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Enhanced glycosyl hydrolase production in *Aspergillus nidulans* using transcription factor engineering approaches

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Abstract

Background: Engineered fungi are attractive platforms for the production of plant cell wall hydrolytic enzymes which, among other biotechnological applications, are required for the efficient conversion of biomass to glucose and other fermentable sugars. As a fungal model system, *Aspergillus nidulans* provides genetic tools that are of relevance in this context and potentially applicable to industrially important filamentous fungi. The goal of this study is to assess the utility of *A. nidulans* as a host for recombinant protein production.

Results: We have successfully applied a transcription factor engineering approach to improve the efficiency of the *A. nidulans* xylanolytic XlnR-xln_p expression system. Specifically, endo-1,4-β-xylanases and an α-L-rhamnosidase were chosen as representatives of endogenous and heterologous glycosyl hydrolases involved in plant cell wall deconstruction. By deregulating the expression of the xylanolytic transcriptional activator XlnR and modulating the activity of the pH regulator PacC we improved protein production and reduced production times. Xylanase activity was about 200-fold greater in $gpdA_p$::$xlnR$ strains compared to controls 4 hours after transfer to inducing conditions, and 10-fold greater after 24 hours. Remarkably, 75% of the xylanase activity was present in the engineered strains within 4 hours. Engineering XlnR expression also had a considerable impact on foreign protein production, especially when the promoter of the 'acidic' $xlnB$ gene was used to express the transgene. α-L-rhamnosidase activity in $xlnB_p$::$rhaA$, $gpdA_p$::$xlnR$ strains was about 19-fold greater than that of controls 72 hours after transfer to xylan (about 85% of the total activity produced), and 10-fold greater at later times (120 hours). The performance of these strains was further enhanced by impeding the proteolytic activation of PacC; introduction of the $palA1$ allele in $xlnB_p$::$rhaA$, $gpdA_p$::$xlnR$ strains resulted in an additional 2.7-fold increase in α-L-rhamnosidase activity by 48 hours (about 87% of the total activity produced) and a 1.7-fold increase at later times.

Conclusions: Our results show that the XlnR-xln_p expression system is a valuable tool for manipulating the production of plant cell wall degrading enzymes in *A. nidulans* and establish the biotechnological potential of the transcription factors XlnR and PacC to boost and control the strength of xylanolytic promoters.

Keywords: Fungal cell factories, *Aspergillus nidulans*, Plant cell wall degrading enzymes, Endo-1,4-β-Xylanase, α-L-rhamnosidase, Protein production, XlnR-xln_p expression system, Xylan/xylose regulator (XlnR), PalA/PacC, Ambient pH regulation

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Background

Lignocellulosic biomass is the most abundant source of carbon and energy on Earth and constitutes a clean alternative to fossil fuels [1]. Its specific composition varies between plant species but mainly consists of polysaccharides (predominantly cellulose and hemicellulose) and lignin [2]. A central challenge in the utilization of this material is its enzymatic conversion into fermentable sugars [3,4]. In this context, saprophytic and phytopathogenic fungi are known to coordinately produce, or to have the capacity to produce, a wide spectrum of lignocellulosic enzymes that can synergistically degrade structural polysaccharides in plant cell walls [5-11]. Filamentous fungi are also attractive expression hosts for recombinant protein production, especially due to their high enzyme secretory capacity [12-14]. Whereas the majority of recombinant proteins manufactured by biotechnological industries are produced using a limited number of fungal species, mainly *Aspergillus niger, Aspergillus oryzae* and *Trichoderma reesei,* on occasions these fungi may not be appropriate hosts, especially where they are covered by patents and intellectual property rights [12]. Engineering other fungal species suitable for high level production of endogenous or heterologous plant cell wall polysaccharide degrading enzymes will therefore provide an alternative to the hosts traditionally used for commercial exploitation.

Although more widely used in basic research, *Aspergillus nidulans*, unlike *A. niger*, *A. oryzae* and *T. reesei*, offers the advantages of a well-characterized sexual cycle and thus a tractable genetic system which, along with the availability of many mutant alleles of wide domain and pathway-specific regulators, provides a major advantage for strain construction and development. Analysis of its genome [15] in combination with proteomic studies [9,16] show it to have a similar polysaccharide degradation potential to that of the industrial fungi, and in addition the regulatory mechanisms involved in controlling the expression of plant cell wall degrading activities are mostly conserved between it and industrially important fungi [17-20]. Finally, the use of *A. nidulans* as a production vehicle could provoke less concern regarding mycotoxin synthesis since its genome encodes the metabolic pathway for the less toxic compound sterigmatocystin compared to certain industrial strains which still retain the capacity to produce very harmful mycotoxins [21,22].

An important factor for the production of recombinant proteins concerns the efficiency of transcription of the encoding genes. In this regard a variety of promoters are available in *A. nidulans* and the usefulness of some of them beyond the fungal kingdom has been demonstrated [23,24]. Both constitutive (such as that of the *gpdA* gene which encodes glyceraldehyde-3 phosphate dehydrogenase) and inducible (for example that of the *alcA* gene which encodes alcohol dehydrogenase I) promoters are utilized in

A. nidulans to drive gene expression, though regulated promoters are frequently preferred when the product is toxic or compromises growth. An ideal inducible promoter for industrial processes should be inexpensive to activate and capable of achieving high levels of expression after induction. In this context, we are exploring the potential of other regulated *A. nidulans* promoters, such as those of the xylanolytic genes *xlnA* (encodes xylanase X_{22}) and *xlnB* (encodes xylanase X_{24}), with the aim of obtaining improvements in expression levels and/or production times of both endogenous and heterologous high-value plant cell wall degrading enzymes. The biotic and abiotic factors influencing the expression of these promoters ($xlnA_{p}$ and $xlnB_{p}$) and the transcriptional repressors and activators associated with them have been thoroughly characterized [18,25-27]. An additional advantage of these alternative promoters is the almost complete lack of basal level of expression in the absence of the inducer. In this regard, the utility of the *A. nidulans* *xlnA* gene promoter has been previously demonstrated for the conditional expression of the Cre/loxP system [28]. The question now is how our current knowledge of the molecular mechanisms regulating the XlnR-*xln*$_p$ expression system can be best employed to further improve productivity.

The activities of the *xlnA* and *xlnB* promoters are tightly regulated by at least three mechanisms: ambient pH regulation mediated by the wide-domain transcriptional factor PacC, carbon catabolite repression (CCR) mediated by the wide-domain repressor CreA and specific induction in the presence of xylan or xylose controlled by the $Zn(II)_2Cys_6$ protein XlnR, which is itself transcriptionally regulated by CreA [18,25-27]. For efficient induction, $xlnA_{p}$ requires both xylose and alkaline pH whereas $xlnB_{p}$ requires xylose and acidic pH [25]. Previous studies that have demonstrated the roles of PacC, CreA and XlnR in the regulation of the *A. nidulans* xylanolytic system led to the identification of genetic backgrounds in which the expression of *xlnA* or *xlnB* was strongly enhanced compared to controls [18,25-27]. Notable examples are $creA^{d}30$ and $gpdA_{p}$::$xlnR$ (*xlnA* and *xlnB* overexpressed), $pacC^{c}14$ (*xlnA* overexpressed) and *palA1* (*xlnB* overexpressed because the proteolytic activation of PacC is impaired) mutant strains. Thus, manipulation of the expression or activity of the regulators CreA, XlnR or PacC (either alone or in combination) could be exploited to improve the production of target proteins, the expression of which is driven by xylanolytic promoters. In this regard, the $creA^{d}30$ mutation has been shown to result not only in considerably elevated transcript levels of the xylanolytic genes under inducing conditions but also in elevated levels of extracellular xylanase activity [29,30]. This could well be a result of the derepression of the regulatory gene *xlnR* [18].

Here we optimize the performance of the *A. nidulans* xylanolytic (XlnR-*xln*$_p$) system to overproduce recombinant proteins. We chose endogenous xylanases and the *Aspergillus aculeatus* α-L-rhamnosidase RhaA as representatives because of their commercial potential in the biofuel, food and feed industries among others [31-33]. Improvements in the production of these enzymes by means of engineering the expression of transcription factors regulating *xlnA* and *xlnB* (XlnR and PacC) is reported. To the best of our knowledge there has been no systematic evaluation of the effect of engineering PacC function (alone or in combination with pathway-specific regulators) to enhance protein yields in fungi.

Results and discussion

CreA-independent overexpression of the xylanolytic activator XlnR leads to enhanced production of native xylanases in *A. nidulans*

It has been previously shown that the three characterized *A. nidulans* xylanolytic genes (*xlnA-C*) are overexpressed under inducing conditions (1% w/v xylose) in the *gpdA*$_p$::*xlnR* genetic background (that is, where the regulatory gene *xlnR* is overexpressed in a CreA-independent fashion) [18]. To evaluate whether this manipulation of the expression of *xlnR* also leads to enhanced protein production from XlnR-regulated promoters we first compared the kinetics of xylanase production in *xlnR*⁺ (AR70) and *gpdA*$_p$::*xlnR* (AR66 and AR67) isogenic strains, as well as in the reference strain *biA1* (AR5). Extracellular xylanase activity was determined at 4, 24, 48 and 72 hours after mycelia transfer to inducing

conditions. A dramatic increase in xylanase yields during the time course of the experiment was found in the *gpdA*$_p$::*xlnR* strains, relative to controls (Figure 1). The maximum level of extracellular xylanase activity in these XlnR engineered strains was achieved after 24 hours of cultivation in the presence of xylose, corresponding to an approximately 10-fold increase compared to controls. Interestingly, about 75% of the maximum activity reached by AR66 and AR67 was attained 4 hours after transfer; at this time, extracellular xylanase activity in AR70 and *biA1* was about 200-fold lower. This rapid enhancement of xylanase production in the *gpdA*$_p$::*xlnR* strains has the additional biotechnological advantage of cost savings as a result of reduced production times. Moreover, with shorter times the presence of undesirable proteins such as proteases can be expected to be lower than at later stages of culture since they are produced in response to nutrient limitation [34]. As expected, similar kinetics and yields were attained in each strain pair (AR66 and AR67 on one hand and the two control strains *biA1* and AR70 on the other). In addition, xylanase activities per mg of total extracellular protein were calculated from the volumetric activity data. A notable difference between the *gpdA*$_p$::*xlnR* and *xlnR*⁺ strains was also observed at 4 and 24 hours (about 49- and 11-fold increases respectively) thus ruling out the possibility that the increases in xylanase activity were simply due to an overall increase in extracellular protein accumulation.

The improvement in xylanase yields achieved in *A. nidulans* is greater than that reported for *Penicillium canescens* in which transformants with an altered copy
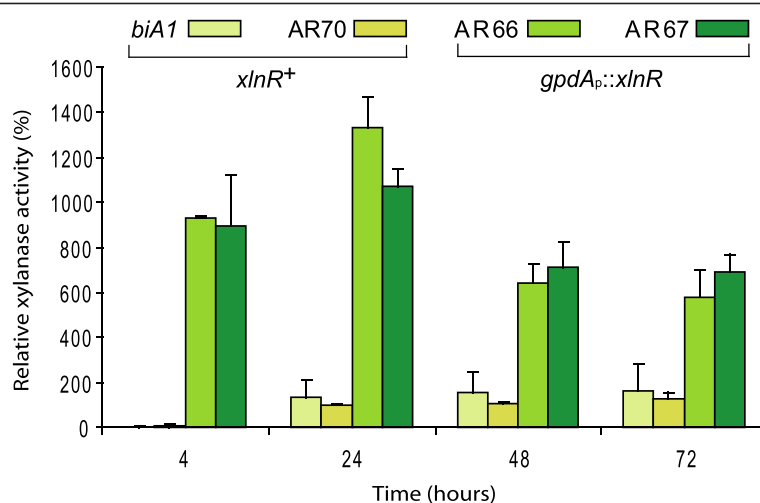
Figure 1 Effect of the *gpdA*$_p$::*xlnR* genetic background on native xylanase activity. Time course analysis of the extracellular xylanase activities in *gpdA*$_p$::*xlnR* strains (AR66 and AR67) and controls (wild type *biA1* and the nutritional arginine prototrophic control AR70). AR66, AR67 and AR70 are isogenic strains bearing respectively one copy of pILJ16_gpdA$_p$::xlnR or pILJ16 integrated at the *argB* locus. The mycelia were grown under non-repressing, non-inducing conditions (0.1% w/v fructose) before being transferred to inducing conditions (1% w/v xylose), and samples were collected at the time points indicated. Values (means ± standard deviation (SD)) are expressed as percentages and referred to that of the AR70 strain at 24 hours. For each strain, two independent experiments with two independent cultures were carried out.

number of the *xlnR* regulatory gene expressed from its own promoter produced up to six times more activity than the wild type [35]. Xylanase production in xylan cultures of a *T. reesei* strain constitutively expressing the xylanolytic activator Xyr1 was also shown to be greater than that of the wild type at relatively early culture stages (about four times after 48 hours), though the maximum levels of activity achieved by both strains were similar after 72 hours [36]. Characteristics specific to each fungal species (for example protein folding, secretory ability and protease activity), differences in XlnR/Xyr1 expression levels, different requirements for XlnR/Xyr1 of the xylanolytic gene promoters and/or differences in the culture conditions (such as the use of distinct inducers) might account for the differences in improvements reported in each study.

The intrinsic efficiency of $xlnA_p$ in driving the expression of heterologous glycosyl hydrolases is greater than that of $xlnB_p$

In order to investigate the potential of the *A. nidulans* xylanolytic system (XlnR-xln_p) for the production of heterologous proteins, the well-characterized promoters of the *xlnA* and *xlnB* genes ($xlnA_p$ and $xlnB_p$) were chosen [18,25-27] and the *rhaA* gene from *A. aculeatus* that encodes an extracellular α-L-rhamnosidase of industrial interest [37] was used as a reporter.

Two expression vectors (pILArhaA and pILBrhaA) comprising in-frame translational fusions of $xlnA_p$ and $xlnB_p$ (respectively) with the *A. aculeatus rhaA* gene were generated (Additional file 1: Figure S1A and B)

and used independently to transform the *A. nidulans* arginine auxotrophic strain AR4 (*argB2*, *metG1*, *biA1*). Arginine prototrophic transformants were selected and tested for α-L-rhamnosidase activity on minimal medium (MM)-xylose plates supplemented with the substrate 4-methylumbelliferyl α-L-rhamnopyranoside (MUR). A total of 18 transformants of pILArhaA and 50 of pILBrhaA were analyzed by Southern blot analysis (Additional file 1: Figure S1C and D). To minimize positional effects that could affect regulatory mechanisms controlling these promoters, transformants carrying single-copy integrations of the expression vectors at the homologous *xlnA* and *xlnB* loci were selected. Two strains (AR137 and AR138), each bearing a single copy of the $xlnA_p$::*rhaA* expression cassette integrated at the *xlnA* locus, and two strains (AR142 and AR143) carrying one copy of $xlnB_p$::*rhaA* located in the *xlnB* locus were chosen for further analyses. α-L-rhamnosidase plate assays confirmed *rhaA* induction by xylose and wild type responses to ambient pH in all (not shown).

The performance of these four recombinant strains was studied by measuring extracellular α-L-rhamnosidase activity under inducing conditions. Xylan from oat spelts (OSX) was used as the sole carbon source. Given that full induction of the *xlnA* gene requires alkaline growth conditions, whereas that of *xlnB* mainly occurs at acidic pH values [25], mycelia grown for 18 hours in fructose (0.1% w/v) were transferred to fresh media buffered to pH 8 (AR137 and AR138) or pH 5.5 (AR142 and AR143) containing 1% (w/v) OSX and incubated for 6 more days. As seen in Figure 2, extracellular
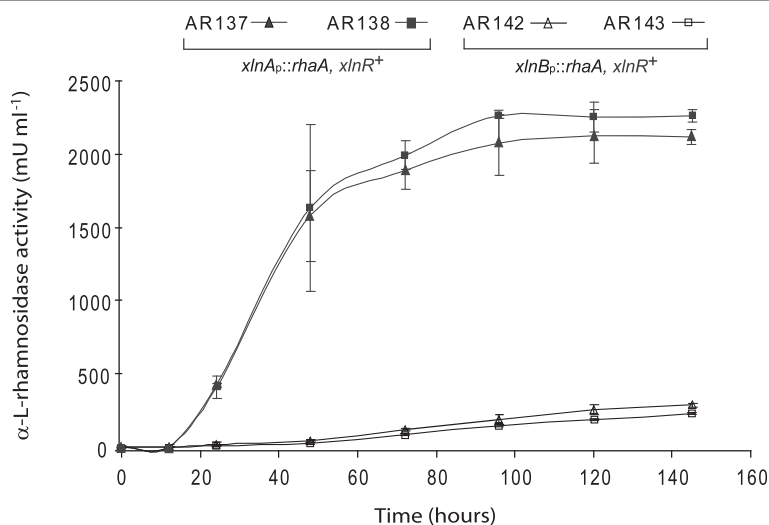
Figure 2 Intrinsic yield potential of $xlnA_p$ and $xlnB_p$ for the production of heterologous proteins. Time course analysis of extracellular α-L-rhamnosidase activities produced by $xlnA_p$::*rhaA* (AR137 and AR138) and $xlnB_p$::*rhaA* (AR142 and AR143) strains. The mycelia were grown under non-repressing, non-inducing conditions (0.1% w/v fructose) before being transferred to their specific inducing conditions (1% w/v oat spelt xylan (OSX) alkaline or acidic), and samples were collected at the time points indicated. Values (mU ml^{-1}) are presented as means ± SD. For each strain, at least two independent cultures were carried out.

α-L-rhamnosidase activity reached values about 8.5-fold higher in the $xlnA_p::rhaA$ strains compared to those carrying $xlnB_p::rhaA$ (2116 mU ml^{-1} AR137; 2262 mU ml^{-1} AR138 versus 288 mU ml^{-1} AR142; 228 mU ml^{-1} AR143). α-L-rhamnosidase production was also faster in the $xlnA_p::rhaA$ strains. The maximum activity produced by AR137 and AR138 was achieved between 72 and 96 hours after mycelial transfer to inducing conditions, whereas that of the $xlnB_p::rhaA$ strains (AR142 and AR143) was achieved after 120 hours. This concords with previous observations in which the $xlnA$ transcript was seen to accumulate to a greater level under inducing conditions than that of $xlnB$ [18,25-27]. Therefore, in a wild type genetic background under these experimental conditions the $xlnA$ gene promoter is more effective for heterologous protein production than $xlnB_p$. As expected, each strain pair (AR137 and AR138 on one hand and AR142 and AR143 on the other) behaved similarly. To discount the possibility of endogenous α-L-rhamnosidase activity in these culture conditions, strain AR70 (which carries a single copy of the empty vector pILJ16 integrated at the $argB$ locus of AR4 (R. Luque and M. Orejas unpublished work)) was grown under the same experimental conditions. No α-L-rhamnosidase activity was detected.

Bearing in mind that the pH of the culture medium could influence the stability, activity and/or interactions of secreted proteins, the *A. nidulans* $xlnA_p$ and $xlnB_p$ promoters provide an additional advantage in facilitating protein production in response to the ambient pH, thereby potentially widening the portfolio of recombinant enzymes that can be synthesized depending on their optimal pH values. Nevertheless, the poor performance of $xlnB_p$ (compared to $xlnA_p$) indicates that further improvements need to be made with this promoter before it can be used to obtain comparable yields of recombinant enzymes.

CreA-independent overexpression of $xlnR$ enhances the activity of $xlnA_p$ and $xlnB_p$ for heterologous protein production

Previous work revealed considerably increased accumulation of both the $xlnA$ and $xlnB$ transcripts in *A. nidulans* $gpdA_p::xlnR$ mutant strains compared to the wild type [18]. Xylanase activity is also greatly enhanced in this genetic background (Figure 1). These data strongly suggest that wild type expression of $xlnR$ would thus constitute a bottleneck for heterologous protein production when driven by the corresponding *A. nidulans* XlnR-regulated promoters ($xlnA_p$ and $xlnB_p$). To address this, strains $xlnA_p::rhaA$, $gpdA_p::xlnR$ (AR210 and AR211) and $xlnB_p::rhaA$, $gpdA_p::xlnR$ (AR201 and AR206) were generated by means of sexual crosses (see the Methods section) and α-L-rhamnosidase activities driven from both promoters were compared between $gpdA_p::xlnR$ and $xlnR^+$ genetic backgrounds. In agreement with the positive effect

shown on xylanase activity, α-L-rhamnosidase activity was enhanced in the $gpdA_p::xlnR$ engineered strains and found to peak earlier under inducing conditions (OSX, and alkaline or acidic buffered media). The maximum levels of RhaA activity reached by AR210 and AR211 (4535 mU ml^{-1} AR210; 3920 mU ml^{-1} AR211) approximately doubled that of the $xlnR^+$ strain AR137 (2100 mU ml^{-1}; Figure 3A), and this was achieved in the period of 48 to 72 hours rather than 72 to 96 hours. Remarkably, the yields of α-L-rhamnosidase activity reached with the $xlnB_p::rhaA$, $gpdA_p::xlnR$ strains (2757 mU ml^{-1} for AR201; 2364 mU ml^{-1} for AR206) were about 10-fold greater than those for AR142 ($xlnB_p::rhaA$, $xlnR^+$; 254 mU ml^{-1}), the differences in production being greater at shorter times (19-fold after 72 hours) when 84% of the total activity had already been produced (Figure 3B). Levels of RhaA activity in relation to total extracellular protein (Figure 3C) throughout the course of the experiment were also greater in the $gpdA_p::xlnR$ mutants compared to the $xlnR^+$ strains, which is consistent with the contribution of deregulated XlnR overexpression to this enhancement. At the periods where volumetric RhaA activities peaked the average specific activities of AR210 and AR211 (14604 ± 1234 and 13755 ± 657 mU mg^{-1} at 48 and 72 hours, respectively) were about 1.6-fold greater than those of AR137 (9248 ± 1373 and 8366 ± 690 mU mg^{-1}, respectively). In addition, at 72 and 96 hours the average specific activities of AR201 and AR206 (12860 ± 1406 and 13416 ± 986 mU mg^{-1}, respectively) were about 10-fold greater than those of AR142 (1186 ± 199 and 1458 ± 218 mU mg^{-1}, respectively).

Of note is the considerable delay in attaining the maximum yield of α-L-rhamnosidase activity in the $gpdA_p::xlnR$ genetic backgrounds compared to that of the xylanase activity produced by the $gpdA_p::xlnR$ strains AR66 and AR67 which reached 75% of their maximum level just four hours after mycelial transfer to inducing conditions. This difference is probably associated with the nature of the inducing carbon sources used for each experiment: the cultures of AR66 and AR67 were performed in minimal medium with xylose (1% w/v), whereas the production of α-L-rhamnosidase was studied in the presence of OSX (1% w/v). The increased levels of extracellular α-L-rhamnosidase activity associated with the $gpdA_p::xlnR$ genetic background shows that the expression of $xlnR$ is also a limiting factor for $xlnA$ and $xlnB$ expression in the presence of xylan. That XlnR overexpression has a stronger positive effect on $xlnB_p$ than on $xlnA_p$ suggests that the XlnR binding sites on $xlnB_p$ are less responsive to the limiting amounts of this transcription factor present in the wild type genetic background. Since it has been shown that CreA-mediated repression of $xlnB$ occurs indirectly
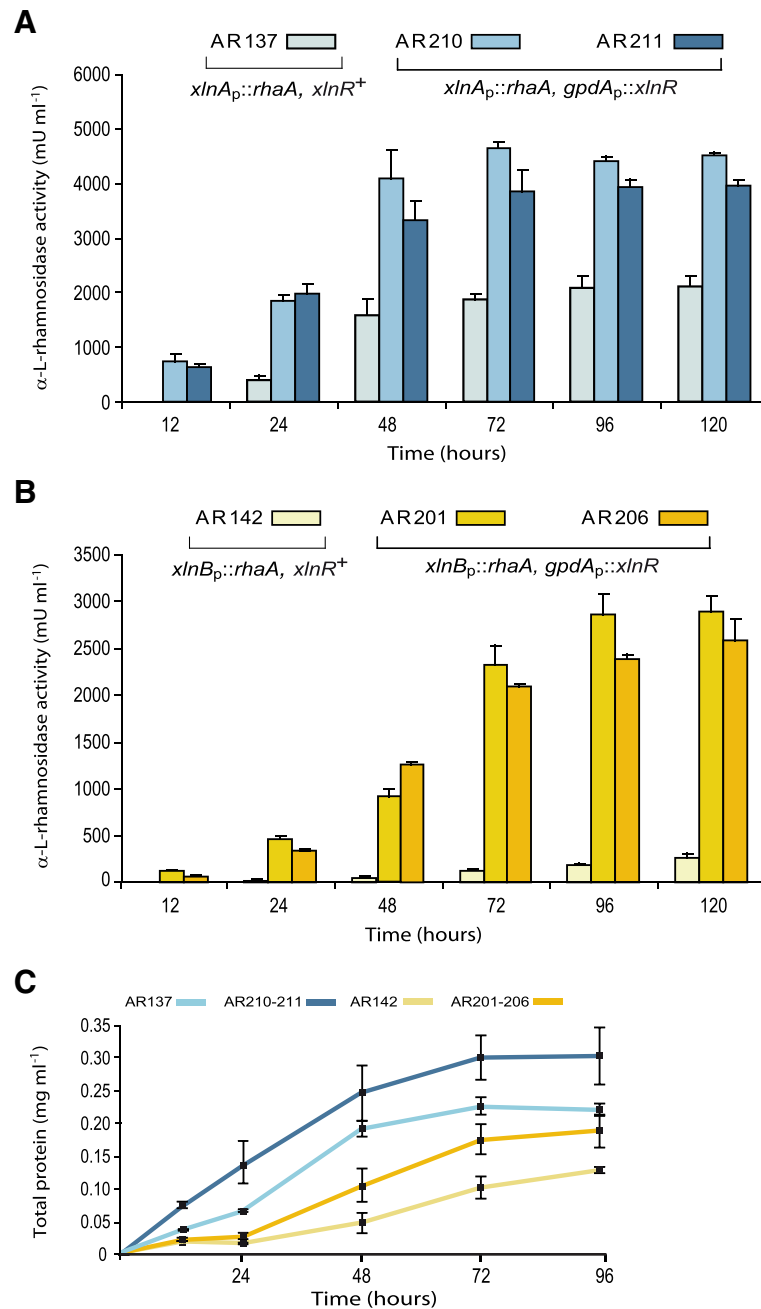
Figure 3 Effect of the *gpdA*$_p$::*xlnR* background on extracellular α-L-rhamnosidase activity. (A) *xlnA*$_p$::*rhaA, gpdA*$_p$::*xlnR* strains (AR210 and AR211) and **(B)** *xlnB*$_p$::*rhaA, gpdA*$_p$::*xlnR* strains (AR201 and AR206). AR137 and AR142 were respectively used as reference strains. AR137, AR210 and AR211 were grown under non-repressing, non-inducing conditions (0.1% w/v fructose) before being transferred to inducing conditions (1% w/v OSX, pH 8). AR142, AR201 and AR206 were grown in 0.1% w/v fructose before being transferred to inducing conditions (1% w/v OSX, pH 5.5). Samples were collected at the time indicated. Values (mU ml^{-1}) are presented as means ± SD. **(C)** Comparison of extracellular protein levels (mg ml^{-1}) accumulated by the *xlnR* engineered strains. For each strain, at least two independent cultures were carried out.

through a transcriptional regulatory cascade via *xlnR* repression [18], complete CreA-mediated derepression can be expected for *xlnB*$_p$ in *gpdA*$_p$::*xlnR* genetic backgrounds. Hence the use of Δ*creA* or *creA*^{d}30 mutant derepressed backgrounds as a means to obtain an additional positive effect on *xlnB*$_p$ activity was not considered.

Introducing the *palA1* allele into *gpdA*$_p$::*xlnR* strains expressing *rhaA* under the control of *xlnB*$_p$ further enhances α-L-rhamnosidase yields

As noted above, expression of the *A. nidulans xlnB* gene is regulated in response to ambient pH via the wide domain transcription factor PacC. Interestingly, acidity-mimicking

mutations (such as *palA1*) in the pH-sensing transduction pathway resulted in a greater elevation of the levels of *xlnB* expression compared to those reached by the wild type strain grown at acidic pH values [25]. As different mutations could act synergistically, the improvements seen in the level of RhaA activity resulting from the expression of the *xlnB*$_p$::*rhaA* cassette in *gpdA*$_p$::*xlnR* mutant strains encouraged us to explore the effect of combining this genetic background with the *palA1* mutation.

Strains of genotype *xlnB*::*rhaA*, *gpdA*$_p$::*xlnR*, *palA1* (AR207 and AR208) were obtained by means of genetic crosses (see the Methods section). Fructose-grown mycelia (0.1%) were transferred to buffered acidic media containing OSX (1%), and the kinetics of extracellular α-L-rhamnosidase activity of these strains was compared to that of AR206 (*xlnB*$_p$::*rhaA*, *gpdA*$_p$::*xlnR*, *palA*$^+$). Figure 4 shows that, under inducing conditions, the maximum level of α-L-rhamnosidase activity in the *palA1* strains (4124 mU ml^{-1} AR207; 3583 mU ml^{-1} AR208) was about 1.7-fold greater than that in AR206 (*palA*$^+$) and was also reached earlier (between 48 and 72 hours instead at 96 hours). Differences in RhaA production between *gpdA*$_p$::*xlnR*, *palA*$^+$ and *gpdA*$_p$::*xlnR*, *palA1* strains are even greater at earlier times, that is, 14-, 4.3- and 2.7-fold higher after 12, 24 and 48 hours respectively. After 48 hours, 87% of the total activity has already been produced.

The extracellular α-L-rhamnosidase titers achieved by AR207 or AR208 (approximately 1300 mU ml^{-1} 13000 mU mg^{-1}) and AR210 or AR211 (approximately 1900 mU ml^{-1}; 15000 mU mg^{-1}) after 24 hours are 2- to 3-fold greater than those reported for the endogenous

rhamnosidase(s) (approximately 588 mU ml^{-1}; 9300 mU mg^{-1}) after 24 hours of growth in L-rhamnose [38] and 10- to 15-fold higher than the maximum levels produced by a *Saccharomyces cerevisiae* strain overexpressing the same *rhaA* gene from a high copy number plasmid (approximately 130 mU mL^{-1}; 2600 mU mg^{-1}) [37]. In addition, the α-L-rhamnosidase maximum yields produced by the engineered strains AR207 and AR208 (approximately 3800 mU ml^{-1}; 15400 mU mg^{-1}), and AR210 and AR211 (approximately 4260 mU ml^{-1}; 13800 mU mg^{-1}) were greater than those reported for commercial preparations of *A. niger* (570 mU mg^{-1}) [39] and *A. aculeatus* (13000 mU mg^{-1}) [40]. It should be mentioned that these filamentous fungi have multiple α-L-rhamnosidase genes. The expression of *rhaA* in the engineered *A. nidulans* XlnR-*xln*$_p$ system also provides a more economical means of induction (xylose or xylan instead of L-rhamnose) which may be especially relevant in the case of large-scale fermentations. The possibility of using hemicellulosic hydrolysates as inducers could also reduce production costs even more. Since carbon catabolite repression (CCR) is a challenge to overcome when plant hydrolysates are used as both substrates for growth and as inducing carbon sources, CreA-independent expression of engineered *xlnB*$_p$::*rhaA*, *gpdA*$_p$::*xlnR*, *palA1*$^-$ strains could facilitate the use of less preferred carbon sources such as xylose in the presence of glucose.

These results clearly demonstrate the potential of engineering the expression (XlnR) or activity (PacC) of key regulators of the *A. nidulans* xylanolytic system to dramatically enhance the production of heterologous glycosyl hydrolases and, by extension, other proteins.
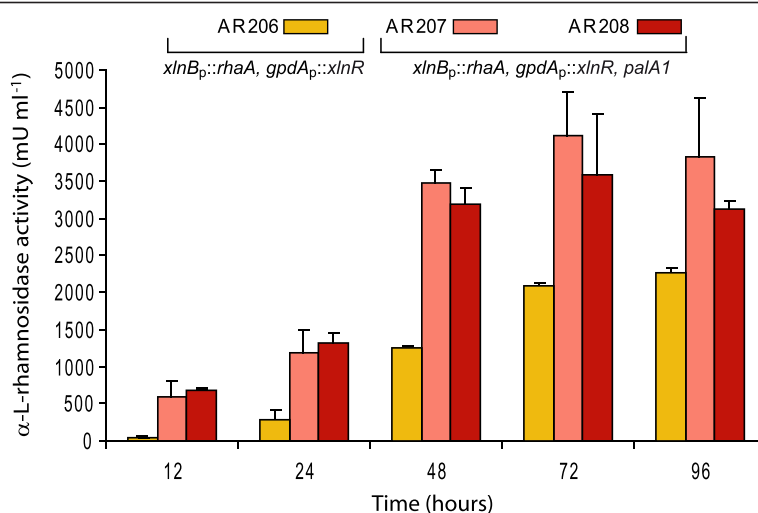
Figure 4 Effect of the *palA1* mutation on extracellular α-L-rhamnosidase activity produced by *xlnB*$_p$::*rhaA*, *gpdA*$_p$::*xlnR* strains. Bar diagram of the α-L-rhamnosidase activities in AR207 and AR208 (*xlnB*$_p$::*rhaA*, *gpdA*$_p$::*xlnR*, *palA1*) and AR206 (*xlnB*$_p$::*rhaA*, *gpdA*$_p$::*xlnR*) strains. Supernatants of the indicated strains were obtained after grown in 1% w/v OSX pH 5.5 at the time indicated. Values (mU ml^{-1}) are presented as means ± SD. For each strain, at least two independent cultures were carried out.

The activity of the *xlnB* promoter in the engineered *palA1*, *gpdA*$_p$::*xlnR*, *xlnB*$_p$::*rhaA* strains remains dependent on the presence of the inducer

Through the combination of different mutations affecting regulatory genes that control the expression of the *A. nidulans* xylanolytic system it has been possible to enhance the efficiency of the *xlnB* gene promoter. To assess whether *xlnB*$_p$ is still a regulated promoter in *gpdA*$_p$::*xlnR*, *palA1* genetic backgrounds, the levels of α-L-rhamnosidase activity produced by AR207 in the presence or absence of an inducing carbon source were analyzed. AR207 was grown in MM with 0.1% fructose for 18 hours, transferred to 1% OSX or 1% glucose media, and extracellular α-L-rhamnosidase activity was evaluated during the 72 hours after mycelium transfer. As shown in Figure 5, α-L-rhamnosidase activity was almost undetectable in cultures containing glucose as the sole carbon source, reaching at most 1% of the maximum activity produced in xylan cultures even at late culture times when glucose is depleted. This result clearly indicates that the protein expression system based on XlnR-*xlnB*$_p$ in the genetic background *gpdA*$_p$::*xlnR*, *palA1* does not obviate the requirement for the inducer and could be used to express genes the products of which could otherwise compromise fungal growth.

Conclusions

In this study a transcription factor engineering approach for strain improvement was successfully applied to the *A. nidulans* XlnR::*xln*$_p$ expression system. Specifically, the *xlnR* gene was overexpressed from the strong constitutive promoter of the *gpdA* gene (instead of from its own weak and CreA-repressed promoter), and this was combined with a second modification altering the activity of the pH master regulator PacC. Overall, improvements were manifest in the yield of endogenous xylanases and reduced production times. Engineering XlnR expression and PacC activity has also been shown to have a considerable impact on heterologous protein production. These results highlight the potential of *A. nidulans* for development as an efficient 'mycofactory' and the importance of engineering transcription factors as a key strategy in achieving overproduction.

Methods

Strains and growth conditions

Escherichia coli DH5α (*endA1*, *hsdR17*, *gyrA96*, *thi-1*, *relA1*, *supE44*, *recA1*, *ΔlacU169* (*Φ80 lacZΔM15*)) was used for cloning experiments and plasmid propagation. It was grown in Luria Broth (LB) medium (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl) with or without 100 μg ml^{-1} ampicillin. For solid media 1.5% w/v agar was added. *A. nidulans* strains used in this study are described in Table 1. These strains were grown in either minimal (MM) or complete (CM) media [41,42] containing 1% w/v of the carbon source unless specified otherwise, and supplemented with appropriate requirements. CM agar-plates were used to generate *A. nidulans* spores. For enzyme production, mycelial biomass was generated from spores inoculated in MM supplemented with 0.09% w/v ammonium tartrate as the nitrogen source, to which 0.1% w/v fructose was added as the sole carbon source, with a final pH value of 6.5. After 18 hours of growth, mycelia were harvested, washed with MM without a carbon source and transferred to the induction media. Induction media was prepared by substituting fructose with either D-xylose or OSX, both at 1% w/v. Acidic MM (buffered with 100 mM monosodium phosphate plus 100 mM NaCl) was used to optimize *xlnB*$_p$ performance, whereas alkaline MM (buffered with 100 mM disodium phosphate) was used to optimize *xlnA*$_p$ [25]. When needed, 1% w/v glucose was used. All the chemicals used were purchased
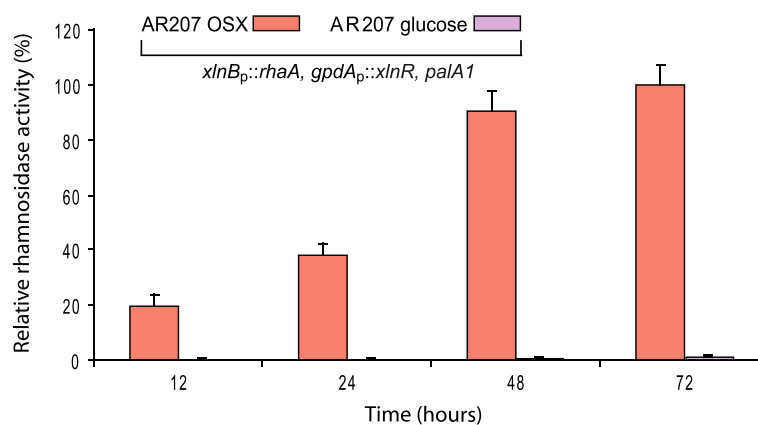
Figure 5 *xlnB*$_p$::*rhaA*, *gpdA*$_p$::*xlnR*, *palA1* strains are still highly dependent on the presence of the inducer. Comparison of the extracellular RhaA activities produced by the AR207 strain grown in 1% w/v OSX pH 5 or 1% w/v glucose pH 5. Values (mU ml^{-1}) are expressed as percentages (means ± SD) and referred to that obtained after 72 hours on OSX. For each strain, two independent cultures were carried out.

Table 1 List of *A. nidulans* strains used in this study

Strain	Relevant genotype	Source
AR4	*argB2, metG1, biA1*	MA Peñalva (CIB/CSIC) (lab strain)
AR5	*biA1*	MA Peñalva (lab strain)
AR66; AR67	[*gpdA*p::*xlnR*/pILJ16]-*argB2, metG1, biA1*	[18]
AR70	*metG1, biA1*, [pILJ16]-*argB2*	M Orejas (unpublished work)
AR83	*pabaA1, yA2, argB2, pacC^c14*	E Espeso (CIB/CSIC) (lab strain)
AR137; AR138	[*xlnA*p::*rhaA*]-*xlnA, argB2, metG1, biA1*	This study
AR142; AR143	[*xlnB*p::*rhaA*]-*xlnB, argB2, metG1, biA1*	This study
AR152	[*xlnA*p::*rhaA*]-*xlnA, pabaA1, biA1, pacC^c14*	This study
AR181	*argB2, pabaA1, yA2, palA1*	E Espeso (lab strain)
AR187	[*xlnB*p::*rhaA*]-*xlnB, pabaA1, yA2, palA1*	This study
AR201	[*xlnB*p::*rhaA*]-*xlnB*, [*gpdA*p::*xlnR*]-*argB2, biA1*	This study
AR206	[*xlnB*p::*rhaA*]-*xlnB*, [*gpdA*p::*xlnR*]-*argB2, metG1, biA1*	This study
AR207	[*xlnB*p::*rhaA*]-*xlnB*, [*gpdA*p::*xlnR*]-*argB2, pabaA1, yA2, palA1*	This study
AR208	[*xlnB*p::*rhaA*]-*xlnB*, [*gpdA*p::*xlnR*]-*argB2, metG1, pabaA1, yA2, palA1*	This study
AR210	[*xlnA*p::*rhaA*]-*xlnB*, [*gpdA*p::*xlnR*]-*argB2, pabaA1, biA1*	This study
AR211	[*xlnA*p::*rhaA*]-*xlnA*, [*gpdA*p::*xlnR*]-*argB2, biA1*	This study

from Conda-Pronadisa (Torrejón de Ardoz, Madrid, Spain), Panreac-Química (Castellar del Vallés, Barcelona, Spain) and Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated.

DNA manipulations and transformations

E. coli plasmid isolation was performed using the GenElute plasmid miniprep kit (Sigma-Aldrich, St. Louis, MO, USA). Other general DNA manipulations were performed as described [43]. Transformation of the *A. nidulans* strain AR4 was carried out as described previously [44]. Restriction enzymes, Expand High Fidelity system, and T4 DNA ligase were purchased from Roche (Roche Diagnostics, Mannheim, Germany) and used as recommended by the manufacturer. Nucleic acid hybridization for Southern analyses was made using Hybond-N⁺ membranes (GE Healthcare, Little Chalfont, UK). DNA probes were labelled using the digoxigenin (DIG) DNA Labeling Kit (Roche). DNA sequencing was carried out using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK) and the ABI PRISM 310 Genetic Analyzer (Applied Biosystems) at the DNA Sequencing Service of the University of Valencia. Chromatograms were analyzed using the program Chromas LITE 2.01(http://technelysium. com.au). Oligonucleotides used in the present study are listed in Table 2.

Construction of plasmids expressing *rhaA* of *A. aculeatus* under the control of the *xlnA* and *xlnB* gene promoters

Promoter regions of *xlnA* (1480 bp before the ATG codon; *xlnA*p) and *xlnB* (1815 bp before the ATG; *xlnB*p)

were obtained by polymerase chain reaction (PCR) from plasmids pBA7.1 and pEA8.1 respectively [45]. XAKpn and XAXhoII oligonucleotides were used to amplify *xlnA*p whereas XBKpn and XBXhoII for *xlnB*p. Primers XAKpn and XBKpn were designed with an artificial *Kpn*I site at the 5' end. XAXhoII and XBXhoII were designed with an additional sequence of 21 nucleotides that encode the 7 first amino acids of RhaA from *A. aculeatus*, and including an artificial *Bam*HI site at the 5' end, compatible with the *Xho*II site present in *rhaA*. A 381 bp fragment corresponding to the 5' end of the *rhaA* open reading frame (ORF) was also amplified by PCR from plasmid pR22 (M. Orejas, unpublished work) using primers RhaATG and Rha381, and it was cloned into the pGEM T-Easy vector (Promega, Madison, WI, USA) generating

Table 2 List of primers used in this study

Name	Sequence (5'-3')[a]
XAKpn	tcttgagctc**ggtacc**TTGACTGTACAATCATG
XAXhoII	ctca**ggatcc**AGGATGACCACAACATTGTGCTGATCCTG
XBKpn	tcttgagctc**ggtacc**AATTCGAAAAGGTAG
XBXhoII	ctca**ggatcc**AGGATGACCACAACATGTTGCCGGTTGTTG
RhaATG	AACCAGCTGTGGTCATCCTGGATC
Rha381	aaca**aagctt**CCTGGTCCATGGCC
PacC923dir	CTGTGGCTGTTGCTAGTGGTCC
PacC1650rev	CCGAGTTACATCATCCTCGCCC
GpdAXho	CCTTCAGTGGACTCGAGTACC
XlnREco	cc**gaattc**TATACAATGCGC

[a]Bold nucleotides are restriction sites added. Nucleotides in italics are those coding for the seven first amino acids of RhaA. Capital letters indicate the nucleotides complementary to the genes.

pGEM-frhaA. The primer Rha381 contains an artificial *Hind*III site to allow an additional cloning step.

$xlnA_p$ and $xlnB_p$ PCR fragments were digested with *Kpn*I and *Bam*HI and ligated together with the 381 bp fragment of *rhaA* (*Xho*II-*Hind*III) into the vector pGEM-7Zf(−) (Promega), previously digested with *Kpn*I and *Hind*III, generating plasmids pGAfrhaA and pGBfrhaA respectively. The rest of the ORF of *rhaA* (1819 bp) was obtained from pR22 through a double restriction *Nco*I-*Bam*HI and it was ligated to the *trpC* gene terminator sequence ($trpC_p$), obtained from pAN7-1 (as a *Bam*HI-*Sph*I fragment of 773 bp), into the pUCMB20 vector (Roche) previously digested with *Nco*I-*Sph*I, thus generating pUCfrhaAT.

Fragments $xlnA_p$::*rhaA*-381 bp and $xlnB_p$::*rhaA*-381 bp were isolated after a *Kpn*I-*Nco*I digestion and ligated to *rhaA*-1819pb::$trpC_t$ (*Nco*I-*Sma*I) into the *A. nidulans* expression vector pILJ16 [46] previously digested with *Kpn*I and *Sma*I to generate the expression vectors pILArhaA and pILBrhaA (Additional file 1: Figure S1A and B respectively).

Construction of different *A. nidulans* strains expressing the *rhaA* gene of *A. aculeatus*

A. nidulans AR4 (*argB2*, *metG1*, *biA1*) was used as the recipient for transformations with the *argB*+-carrying plasmids pILArhaA and pILBrhaA (Additional file 1: Figure S1A and B), to generate AR137 and AR138 (relevant genotype $xlnA_p$::*rhaA*) and AR142 and AR143 ($xlnB_p$::*rhaA*), respectively. Transformant AR137 was crossed to AR83 (*pabaA1*, *yA2*, *argB2*, $pacC^c14$) to obtain the strain AR152 (relevant genotype $xlnA_p$::*rhaA*, $pacC^c14$), whereas transformant AR142 was crossed to AR181 (*pabaA1*, *yA2*, *argB2*, *palA1*) to isolate AR187 ($xlnB_p$::*rhaA*, *palA1*). Once xln_p::*rhaA* expressing strains with mutations in the pH regulatory circuit were isolated, AR152 and AR187 were subsequently crossed to AR66 (relevant genotype $gpdA_p$::*xlnR-argB2*) [18]. Four descendants co-expressing $xlnA_p$::*rhaA* and $xlnB_p$::*rhaA* together with $gpdA_p$::*xlnR* were isolated: AR210 ($xlnA_p$::*rhaA*, $gpdA_p$::*xlnR*, *argB2*, *pabaA1*, *biA1*), AR211 ($xlnA_p$::*rhaA*, $gpdA_p$::*xlnR*, *argB2*, *biA1*), AR201 ($xlnB_p$::*rhaA*, $gpdA_p$::*xlnR*, *argB2*, *biA1*) and AR206 ($xlnB_p$::*rhaA*, $gpdA_p$::*xlnR*, *argB2*, *metG1*, *biA1*). In addition, two strains (AR207 and AR208) with the $xlnB_p$::*rhaA* cassette in $gpdA_p$::*xlnR*, *palA1* genetic backgrounds were also isolated. For yet unknown reasons, in repeated attempts, we were unable to obtain strains with the $xlnA_p$::*rhaA* expression cassette in $gpdA_p$::*xlnR*; $pacC^c14$ backgrounds.

α-L-rhamnosidase production was tested in plate assays using MM-xylose plates supplemented with 40 μM 4-methylumbelliferyl α-L-rhamnopyranoside (MUR) and 1 mM McIlvaine (citrate-phosphate) buffer pH 4.0. After incubation, hydrolysis of MUR was visualized under ultraviolet light. The $pacC^c14$ mutation was identified by sequence analysis using oligonucleotides PacC923dir and PacC1650rev. *palA1* mutations were confirmed by testing their inability to grow at alkaline pH. The rapid DNA extraction procedure for PCR analysis developed in *Aspergillus parasiticus* [47] was applied to *A. nidulans* descendants to confirm the presence of the $gpdA_p$::*xlnR* cassette (using oligonucleotides GpdAXho and XlnREco). All selected strains were also analyzed by Southern analysis to further confirm the presence of one copy of the $xlnA_p$::*rhaA*, $xlnB_p$::*rhaA* and $gpdA_p$::*xlnR* cassettes integrated into the expected loci (*xlnA*, chromosome II; *xlnB*, chromosome VIII; and *argB*, chromosome III, respectively). In this analyses, genomic DNAs from the untransformed (AR4), pILArhaA transformant and descendent strains were digested with *Bam*HI. After probing the blots with a DIG-labeled probe corresponding to 680 bp of the *xlnA* gene promoter, the AR137, AR138, AR152, AR210 and AR211 strains displayed 11,3 and a 3,9 kb bands corresponding to the insertion of the pILArhaA plasmid into *xlnA* whereas the untransformed (AR4) strain yielded a 6 kb band. A similar approach was used to analyze strains bearing the $xlnB_p$::*rhaA* cassette: their genomic DNAs were digested with *Bgl*II, and the Southern analyses showed 4.4 and 3 kb bands when the pILBrhaA plasmid was integrated into *xlnB* (strains AR142, AR143, AR187, AR201, AR206, AR207 and AR208, whereas the AR4 strain displayed a single 3.8 kb band. In this case, a $xlnB_p$ 645 bp fragment was used as a probe. The correct integration of the $gpdA_p$::*xlnR* into *argB2* was also confirmed by Southern analysis in AR201, AR206, AR207, AR208, AR210 and AR211, using an 800 bp fragment from the *argB* gene as a probe. The genomic DNA of the mentioned strains and that of the parental strain (AR66) were double digested with *Bam*HI and *Eco*RI. All strains tested showed 7.7 and 3.8 kb bands and the descendants showed one additional band of 6.7 kb (AR210 and AR211) or 9.4 Kb (AR201, AR206, AR207 and AR208) corresponding to the *argB* selection marker within the pILArhaA and pILBrhaA plasmids.

Enzyme activity assays

Endo-1,4-β-xylanase (xylanase) activity was measured using azo-xylan from birchwood (Megazyme, Warriewood, Sydney, Australia) as a substrate. The reaction mixture contained 250 μl of azo-xylan (20 mg ml^{-1}) in 50 mM succinic buffer pH 5.5 and 100 μl of culture supernatant. After 10 minutes of incubation at 40°C, the non-hydrolyzed xylan was precipitated with 1 ml of ethanol (96% v/v) followed by a centrifugation step (3000 rpm during 10 minutes).

α-L-rhamnosidase (rhamnosidase) activity was quantified using *para*-nitrophenyl α-L-rhamnopyranoside (*p*NPR) as a substrate. The assay was performed in a final volume of

250 μl, in a reaction mixture containing 1.4 mM of *p*NPR in 250 mM McIlvaine buffer pH 5.0 for 15 minutes at 50°C. Release of *p*-nitrophenol was measured spectrophotometrically at 400 nm. One unit of enzyme activity was defined as the amount of enzyme that releases 1 mmol of *p*-nitrophenol per minute in the same conditions. MM-xylose plates supplemented with 40 μM 4-methylumbelliferyl α-L-rhamnopyranoside (MUR) were used for plate assays of α-L-rhamnosidase activities. After incubation, hydrolysis of MUR was assessed under ultraviolet light.

Protein concentrations were determined using the Bradford assay and bovine serum albumin (BSA; Roche) as standard.

Additional file

Additional file 1: Figure S1. Construction and gene replacement analyses of the *xln*ₚ::*rhaA* strains. (**A** and **B**) Maps of the vectors pILArhaA and pILBrhaA used to generate the *xlnA*ₚ::*rhaA* (AR137, AR138) and *xlnB*ₚ::*rhaA* (AR142 and AR143) strains, respectively. Schematic diagrams of the *xlnA* and *xlnB* loci and gene replacement events are shown. (**C** and **D**) Southern analyses of gene targeting events. Genomic DNAs from transformants were digested with *Bam*HI (pILArhaA) or *Bgl*II (pILBrhaA), electrophoresed, blotted and hybridized with specific probe fragments of *xlnA*ₚ (**C**) or *xlnB*ₚ (**D**). As a control, genomic DNA from the untransformed AR4 strain, digested with the same enzymes, was included. The sizes of the fragments are presented on the right of each blot.

Abbreviations

BSA: Bovine serum albumin; CCR: Carbon catabolite repression; CM: Complete medium; GH: Glycosyl hydrolase; MM: Minimal medium; MUR: 4-methylumbelliferyl α-L-rhamnopyranoside; ORF: Open reading frame; OSX: Oat spelt xylan; PCR: Polymerase chain reaction; *p*NPR: *p*-nitrophenyl α-L-rhamnopyranoside; SD: standard deviation; XlnR/Xyr1: Xylan regulator.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JAT performed the experiments, analyzed the data and drafted the manuscript. MO conceived the study, designed and supervised the experiments, analyzed the data and wrote the manuscript. Both authors read and approved the final manuscript.

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