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A plant peptide: *N*-glycanase orthologue facilitates glycoprotein ER-associated degradation in yeast

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ABSTRACT

Background: The cytoplasmic peptide:*N*-glycanase (PNGase) is a deglycosylating enzyme involved in the ER-associated degradation (ERAD) process, while ERAD-independent activities are also reported. Previous biochemical analyses indicated that the cytoplasmic PNGase orthologue in *Arabidopsis thaliana* (AtPNG1) can function as not only PNGase but also transglutaminase, while its *in vivo* function remained unclarified.
Methods: AtPNG1 was expressed in *Saccharomyces cerevisiae* and its *in vivo* role on PNGase-dependent ERAD pathway was examined.
Results: AtPNG1 could facilitate the ERAD through its deglycosylation activity. Moreover, a catalytic mutant of AtPNG1 (AtPNG1(C251A)) was found to significantly impair the ERAD process. This result was found to be *N*-glycan-dependent, as the AtPNG(C251A) did not affect the stability of the non-glycosylated RTAΔ (ricin A chain non-toxic mutant). Tight interaction between AtPNG1(C251A) and the RTAΔ was confirmed by co-immunoprecipitation analysis.
Conclusion: The plant PNGase facilitates ERAD through its deglycosylation activity, while the catalytic mutant of AtPNG1 impair glycoprotein ERAD by binding to *N*-glycans on the ERAD substrates.
General significance: Our studies underscore the functional importance of a plant PNGase orthologue as a deglycosylating enzyme involved in the ERAD.

1. Introduction

Peptide:*N*-glycanase (PNGase) is a deglycosylating enzyme that cleaves the β-aspartyl glycosylamine bond of *N*-linked glycoproteins, releasing intact *N*-glycans from proteins. PNGase activity was originally discovered in almond [1] and subsequently confirmed in bacteria [2]. Since then, this enzyme has been widely used as a tool to analyse the structure and functions of *N*-linked glycans on glycoproteins. The presence of PNGase activity in animals was first reported in Medaka fish (*Oryzias latipes*) [3], and later in various mammalian-derived cultured cells [4,5]. While the fish enzyme is believed to be of lysosomal origin [6], mammalian PNGase is localised in the cytosol (hence, cytoplasmic PNGase) and its optimal activity is at neutral pH [5]. A gene encoding cytoplasmic PNGase (*PNG1*) was identified in *Saccharomyces cerevisiae* [7]. The orthologous genes of *PNG1* are widely distributed throughout eukaryotes.

Endoplasmic reticulum (ER)-associated degradation (ERAD) is a component of the quality control system for newly synthesised proteins. In this system, proteins that fail to fold correctly are degraded, while functional proteins are delivered to their intended destinations through the secretory pathway [8,9]. ERAD involves the extraction of proteins from the ER to the cytosol, followed by proteasomal degradation. Cytoplasmic PNGase is involved in the efficient degradation of some ERAD substrates [10–12]. The PNGase-mediated deglycosylation during ERAD was also suggested to play an important role in antigen presentation by class I major histocompatibility complex in mammalian cells [13–16]. However, some of the PNGase orthologues were reported to be catalytically inactive, while its mutant exhibited severe phenotypic consequences [17,18], raising the possibility that, in addition to its enzyme activity, PNGase orthologues might have significant enzyme-independent roles.

From an evolutionary view, cytoplasmic PNGase is an interesting protein with a diverse structural arrangement [19,20]. The core catalytic domain of cytoplasmic PNGase is highly conserved throughout eukaryotes and, because of its homology with transglutaminase (TGase), cytoplasmic PNGase was categorised as a member of the TGase superfamily [19,21]. The plant orthologue of cytoplasmic PNGase was first identified in *Arabidopsis thaliana* (AtPNG1), based on the homology of a TGase domain [22], while the regions

Abbreviations: PNGase, peptide:*N*-glycanase; ERAD, ER-associated degradation; TG and TGase, transglutaminase; RTAΔ, ricin A chain non-toxic mutant; RTL, RTAΔ-transmembrane-Leu2; SC, synthetic complete; endo H, endoglycosidase H

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outside the TGase domain are quite unique and show no apparent homology with the primary structures of animal/fungal orthologues (Fig. 1A). This indicates that the plant cytoplasmic PNGase may have followed a distinct evolutional route from others to acquire its unique function. Interestingly, AtPNG1 was reported to possess TGase activity [23], besides PNGase activity [24], *in vitro*. However, the functional role of this plant protein *in vivo* remains poorly understood.

In this study, we utilized the assay system for PNGase-dependent ERAD in yeast, using RTAΔ (ricin A chain non-toxic mutant; [10]) or RTL (RTAΔ-transmembrane-Leu2; [12]) as substrates to examine if AtPNG1 can function as PNGase *in vivo*. Our results clearly indicated that AtPNG1, when expressed in yeast, can act as a deglycosylating enzyme and facilitate the degradation of RTAΔ/RTL. Interestingly, its catalytic mutant, AtPNG1(C251A), was found to significantly stabilise the RTAΔ. Most importantly, these results were found to be *N*-glycan specific, since non-glycosylated RTAΔ did not require AtPNG1 for efficient degradation. Consistent with this finding, stable binding of AtPNG1(C251A) to RTAΔ was confirmed by co-immunoprecipitation experiments. Taken all results together, we demonstrated that AtPNG1 can function as a PNGase *in vivo* and facilitate the degradation of ERAD substrate in an *N*-glycan-dependent fashion.

2. Material and methods

2.1. Yeast strains and media

We used the following yeast strains: *cim5-1 png1*Δ cells (*MAT***a** *cim5-1 png1*:: *URA3 ura3-52 leu2Δ1 his3Δ200* FOA^R; [25]) and *png1*Δ cells (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 png1*Δ::*kanMX4*; [7]). Standard yeast media and genetic techniques were used [26,27].

2.2. Plasmid construction

Mutation of the catalytic Cys residue 251 to Ala was introduced to the pET28b-AtPNG1 plasmid [23] using QuikChange (Stratagene) to establish pET28b-AtPNG1(C251A). To construct pRS423$_{GPD}$-AtPNG1-FLAG and pRS423$_{GPD}$-AtPNG1(CA mutant)-FLAG, cDNA of *A. thaliana* AtPNG1 and AtPNG1(C251A) were amplified from pET28b-AtPNG1 and pET28b-AtPNG1(C251A), respectively, using the following primers, 5′-CACCA-TGGGAGAGGTATACGAA-3′ and 5′- ATGCGGCCGCCTACTTATCGTCGTC-ATCCTTGTAATCCTGGTGACTTCTGTACAGAT-3′, in which the second primer was designed to add a C-terminal FLAG tag, and were cloned into pENTR™/D-TOPO (Invitrogen). The DNA sequences of the constructs were confirmed using BigDye ver. 3.1 and an ABI DNA sequencer (3730xl). The AtPNG1 and AtPNG1(C251A) genes in the pENTR vectors

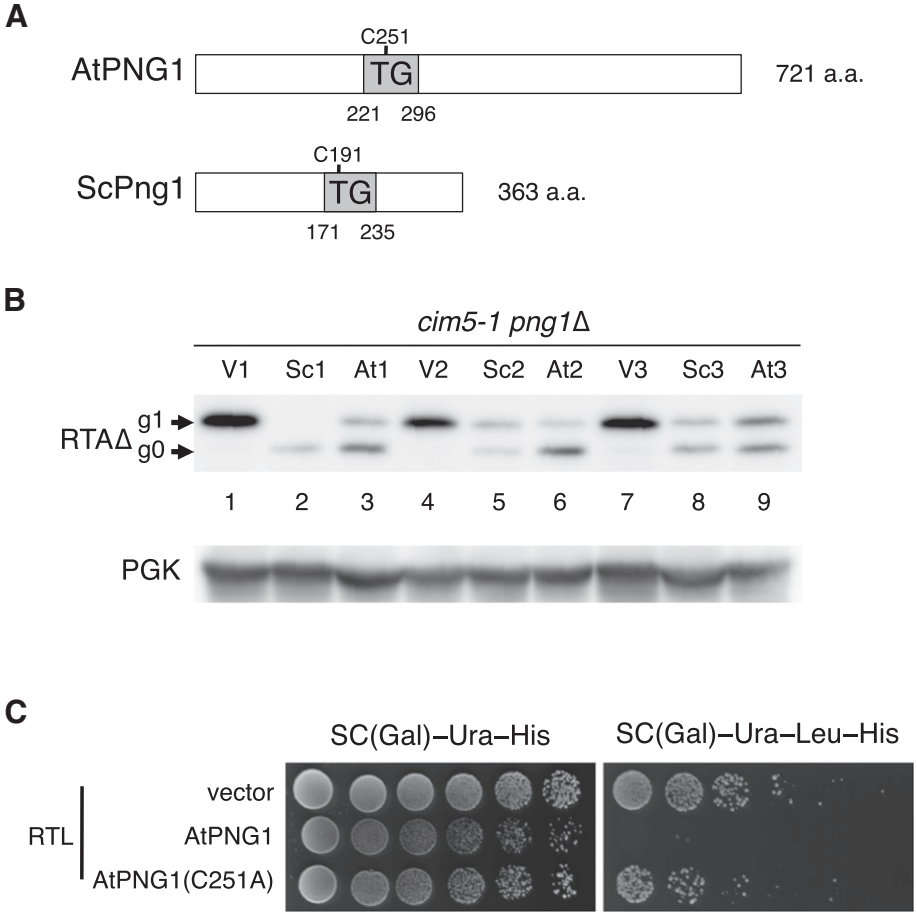

Fig. 1. Heterologous expression of AtPNG1 and deglycosylation of RTAΔ. (A) Schematic representation of AtPNG1 and ScPng1 (*S. cerevisiae* Png1). The transglutaminase (TG) domain (catalytic PNGase domain) is indicated. (B) Western blotting analysis of RTAΔ. RTAΔ was co-expressed with an empty vector (control; V), ScPng1 (Sc) or AtPNG1 (At) in *cim5-1 png1*Δ cells. Cell extracts were resolved by SDS-PAGE and RTAΔ was visualised by immunoblotting using an anti-ricin antibody. The arrows indicate RTAΔ modified with one (g1) or no (g0) glycan. The immunoblot was also probed with anti-PGK antibody as a loading control. At, *Arabidopsis thaliana*; Sc, *Saccharomyces cerevisiae*. (C) RTL assay for *png1*Δ cells expressing AtPNG1 or its catalytic mutant, AtPNG1(C251A). AtPNG1 or AtPNG1(C251A) were co-expressed with RTL in *png1*Δ cells. Cells were plated on SC-galactose medium with or without leucine. The plates were incubated for 3 days at 30 °C.