NOTE

Direct evidence of cytosolic PNGase activity in *Arabidopsis thaliana*: *in vitro* assay system for plant cPNGase activity

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ABSTRACT

Cytosolic peptide:*N*-glycanase (cPNGase), which occurs ubiquitously in eukaryotic cells, is involved in the de-*N*-glycosylation of misfolded glycoproteins in the protein quality control system. In this study, we aimed to provide direct evidence of plant cPNGase activity against a denatured glycoprotein using a crude extract prepared from a mutant line of *Arabidopsis thaliana* lacking 2 acidic PNGase genes.

Keywords: cytosolic PNGase, acidic PNGase, PNGase assay, free *N*-glycan, *Arabidopsis thaliana*

Abbreviations: aPNGase: acidic peptide:*N*-glycanase; cPNGase: cytosolic peptide:*N*-glycanase; DEAE: diethylamino ethyl; DKO: double knock out; DTT: 1,4-dithiothreitol; ERAD: endoplasmic reticulum associated degradation; ESI-MS: electrospray ionization mass spectrometry; FNG: free *N*-glycan; Gal: D-galactose; GlcNAc: *N*-acetyl-D-glucosamine; HPLC: high-performance liquid chromatography; Man: D-mannose; NeuNAc: *N*-acetyl-D-neuraminic acid; NeuNAc$_2$Gal$_2$GlcNAc$_2$Man$_3$GlcNAc$_2$: NeuNAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6(NeuNAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc; NeuNAc$_2$Gal$_2$GlcNAc$_2$Man$_3$GlcNAc$_1$: NeuNAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6(NeuNAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAc; PA-: pyridylamino; rcm-transferrin: reduced and *S*-carboxymethylated human transferrin; RP-HPLC: reversed-phase HPLC; SF-HPLC: size-fractionation HPLC; TKO: triple knock out

Cytosolic peptide:*N*-glycanase (cPNGase) is responsible for protein quality control system or ERAD system and is involved in the de-*N*-glycosylation of misfolded glycoproteins (Suzuki *et al.* 2002). Genes encoding animal cPNGases have been previously identified (Suzuki *et al.* 2002; Kato *et al.* 2007), and the relevant enzymatic properties have been characterized (Seko *et al.* 1991, Suzuki *et al.* 1993, 1994). Defects in the animal cPN-Gase gene have recently been shown to cause severe diseases (Enns *et al.* 2014; Caglayan et al. 2015; Fujihira *et al.* 2020); however, no significant phenotypic difference was observed in case of plants, in which the cPNGase genes had been knocked-out, under normal growth conditions or under salt stress (Shirai *et al.* 2019). The gene encoding *Arabidopsis thaliana* PNGase (AtPNG1, AT5G49570) had been reported earlier, and PNGase activity of the AtPNG1-protein expressed in yeast has also been reported (Diepold *et al.* 2007; Masahara-Negishi *et al.* 2012). In this report,

we use the term "cytoplasmic PNGase (cPNGase)" to emphasize the cytoplasmic function of AtPNG1 (*AT5G49570*) in contrast to the plant specific acidic PNGase functioning in the acidic environment, because the previous studies have suggested that AtPNG1 has ERAD-related PNGase activity. The direct evidence of cPNGase activity against a denatured glycoprotein in plants, however, has not been proven by any *in vitro* assay system yet. In this study, we aimed to investigate the plant cPNGase activity in an extract from a mutant line of *Arabidopsis thaliana*, in which 2 acidic PNGase (aPNGase) genes had been knocked out.

To detect the plant cPNGase activity, we constructed a mutant line of *Arabidopsis thaliana* lacking 2 aPNGase genes, namely *At3g14920* and *At5g05480,* and confirmed the complete deletion of aPNGase activity (Uemura *et al.* 2018). Since aPNGase, which ubiquitously occurs in plants and is responsible for de-*N*-glycosylation of glycopeptides rather than glycoproteins in the acidic environment, hampers the specific assay of cPNGase activity, direct evidence of *in vitro* activity of cPNGase in plant materials has never been reported till date.

As a substrate of cPNGase, human transferrin (holo) was used in this study (FUJIFILM Wako Pure Chemicals Co., Osaka, Japan). Since animal cPNGase is believed to require denatured or misfolded glycoproteins as substrates, we prepared reduced and S-carboxymethylated transferrin (rcm-transferrin) using the method of Crestfield (Crestfield, Moore and Stein 1963).

Crude enzyme solutions were prepared from rosette leaves (0.5 g) of wild-type (WT) *Arabidopsis thaliana,* and those (0.5 g) of aPNGase double knockout (aPNGase-DKO; *At3g14920* and *At5g05480*) lines (Uemura *et al.* 2018). After grinding each sample in liquid nitrogen, the homogenates were suspended in approximately 800 μL of 50 mM Tris-HCl (pH 7.5) and centrifuged at $20\,600 \times g$ for 10 min at 4 °C. After centrifugation, the supernatant was dialyzed against 1 L of 50 mM Tris-HCl (pH 7.5) for 5 h at 4 °C. The resulting dialysate was used as the crude enzyme solution. Protein concentrations were measured using the Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA).

A mixture of the substrate (rcm-transferrin; approximately 100 μg) and internal standard PA-sugar chain (NeuNAc$_2$Gal$_2$GlcNAc$_2$Man$_3$GlcNAc$_1$-PA, approximately 0.5 nmol) was treated with the crude enzyme solution (900 μg protein) from rosette leaves of *Arabidopsis thaliana* (WT and DKO lines) in 300 μL of 50 mM Tris-HCl (pH 7.5) containing 10 mM DTT at 25 °C for 18 h. After boiling the reaction mixture for 5 min, followed by centrifugation ($20\,600 \times g$, 10 min), the supernatant was lyophilized. The lyophilizates were pyridylaminated, as described previously (Natsuka and Hase 1998). After gel-filtration through a Sephadex G-25 column (1.2 × 17 cm) in 0.1 N NH$_4$OH, the FNG-containing fraction (elution volume, from 7 to 10 mL) was evaporated to dryness using a rotary evaporator and dissolved in 150 μL of distilled water. One hundred milliliters of PA-FNGs solution were mixed with 50 μL of diluted ammonia water (pH 8.5) (solvent A) and loaded into DEAE-HPLC using a TSK-GEL DEAE-5PW (7.5 × 75 mm; Tosoh, Tokyo, Japan). The solvents used were solvent A, diluted ammonia water (pH 8.5), solvent B, and 0.25 M ammonium acetate (pH 8.0). The aPNGase products, PA-FNGs carrying sialic acids, which were bound to the DEAE-column, were eluted by increasing the concentration of solvent B from 0 to 20% for 40 min at a flow rate of 0.5 mL/min. As shown in Figure 1, the fraction containing sialylated PA-FNGs (both NeuNAc$_2$Gal$_2$GlcNAc$_2$Man$_3$GlcNAc$_2$-PA and NeuNAc$_2$Gal$_2$GlcNAc$_2$Man$_3$GlcNAc$_1$-PA), indicated by a horizontal bar, was pooled, and concentrated to dryness using a rotary evaporator. The PA-FNGs were further analyzed by RP-HPLC, as described previously (Maeda *et al.* 2017).
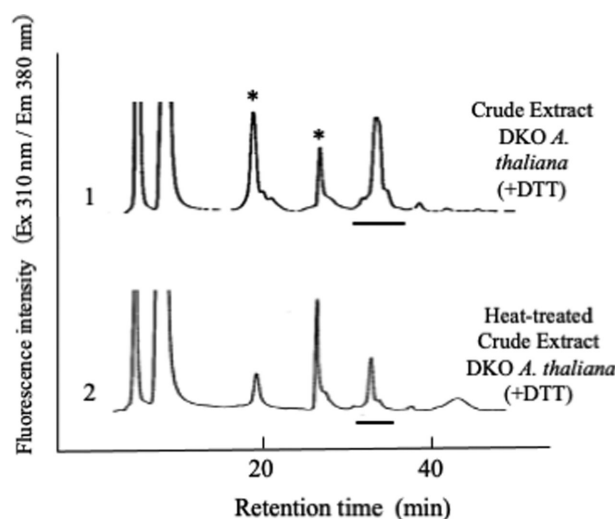

Figure 1. DEAE-HPLC profile of pyridylaminated FNGs produced in the cPNGase assay using rcm-transferrin and crude extracts of aPNGase-DKO *A. thaliana* leaves. 1, Pyridylaminated FNGs released from the reaction of rcm-transferrin with rosette leaf extract from the mutant line. 2, Pyridylaminated FNGs in the reaction mixture using heat-treated extract from *A. thaliana* leaves. The 2 asterisk marks mean that these peaks were unknown fluorescent compounds from the plant extracts but not *N*-glycans. The PA-oligosaccharide fraction was pooled as indicated by a horizontal bar.

As shown in Figure 2I-1, the cPNGase products (sialylated PA-FNGs) were observed when denatured glycoprotein (substrate) was treated with the crude extracts from rosette leaves of aPNGase-DKO line in the presence of 10 mM DTT; no product was found when the substrate was treated with the extract and incubated in the absence of DTT (Figure 2I-2) or when the crude extract was heat-treated (Figure 2I-3). Structure of the cPNGase product was confirmed by sialidase digestion (Figure 2II) and ESI-MS analysis (Figures S1 and S2). The cPNGase-product was digested by α2-3/6 sialidase (0.01 U, *Vibrio cholerae*, Sigma-Aldrich, St. Louis, MO), indicating that the FNG was released from the animal glycoprotein (human transferrin) but not endogenous glycoproteins in the mutant line of *A. thaliana*. ESI-MS analysis of the cPNGase product indicated that the PA-FNGs produced by cPNGase was the sialylated *N*-glycan from rcm-transferrin. These results clearly indicated that the plant cPNGase requires a reducing condition for its full activity, a property similar to that of animal cPNGases (Suzuki *et al.* 1993, 1994; Diepold *et al.* 2007).

Interestingly, when a mutant line of *Arabidopsis thaliana* lacking the cPNGase gene (*At5g49570,* SALK_098451) was used for the cPNGase assay, the PNGase product, NeuNAc$_2$Gal$_2$GlcNAc$_2$Man$_3$GlcNAc$_2$-PA, was unexpectedly detected as shown in Figure 3II-1, suggesting that aPNGase might have released the *N*-glycan from glycopeptides produced from rcm-transferrin by endogenous proteases. To eliminate the possibility of the aPNGase activity, therefore, we have constructed a triple-deficient line (TKO line) lacking 1 cPNGase gene and 2 aPNGase genes (Figure 3I). The *Arabidopsis* Columbia T-DNA insertion mutant lines SALK_098451 (*AT5G49570*) was obtained from the *Arabidopsis* Biological Resource Center. These homozygous lines (*At5g49570* and *At3g14920*/*At5g05480*) were crossed, and the resulting double-heterozygote was selfed, and the F1 self-plants were screened for *At5g49570*/*At3g14920*/*At5g05480* triple-knockout by examining T-DNA insertions in the 1 cPNGase gene and 2 aPNGase genes. The loci of the T-DNA insertions were confirmed by PCR using the following primers: SALK_098451 -LP, 5'-AAAAATATGGCACATGTCAGC-3'; SALK_098451 -RP,
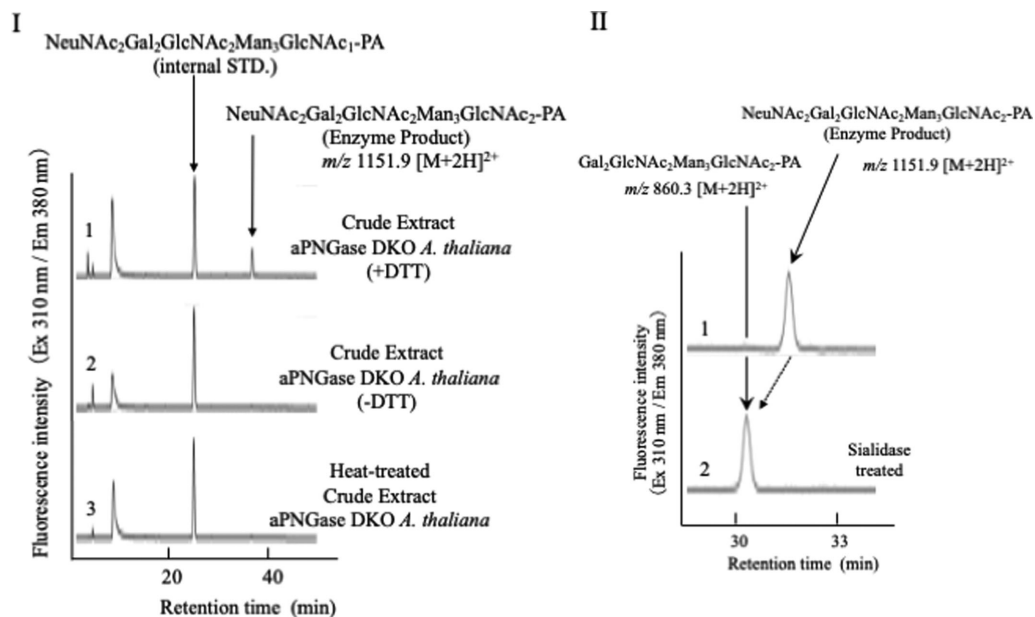
Figure 2. RP-HPLC profile of pyridylaminated FNGs obtained in Figure 1. I. RP-HPLC profiles of FNGs produced by cPNGase in the crude extracts of *A. thaliana* leaves. 1, Pyridylaminated FNGs obtained from the reaction mixture when rcm-transferrin (substrate) was treated with the crude extract of aPNGase-DKO line in the presence of DTT (10 mM). 2, Pyridylaminated FNGs obtained from the reaction mixture when rcm-transferrin (substrate) was treated with the crude extract without DTT. 3, Pyridylaminated FNGs obtained from the reaction mixture when rcm-transferrin (substrate) was treated with heat-treated crude extract and 10 mM DTT. II. α2-3/6 Sialidase digestion of FNGs obtained in I-1.1, RP-HPLC profile of PA-FNGs obtained in I-1.2, RP-HPLC profile of PA-FNGs treated with the α2-3/6 sialidase (*Vibrio cholerae*).
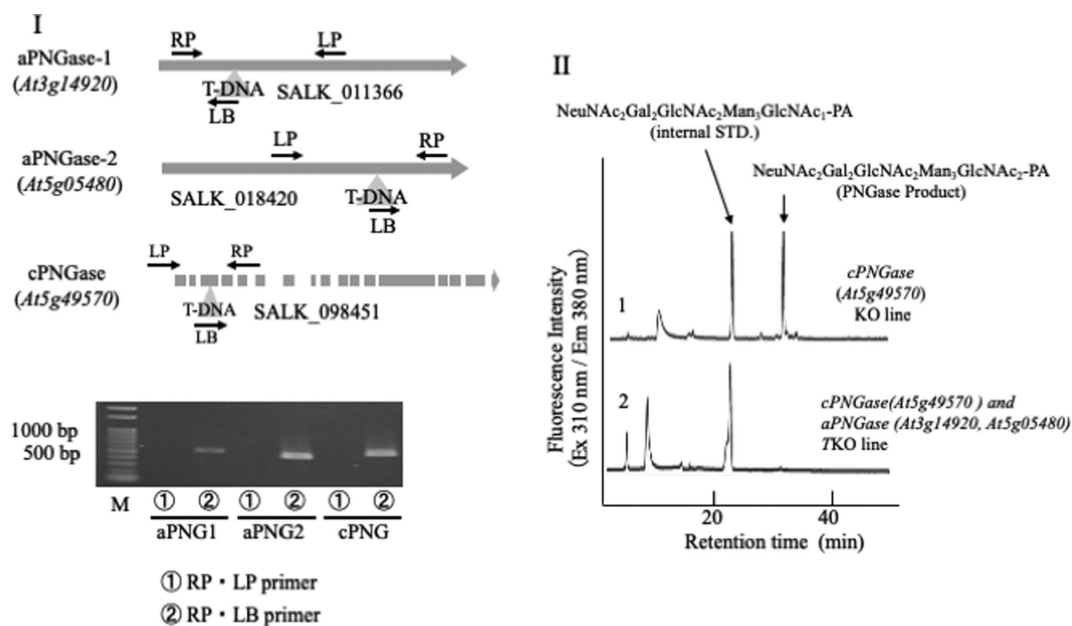

Figure 3. PCR analysis of triple knockout line (*At3g14920*, *At5g05480*, and *At5g49570*) and RP-HPLC profile of PNGase-products obtained from cPNGase single knockout line and cPNGase/aPNGase TKO line. I. PCR analysis of TKO line (*At3g14920*, *At5g05480*, and *At5g49570*). II. RP-HPLC profile of PNGase-products. 1, Pyridylaminated FNGs obtained from the reaction mixture when rcm-transferrin was treated with the crude extract of cPNGase knockout line. 2, Reaction product obtained from the reaction mixture when rcm-transferrin was treated with the crude extract of cPNGase/aPNGase knockout line.

5′-AGTCTTTCGAGCAGCATCTTG-3′; SALK_011366-LP, 5′-TTC GTGGTGAAGTTTCCATTC-3′; SALK_011366 -RP, 5′-CTTCGAGG TTCAAAAACCTCC-3′; SALK_018420 -LP, 5′-TCTGGTTCATGAT CGAGAACC-3′; SALK_018420-RP, 5′-ACTCTGTTTTGTGCTCG CTTC-3′. As shown in Figure 3II-2, the PNGase product was not detected in the extract from the cPNGase/aPNGase TKO-line, suggesting that AtPNG1 carry the cPNGase activity in plant and the product found in the cPNGase single knockout line must be an aPNGase product. However, no significant phenotypic differences between the mutant line and wild-type *A. thaliana* was observed under the normal growth conditions.

In conclusion, using the crude extract from aPNGase-DKO line of *Arabidopsis thaliana* as the enzyme and rcm-transferrin as the substrate, we confirmed the cPNGase activity by an *in vitro* assay. In this method, however, it would be difficult to accurately quantify the cPNGase activity in the crude extracts, since the endogenous protease/peptidase activities would

degrade some of the substrate glycoproteins to small glycopeptides prior to the action of cPNGase. Nevertheless, the addition of the internal standard PA-glycans allows us to compare the differences in the PNGase activities among different plant extracts. To the best of our knowledge, this is the first report showing direct evidence of cPNGase activity in plants. The structural features of FNGs occurring in the aPNGase/cPNGase TKO line and the details of the phenotype analysis will be described in the next paper.

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Supplementary material

Supplementary material is available at *Bioscience, Biotechnology, and Biochemistry* online.

Data availability

The data relevant to this article are available in Bioscience Biotechnology and Biochemistry 2018;82:1172-5, https://doi.org/10.1080/09168451.2018.1459464.

Author contribution

Y.K. shared the responsibility of writing the manuscript with S.S., R.U., M.M. H.K., R.M., and K.F. All authors were responsible for the study concept and design. S.S., R.U., and M.M. performed all the experiments. All authors contributed to the critical revision of the manuscript.

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Disclosure statement

No potential conflict of interest was reported by the authors.

References

Caglayan A, Comu S, Baranoski JF *et al*. NGLY1 mutation causes neuromotor impairment, intellectual disability, and neuropathy. *Eur J Med Genet* 2015;**58**:39-43.

Crestfield AM, Moore S, Stein WH. The preparation and enzymatic hydrolysis of reduced and S-carboxymethylated proteins. *J Biol Chem* 1963;**238**:622-7.

Diepold A, Li G, Lennarz WJ *et al*. The Arabidopsis AtPNG1 gene encodes a peptide:N-glycanase. *Plant J* 2007;**52**:94-104.

Enns G M, Shashi V, Bainbridge M *et al*. Mutations in NGLY1 cause an inherited disorder of the endoplasmic reticulum-associated degradation pathway. *Genet Med* 2014;**16**:751-8.

Fujihira H, Masahara-Negishi Y, Akimoto Y *et al*. Liver-specific deletion of Ngly1 causes abnormal nuclear morphology and lipid metabolism under food stress. *Biochim Biophys Acta Mol Basis Dis* 2020;**1866**:165588.

Kato T, Kawahara A, Ashida H *et al*. Unique peptide:N-glycanase of *Caenorhabditis elegans* has activity of protein disulphide reductase as well as of deglycosylation. *J Biochem* 2007;**142**:175-81.

Maeda M, Ebara N, Tani M *et al*. Occurrence of plant complex type free N-glycans carrying a single GlcNAc residue at the reducing end in a fresh-water plant, *Egeria densa*: a possible mechanism for biosynthesis of free N-glycans in plants. *Glycoconj J* 2017;**34**:229-40.

Masahara-Negishi Y, Hosomi A, Della Mea M *et al*. A plant peptide: N-glycanase orthologue facilitates glycoprotein ER-associated degradation in yeast. *Biochim Biophys Acta* 2012;**1820**:1457-62.

Natsuka S, Hase S. Analysis of N- and O-glycans by pyridylamination. *Methods Mol Biol* 1998;**76**:101-13.

Seko A, Kitajima K, Inoue Y *et al*. Peptide:N-glycosidase activity found in the early embryos of *Oryzias latipes* (Medaka fish). The first demonstration of the occurrence of peptide:N-glycosidase in animal cells and its implication for the presence of a de-N-glycosylation system in living organisms. *J Biol Chem* 1991;**266**:22110-4.

Shirai S, Uemura R, Maeda M *et al*. Structural features of FNGs and salt sensitivity of ENGase and cPNGase/ENGase knocked-out *Arabidopsis thaliana*. *Glycoconj J* 2019;**36**:365.

Suzuki T, Park H, Lennarz WJ. Cytoplasmic peptide: N-glycanase (PNGase) in eukaryotic cells: occurrence, primary structure, and potential functions. *FASEB J* 2002;**16**:635-41.

Suzuki T, Seko A, Kitajima K *et al*. Identification of peptide:N-glycanase activity in mammalian-derived cultured cells. *Biochem Biophys Res Commun* 1993;**194**:1124-30.

Suzuki T, Seko A, Kitajima K *et al*. Purification and enzymatic properties of peptide:N-glycanase from C3H mouse-derived L-929 fibroblast cells. Possible widespread occurrence of post-translational remodification of proteins by N-deglycosylation. *J Biol Chem* 1994;**69**:17611-8.

Uemura R, Ogura M, Matsumaru C *et al*. Novel assay system for acidic peptide: N-glycanase (aPNGase) activity in crude plant extract. *Biosci Biotechnol Biochem* 2018;**82**:1172-5.