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Endo-β-*N*-acetylglucosamidases (ENGases) in the fungus *Trichoderma atroviride*: Possible involvement of the filamentous fungi-specific cytosolic ENGase in the ERAD process

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ABSTRACT

N-Glycosylation is an important post-translational modification of proteins, which mainly occurs in the endoplasmic reticulum (ER). Glycoproteins that are unable to fold properly are exported to the cytosol for degradation by a cellular system called ER-associated degradation (ERAD). Once misfolded glycoproteins are exported to the cytosol, they are subjected to deglycosylation by peptide:*N*-glycanase (PNGase) to facilitate the efficient degradation of misfolded proteins by the proteasome. Interestingly, the ortholog of PNGase in some filamentous fungi was found to be an inactive deglycosylating enzyme. On the other hand, it has been shown that in filamentous fungi genomes, usually two different fungi-specific endo-β-*N*-acetylglucosamidases (ENGases) can be found; one is predicted to be localized in the cytosol and the other to have a signal sequence, while the functional importance of these enzymes remains to be clarified. In this study the ENGases of the filamentous fungus *Trichoderma atroviride* was characterized. By heterologous expression of the ENGases Eng18A and Eng18B in *Saccharomyces cerevisiae*, it was found that both ENGases are active deglycosylating enzymes. Interestingly, only Eng18B was able to enhance the efficient degradation of the RTL protein, a PNGase-dependent ERAD substrate, implying the involvement of this enzyme in the ERAD process. These results indicate that *T. atroviride* Eng18B may deglycosylate misfolded glycoproteins, substituting the function of the cytoplasmic PNGase in the ERAD process.
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1. Introduction

N-Glycosylation occurs in the endoplasmic reticulum (ER) and is one of the most important posttranscriptional modifications of proteins in eukaryotic cells [1]. During this process, the oligosaccharyltransferase transfers an oligosaccharide (Glc3Man9GlcNAc2 in the case of *Saccharomyces cerevisiae*) from dolichol-linked donors to an Asn residue in the sequon Asn-X-Ser/Thr (where X can be any amino acid except proline) of a nascent protein [2]. *N*-glycosylation plays an important role in physicochemical/physiological properties of proteins, such as protein stability, secretion and correct folding [3]. Once *N*-glycans are linked covalently to nascent proteins, they are trimmed by glucosidases I and II allowing the monoglucosylated glycans to be bound to chaperones calnexin/calreticulin that are responsible for their correct folding [4]. Then, glucosidase II cleaves the glucose residue and the correctly folded glycoproteins are transferred to the Golgi apparatus for final modification and translocation. Glycoproteins that fail to fold properly are reglucosylated and rebound to the calnexin/calreticulin cycle [5], or accumulate in the ER causing the induction of the unfolded protein response (UPR) [6]. In glycoproteins that consistently fail to acquire the correct folding state, *N*-glycans are trimmed by ER α-mannosidase I and/or EDEMs (ER-degradation α-mannosidase-like proteins) [7]. The trimmed glycans serve as a degradation signal, so that carrier proteins are retrotranslocated from the ER to the cytosol for degradation by the proteasome. This process is often called ER-associated degradation or ERAD in short [8].

In the ERAD process, misfolded glycoproteins are subjected to deglycosylation by the peptide:*N*-glycanase (PNGase). This enzyme cleaves the amide bond between the proximal GlcNAc and the linker asparagine residue, releasing free oligosaccharides with a *N*,*N*′-diacetylchitobiose structure at their reducing termini

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(fOS-GN2) [9]. The enzyme activity of PNGases has been characterized in a variety of eukaryotes such as yeasts, animals and plants [10,11]. However, the situation seems to be different in filamentous fungi since the cytosolic PNGase PNG1 in *Neurospora crassa* is not an active enzyme [12]. Free oligosaccharides can also be generated from dolichol-linked oligosaccharides, a donor substrate for *N*-glycosylation, by the hydrolytic activity of oligosaccharyltransferase [13], or by a putative pyrophosphate releasing phosphorylated free oligosaccharides [14].

In most eukaryotes, once fOSs-GN2 are formed in the cytosol, they are trimmed by the cytoplasmic endo-β-*N*-acetylglucosamidase (ENGase). This enzyme is a glycosidase responsible for cleaving the glycoside bond in the *N*,*N*′-diacetylchiotobiose moiety of fOS-GN2 [15], resulting in the generation of fOSs with a single GlcNAc residue at their reducing termini (fOS-GN1). ENGases can be classified into 2 different glycoside hydrolase (GH) families, 18 and 85 according to the carbohydrate-active enzymes database (CAZy) classification [16]. In terms of ENGases from filamentous fungi, the secreted ENGase Eng18A (also known as Endo-T) from *Trichoderma reesei* has been characterized, and is shown to be able to deglycosylate the RNase B protein [17,18]. On the other hand, the GH85 Endo-M ENGase from the zygomycete *Mucor hiemalis* is shown to deglycosylate not only high mannose-type glycans but also sialylated, biantennary complex type glycans [19].

Trichoderma atroviride is a filamentous ascomycete fungus that lives as a parasite on other fungi (mycoparasitism) and contains 2 putative GH18 ENGase genes, *Eng18A* and *Eng18B*. Eng18A is the ortholog to the secreted Eng18A in *T. reesei* and it is putatively secreted due to the presence of an N-terminal signal peptide, while the Eng18B is predicted to be localized in the cytosol [20]. Deletion of the *Eng18B* gene has a severe impact on the phenotype of *T. atroviride*. Interestingly, deletion of the *gh18-10* gene, encoding the *Eng18B* ortholog in *N. crassa*, resulted in similar phenotypes [21] suggesting an important and conserved role of this enzyme in fungal physiology.

In the present study, we characterized the enzymatic function of Eng18A and Eng18B found in *T. atroviride* by heterologous expression in *S. cerevisiae*, and by their ability to disrupt the RTA-transmembrane-Leu2 (RTL) protein, a model ERAD substrate known to be degraded in a PNGase-dependent manner [11,22]. Our data showed that both Eng18A and Eng18B are active deglycosylating ENGases, whereas only Eng18B was able to enhance the efficiency of RTL degradation, suggesting that this enzyme is indeed involved in the ERAD process. These results imply that filamentous ascomycetes employ different mechanisms to cope with misfolded glycoproteins as compared with yeasts and other eukaryotic cells.

2. Materials and methods

2.1. T. atroviride genome analysis

The *T. atroviride* genome sequence [23] was screened for the presence of ENGases and PNGases, and used for sequence retrieval. Presence of GH85 ENGases was investigated using the *M. hiemalis* Endo-M (GenBank acc. No. AB060586) as a query in BlastP [19] searches, while presence of PNGases was investigated using the PNG1 (GenBank acc. No. BAI53085) and NCU04643 (GenBank acc. No. XP_959186) from *N. crassa* as queries. Alignment of PNGase amino acid sequences was carried out using the Clustal W software [24].

2.2. Strains, construction of plasmids and yeast transformation

*S. cerevisiae png1*Δ cells were maintained on SC (synthetic complete) medium with appropriate dropout supplements at 30 °C, unless noted otherwise. *T. atroviride* Eng18A (GenBank acc. No. EHK47006) and Eng18B (GenBank acc. No. EHK41777) were expressed in a *S. cerevisiae png1*Δ strain (*MATa his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *png1*::*hphNT1*). The Eng18A gene was PCR amplified from *T. atroviride* DNA using the primers Eng18Afor, 5′-TGAAGTCGGCATTTGTCT-3′ and Eng18Arev: 5′-CTACGCATTCACCCACTTC-3′, as described previously [20], and cloned in a TOPO-TA vector according to manufacturer's instructions (Invitrogen, Carlsbad, CA). ENGase genes were amplified from TOPO-TA-Eng18A and TOPO-TA-Eng18B [20] plasmids using the following primers: Eng18Afor, 5′-CACCATGAAGTCGGCATTTGTCTTTCTAGC-3′, Eng18Arev: 5′ CGCATTCACCCACTTCGAGTAGTT-3′, Eng18Bfor: 5′-CACCATGCCGCCAATTCAACCGG-3′ and Eng18Brev: 5′-GTTATCTGATCCGTCTGAGTAGTAATCGA-3′. Entry clones for gateway cloning were constructed using the pENTR™-D-TOPO vector (Invitrogen, Carlsbad, CA). Kanamycin resistant *Escherichia coli* colonies carrying the appropriate clones were confirmed by sequencing. Entry clones were used for the construction of expression clones in the pYES-DEST52 gateway destination vector (URA3 2 μ origin) using the LR Clonase II reaction (Invitrogen, Carlsbad, CA). Yeast transformation was carried out as described previously [25] and cells were then spread on SC-Ura agar plates containing 2% glucose and incubated at 30 °C.

2.3. Protein extraction and Western blot analysis

Heterologous expression of Eng18A and Eng18B in yeast strains was confirmed by Western blot analysis. Yeast cells were grown in SC-Ura-His media supplemented with 2% raffinose overnight at 30 °C on rotary shaker. Since heterologous expression was driven by the *GAL1* promoter, the expression was triggered by incubation of yeast strains in SC-Ura-His supplemented with 2% galactose for 6 h at 30 °C. Protein extraction was carried out essentially as described previously [26]. In brief, approximately 10^8 yeast cells were harvested and suspended in 0.1 M NaOH followed by incubation for 10 min at room temperature. Then, cells were collected by centrifugation at 3000*g* for 1 min and pellets were re-suspended in 100 μl SDS buffer containing 62.5 mM Tris–HCl (pH 6.8), 5% β-mercaptoethanol, 2% SDS, 5% sucrose and 0.04% bromophenol blue. Samples were heated for 5 min at 95 °C and cell debris were removed by centrifugation at 12,000*g*. Samples were analyzed by 15% SDS–polyacrylamide gel and transferred to Immobilon-P polyvinylidene difluoride (PVDF) membranes (Millipore, Beverly, MA). Blots were incubated with mouse anti-V5 monoclonal antibody (Invitrogen, Carlsbad, CA) diluted 1:10,000 in 1% skimmed milk in TBS-T (25 mM Tris–HCl (pH 8.0), 137 mM NaCl and 0.05% Tween 20), washed 3 times and then incubated with goat horseradish peroxidase-linked anti-mouse IgG secondary antibody (Rockland, Gilbertsville, PA) diluted 1:10,000 in 1% skimmed milk in TBS-T. The secondary antibody was detected with ImmobilonTM Western blot chemiluminescent horseradish peroxidase substrate according to manufacturer's instructions (Millipore, Beverly, MA). For deglycosylation of proteins, 10 μl of samples were treated with 2.5 mU of Endo-H (Roche, Basel, Switzerland) in 20 μl reaction at 37 °C overnight. The membrane was also probed with anti-Dpm1 antibody (5C5, Invitrogen, 1:10,000 dilution) for loading controls.

2.4. Enzymatic assay of Trichoderma ENGases

The deglycosylation assay using *S*-alkylated RNase B [27] was carried out to examine the *in vitro* deglycosylation activity of Eng18A and Eng18B. Yeast cells expressing the Eng18A and Eng18B were grown, and cells equivalent to 10 OD_{600} units were collected. Pellets were washed twice with distilled water and suspended in 1 ml lysis buffer (10 mM Tris–HCl, 1 mM EDTA, 250 mM sucrose and 1 mM DTT) containing 1X complete™ protease inhibitor

cocktail (Roche, Basel, Switzerland) and 1 mM Pefabloc (Roche, Basel, Switzerland). Cells were disrupted with glass beads using Multi-beads Shocker (Yasui Kikai, Osaka, Japan), and the supernatant was ultra-centrifuged for 1 h at 100,000*g*. Nine microlitres of yeast extract was then mixed with 1 μl of *S*-alkylated RNase B (2 mg/ml) and incubated overnight at 30 °C in the presence of 1X complete™ protease inhibitor cocktail and 1 mM Pefabloc. For the deglycosylated positive control, 1 μl of *S*-alkylated RNase B was treated with 200 mU of PNGase F (Roche, Basel, Switzerland), including 1X complete™ protease inhibitor cocktail and 1 mM Pefabloc as well, filled with lysis buffer up to 10 μl. Furthermore, in order to confirm that any band shifting was attributed to ENGase deglycosylation activity, the Eng18A and Eng18B samples were also treated with PNGase F. The *S*-alkylated RNase B was prepared as previously described [27], except for reduction of protein that was carried out on 10 mM DTT/8 M urea and 0.1 M Tris–HCl buffer (pH 8.0). The deglycosylation pattern of RNase B was monitored by Western blot analysis as described above using rabbit anti-RNase B antibody (Rockland, Gilbertsville, PA) diluted 1:5000 and donkey horseradish peroxidase-linked anti rabbit IgG antibody (General Electric, Fairfield, CT) diluted 1:5000. *S*-Alkylated RNase B incubated with lysis buffer was used as a negative control.

2.5. RTL spotting assay

The RTL assay was performed as described previously [22]. In short, *S. cerevisiae png1Δ* strains expressing RTL and the Eng18A or Eng18B proteins from *T. atroviride* were grown overnight in liquid SC-Ura-His supplemented with 2% raffinose at 30 °C on rotary shaker. A 4-fold dilution series of each cell suspension were spotted onto SC-Ura-His-Leu + 2% galactose and to SC-Ura-His + 2% galactose and were incubated at 30 °C for 60 h. A complemented *png1ΔS. cerevisiae* strain expressing the Png1 protein (ScPng1), and a strain containing only the pRS316 empty vector were used

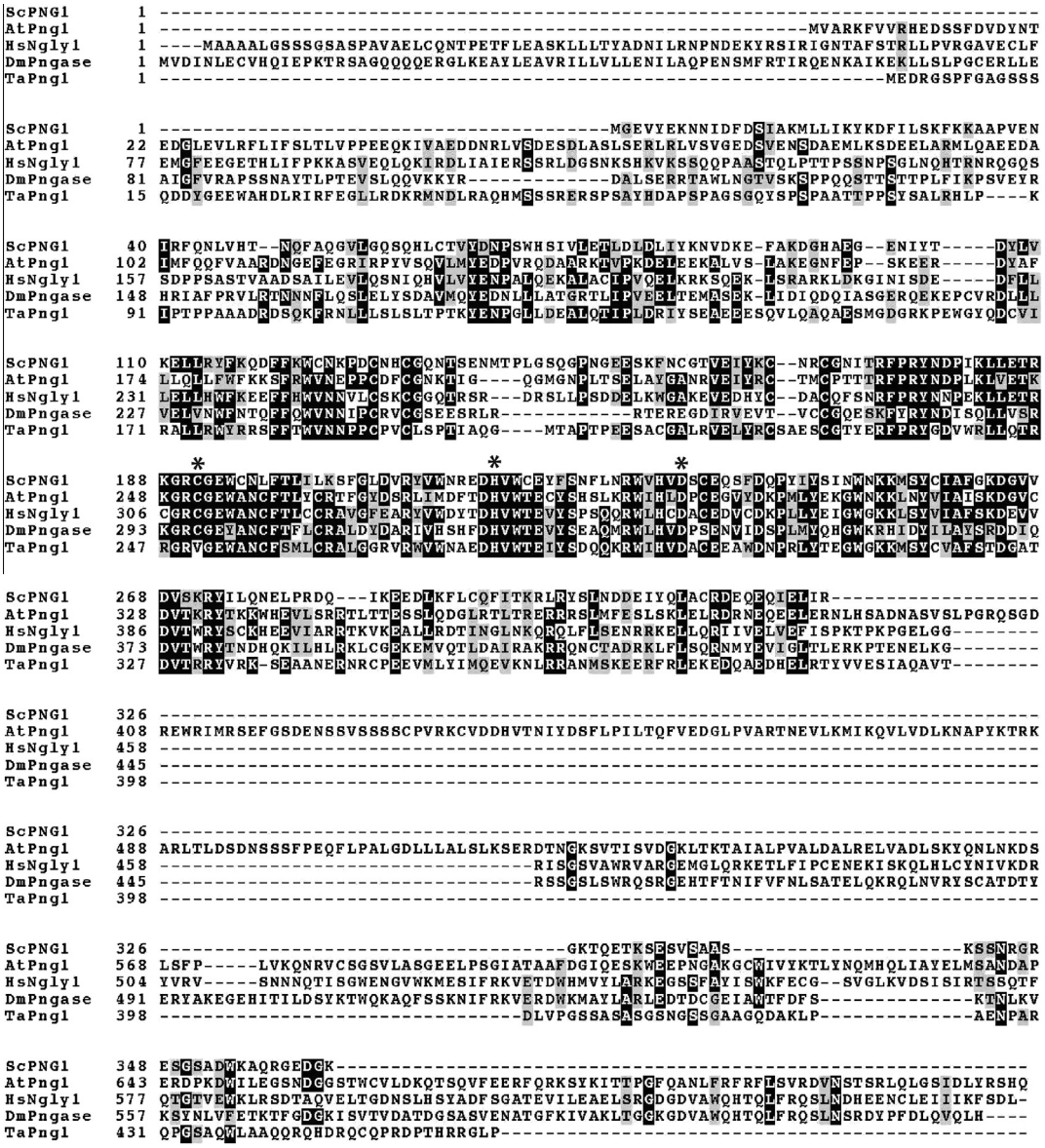

Fig. 1. Sequence alignment of the cytoplasmic PNGase orthologs. The *Trichoderma atroviride* Png1 was aligned with functional PNGases from eukaryotic organisms using Clustal W. Amino acids corresponding to the catalytic triad are marked with asterisks (Cys, His and Asp). *Abbreviations:* Sc, *Saccharomyces cerevisiae*; At, *Arabidopsis thaliana*; Hs, *Homo sapiens*; Dm, *Drosophila melanogaster*; Ta, *Trichoderma atroviride*.

as positive and negative controls, respectively. Plates were visualized using FUJIFILM LAS-3000 mini (Fujifilm Co., Tokyo, Japan).

3. Results

3.1. T. atroviride genome analysis

In this study, we aimed to characterize the deglycosylating enzymes of *T. atroviride*. BlastP analysis of the genome sequence of *T. atroviride* revealed no putative GH85 ENGases when the Endo-M ENGase from *M. hiemalis* was used as a query. A single gene (*Png1*) putatively encoding a PNGase enzyme (Protein ID. 48982) was identified when using the PNG1 PNGase from *N. crassa* [12] as a query. Additionally, a mutation was observed (Fig. 1) in the catalytic triad (Cys, His, Asp) that previously has been reported to be essential for PNGase activity [10]. Specifically, Cys250 (corresponding to Cys191 in *S. cerevisiae*) was substituted by Val, implying that the enzymatic function of Png1 in *T. atroviride* was abolished.

3.2. ENGase enzymatic assay

As no active PNGase appears to exist in *T. atroviride*, we then focused on the characterization of ENGases in this fungus. It has already been shown that this fungus has two putative ENGases, one predicted to be targeted to the ER (Eng18A) and the other to the cytosol (Eng18B) [20]. We wondered if one of these enzymes, especially the cytosolic Eng18B, can replace the deglycosylation function of the cytoplasmic PNGase in the ERAD process. To validate our hypothesis, we first aimed to test the *in vitro* activity of *T. atroviride* ENGases by expressing them in *S. cerevisiae*. To this end, *T. atroviride* Eng18A and Eng18B were heterologously expressed in the *S. cerevisiae png1*Δ strain. As Png1 is the only PNGase detected in *S. cerevisiae* [28] and this organism is devoid of ENGase activity [15], the *png1*Δ strain is expected to be devoid of any deglycosylation activity toward *N*-glycoproteins. A Western blot analysis targeting the V5 epitope-tag revealed that both Eng18A and Eng18B were successfully expressed in the yeast cells (Fig. 2A). Eng18A appeared to be *N*-glycosylated, since it was detected as multiple bands on SDS–PAGE, which were shifted to a single band after Endo-H treatment (Fig. 2B). In contrast, no difference in the molecular weight of Eng18B was observed before and after Endo-H treatment (Fig. 2C). This result is consistent with the prediction that the Eng18B is localized in the cytosol.

Having confirmed the expression of Eng18A/Eng18B in yeast, we next tried to detect the *in vitro* enzyme activity of *T. atroviride* Eng18A and Eng18B. *S*-alkylated RNase B was incubated with the cell extract of *S. cerevisiae png1*Δ cells expressing either Eng18A or Eng18B. The results clearly showed that both Eng18A and Eng18B exhibited deglycosylation activity as judged by the mobility shift of the *S*-alkylated RNase B (Fig. 3). The migration positions of the reaction products by Eng18A and Eng18B was similar to that of the positive control. Moreover, no additional shift was observed when reaction mixtures were further treated with PNGase F (Fig. 3). On the other hand, incubation of a control cell extract (i.e. without Eng18A/Eng18B expression) with *S*-alkylated RNase B showed no sign of deglycosylation reaction (Fig. 3). These results clearly suggest the *in vitro* ENGase activities of Eng18A/Eng18B when expressed in yeast.

3.3. RTL spotting assay

As described above, the cytoplasmic PNGase in *T. atroviride* was predicted to be an inactive deglycosylating enzyme. Having detected the *in vitro* ENGase activity of Eng18A/Eng18B, we next attempted to examine if these enzymes can substitute the role of the cytoplasmic PNGase in the ERAD process in *S. cerevisiae*. To this end, we took advantage of the *in vivo* RTL assay system [11,22]. RTL is a membrane protein that consists of a luminal RTA, transmembrane domain of Pdr5 and a cytoplasmic Leu2 protein [22]. The RTL assay is a method for detection of defective ERAD assessed by yeast cell growth. The logic behind this assay is that, for a strain with a *leu2* mutant allele (and thereby unable to grow in media lacking leucine), cells can be provided with more Leu2 protein (in the form of RTL) when the RTL protein remains intact. Therefore, cells bearing RTL-expression plasmids can grow in media without leucine only when RTL degradation is impaired.

To examine the effect of Eng18A/Eng18B expression on RTL degradation, Eng18A and Eng18B were expressed in *png1Δ* strains simultaneously expressing the RTL protein. As shown in Fig. 4, yeast cells expressing Eng18B were not able to grow on media lacking leucine, indicating that Eng18B was able to enhance the degradation of the RTL protein to a similar extent as the yeast Png1. In contrast, yeast strains expressing Eng18A were able to

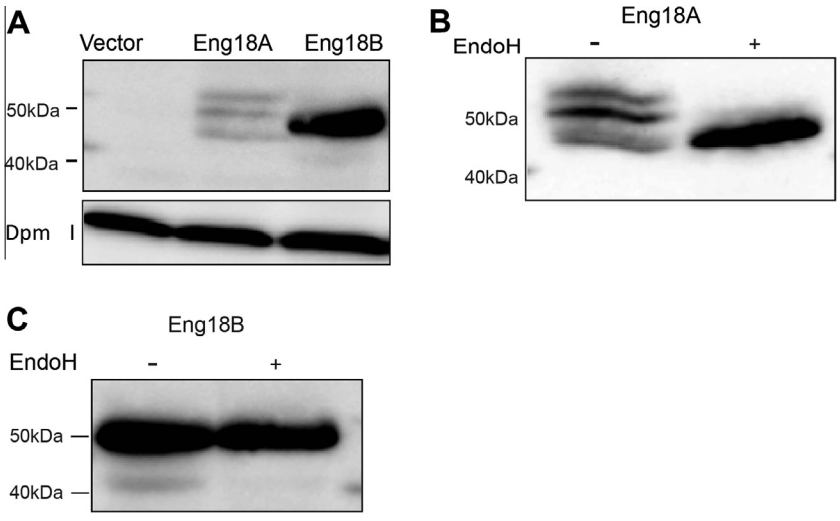

Fig. 2. Heterologous expression of *Trichoderma atroviride* ENGases in *Saccharomyces cerevisiae*. (A) *Trichoderma atroviride* Eng18A and Eng18B were heterologously expressed in *Saccharomyces cerevisiae png1*Δ cells. The Dpm1 protein was used as a loading control. (B) Treatment of Eng18A with (+) or without (−) Endo-H. (C) Treatment of Eng18B with (+) or without (−) Endo-H.

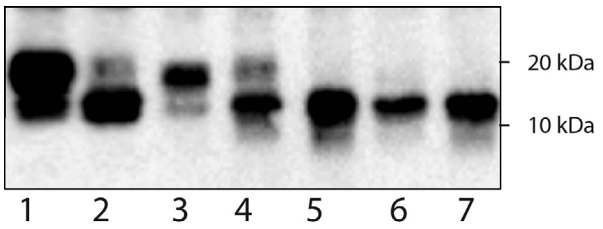

Fig. 3. *In vitro* deglycosylation activity of *Trichoderma atroviride* ENGases. *Trichoderma atroviride* Eng18A and Eng18B were expressed in *png1*Δ cells. Proteins were extracted from each strain and incubated with *S*-alkylated RNase B. Deglycosylation activity resulted in decreased molecular weight and can be detected by a shift in migration on SDS–PAGE. 1. Negative control (*S*-alkylated RNase B + lysis buffer), 2. Positive control (*S*-alkylated RNase B + PNGase F), 3. *S*-alkylated RNase B + empty vector, 4. *S*-alkylated RNase B + Eng18A, 5. *S*-alkylated RNase B + Eng18A + PNGase F, 6. *S*-alkylated RNase B + Eng18B, 7. *S*-alkylated RNase B + Eng18B + PNGase F.

grow on media lacking leucine, similar to ones expressing only the empty vector (negative control, pRS316). These results clearly indicate that Eng18A is not involved in the degradation of RTL.

4. Discussion

While the cytoplasmic PNGase was found to be evolutionally conserved throughout eukaryotes [10], it was found that the ortholog in some filamentous fungi displays no enzymatic activity due to the intrinsic mutation in the catalytic residues [12], raising questions about the mode of function of the glycoprotein ERAD process in these filamentous fungi. In this study, we have clearly shown that the fungi-specific cytosolic GH18 ENGase can substitute the function of the cytoplasmic PNGase in the ERAD process.

Filamentous ascomycetes and basidiomycetes are previously reported to contain GH18 ENGases [17,29], while the filamentous zygomycete *M. hiemalis* is reported to contain a GH85 ENGase [19]. The failure to detect GH85 ENGase genes in *T. atroviride* suggests that the GH18 Eng18A and Eng18B are the only proteins responsible for ENGase activity in this species. By heterologous expression in *S. cerevisiae* and a mobility shift assay of *S*-alkylated RNase B, we confirmed that both Eng18A and Eng18B are active ENGases. The Eng18A in *T. reesei*, ortholog to the *T. atroviride* Eng18A, is a secreted ENGase that hydrolyses high mannose-type glycans present in fungal glycoproteins, and it is responsible for the partial de-*N*-glycosylation of cellulases and other proteins in *T. reesei* [17]. Based on the orthologous status and high sequence similarity between these two GH18 ENGases, it is reasonable to assume a similar function of the Eng18A in *T. atroviride*.

Under the hypothesis that Eng18B is an intracellular ENGase involved in the ERAD process in *T. atroviride*, we employed the RTL assay to show that Eng18B, but not Eng18A, enhances the efficiency of RTL degradation. The wide distribution of Eng18B orthologs in filamentous ascomycetes [17] and the fact that the *Trichoderma* spp. Eng18B orthologs evolve under purifying selection [20], supports a conserved function of these proteins in ascomycete physiology. Deletion of this gene in *T. atroviride* and the ortholog *gh18-10* in *N. crassa* results in highly similar phenotypes: slower growth rate on carbon rich media, increased tolerance to abiotic stress and higher conidiation rates [20,21]. In addition, reduced protein secretion and, for *T. atroviride*, reduced mycoparasitic ability against *Botrytis cinerea* were also observed [20,21]. The situation is in contrast with the cases of cytosolic ENGases in other organisms; for instance, single and double deletions of GH85 ENGases do not have any impact on *Arabidopsis thaliana* morphology [30]. Similarly, no phenotypic effect was observed in a GH85 ENGase deletion strain in the yeast species *Ogataea minuta* [31]. Therefore, the functional importance of the cytosolic ENGases in fungi may be a special feature for the fungi-specific GH18 ENGases.

In summary, we showed that both Eng18A and Eng18B in *T. atroviride* are active deglycosylating enzymes. We also show that Eng18B is able to promote the degradation of the RTL, a Png1-dependent ERAD substrate. These data provide strong indications that Eng18B, and its fungal orthologs, may be involved in the degradation of misfolded glycoproteins in filamentous ascomycetes. It is tempting to speculate that filamentous fungi may have acquired the fungi-specific GH18 cytosolic ENGase to substitute the PNGase deglycosylation function.

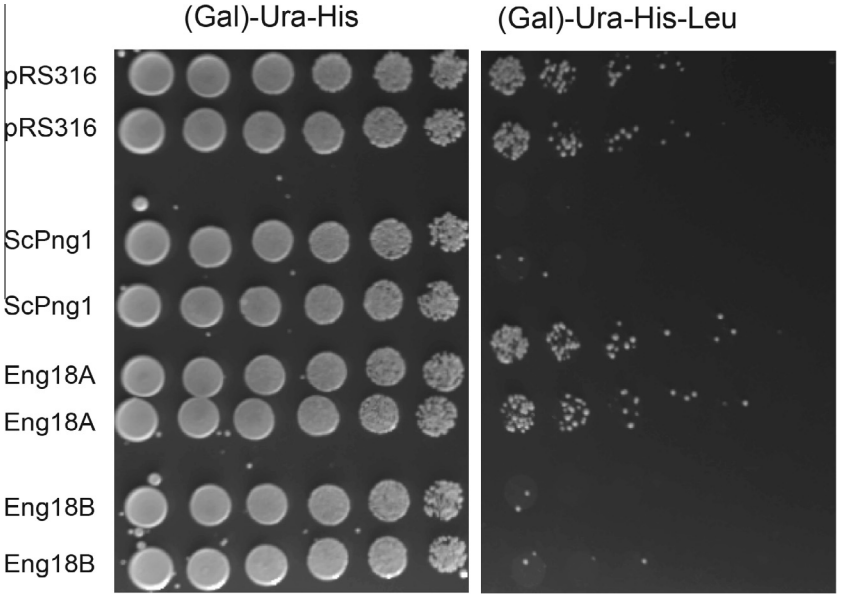

Fig. 4. Effect of *Trichoderma atroviride* ENGases on RTL stability. *Trichoderma atroviride* ENGases were coexpressed with the RTL in yeast *png1*Δ cells. Yeast strains were cultured in SC medium (-Ura-His) supplemented with 2% raffinose overnight at 30 °C. Four-fold serial dilutions of each cell suspension were spotted onto SC-Ura-His + 2% galactose plate and to SC-Ura-His-Leu + 2% galactose plate. Yeast strains expressed only the empty vector (pRS316) were used as a negative control exhibiting a defective ERAD (and thereby growth on SC-Ura-His-Leu + 2% galactose plate), while the *Png1* complemented yeast strains (ScPng1) were used as a control exhibiting efficient degradation of RTL (and thereby no growth on SC-Ura-His-Leu + 2% galactose plate).

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