Running title: PNGase activity in crude plant extract

Novel Assay System for Acidic Peptide:*N*-glycanase (aPNGase) Activity in Crude Plant Extract.

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Abbreviations: FNG, free *N*-glycan; Fuc, L-fucose; Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; HPLC, high-performance liquid chromatography; Man, D-mannose; NeuNAc, *N*-acetylneuraminic acid; $NeuNAc_2Gal_2GlcNAc_2Man_3GlcNAc_2$, NeuNAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6(NeuNAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc; $NeuNAc_2Gal_2GlcNAc_2Man_3GlcNAc_1$, NeuNAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6(NeuNAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAc; PA-, pyridylamino; PNGase, peptide:*N*-glycanase; PNGase-A, aPNGase from almond seed; PNGase-Le, aPNGase from tomato (*Solanum lycopersium* L.*)*; PTC, plant complex type; RCA120, *Ricinus communis* agglutinin (120 kDa); RP-HPLC, reversed-phase HPLC; SF-HPLC, size-fractionation HPLC; Xyl, D-xylose

Abstract

Acidic peptide:*N*-glycanase (aPNGase) plays a pivotal role in plant glycoprotein turnover. For the construction of aPNGase-knockout or -overexpressing plants, a new method to detect the activity in crude plant extracts is required because endogenous peptidases present in the extract hamper enzyme assays using fluorescence-labeled *N*-glycopeptides as a substrate. In this study, we developed a new method for measuring aPNGase activity in crude extracts from plant materials.

Keywords: acidic PNGase, free *N*-glycans, transgenic plant, enzyme assay, affinity chromatography

Peptide:*N*-glycanase (PNGase, peptide-N^4-(*N*-acetyl-β-D-glucosaminyl)asparagine amidase; EC 3.5.1.52) is an enzyme that hydrolyzes the β-aspartyl-glycosylamine bond of *N*-linked glycopeptides, and is involved in the degradation of misfolded or function-lost glycoproteins. It is widely distributed in plants, animals, and microorganisms.[1–4] PNGases are classified into two types based on their optimum pH: neutral or cytosolic PNGase (cPNGase) and acidic PNGase (aPNGase). cPNGase is found ubiquitously in eukaryotic cells and is believed to be involved in the protein quality control system, while aPNGase, found mainly in plants, is involved in the release of *N*-glycan units from various glycopeptides produced in the degradation process of function-lost or aged glycoproteins.

As for the physiological function(s) of free *N*-glycans (FNGs) produced by aPNGase, it has been postulated that tomato fruit ripening and hypocotyl elongation may be stimulated by treatment with high-mannose-type and/or plant complex-type FNGs.[5,6] However, biochemical or molecular biological proof of these hypothetical functions has not been reported to date. To clarify the biological function(s) of FNGs produced by aPNGase in fruit ripening and hypocotyl elongation, the construction of transgenic aPNGase-knockout and -overexpression lines is prerequisite. In the construction of aPNGase-knockout plants, evaluation of PNGase activity in

crude plant extracts is imperative; however, endopeptidases present in the extract hamper conventional enzyme assays that use fluorescently or PICT-labeled *N*-glycopeptides as a substrate. As shown in Supplemental Fig. 1-II, when purified aPNGase from almond seed (PNGase-A) was used to release sialylated animal-type *N*-glycan(s), the deglycosylated peptide was successfully detected as one of the products. However, when crude plant extract was used as an enzyme source, many peptide fragments that were produced by the contaminative endopeptidase(s) were detected, but the deglycosylated peptide (K-V-A-D-K-T, in which glycosylated Asn was converted to Asp) was not (Supplemental Fig. 1-III). To address this issue, in this study, we developed a novel enzyme assay to analyze PNGase activity in crude extracts prepared from wild-type or genetically modified plants. In the newly developed method, the deglycosylated peptide is not the target for detection of the enzyme activity, but FNGs released from the substrate glycopeptide by the aPNGase activity are detected as fluorescence-labeled products.

As a substrate, we used a sialylated animal complex-type *N*-glycopeptide, since it has been reported that plant aPNGase is active towards sialylated *N*-glycans in addition to plant complex-type and high-mannose-type *N*-glycans.[7] The disialylated *N*-glycopeptide, K-V-A-N(CHO)-K-T, in which N is glycosylated with $NeuNAc_2Gal_2GlcNAc_2Man_3GlcNAc_2$ ($NeuNAc_1Gal_2GlcNAc_2Man_3GlcNAc_2$ was also found as a minor component), was purified from hen's egg yolk by the method of Seko *et al*.[8] The glycopeptide was dansylated by a previously described method.[9] Pyridylaminated internal standard *N*-glycan, $NeuNAc_2Gal_2GlcNAc_2Man_3GlcNAc_1$-PA, was prepared from the substrate glycopeptide by prolonged hydrazinolysis (100°C for 48 h), and the structure was checked by mass spectrometry. RCA120, which recognizes the β1-4 galactosylated *N*-glycans lacking in plant glycoproteins, was purified from *Ricinus communis* seeds in 1985 as described in our previous paper,[10] and had been stored as ammonium sulfate precipitate at 4°C. RCA120 (~30 mg) was coupled to CNBr-activated Sepharose 4B (10 mL) by the method of Axén *et al*.[11]

Rosette leaves of *Arabidopsis thaliana* (0.5 g) or mature green tomato fruits (0.5 g) were homogenized in 1.5 mL of 50 mM ammonium acetate buffer, pH 4.0. After centrifugation at 12,000 ×*g* for 15 min, the supernatant was dialyzed against the same buffer (1 L) at 4°C

overnight, and the dialysate was used as crude enzyme solution. A mixture of the substrate glycopeptide (~50 μg) and the internal standard PA-sugar chain (~5 nmol) was treated with the crude enzyme solution (~120 μg protein) in 550 μL of 50 mM ammonium acetate buffer (pH 4.0) at 37°C for 1 h. After boiling the reaction mixture for 3 min and centrifugation, the supernatant was lyophilized. The lyophilyzate was pyridylaminated as described in the previous paper.[12] Significant changes in the pyridylamination efficiency of FNGs produced by aPNGase were not observed in comparison with the pyridylamination procedure that we have used for structural analyses of *N*-glycans. After filtration through a Sephadex G-25 column (1.5 × 16 cm), PA-sugar chains were treated with α-sialidase (*Vibrio cholerae*, 40 mU) in 0.1 M Na-acetate buffer (pH 5.0) at 37°C overnight. After boiling the reaction mixture for 3 min and centrifugation (12,000 ×*g*, 5 min), the supernatant was mixed with 500 μL of 0.1 M Tris-HCl (pH 8.0), and applied onto an RCA120 Sepharose 4B column (1.5 × 5 cm) equilibrated with 50 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl. The bound PA-sugar chains were eluted with the same buffer containing 0.2 M lactose and desalted using a Sephadex G-25 column (2.6 × 32 cm) with 0.1 N NH_4OH. The RCA120 bound PA-sugar chains were analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) using a Cosmosil 5C18-AR column (6.0 × 250 mm), as described in our previous paper.[13] This procedure is outlined in Fig. 1. Fig.1

We generated an *A. thaliana* aPNGase-knockout line; details of the construction of the transgenic plant, phenotypic descriptions, and structural features of FNGs generated in the transgenic plant will be described elsewhere. In this short communication, we report that the aPNGase activities in the leaves of *A. thaliana* and tomato fruits can be detected using this new method. As shown in Fig. 2, in this new method, the contaminative endogenous peptidase(s) did not hamper the aPNGase assay and the aPNGase products were detected as fluorescence-labeled FNGs only when the substrate was treated with crude extracts prepared from fruits of Micro-Tom (Fig. 2-I) and leaves of wild-type *A. thaliana* (Fig. 2-II). As mentioned above, we constructed a transgenic *A. thaliana* line, in which two aPNGase genes (*At3g14920* and *At5g05480*) were knocked out (Supplemental Fig. 2). The *Arabidopsis* Columbia T-DNA insertion mutant lines SALK_011366 (*At3g14920*) and SALK_018420 (*At5g05480*) were obtained from the *Arabidopsis* Biological Resource Center. These homozygous lines were Fig. 2

crossed and the resulting double-heterozygote was selfed, and the F1 self plants were screened for *At3g14920/At5g05480* double-knockout by examining T-DNA insertions in the two aPNGase genes. The loci of the T-DNA insertions were confirmed by PCR using the following primers: SALK_011366-LP, 5′-TTCGTGGTGAAGTTTCCATTC-3′; SALK_011366-RP, 5′-CTTCGAGGTTCAAAAACCTCC-3′; SAIL_018420-LP, 5′-TCTGGTTCATGATCGAGAACC-3′; SAIL_018420-RP, 5′ACTCTGTTTTGTGCTCGCTTC-3′; LBb1, 5′-GCGTGGACCGCTTGCTGCAACT-3′.

As shown in Fig. 2-II-2, the aPNGase products were not detected in the extract from the aPNGase double knock-out line, while significant amounts of two aPNGase products ($Gal_2GlcNAc_2Man_3GlcNAc_2$-PA and $NeuNAc_1Gal_2GlcNAc_2Man_3GlcNAc_2$-PA) were detected in wild-type plant extracts (Fig. 2-II-1). The structures of these products were confirmed by ESI-MS analysis; *m/z* 860.3 $[M+2H]^{2+}$ for $Gal_2GlcNAc_2Man_3GlcNAc_2$-PA and *m/z* 1005.8 $[M+2H]^{2+}$ for $NeuNAc_1Gal_2GlcNAc_2Man_3GlcNAc_2$-PA (an incomplete disialylated product). It is worth noting that, in this study, we used sialylated animal complex-type *N*-glycopeptides as the substrate for acidic PNGase, as plants lack α-sialidase activity and the *N*-glycan structure of the substrate glycopeptides must not be modified by endogenous β-galactosidase and β-N-acetylglucosaminidase. Furthermore, we used RCA120 affinity chromatography to detect aPNGase products after desialylation by α-sialidase, because pectin fragments are present in considerable quantities in plants (especially in tomato fruits) and these acidic oligosaccharides as well as sialylated *N*-glycans may bind to an anion-exchange HPLC column and hamper the enzyme assay. Further, we analyzed the cPNGase activity using the glycopeptide substrate at neutral pH (pH 7, 1 h) but glycan products were not detected, suggesting that cPNGase does not hamper this assay method for plant aPNGase activity. To date, plant cPNGase activity toward glycopeptide substrates has not been reported, and the activity has been proved only by the indirect method.[14,15)]

In conclusion, using the novel method, we succeeded in confirming the complete deletion of aPNGase activity in the transgenic *A. thaliana*[16)] and in tomato fruits. Details on the construction and phenotypic analyses of the transgenic line will be described elsewhere.

Author contribution

Y.K. shared responsibility for the writing of the manuscript with R.U., M.O., C.M., T.A., and M.M. All authors were responsible for the study concept and design. R.U., M.O., C.M., T. A. carried it out. All authors contributed to the critical revision of the manuscript.

Acknowledgments

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192 **Legends to figures**

193

194 Fig. 1. Schematic representation of the aPNGase assay system developed in this study.

195

196 Fig. 2. aPNGase assay using crude extracts from tomato (Micro-Tom) fruits and leaves of *A.*
197 *thaliana*.
198 I. RP-HPLC of FNGs produced by aPNGase in the crude extract of Micro-Tom fruits. 1. The
199 substrate glycopeptide was treated with heat-treated crude extract; 2, the substrate glycopeptide
200 was treated with the crude extract.
201 II. RP-HPLC of FNGs produced by aPNGase in the crude extract of *A. thaliana.* 1, The
202 substrate glycopeptide was treated with crude extract from wild-type *A. thaliana*; 2, the
203 substrate glycopeptide was treated with crude extract from leaves of a double-aPNGase gene
204 knockout line. Asterisk means a minor product, mono sialylated N-glycan, from the substrate
205 N-glycopeptide.

Legends to supplemental figures

Supplemental Fig. 1. RP-HPLC of dansylated glycopeptides treated with PNGase-A and crude plant extract. 1, Dansylated glycopeptides; 2, the substrate glycopeptide was treated with PNGase-A; 3, the substrate glycopeptide was treated with crude extract from *A. thaliana.* The glycopeptides purified from egg yolk[8] were dansylated as described previously.[9] The substrate glycopeptides (~1 nmol) were treated with PNGase A (almond glycopeptidase, Seikagaku Kogyo, Japan) or crude extract prepared from *A. thaliana*, and the reaction products were analyzed on a Cosmosil 5C18 AR column (4.6 × 250 mm). The dansylated glycopeptides were eluted and detected as described previously.[9] a, elution position of the deglycosylated peptide (dansyl-K-V-A-D-K-T).

Supplemental Fig. 2. PCR Analysis of *At3g14920* (single-knockout)*, At5g05480* (single-knockout)*,* and *At3g14920*/*At5g05480* (double-knockout).

I, *At3g14920* single-knockout line. II, *At5g05480* single-knockout line. III, *At3g14920*/*At5g05480* double-knockout line.

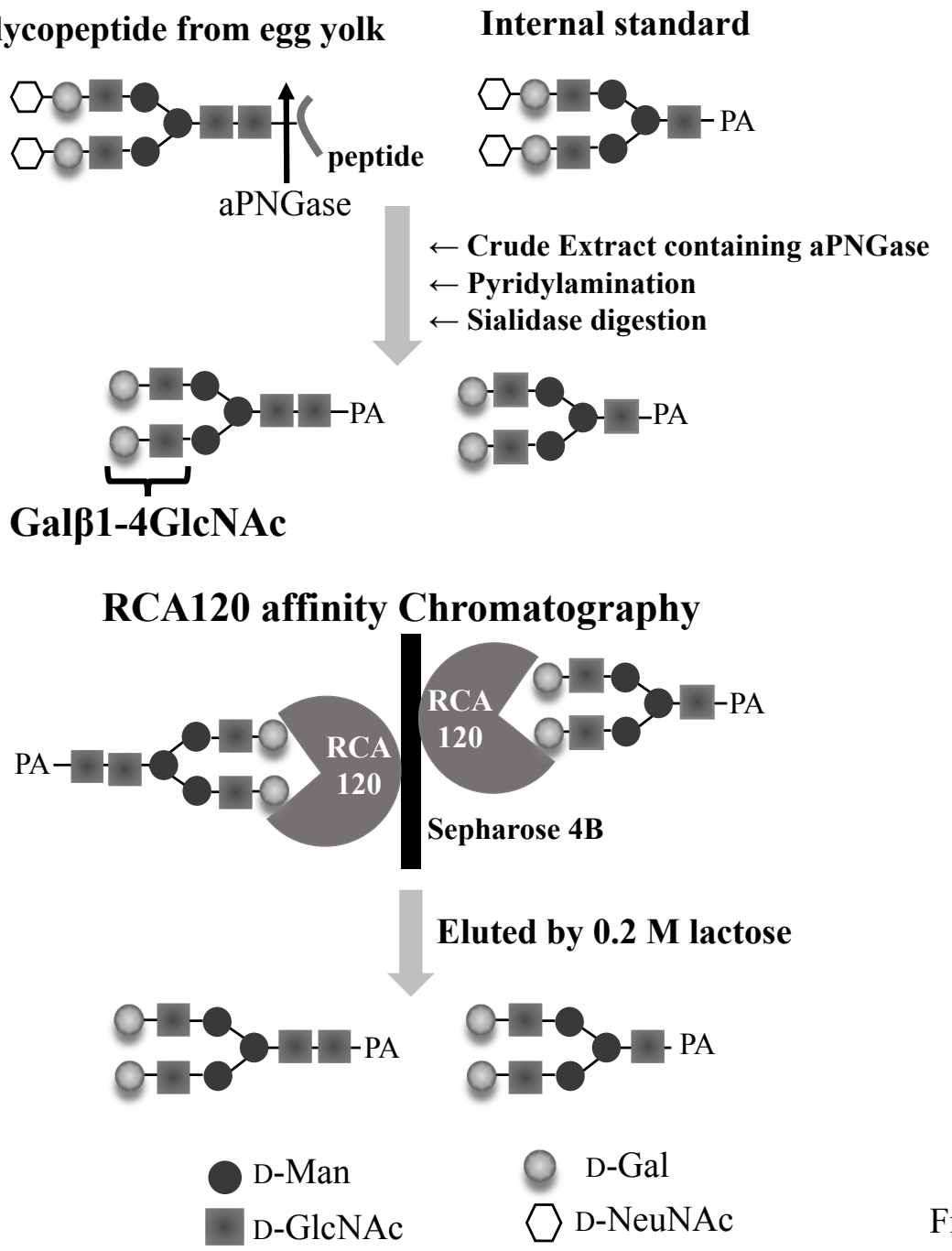


Fig. 1. Uemura, R., *et al*

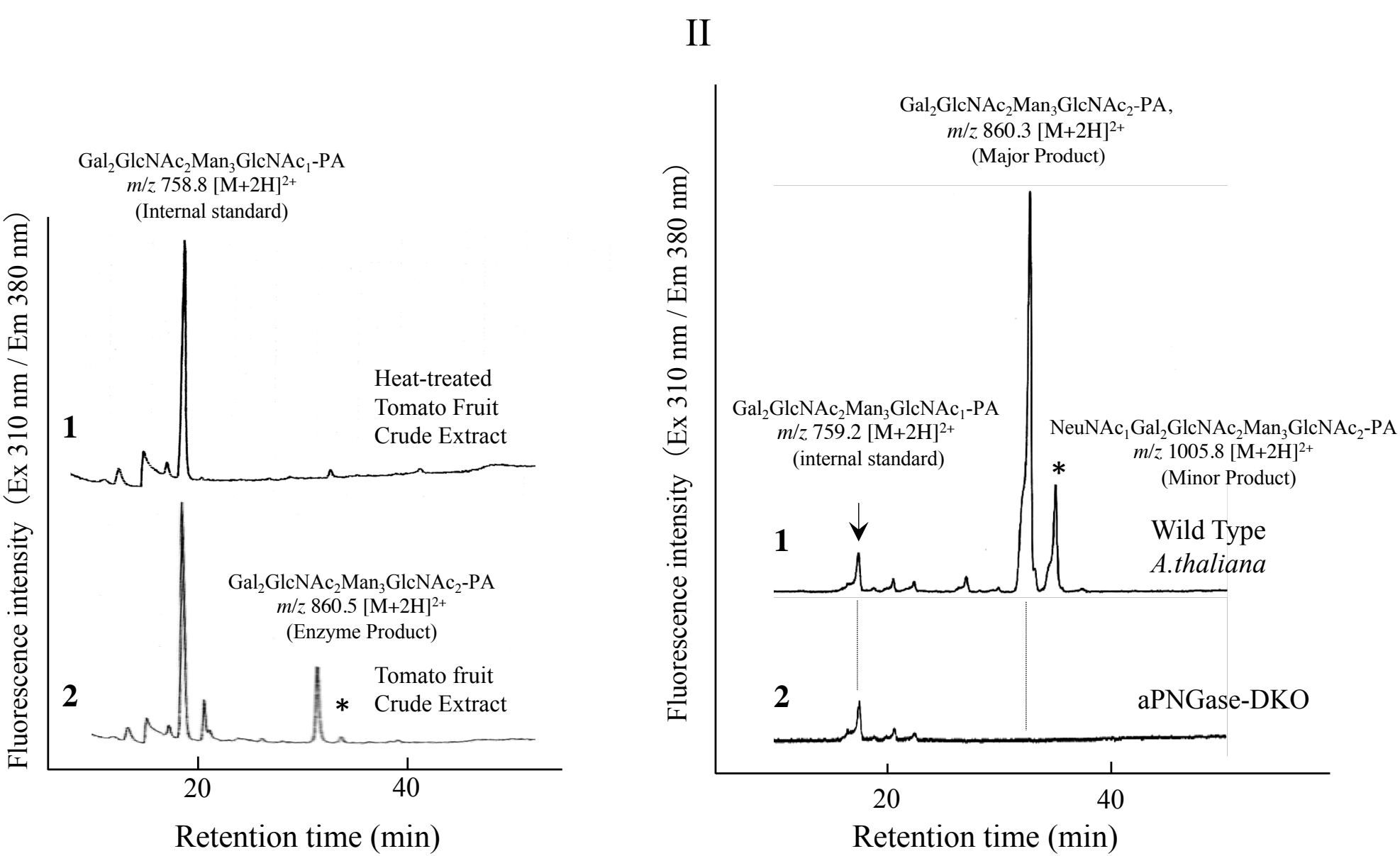
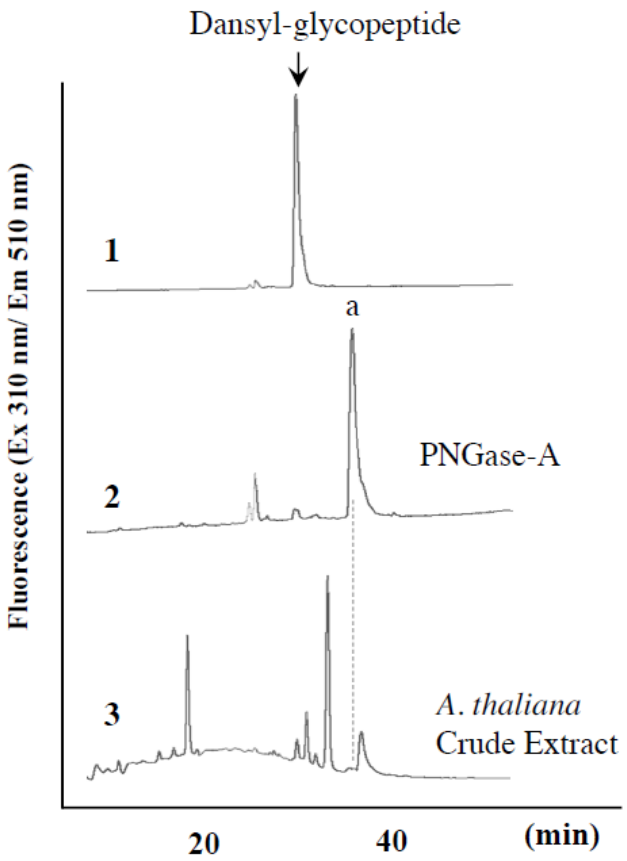


Fig. 2. Uemura, R., *et al*



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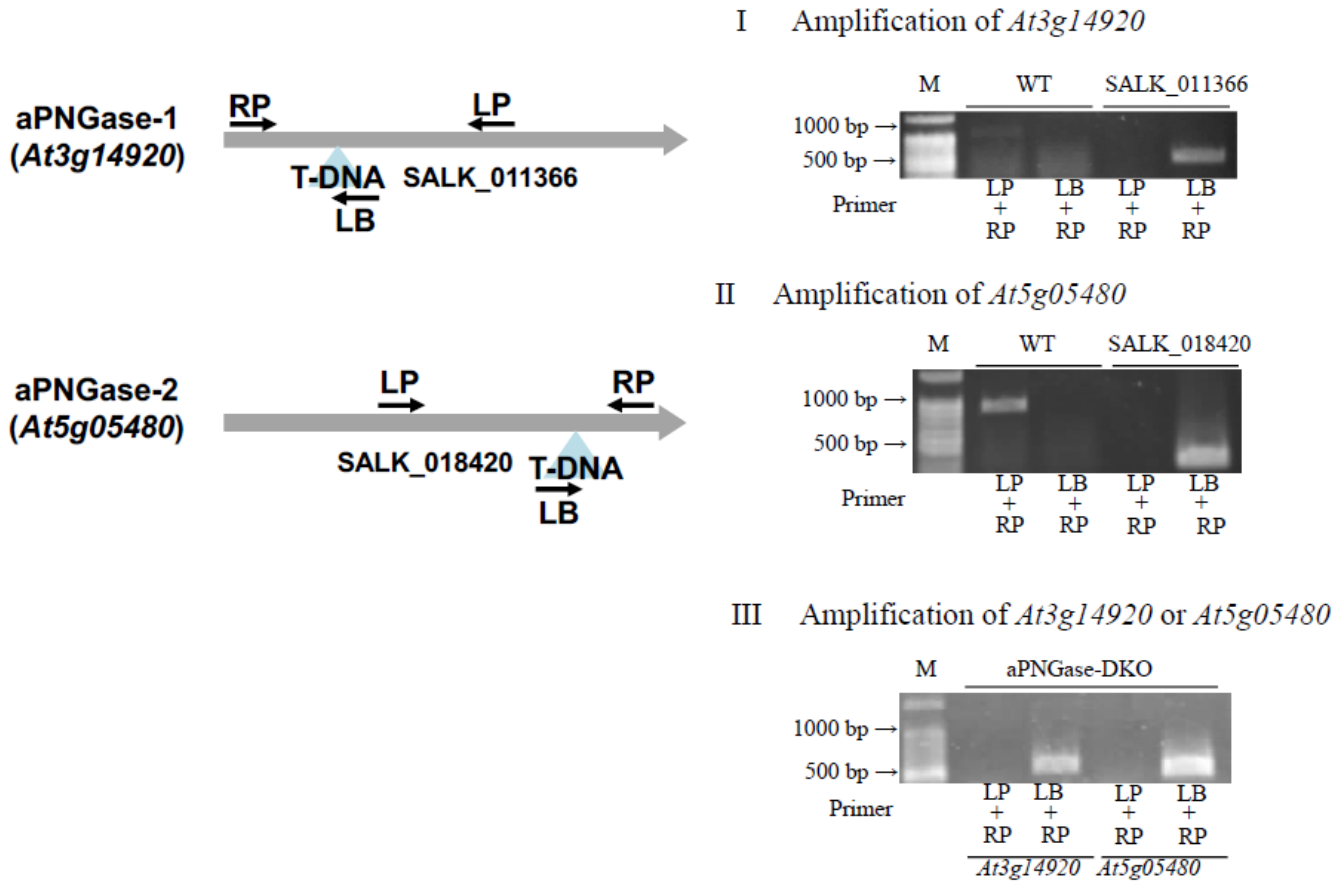

Supplemental Fig. 2. PCR Analysis of *At3g14920* (single-knockout), *At5g05480* (single-knockout), and *At3g14920*/*At5g05480* (double-knockout).

I, *At3g14920* single-knockout line. II, *At5g05480* single-knockout line. III, *At3g14920*/*At5g05480* double-knockout line.