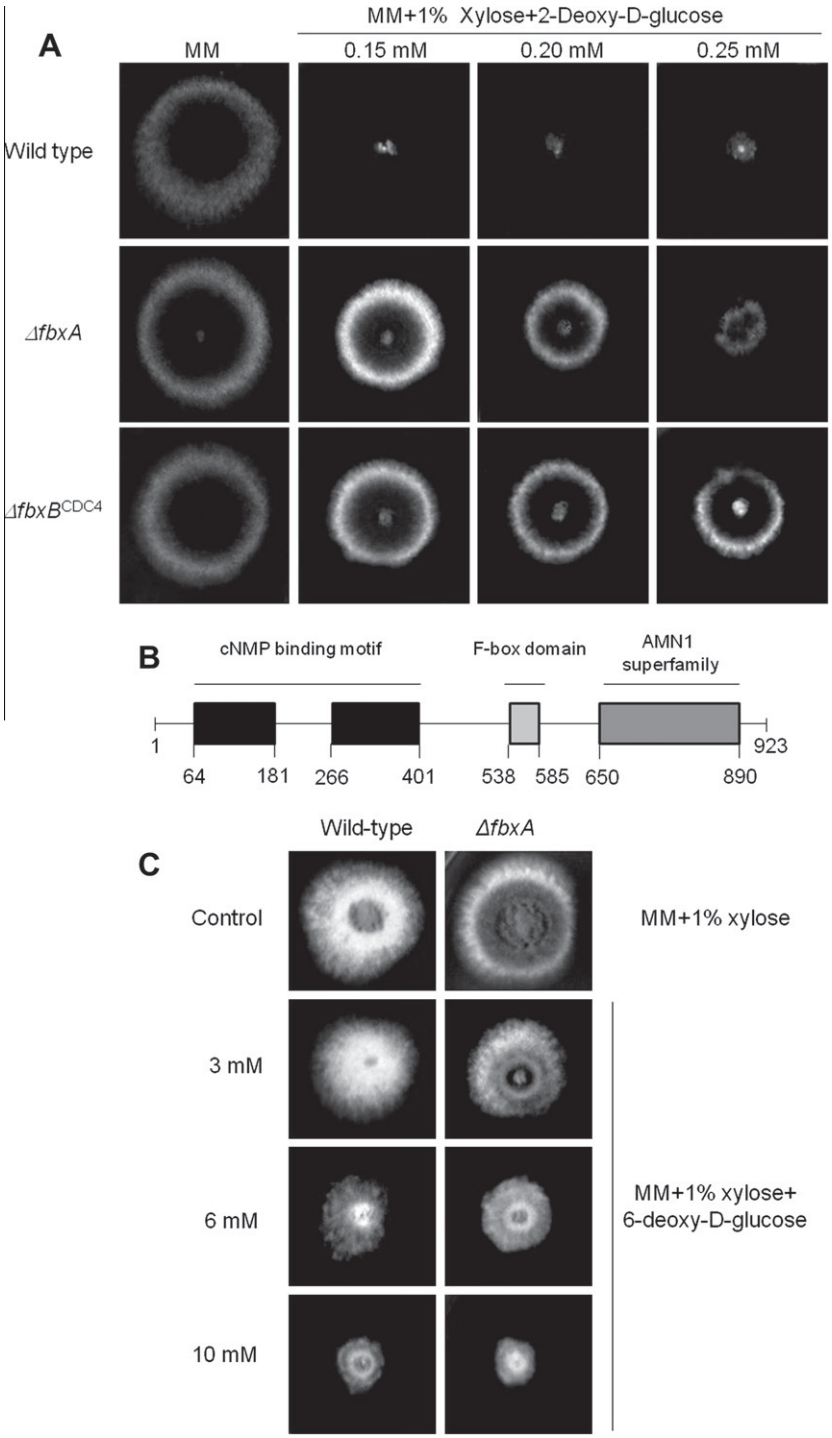

Fig. 1. The *A. nidulans ΔfbxA* mutant is 2-DG-resistant. (A) The wild-type, *ΔfbxA*, and ΔfbxB^{CDC4} mutant strains were grown for 72 h at 37 °C in MM + 1% xylose with 0, 0.15, 0.20, and 0.25 mM 2-deoxy-D-glucose. (B) FbxA protein structure. (C) The wild-type and *ΔfbxA* mutant strains were grown for 72 h at 37 °C in MM + 1% xylose with 0, 3, 6, and 10 mM 6-deoxy-D-glucose.

homologs in higher eukaryotes (Supplementary Fig. S2). In contrast, FbxB is the homolog of *S. cerevisiae* Cdc4p and among many functions Cdc4p mediates the degradation of the Hac1p transcription factor that regulates the unfolded protein response (UPR) in *S. cerevisiae* (Pal et al., 2007). It is quite likely the *ΔfbxB* was identified in our screening because 2-DG is an agent that induces UPR (Back et al., 2005). Thus, we concentrated our attention in the *fbxA* characterization.

To demonstrate that FbxA is a functional F-box protein (FBP) we analyzed if FbxA associates to the *A. nidulans* Skp1 protein, SkpA. We generated a GST fusion FbxA protein lacking the N-terminal 132 amino acid of FbxA (aa 1–829, F), assuming that this deletion does not impair the SCF complex assembly. As a negative control, we produced a truncated version of FbxA in which the F-box domain was completely deleted (aa 1–538, ΔF). The constructs of GST-FbxA (F and ΔF) were coexpressed with 6XMyc-SkpA in HEK293T cells and the cell lysates were subjected to a GST pull-down assay. As seen in Fig. 2, the FbxA-F, but not the version of FbxA with the F-box domain deleted (ΔF) was precipitated together with SkpA, indicating that this protein may act as component of the SCF complex.

To investigate if this effect requires the metabolism of xylose, we investigated the effect of another non-metabolizable glucose analog, 6-deoxyglucose (6-DG). Both analogs are transported by the *A. nidulans* glucose transporter but are trapped at different steps of further glucose metabolism (6-DG cannot be phosphorylated and thus not metabolized, whereas 2-DG will be phosphorylated but cannot be isomerized further). A comparison of the effect of these two analogs therefore estimates the relative importance of glucose uptake and glucose phosphorylation respectively (Strauss et al., 1999). The wild-type and the *ΔfbxA* mutant strains displayed the same degree of 6-DG-sensitivity (Fig. 1C), which suggests the *fbxA* absence does not confer any defect related to glucose transport. However, additional transport kinetics experiments with xylose should be performed in order to confirm this hypothesis. Next, we investigated if the *ΔfbxA* strain was really carbon catabolite repression resistant by measuring the mRNA accumulation of three genes involved in xylan utilization, the transcriptional activator *xlnR*, and *xlnA* and *xlnD* encoding 1,4-beta-D-xylan xylanohydrolase and 1,4-beta-D-xylan xylohydrolase, respectively. We have grown the wild-type and the the *ΔfbxA* strains for 12 h in either MM + 1% xylose or MM + 1% xylose + 0.25 mM 2-DG (Table 2). As expected, in the wild-type strain *xlnR*, *xlnA*, and *xlnD* showed increased mRNA accumulation upon growth on 1% xylose and had their mRNA accumulation dramatically decreased upon growth on 1% xylose + 0.25 mM 2-DG (Table 2). As a control, the *creAd-30* strain, that has a truncated non-functional version of the *creA* gene (Arst et al., 1990), showed to have increased mRNA accumulation of all three genes in both 1% xylose and 1% xylose + 0.25 mM 2-DG (Table 2). Surprisingly, the *ΔfbxA* strain had a much lower mRNA accumulation of these three genes when grown in 1% xylose comparatively to the wild-type strain (Table 2). However, in the *ΔfbxA* strain the mRNA accumulation of these genes is not reduced upon growth on 1% xylose + 0.25 mM 2-DG when compared to the 1% xylose control (Table 2). The lower *xlnR*, *xlnA* and *xlnD* mRNA levels in the presence of xylose for Δ*fbxA* compared with wild type, suggest that *fbxA* is required for xylose induction.

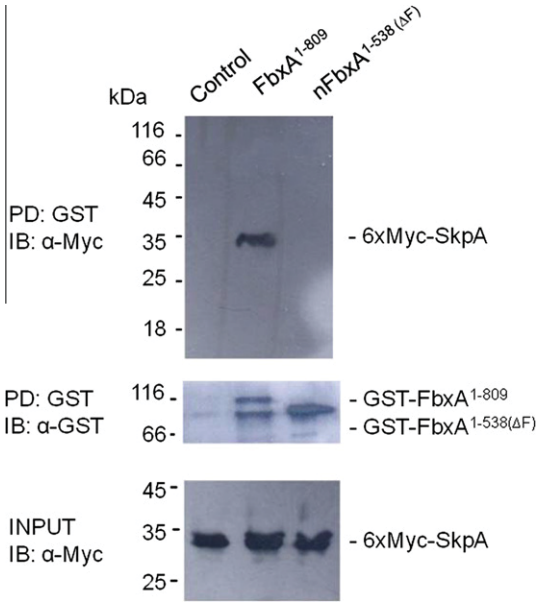

Fig. 2. FbxA interacts with SkpA. HEK293T cells were cotransfected with 6XMyc-SkpA and pCDNA3.1 (mock Control) or GST-FbxA-F or GST-FbxA-ΔF (negative control). The lysates (INPUT) were subjected to pull-down assay (PD) using glutathione-sepharose beads. The input and the eluted were resolved in 10% SDS–PAGE and subsequently processed for immunoblotting (IB) with anti-GST and anti-Myc antibodies. SkpA coprecipitated with GST-FbxA-F, but not with FbxA-ΔF.

3.2. Xylanase activity is decreased in the ΔfbxA mutant strain

The decreased mRNA accumulation of genes involved in xylan utilization upon xylose induction suggested the xylanase activity was reduced in the *ΔfbxA* mutant strain. Further measurement of xylanase activity confirmed this hypothesis, since there is a 25%, 30%, and 70% reduction in the xylanase activity in the *ΔfbxA* mutant when compared to the wild-type strain at 24, 48, and 72 h growth on 1% xylose, respectively (Fig. 3A). Interestingly, the *fbxA* mRNA levels had an increase at 6 h growth upon 1% xylose, but decreased considerably at 12 and 24-h growth (Fig. 3B). We complemented the *ΔfbxA* mutant with the wild-type *fbxA* gene and were able to fully restore the xylanase activity to the wild-type levels, indicating that the *ΔfbxA* mutant defect is due to *fbxA* absence and not other secondary phenotypes in the deletion strain (Supplementary Figs. S3 and S4). We constructed a FbxA::GFP strain that showed comparable levels of xylanase activity with the wild-type strain (data not shown). When grown on MM + fructose and transferred to MM + 1% xylose, the FbxA::GFP was localized to the cytoplasm (Fig. 4). This sub-cellular localization was not changed either by incubating up to 4 h with xylose or by adding glucose 1% (data not shown). Taken together these data suggest FbxA can influence *A. nidulans* xylanase activity.

Table 2
The *xlnA*, *xlnD*, and *xlnR* mRNA accumulation in *A. nidulans* strains. The results are expressed as the number of copies of cDNAs from either *xlnA*, or *xlnD*, or *xlnR* divided by the number of copies of *tubC* (β-tubulin). The relative quantitation of *xlnA*, *xlnD*, and tubulin gene expression was determined by a standard curve (i.e., C_T –values plotted against logarithm of the DNA copy number). The results are the means ± standard deviation of four sets of experiments experiments (X12, growth for 12 h in 1% xylose, and X12 + 2-DG, growth for 12 h in 1% xylose + 0.25 mM 2-DG).

	Control	X12	X12 + 2-DG
xlnA			
Wild-type	0.003 ± 0.0003	10.83 ± 1.78	0.87 ± 0.01
creAd-30	0.56 ± 0.13	90.7 ± 17.9	148.6 ± 15.7
ΔfbxA	0.033 ± 0.011	0.611 ± 0.007	0.787 ± 0.135
xlnD			
Wild-type	0.060 ± 0.001	140.7 ± 8.63	3.83 ± 0.04
creAd-30	0.111 ± 0.022	27.49 ± 7.57	53.0 ± 9.32
ΔfbxA	0.05 ± 0.002	0.71 ± 0.004	1.94 ± 0.07
xlnR			
Wild-type	0.041 ± 0.007	0.21 ± 0.08	0.08 ± 0.02
creAd-30	0.84 ± 0.14	0.90 ± 0.08	0.45 ± 0.01
ΔfbxA	0.02 ± 0.003	0.048 ± 0.005	0.094 ± 0.003

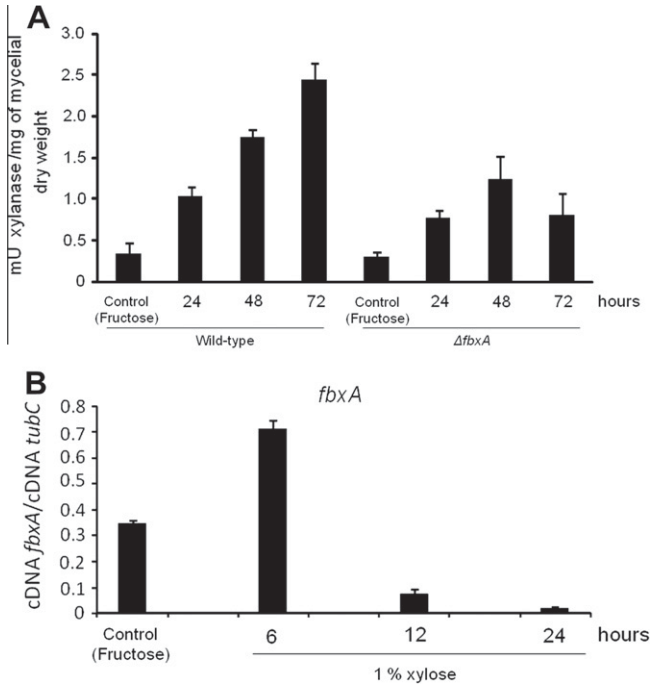

Fig. 3. The *A. nidulans ΔfbxA* mutant has decreased xylanase activity. (A) The wild-type and *ΔfbxA* mutant strains were grown for 24 h at 37 °C in MM + fructose. Mycelia was transferred to MM + 1% xylose and grown for 24, 48, and 72 h. The results are the average ± standard deviation of three repetitions, and they are expressed as mU xylanase/mg dry weight mycelia. One unit of enzyme activity is defined as the amount of enzyme required to release μmole of D-xylose reducing-sugar equivalents from arabinoxylan, at pH 4.5 per minute at 40 °C. (B) The wild-type was grown as in (A) and mRNA was isolated and the relative quantitation of *fbxA* and tubulin gene expression was determined by a standard curve (i.e., C_T – values plotted against logarithm of the cDNA copy number). The results are the means ± standard deviation of four sets of experiments.

3.3. Overexpression of XlnR in the ΔfbxA mutant strain restores partially the wild-type phenotype for xylanase expression

The fact that the xylanase activity was reduced in the *ΔfbxA* mutant strain could mean that *xlnR* mRNA levels were decreased in this strain. Actually, there is a progressive *xlnR* mRNA increase from 6 to 24-h (up to 4-fold) when the wild-type strain was grown on 1% xylose while there is a reduced and constant non-induced *xlnR* mRNA accumulation when *ΔfbxA* mutant strain was grown on 1% xylose (Fig. 5A). Interestingly, the *creA* mRNA levels were reduced from 6 to 24-h (about 4-fold) when the wild-type strain was grown on 1% xylose while the *creA* mRNA levels were constant and high when *ΔfbxA* mutant strain was grown on 1% xylose (Fig. 5C). We assumed that by overexpressing *xlnR*, we would be able to restore the xylanolytic activity of the *ΔfbxA* mutant strain to levels comparable to the wild-type activity levels. Thus, we overexpressed *xlnR* by fusing the whole ORF of this gene plus 1-kb from 5′- and 3′-ends to an autonomous multicopy vector with AMA1. This construct was functional and when transformed in the wild-type and *ΔfbxA* mutant strains, it was able to provide at least a two to threefold increase of *xlnR* mRNA accumulation (Fig. 5C). The wild-type and *ΔfbxA* mutant strains harboring either the empty control plasmid or AMA1::XlnR were grown on 1% xylose for 6, 12, and 24 h and the mRNA accumulation of *xlnA* and *xnlD* were assessed by real-time RT-PCR (Fig. 5D and E). As expected and previously shown by Tamayo et al. (2008) for the wild-type strain, increased *xlnR* copy number was able to increase the *xlnA* and *xlnD* mRNA accumulation in the wild-type and *ΔfbxA* mutant strains (Fig. 5D and E). However, this effect is more noticeable from 6 to 24 h in the *xlnA* gene than in the *xlnD* gene, where a large increase is observed only at 24 h-growth (Fig. 5D and E). Moreover, although the *xlnR* copy number is at least threefold higher in the *ΔfbxA* mutant than in the wild-type strain, the *xlnA* and *xlnD* mRNA levels are lower in the mutant strain than in the wild-type. Taken together these results suggest that increased *xlnR* mRNA levels are able to partially alleviate the *ΔfbxA* mutant defect.

3.4. The creAd-30 mutation can suppress the decreased xylanase activity in the ΔfbxA mutant strain

The reduced and increased mRNA levels of *xlnR* and *creA*, respectively, in the *ΔfbxA* mutant strain suggested *ΔfbxA* mutation is imposing a repression in the xylanase genes expression mediated via CreA. Recently, Jonkers and Rep (2009b) identified an F-box protein Frp1 required for tomato pathogenicity in *Fusarium oxysporum f.* sp. *lycopersici* and verified that the *Δfrp1* mutant is deficient in expression of genes for cell wall-degrading enzymes. These authors observed that Frp1 and Cre1 (the CreA homolog)

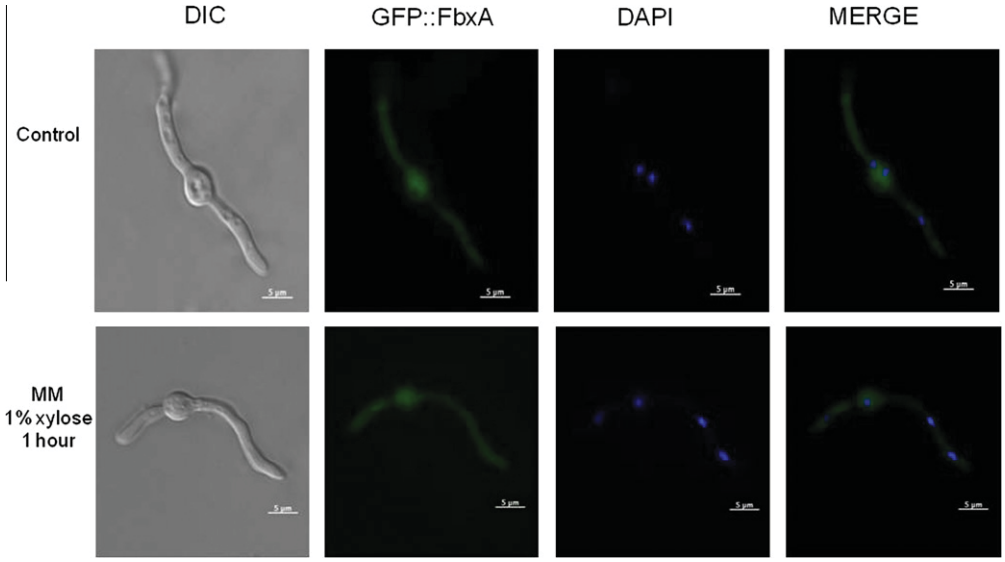

Fig. 4. FbxA::GFP localizes in the cytoplasm. FbxA::GFP conidia were grown for 16 h at 30 °C in MM + fructose and transferred for 1 h to MM + 1% xylose.

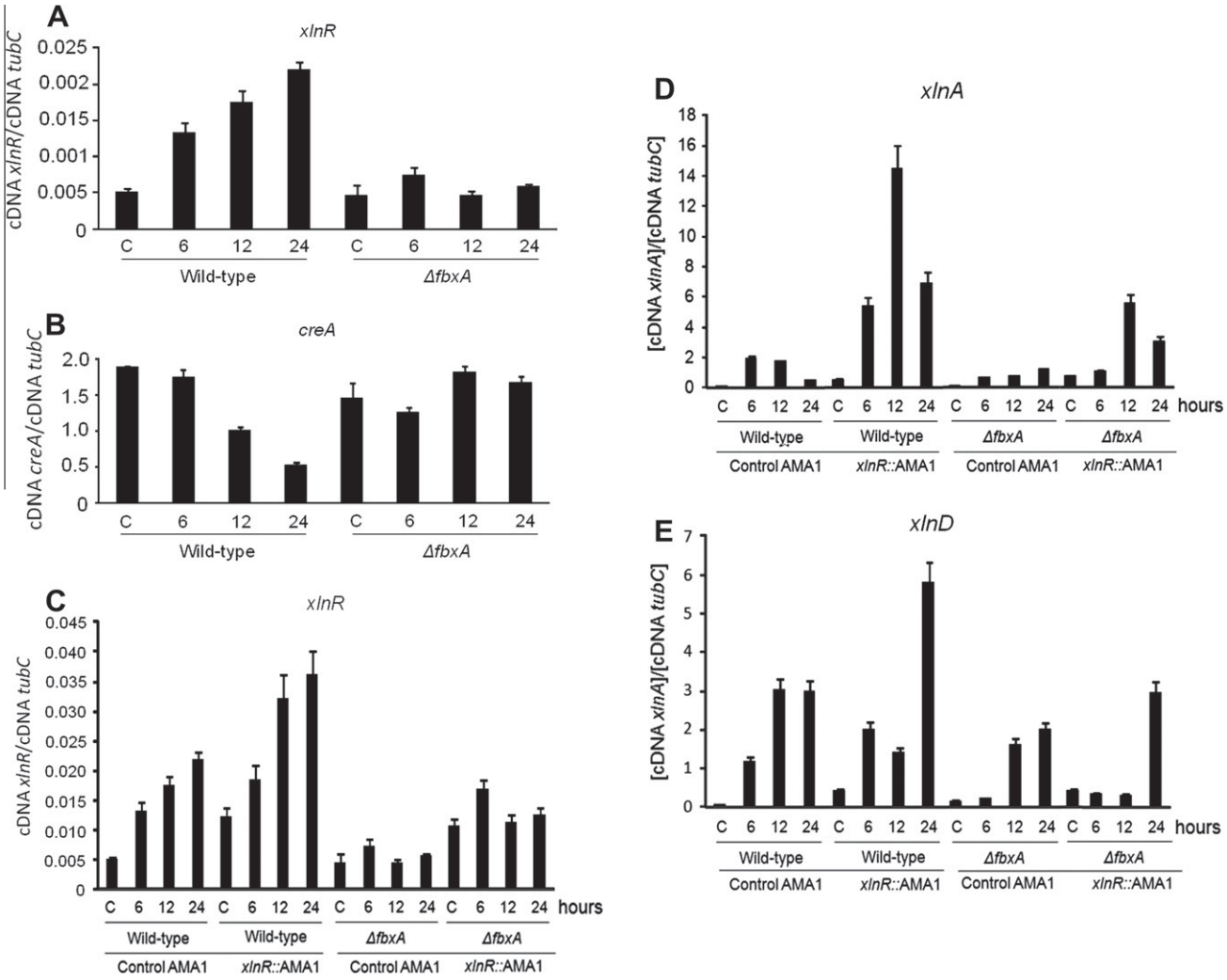

Fig. 5. The *A. nidulans ΔfbxA* mutant has decreased *xlnR* mRNA accumulation. The wild-type and *ΔfbxA* mutant strains were grown for 24 h at 37 °C in MM + fructose. Mycelia was transferred to MM + 1% xylose and grown for 6, 12, and 24 h. The relative quantitation of *xlnR* (A) and *creA* (B) and tubulin gene expression was determined by a standard curve (i.e., C_T –values plotted against logarithm of the DNA copy number). The results are the means ± standard deviation of four sets of experiments. (C) The wild-type AMA1, AMA1::XlnR, the *ΔfbxA* mutant AMA1 and AMA1::XlnR strains were grown for 24 h in MM + fructose at 37 °C. The relative quantitation of *xlnR* and tubulin gene expression was determined as in (A). Mycelia was transferred to MM + 1% xylose and grown for 6, 12, and 24 h. The relative quantitation of *xlnA* (D) and *xlnE* (E) and tubulin gene expression was determined as in (A). (C = control, fructose).

both control the repression/derepression state of such genes and replacement of CRE1 with GST::CRE1 in the *Δfrp1* mutant restored pathogenicity and expression of genes encoding cell wall-degrading enzymes. We hypothesize that a similar situation could happen in *A. nidulans ΔfbxA* mutant strain and decided to construct a double *ΔfbxA creAd-30* mutant strain by sexually crossing *A. nidulans ΔfbxA* mutant with *creAd-30* mutant strains. Interestingly, the double mutant restored the 2-DG-sensitivity and the xylanase activity to wild-type levels (Figs. 6 and 7C). Even more interestingly, colonies of the double *ΔfbxA creAd-30* mutant strain are much larger than the *creAd-30* mutant strain, with a comparable growth to the wild-type strain, suggesting that absence of *fbxA* alleviates the growth defect caused by *creAd-30* mutation. We have also seen that *xlnA* and *xlnD* mRNA accumulation in *ΔfbxA creAd-30* mutant strain is decreased to levels closer to the wild-type strain in the presence of 1% xylose for 1 and 4 h (Table 3). However, there are variable levels of glucose catabolite repression in the presence of 1% xylose + glucose 1% for 1 and 4 h (Table 3). Taken together, these results suggest a genetic interaction between *fbxA*, *xlnR*, and *creA*.

To extend our genetic analysis, we constructed by sexual crosses double mutants *ΔfbxA creB15* and *ΔfbxA creC27*. The *creB* and *creC* genes, when mutated, affect expression of many genes in both carbon catabolite repressing and derepressing conditions (Arst, 1981; Hynes and Kelly, 1977). We first evaluated the growth of these double mutants on MM + 1% xylose + 0.14 mM 2-DG (Fig. 7A). As expected, *ΔfbxA*, *creA*d-30, *creB*, and *creC* were more 2-DG-resistant while the wild-type showed to be 2-DG-sensitive (Fig. 7A). Interestingly, the double *ΔfbxA creB* and *ΔfbxA creC* mutants showed an intermediate degree of 2-DG-sensitivity when compared to the *ΔfbxA*, *creB*, and *creC* mutant strains, suggesting a genetic interaction among them (Fig. 7A). As previously observed (Fig. 6A), the *ΔfbxA creA*d-30 reversed the 2-DG-sensitivity to the levels closer to wild-type strain (Fig. 7A).

Wild-type strains are resistant to the presence of allyl alcohol (AA) in 1% glucose medium, as a result of complete carbon catabolite repression of the gene encoding the alcohol dehydrogenase, which is required to convert AA to a toxic compound, acrolein (34). Growth on allyl alcohol is an indicator for repression of the alcohol dehydrogenase (*ADH*) genes in fungi. As expected the wild-type strain is AA-resistant while the *creAd-30*, *creB*, and *creC* are AA-sensitive (Fig. 7B). Interestingly, the double mutants *ΔfbxA creAd-30*, *ΔfbxA creB*, and *ΔfbxA creC* showed increasing levels of AA-resistance, suggesting these mutations are suppressing the

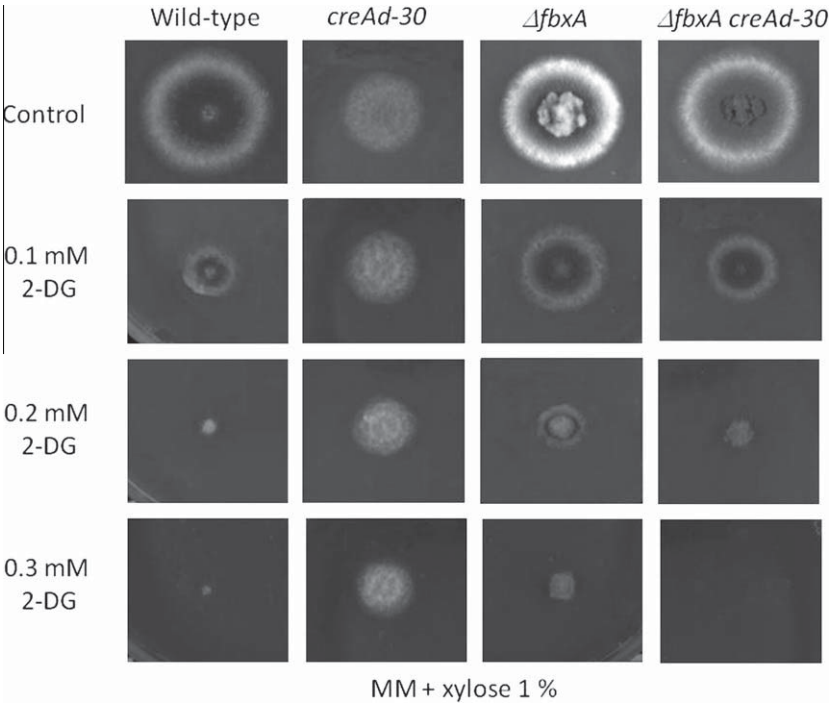

Fig. 6. The *creAd-30* mutation can suppress the decreased xylanase activity in the *ΔfbxA* mutant strain. (A) The wild-type, *creAd-30*, *ΔfbxA*, and *ΔfbxA creAd-30* mutant strains were grown for 72 h in MM + 1% xylose with 0, 0.1, 0.2, and 0.3 mM 2-DG. (B) The wild-type, *creAd-30*, *ΔfbxA*, and *ΔfbxA creAd-30* mutant strains were grown for 24 h at 37 °C in MM + fructose (control). Mycelia was transferred to MM + 1% xylose and grown for 24 h (xylose). The results are the average ± standard deviation of three repetitions, and they are expressed as mU xylanase/mg dry weight mycelia.

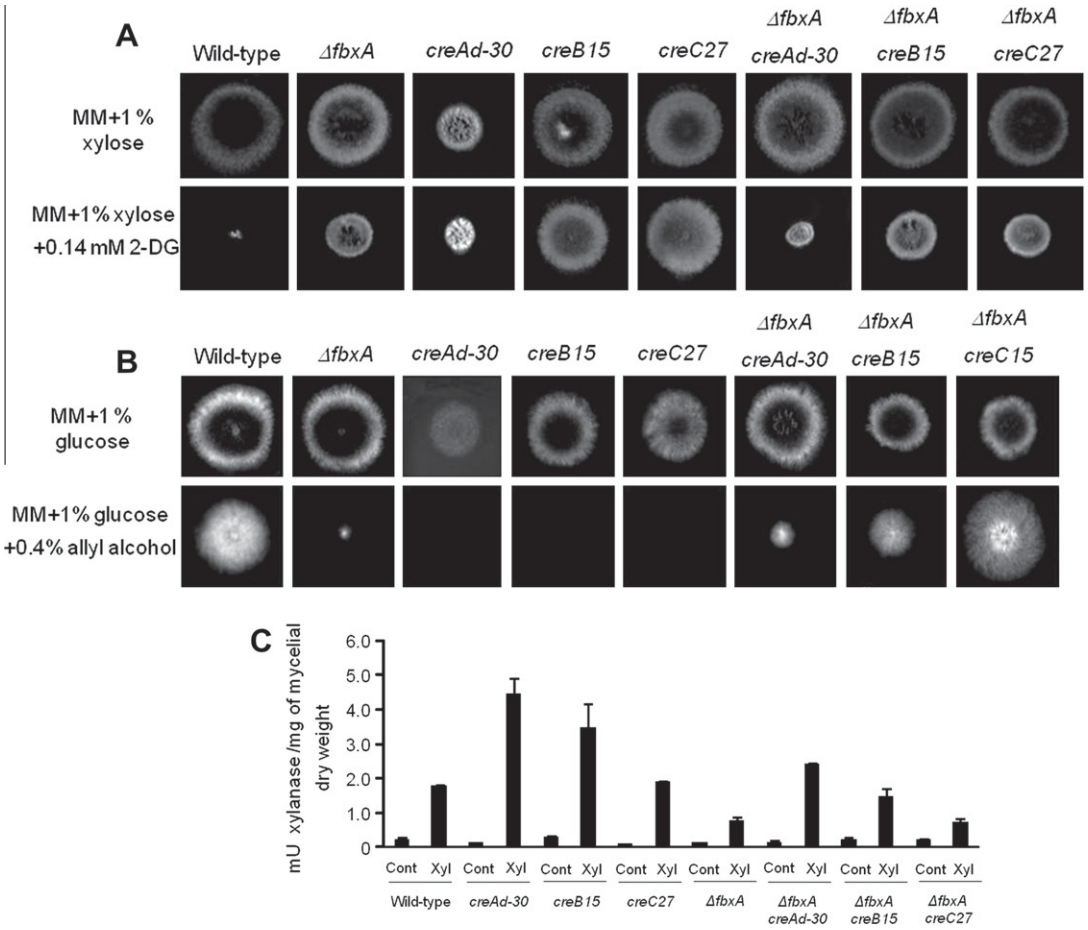

Fig. 7. There is a genetic interaction among *A. nidulans ΔfbxA*, *creAd-30*, *creB*15, and *creC27* mutations. The wild-type, *creAd-30*, *creB15*, *creC27*, *ΔfbxA*, *ΔfbxA creAd-30*, *ΔfbxA creB15*, and *ΔfbxA creC27* mutant strains were grown for 72 h in MM + 1% xylose with 0 and 0.14 mM 2-DG (A) or MM + glucose 1% with 0 and 0.4% allyl alcohol (B).

Table 3
The *xlnA* and *xlnD* mRNA accumulation in *A. nidulans* strains. The results are expressed as the number of copies of cDNAs from either *xlnA* or *xlnD* divided by the number of copies of *tubC* (β-tubulin). The relative quantitation of *xlnA*, *xlnD*, and tubulin gene expression was determined by a standard curve (i.e., C_T –values plotted against logarithm of the DNA copy number). The results are the means ± standard deviation of four sets of experiments (X1 and X4, growth for 1 h in 1% xylose, respectively and X1 + G1 and X4 + G4, growth for 4 h in 1% xylose + glucose 1%).

	Control	X1	X1 ± G1	X4	X4 ± G4
xlnA					
Wild-type	0.04 ± 0.00	3.82 ± 0.02	0.92 ± 0.15	1.14 ± 0.06	0.43 ± 0.03
creAd-30	2.96 ± 1.18	546.0 ± 26.2	51.6 ± 7.6	238.0 ± 40.0	198.0 ± 3.00
ΔxlnR	0.004 ± 4e-4	0.004 ± 0.001	0.002 ± 6e-5	0.023 ± 0.001	0.012 ± 0.002
ΔfbxA	0.007 ± 0.00	0.92 ± 0.02	0.31 ± 0.08	1.10 ± 0.09	0.35 ± 0.09
ΔfbxA creAd-30	0.43 ± 0.03	7.81 ± 1.00	9.95 ± 0.07	5.87 ± 1.16	8.06 ± 1.25
xlnD					
Wild-type	0.07 ± 0.007	7.00 ± 0.60	0.4 ± 0.1	2 ± 0.06	0.19 ± 0.005
creAd-30	0.08 ± 0.07	36.0 ± 2.00	3.00 ± 0.40	10.0 ± 0.8	3.80 ± 0.10
ΔxlnR	0.01 ± 3e-4	0.007 ± 0.002	0.003 ± 6e-4	0.02 ± 9e-4	0.006 ± 7e-4
ΔfbxA	0.12 ± 0.005	1.9 ± 0.1	0.1 ± 0.005	0.6 ± 0.01	0.02 ± 5e-4
ΔfbxAcreAd-30	0.16 ± 0.01	48.5 ± 5.0	0.39 ± 0.02	1.28 ± 0.24	1.84 ± 0.92

ΔfbxA AA-sensitivity (Fig. 7B). These growth assays indicate that both derepression and repression are defective in the *ΔfbxA* strain and could be suppressed to different extents by *creAd-30*, *-B15*, *-C27* mutations. We also examined the effects of the *creB15* and *creC27* mutations on xylanase activity (Fig. 7C). The *creAd-30* and *creB* mutants have higher xylanase activity than the wild-type strain; the *creC* mutant has xylanase activity comparable with the wild-type strain (Fig. 7C). As previously shown *creAd-30* can restore the xylanase activity to the levels of the wild-type activity in the double mutant *ΔfbxA creAd-30* (Fig. 7C). However, *creB and creC* mutations reduce the xylanase activity in the *ΔfbxA creB* and *ΔfbxA creC* double mutants (Fig. 7C).

Taken together, these results emphasize again the involvement of FbxA on carbon catabolite repression of xylanase induction and suggest a genetic interaction among *fbxA*, *creB*, and *creC*.

4. Discussion

Fungi produce hydrolytic enzymes able to degrade complex polysaccharides, such as hemicelluloses. The preferred energy source is glucose and the genes that are required for the use of alternative carbon sources are not transcribed when glucose is available. It is well established that the genes encoding these enzymes are subjected to CCR. Here, we investigated the influence of the absence of specific genes encoding F-box proteins on the deregulation of xylanase induction. We are interested on understanding xylanase induction and that was the motivation to screen for mutants that are able to grow on 1% xylose with different 2-DG concentrations. We identified two mutants, one that is complemented by a gene *fbxB* that encodes a putative homolog of *S. cerevisiae* Cdc4p, and another one that encodes a novel protein with homologs only in fungi. FbxA is able to interact with SkpA, in the presence of F-box domain, suggesting the assembly into an SCF-type complex. The F-box hypothesis states that F-box proteins work as scavengers in the cell, collecting proteins that have to be degraded and recycled, distributing them to the SCF complex, to which they dock through their F-box domain (Skowyra et al., 1997; Patton et al., 1998). In the SCF complex, these garbage proteins are labeled with a chain of ubiquitin leading to destruction in the proteasome. The targets of these F-box proteins are commonly first phosphorylated before being recognized and ubiquitinated by the SCF complex (Jonkers and Rep, 2009a). Thus, genes encoding F-box proteins have already been identified that are responsible for multiple targets, such as *S. cerevisiae CDC4* and *GRR1* or single targets such as *MET30/Scon2/SconB* in *S. cerevisiae/N. crassa/A. nidulans* (Jonkers and Rep, 2009a). Since SCF complexes are E3 ligases involved in ubiquitination of proteins, FbxA could be a specificity factor recruiting proteins to the SCF complex aiming their labelling with ubiquitin (see references (for reviews, see Skowyra et al., 1997; Jonkers and Rep, 2009a). Besides 2-DG-resistance, the *fbxA* null mutant also showed increased-sensitivity to allyl alcohol when grown in the presence of glucose. These two phenotypes are CCR hallmarks for filamentous fungi.

Glucose sensing and signalling pathways involving hexokinases, tranporters-like sensors, and G-coupled protein receptors have been well characterized in *S. cerevisiae* (for reviews, see Santangelo, 2006; Gancedo, 2008; Zaman et al., 2008; Turcotte et al., 2010). In yeast, glucose is the preferred carbon source and components of the CCR, such as the transcription factor Mig1p have been extensively studied (for reviews, see Santangelo, 2006; Gancedo, 2008; Zaman et al., 2008; Turcotte et al., 2010). It is clear that the situation is considerably more complex in filamentous fungi than in yeast. This could be due to the preference of yeast for glucose fermentation instead of aerobic metabolism via Krebs cycle as in filamentous fungi. The most important components of fungal CCR are the proteins CreA,-B,-C,-D. The *creA* gene has some identity with *MIG1* and encodes a Cys2-His2 DNA-binding protein of the zinger finger class (Arst and Cove, 1973; Hynes and Kelly, 1977; Dowzer and Kelly, 1989, 1991). How CreA mediates gene repression and derepression in filamentous fungi is not fully understood. Recently, Roy et al. (2008) showed that CreA-mediated repression in *A. nidulans* does not require transcriptional auto-regulation, regulated intracellular localization or degradation of CreA. CreB (Lockington and Kelly, 2001) and CreC (Todd et al., 2000) make a complex *in vivo*, and Lockington and Kelly (2002) have proposed that the CreB–CreC deubiquitination complex removes ubiquitin moieties from CreA and other substrates, thus modifying or stabilizing these proteins. It is known that creD34 mutation suppresses the phenotypic effects of mutations in *creC* and *creB* (Kelly and Hynes, 1977). CreD encodes a protein that contains arrestin domains and PY motifs and is highly similar to the Rod1p and Rog3p proteins from *S. cerevisiae*. This suggests that ubiquitination is involved in the *A. nidulans* CCR.

The *ΔfbxA* mutant strain has reduced xylanase activity and mRNA accumulation of genes important for xylanase induction, such as *xlnR*,*-A*,*-D*. Interestingly, when the *A. nidulans* wild-type is grown in the presence of xylose it has reduced *fbxA* mRNA accumulation. Upon xylose induction, *xlnR* and *creA* mRNA accumulation showed conflicting phenotypes in the wild-type and *ΔfbxA* mutant strains, i.e., *xlnR* has increased and decreased mRNA accumulation in the wild-type and *ΔfbxA* mutant strain, respectively, while *creA* has decreased and increased mRNA accumulation in the wild-type and *ΔfbxA* mutant strain, respectively. Since XlnR

is the single transcription factor responsible for its transcriptional self-activation and *xlnA,-D* activation (Stricker et al., 2008), these results suggest that XlnR is not functional either because XlnR is not able to bind its binding-site and/or XlnR is degraded. Thus, we decided to increase XlnR expression to investigate if *xlnA,-D* mRNA accumulation and xylanase activity could be restored in the *ΔfbxA* mutant. Increased *xlnR* mRNA levels are able to partially suppress the xylanase deficiency in the *ΔfbxA* mutant defect. Therefore, these results did not allow us to distinguish if XlnR is not able to bind its binding-sites and/or is degraded. Additional evidence for *fbxA* influencing CCR comes from the interaction among *ΔfbxA* and *creAd-30,-B,-C* mutant strains. The *creAd-30,-B,-C* mutations suppress *ΔfbxA* sensitivity to 2-DG and allyl alcohol and restore its xylanase activity. Taken together, these data strongly indicate that FbxA modulates the XlnR and CreA mRNA accumulation and the *ΔfbxA* resistance to CCR is reversed by the *creAd-30,-B,-C* mutations. A similar phenotype to what it is described here was observed by Jonkers and Rep (2009b). The F-box protein Frp1 is required for pathogenicity of *F. oxysporium* towards tomato (Duyvesteijn et al., 2005). The *Δfrp1* mutant is lacking cell wall-degrading enzymes and a double mutant *Δcre1 Δfrp1* restored pathogenicity and expression of cell wall-degrading enzymes, strongly indicating that there is a constitutive repression by Cre1 in the *Δfrp1* mutant (Jonkers and Rep, 2009b). Frp1 and FbxA are not homologs and there is no apparent Frp1 homolog in *A. nidulans*.

Our work does not provide details of how FbxA influences *A. nidulans* xylanase induction and CCR. An essential pathway of targeted protein degradation includes ubiquitination and the 26S proteasome (Tyers and Jorgensen, 2000). The conserved ubiquitin is a 76-amino acids protein that is attached covalently to lysine residues of substrate proteins. Ubiquitination can either reduce stability or change activity or localization of a protein. FbxA encodes an F-box protein that is together with CreA required for the expression of xylanase genes. It is possible FbxA ubiquitinates CreA and/or a (yet unidentifed) protein which is controlling xylan-degradation genes as well as glucose repression genes. Transcription factors are ubiquitin-labeled in the nucleus and some of them are degraded and others, like *S. cerevisae* Met4p are stored when ubiquitinated (for a review, see Freiman and Tjian, 2003). There is also mono-ubiquitylation which can occur at the nuclear localization signal resulting in impairment of nuclear import (Trotman et al., 2007). FbxA::GFP is located in the cytoplasm in germlings when grown either in repressing or derepressing conditions. In contrast, CreA is located in the nucleus in both repressing and derepressing conditions (Roy et al., 2008) and XlnR is possibly translocated to the nucleus upon xylose induction. If FbxA interacts with transcription factors in the nucleus, there are two possibilities: (i) it interacts with them before they can enter the nucleus (and prevents nuclear entry until the situation has changed or until they will be degraded or might mislocalize them) or (ii) it interacts with transcription factors which have been exported from the nucleus (with similar consequences as before). In both cases all processes could be reversible because the cell is plenty of deubiquitinating enzymes.

Our work emphasizes the importance of the ubiquitination in the *A. nidulans* xylanase induction and CCR. The identification of FbxA provides another layer of complexity to both phenomena in filamentous fungi. Further work will concentrate on the identification of FbxA targets and how they interact with XlnR and CreA.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2011.11.004.

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