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Molecular identification and characterization of an acidic peptide:*N*-glycanase from tomato (*Lycopersicum esculentum*) fruits*

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Plant acidic peptide:*N*-glycanase (PNGase) is one of the deglycosylation enzymes and has been considered to be involved in the catabolism of glycoproteins in plant cells. However, the tangible physiological significance involved in plant differentiation or growth is yet unclear. In this study, as a first step to elucidate the physiological role of free *N*-glycans and the de-*N*-glycosylation machinery working in developing plant cells, we have succeeded in expressing a cDNA from tomato fruits in *Pichia pastoris* and identified an acidic peptide:*N*-glycanase in the culture supernatant. The PNGase-gene-encoded protein is a single polypeptide chain of 588 amino acids with a predicted molecular mass of 65.8 kDa. The deduced amino acid sequence showed 57.9% similarity with almond PNGase A. The recombinant tomato PNGase showed optimum activity at pH 4.5 and 40°C. It did not require any metal ions for full enzymatic activity and could release the complex-type *N*-glycan from glycopeptides. Our phylogenetic analysis reveals that the plant acidic PNGase is completely different from the ubiquitous cytosolic PNGase and is involved in a different de-*N*-glycosylation mechanism associated with plant growth and development.

Keywords: Acidic peptide/*N*-glycanase/Lycopersicum esculentum/plant *N*-glycan/tomato fruit.

Abbreviations: PNGase-Le, Peptide:*N*-glycanase from *Lycopersicum esculentum*; ENGase, endo-β-*N*-acetylglucosaminidase; Endo-LE, ENGase from *Lycopersicum esculentum*; *Endo-OS*, ENGase from *Oryza sativa*; RP-HPLC, reverse-phase HPLC; rPNGase-Le, recombinant PNGase-Le; YPDS, Yeast Extract Peptone Dextrose Medium; BMGY, Buffered Glycerol-complex Medium; BMMY, Buffered Methanol-complex Medium; L-929 PNGase, PNGase from C3H mouse derived L-929 fibroblast cells.

Peptide:*N*-glycanase (PNGase or glycoamidase; peptide-N^4-(*N*-acetyl-β-D-glucosaminyl)asparagine amidase; EC 3.5.1.52), which hydrolyses the β-aspartyl-glycosylamine bond of N-linked glycoproteins/glycopeptides and releases free *N*-glycans, is a widely distributed enzyme in plants, animals, and microorganisms (*1–9*). On the basis of optimum pH values for their activities, PNGases can be classified into two types: neutral or cytosolic PNGase and acidic PNGase. The former PNGase is a ubiquitous enzyme that is recognized as a component of the machinery for endoplasmic reticulum-associated degradation (ERAD), *i.e.*, proteasomal degradation of misfolded, newly synthesized (glyco) proteins that have been exported from the ER (*9*). From the viewpoint of protein structural features, such cytosolic PNGase consists of a transglutaminase-like domain, a zinc-binding domain, and a RAD23/HR23-binding module (*9–11*), and requires a -SH group and neutral pH for optimum activity. The latter one, acidic PNGase, found ubiquitously in plants, is capable of releasing *N*-glycan units bearing of high-mannose type, plant complex type and also hybrid type from various glycopeptides but shows rather weak activity towards glycoproteins (*2*, *7*, *8*). Since the acidic plant PNGase can release the xylose/fucose-containing complex-type *N*-glycans from glycopeptides, in addition to the high-mannose-type structures, under acidic condition, it has been postulated that this type of PNGase may be involved in the turnover of glycoproteins that have lost their biological roles in acidic organelles such as the vacuole. However, their accurate localization has not been determined and their physiological role for plant development or fruit maturation remains to be elucidated.

Meanwhile, it has been reported that free *N*-glycans released from glycoproteins/glycopeptides are ubiquitous at micromolar concentrations in various plant cells during the differentiation, growth and fruit maturation stages (*12–20*), and their auxinlike activity has been postulated (*21–23*). On the basis of the structural features of such *N*-glycans found in developing seeds and hypocotyls of ripening fruits (*14–19*), it is presumed that high-mannose type free *N*-glycans having one GlcNAc residue (also known as GN1 type) at the reducing end could be produced by

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endo-β-*N*-acetylglucosaminidase (ENGase). On the other hand, the plant-complex-type free *N*-glycans with xylose/fucose residues with chitobiosyl unit (GN2 type) could be produced by PNGase. These enzyme's activities also appear to be regulated in a developmental stage-dependent manner (*5*, *6*, *24*). It has been proposed that the de-*N*-glycosylation of plant glycoproteins by PNGase or ENGase is a possible mechanism for the release of free *N*-glycans having important biological activities, and the removal of *N*-glycans could regulate the activity of proteins (*25*).

Although several acidic PNGases have been isolated from plants and microbes and characterized, most of their molecular identities as well as their physiological functions still remain unknown except for a microbial PNGase F (*1–8*). As part of a study to elucidate the role of free *N*-glycans and the significance of the de-*N*-glycosylation mechanism working at the differentiation and development stages of plant cells, the molecular cloning and gene isolation of de-*N*-glycosylation enzymes are prerequisites. We have recently succeeded in identifying and expressing the endo-β-*N*-acetylglucosaminidase genes of rice (endo-OS) and tomato (endo-LE) in *E. coli*. We have found that plant ENGases are expressed in growing tissues of the hypocotyls of rice seedlings (*26–28*). Our gene expression analysis by real-time PCR revealed that the tomato Endo-Le gene expression level did not vary significantly with the tomato fruit ripening process, suggesting that Endo-Le is ubiquitously expressed (*29*), although the amount of high-mannose-type *N*-glycans increases significantly during tomato fruit ripening. To elucidate the putative physiological function of free *N*-glycans accumulated during plant development or fruit maturation, the construction of a transgenic plant in which the PNGase gene is knocked-out, is also a prerequisite. For this purpose, in this study, we started to analyse the plant PNGase gene using tomato as a model plant. Firstly, we expressed a putative PNGase-Le (PNGase from *Lycopersicum esculentum*) gene in *E. coli* BL21 (DE3) cells using pET41b(+) expression vector but the recombinant enzyme had no PNGase activity (data not shown). Finally, we succeeded in expressing the PNGase cDNA of *Lycopersicum esculentum* (PNGase-Le) with strong PNGase activity against human transferrin glycopeptides in the Pichia system. Therefore, in this study we describe the cDNA cloning, sequencing and heterologous expression of PNGase-Le gene from mature red tomato fruits in *Pichia pastoris* and the characterization of the recombinant enzyme.

Materials and methods

Mature red tomato (*Lycopersicum esculentum* mill. Cv Momotaro) fruits were harvested from a local commercial farm. All synthetic oligonucleotide primers used in this study were obtained from Operon Biotechnologies, Tokyo, Japan. A Cosmosil C18-AR column (6 × 250 mm) was purchased from Nacalai Tesque (Kyoto, Japan).

RNA extraction and Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from mature red tomato by the hot borate method (*30*). Poly (A)$^+$ RNA was isolated using Oligotex-dT30 (Takara, Kyoto, Japan) in accordance with the manufacturer's protocol. The first strand of cDNAs synthesized by RT-PCR from 2 μg of poly (A)$^+$ RNA isolated from mature red tomato fruit were used as a template for RT-PCR with primers A and B (Table 1). The gene-specific primers were designed on the basis of the nucleotide sequence information of tomato EST (SGN-U330110) found in the Sol genomics network. Reactions for the RT-PCR mentioned above were carried out for 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 3 min.

Table 1. Oligonucleotide primers used for cloning of PNGase-Le gene.

	Name	Oligonucleotide sequences	Uses
A	F1 primer	cgagggctgcctcctcttcc	RT-PCR/RACE-PCR
B	R1 Primer	caaccccactaacccaaacacca	RT-PCR/RACE-PCR
C	F2 Primer	ggtgtttgggttagtggggttgag	3′ Nested-PCR
D	R2 primer	gaagtactgtggaggttgctgtgg	5′ Nested-PCR
E	Primer-F	gcggccgccatggctgcctcctcttc	PCR amplification
F	Primer-R	tctagaaccaggaagagaccgc tttttg	PCR amplification
G	Primer-F1	caagaagctgcactgctgaac	DNA Sequencing
H	Primer-R1	ggtctccaaaagagggggatt	DNA Sequencing
I	Primer-F2	ttcagggaggtggtggttag	DNA Sequencing
J	Primer-R2	gcgtcgtggcataaacagra	DNA Sequencing
K	Primer-F3	cagccccagaatctaatcca	DNA Sequencing
L	Primer-R3	gggaaatgaagggaacttgc	DNA Sequencing

Amplification of full-length cDNA of PNGase-Le by Rapid amplification of cDNA end (RACE)-PCR

To determine the full-length nucleotide sequences of putative PNGase-Le, RACE-PCR was performed using a cDNA amplification kit (Marathon, Clontech, Palo Alto, CA) according to the manufacturer's instructions. The 5′- and 3′-end fragments were amplified using gene-specific primers A and B, respectively (Table 1). Two other gene-specific primers, C and D were used for Nested-PCR for the amplification of the 5′- and 3′-end fragments (Table 1). Each gene-specific primer was designed on the basis of the nucleotide sequence information of tomato EST (SGN-U330110) found in the Sol genomics network.

cDNA cloning and sequencing of tomato acidic peptide: N-glycanase

The PCR products obtained from RACE-PCR were ligated into a TA-cloning vector and pGEM-T easy vector (Invitrogen, Carlsbad, CA) and transformed into *E. coli* JM109 competent cells. After blue and white screening, the target cDNAs were sequenced with the M13 and various other sequencing primers (Table 1, G-L) using a DNA sequencer (ABI Prism 3100-Avant Genetic Analyzer, Applied Biosystems, USA) according to the manufacturer's instructions. At least three colonies of each of the 5′- and 3′-end RACE-PCR fragments were sequenced for the correct determination of the cDNA sequence.

Construction of an expression plasmid for PNGase-Le

To facilitate the detection and purification of recombinant PNGase-Le, a hexa-His tag was fused with the open reading frame of the PNGase-Le gene at the C-terminal. The PNGase-Le cDNA was amplified by PCR using the oligonucleotide primers, 5′-GCG GCCGCCATGGCTGCCTCCTCTTC- 3′ (sense primer containing ATG start codon) and 5′TCTAGAACCAGGAAGAGACCGCTT TTTG-3′(antisense primer). The *NotI* and *XbaI* restriction sites were designed into the sense and antisense primer, respectively. The primers used for PCR introduced *NotI* and *XbaI* restriction sites permitting the directional cloning of the amplified DNA in frame with the α-factor leader sequence in the pPICZαC expression vector. The PCR reaction was carried out using PrimeSTAR-HS DNA polymerase (Takara Bio Inc., Japan) for 30 cycles. The conditions for each cycle were as follows: denaturation at 98°C for 10 s, annealing at 57°C for 30 s, and extension at 72°C for 2.5 min. For cloning into the pGEM-T easy vector, the PCR products were subjected to phenol-ethanol precipitation and the adenine nucleotide was

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subsequently joined at the 3′-end using Takara DNA polymerase at 72°C for 30 min. The PCR fragment of 1764 bp was ligated into the pGEM-T easy vector and then transformed in *E. coli* JM109 cells with proper antibiotic selection. The recombinant vector pGEM-T/PNGase-Le easy was isolated from *E. coli* cells and analysed cDNA sequence with various sequencing primers using an ABI Prism 3100-Avant Genetic Analyzer, Applied Biosystems, USA (Table 1). The PNGase-Le cDNA insert obtained from the digestion of the recombinant vector pGEM-T/PNGase-Le easy with *NotI* and *XbaI* was ligated into corresponding sites of pPICZα C (Fig. 4A). The recombinant vector, pPICZα C/PNGase-Le was transformed into competent cells of *E. coli* JM109 in low-salt LB medium with zeocin. The correct orientation of the PNGase-Le insert was further confirmed with various sequencing primers using the same DNA sequencer (Table 1). Unless otherwise stated, standard methods for the small-scale preparation of plasmids, digestion with restriction enzymes, ligation and transformation were used.

Transformation, screening and expression of PNGase-Le

Ten micrograms of pPICZα C/PNGase-Le DNA was isolated and linearized with SacI. The digested DNA was transformed into yeast *Pichia pastoris* GS115 strain using Pichia EasyComp kit in accordance with the manufacturer's instructions. Aliquots (150 µl) were spread on YPDS plates containing 100 µg/ml zeocin, and then the plates were incubated at 30°C for 4 days. The integration of PNGase-Le cDNA into the genome of *P. pastoris* was confirmed by PCR using 5′AOX1 and 3′AOX1 primers. *P. pastoris* GS115 albumin (Mut^S) and GS115/pPICZ/lacZ(Mut^+) were used as control strains for the determination of Mut phenotype. A single colony of transformants, which confirmed as Mut^+ phenotype, was grown in 25 ml of BMGY medium. The yeast was grown at 30°C in an incubator shaker at 250 rpm for about 24 h until the culture reached an $OD_{600} = 5.0$. The culture yeast was harvested by centrifugation at 3000 *g* for 5 min at room temperature. To induce expression of recombinant PNGase-Le, the pellet was resuspended in 50 ml of BMMY medium and grown at 30°C with 250 rpm shaking for 7 days. Every 24 h, methanol (100%) was added to a final concentration of 0.5% to maintain induction, and at the same time, 1 ml of culture was collected for the expression detection and activity assay. Yeast growth media were prepared following the Pichia expression system protocol from Invitrogen (USA).

Purification of recombinant PNGase-Le

After confirming the period of the highest expression and activity of the secreted recombinant PNGase-Le the 96 h (4 days)-incubated Pichia culture medium was collected and centrifuge at 4000*g* for 15 min. The collected supernatant was concentrated using the Amicon ultra-centrifugal filter device (Millipore Ultra-15). The resulting concentrated enzyme solution was dialyzed against 20 mM Tris-HCl, pH 7.8 with three changes and finally used as a crude enzyme of PNGase-Le. The crude enzyme solution was applied onto a Ni-NTA column for affinity purification according to manufacturer's instructions (Novagen, USA).

SDS-polyacrylamide gel electrophoresis

The expression of rPNGase-Le was analyzed by SDS-PAGE (12.5% polyacrylamide) in 0.1 M Tris-glycine buffer (*31*). Twenty microliters of the supernatant was added to each lane of the gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 and de-stained by washing with a mixture of acetic acid-methanol-water (10 : 25 : 65, v/v/v). The marker proteins used for molecular weight determination were Precision Plus Protein^{TM} Standards (250, 150, 100, 75, 50, 37, 25, and 20 kDa) (Bio-Rad).

PNGase-Le activity assay system

The dansylated glycopeptides used as a substrate for the enzyme activity assay were prepared from human transferrin glycopeptides (cmCys-Gly-Leu-Val-Pro-Val-Leu-Ala-Glu-Asn-Tyr-Asn($Man_3Gal_2GlcNAc_4NeuAc_2$)-Lys following the procedure described in our - previous paper (*7*). A recombinant enzyme solution (50 µl) was mixed with 5 µl of the human transferrin glycopeptides ($Man_3Gal_2GlcNAc_4NeuAc_2$-dansylated peptide: 100–200 pmol) as a substrate in 50 µl of 0.1 M citrate buffer, pH 4.0 containing 5 mM EDTA and 5 mM PMSF. After incubation at 37°C for 18 h, a sample (90 µl) of the resulting supernatant was analyzed by RP-HPLC using a Cosmosil 5C18-AR column (6 × 250 mm). The glycopeptide substrates and the resulting deglycosylated peptides were eluted and separated by increasing the acetonitrile content in the 0.05% TFA/water from 5 to 70% for 45 min at a flow rate of 1.20 ml/min. The eluate was monitored with the spectrofluorometer at Ex 310 nm/Em 510 nm.

Recombinant enzyme characterization

Estimations of the recombinant PNGase activities at various pHs and temperatures were conducted using the crude enzyme. To determine the optimal pH, buffers with pHs ranging from 3.5–8.0 were used for the activity assay. Enzyme and substrate (human transferrin glycopeptides) were incubated with the buffers of various pHs (pHs 3–5, 0.1 M citrate buffer; pHs 5.5–6.5, 0.1 M MES buffer; pHs 7–8, 0.1 M HEPES buffer) containing 5 mM EDTA and 5 mM PMSF at 37°C for 14 h. The rest of the procedure was the same as that described in the standard activity assay section. The optimal temperature was determined by the standard activity assay at temperatures ranging from 20 to 80°C in 0.1 M citrate buffer, pH 4.5. To investigate the effects of different metal ions and EDTA on the recombinant PNGase activities, 10 mM $CuSO_4$, 10 mM $ZnSO_4$, 10 mM $CaCl_2$, 10 mM $FeCl_2$, 10 mM $FeCl_3$, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 10 mM $CoCl_2$, and 10 mM EDTA were added separately to the reaction solution, and PNGase activities were then measured using the standard assay as described earlier in the section of PNGase activity assay.

Bioinformatics analysis

The signal sequence was detected using PrediSi (*PREDIction of SIgnal peptides)*, Germany (http://www.predisi.de/). Another online software, PredictProtein (*32*) was used to identify the *N*-glycosylation sites in the deduced amino acid sequence of PNGase-Le (http://www.predictprotein.org/). The PNGase-Le sequence was compared with that of Almond PNGase A using on online multiple-alignment programs, ClustalW 2.0.3 (*33*). To construct a phylogenetic tree, the PNGase sequences were compared with the nonredundant sequence database at the National Center for Biotechnology Information (NCBI) using Blastp. The query amino acid sequence was PNGase-Le for the searching the acidic PNGase homologous genes. The amino acid sequence homologues of PNGase were completely aligned using ClustalX (2.0.6) program. Phylogenetic reconstruction of the sequences was carried out using the neighbor-joining programs, ClustalX (2.0.6) (*34*).

Results and discussion

Cloning, sequencing and identification of PNGase-Le cDNA

The amino acid sequence information of almond PNGase A (Genbank accession no. P81898) was used as a reference sequence for tblastn search in the Sol genomic network to detect putative candidate genes. We predicted that the two EST cDNA clones, SGN-U330110 and SGN-U333183, could be candidate genes of acidic PNGase in *Lycopersicum esculentum*. Two gene-specific primers, A and B, were designed on the basis of the nucleotide sequence information of the EST clone, SGN-U330110. The RT-PCR sample showed the amplification of 375 bp fragments in 1% agarose gel electrophoresis (Fig. 1). Moreover, at the same time genomic DNA isolated from mature red tomato was also used for PCR amplification using the A and B primers. PCR products of genomic DNA having the same size as the RT-PCR products on the 1% agarose gel confirmed the existence of the PNGase-Le gene in the whole genomic DNA (Fig. 1) To determine the full-length cDNA of PNGase-Le, the 5′- and 3′-end fragments amplified by RACE and nested-PCR using gene-specific primers were cloned into pGEM-T easy vector. The analysed DNA sequences found in our clones showed 100%

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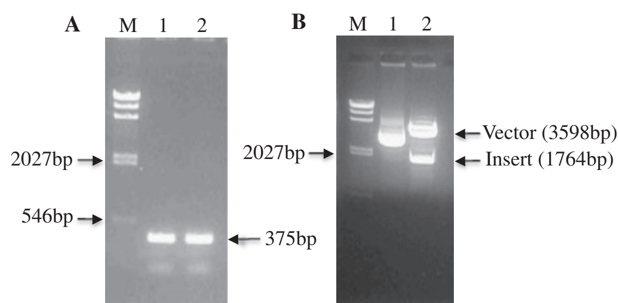

Fig. 1 1% Agarose/ethidium bromide gel image of RT-PCR products and vector-insert. (A) Image of RT-PCR and genomic DNA amplification by PCR. Lane M, marker λ-DNA digested by *HindIII*; lane 1, RT-PCR; lane 2, genomic DNA. (B) Image of vector and insert. Lane M, marker λ-DNA digested by *HindIII*; lane 1, pPICZαC(3598 bp); lane 2, PNGase-Le insert (1764 bp).

identities of our predicted ESTs, SGN-U330110 and SGN-U333183, available in the Sol genomic network. The full-length cDNAs of the PNGase-Le obtained by RACE and nested-PCR was found to be 1941 bp (registered under accession no. FJ804752). These results suggest that our predicted EST clones, SGN-U330110 and SGN-U333183, are parts of the full-length cDNA of PNGase-Le gene. Blastn analyses have identified a putative acidic PNGase orthologue in *Arabidopsis thaliana* (accession number NM 112354) and *Oryza sativa* japonia (accession number NC 008394). Some of closely related genes are represented in the EST collection from other plant species (e.g., *Nicotiana tabacum, Solanum tuberosum, Capsicum annuum,* and *Coffea canephora*).

Analysis of predicted amino acid sequence of PNGase-Le

The PNGase-Le cDNA of 1941 bp isolated from tomato fruits has an open reading frame (ORF) of 1764 bp encoding a sequence of 588 amino acids with a predicted molecular weight of 65.8 kDa. A typical poly $(A)^+$ tail was found at the 3′ untranslated region. Further analysis of the amino acid sequence of PNGase-Le predicted an *N*-terminal signal sequence of 21 amino acids and 10 *N*-glycosylation sites (Fig. 2). The predicted *N*-glycosylation sites are in the positions of 220, 266, 386, 397, 447, 481, 489, 551, 556 and 570 in the deduced polypeptide chain. Sequence alignment showed identities of about 57.9% with almond PNGase A, and the conserved regions are enclosed in boxes in Fig. 3. The active site of PNGase-Le probably resides in these conserved regions.

Expression of PNGase-Le cDNA in Pichia pastoris

The full-length PNGase-Le cDNA amplified by PCR using the oligonucleotide primers, E and F, was successfully cloned into the pPICZα C expression vector in frame with the α-factor leader sequence. A hexa-histidine tag was attached to the C-terminal of PNGase-Le for easy purification using Ni column. The PNGase-Le expression construct is shown in Fig. 4A. The recombinant vector, pPICZα C/PNGase-Le was transformed into competent cells of *E. coli* JM109. The inserted DNA sequence containing 1764 bp was analyzed and further confirmed by PCR amplification and sequence analysis. The recombinant plasmid, pPICZα C/PNGase-Le DNA isolated and linearized with *SacI*, was used for transformation into *Pichia pastoris* GS115 strain with proper antibiotic screening. A single colony transformants expressing the Mut^+ phenotype was grown in BMGY medium, and finally, expression was induced by methanol in BMMY medium. The resulting cell supernatant, as well as lysate, was analysed for PNGase activity *in vitro* using fluorescence-labeled glycopeptides bearing the complex-type *N*-glycan from human transferrin as a substrate. While high enzyme activity was found in the culture supernatant, only marginal activity, which might be endogenous cytosolic PNGase activity, could be detected in the Pichia cell lysate. In contrast, the culture supernatant from Pichia with the expression vector pPICZα C (without PNGase-Le cDNA insert) had no significant expression and no PNGase activity. A typical RP-HPLC profile of the glycopeptides substrate treated with crude PNGase-Le is shown in Fig. 4B. In addition to major substrate glycopeptide (Peak A), Peak X was also digested by the tomato PNGase, suggesting that this peak might be a contaminative human transferrin glycopeptides but the structure of *N*-glycan moiety might be slightly different from that of Peak A. Due to small amount of this contaminative peak, the structural analysis of *N*-glycan moiety of the contaminative glycopeptide could not be done in this study.

We carried out a time-course study to optimize secretion of rPNGase (recombinant PNGase)-Le in media by adding 0.5% methanol every 24 h. Ninety six hours (4 days) was found to be the optimum incubation time for maximizing the secreted rPNGase-Le in BMMY medium after methanol-induction (Fig. 5). Although our PNGase-Le expression construct contained a hexa-histidine tag in the C-terminal of its coding sequence, we could not successfully purify rPNGase-Le by affinity chromatography using a Ni column. The run-through fraction of the Ni column that showed the PNGase activity suggested that the poly-His tag might either be cut off from the recombinant PNGase-Le by certain endogenous protease(s) during secretion in media or remain inside the properly folded PNGase-Le. We also carried out western blotting with anti-His (C-term)-HRP antibody but could not successfully identify the His tag. Probably, the C-terminal His tag did not remain on the surface of the PNGase-Le (data not shown). SDS-PAGE of the culture supernatants containing secreted proteins showed a major band at about 65 kDa and some minor protein bands by SDS–PAGE (Fig. 5B), suggesting this major band might be recombinant PNGase-Le, and the apparent molecular mass seemed to be good agreement with the theoretical molecular mass of non-glycosylated PNGase-Le. But decrease of the enzyme activity in the supernatant after 5 days-culture was observed in spite of constant expression of the major protein, suggesting inactivation of the secreted rPNGase-Le occurred after

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1 – ATGGCTGCCTCCTCTTCCATTTTCTTAGTTTATTTCTTGCTACCCTTATTTTCCACAGCAACCCTCCACAGTACTTCTTCT
1 - M A A S S S I F L V Y F L L P L F S T A T L H S T S S
82 – CTTTATAGATCCCAACTCATCACTCAGCAAGAATCTTCACCCAAAAATGCCACCCCCACAACATTTTTTGAAGTCACAAAA
28 - L Y R S Q L I T Q Q E S S P K N A T P T T F F E V T K
163 – CCTATAAAATTACCCAAAACCAAACCTTTTTCACACCTAATTCTTGAACATGATTTTGGTTCCACCTACAGAAAACCCCCA
55 - P I K L P K T K P F S H L I L E H D F G S T Y R K P P
244 – ATTCTTGCTAACTACACACCCCCTTTTAATTGCCCATCTCAGAAATTTTCCAAAATTGTGCTGGAATGGAGAGCAACTTGT
82 - I L A N Y T P P F N C P S Q K F S K I V L E W R A T C
325 – AAAGGTAGACAATTTGATAGAATTTTTGGTGTTTGGGTTAGTGGGGTTGAGATTTTCAGAAGCTGCACTGCTGAACCAACT
109 - K G R Q F D R I F G V W V S G V E I F R S C T A E P T
406 – AAAAATGGGATCTTTTGGACTGTCAAGAAGGACATTACTAGGTATTCTTCTTTGCTTATGAAAAATCAAATCTTTGCTGTT
136 - K N G I F W T V K K D I T R Y S S L L M K N Q I F A V
487 – TATTTAGGGAATATTGTTGATAGTACATATACTGGTGTGTACCATGTGGAAATTTTTGTTCACTTTTATCCTGCTAAAGTG
163 - Y L G N I V D S T Y T G V Y H V E I F V H F Y P A K V
568 – AGATTGGGTGGATTAGATTCTGGGGCTGATTTAATTGTTCCCATTTCAAGAAATATGCATTTGAATGATGGGTTGTGGTTT
190 - R L G G L D S G A D L I V P I S R N M H L N D G L W F
649 – GAGATTGAAAATTCAACAGATGTACAGTCAAAGGATTTCAAGATTCCCCCAAATGTGTATAGGGCTGTATTGGAAGTTTAT
217 - E I E **N S T** D V Q S K D F K I P P N V Y R A V L E V Y
730 – GTTTCATTTCATGAGAATGATGAGTTTTGGAATGGGAATCCACCTAATGAGTATATAAGTTCGAATAATCTTAGTATCGCG
244 - V S F H E N D E F W N G N P P N E Y I S S N **N L S** I A
811 – GGGAATGGAGCTTTCAGGGAGGTGGTGGTTAGTTTGGATGAAATGGTAGTTGGTGTAGTTTGGCCTTTTACTGTGATCTAT
271 - G N G A F R E V V V S L D E M V V G V V W P F T V I Y
892 – ACTGGGGGTGTTAATCCCCTCTTTTGGAGACCGATTAGTGGAATTGGATCGTTCGATCTTCCTTCTTATGACATTGAAATT
298 - T G G V N P L F W R P I S G I G S F D L P S Y D I E I
973 – ACCCCGTTGTTAGGAAAGATTTTAGATGGAAATAGTCATAAGATTTCATTTGGAGTCACGGATGCTCTGAACGTGTGGTAT
325 - T P L L G K I L D G N S H K I S F G V T D A L N V W Y
1054 – GTTGATGCAAATTTGCATCTTTGGTTGGACGGGAAAAGTAAGAAAAACGGAAGGGAAGTTGTTGAGATACAGCTTATTGCCC
352 - V D A N L H L W L D G K S K K T E G K L L R Y S L L P
1135 – CTTTCTTTATCGGTGCTGACCAATTTTACAGGTTTTGATGGATCCTTTATCACGAATGCTAGTAGGTCCATCACATTGACC
379 - L S L S V L T **N F T** G F D G S F I T **N A S** R S I T L T
1216 – GGAATGGTGAAGTCGTCTTATGGAACTATCACTACTAAGTCGTCTCAAAGTTTAAGTTATAGCAACCATATGGTAATGGGA
406 - G M V K S S Y G T I T T K S S Q S L S Y S N H M V M G
1297 – AATGAAGGGAACTTGCAAATAGTGGATCAGATAATCGAATTCAATGATACTGTTTATGCCACGACGCCATCTTCTTATGTT
433 - N E G N L Q I V D Q I I E F **N D T** V Y A T T P S S Y V
1378 – CACTCGCTTGAGTCCTTCAAAAAGTATCTGTTGAAGTTGTATTCTGACAATGTAGATCAAGGAAACCAAAGCTATACCTCG
460 - H S L E S F K K Y L L K L Y S D N V D Q G **N Q S** Y T S
1459 – ATATCAAACTTGACGTTGGGATTAGATGACAAGAGGGTAAAGGGTTCCAAGTATGGATCCTCAGTCAGCTCTGTGAATAAT
487 - I S **N L T** L G L D D K R V K G S K Y G S S V S S V N N
1540 – TTGCAAAACGCGCAGGGCTATATGATTGTAAAGGGCCACTTAGTAGTCAAAGGACTTGGAAGTACCCAACAAGTATATAAA
514 - L Q N A Q G Y M I V K G H L V V K G L G S T Q Q V Y K
1621 – CATAACGATGATTCGTGCTGCTACTCCAGGAACATAAGCAGCTCAAATTACACAATACTTTACGATAAGGTAAGCAATAGC
541 - H N D D S C C Y S R **N I S** S S **N Y T** I L Y D K V S N S
1702 – TGCAGCAATAGTACTTGGTCTCATTGGCCTTTGAGGATTGACAAAAAGCGGTCTCTTCCTGGTTGAAGAGTCCTTTTTTGC
568 - C S **N S T** W S H W P L R I D K K R S L P G *
1783 – AATCTCATCTTGACTCTTCCNGAATTCCACTTTCACTCATTTGCTGNCTAAGCTACTTATTATTATGATGCAAATGCACCA

1864 – CTGCAGTGCCTCTTGTAATCTTGGGACTGGTTATTTTTGCCAATATGCAGGCCGGTTTTGCTAAAAAAAAAAA - 1936

Fig. 2 Nucleotide sequence of PNGase-Le cDNA and deduced amino acid sequence. The nucleotide and amino acid sequences are numbered from the left beginning with the first start codon. The signal sequence finishes at 21 and is underlined. Putative ten *N*-glycosylation sites are indicated by boldface.

prolonged culture period. Since the rPNGase-Le could not be purified by the affinity chromatography, it is not clear at this time whether the major protein band with the molecular mass of 65 kDa is the secreted rPNGase-Le with *N*-glycans(s) or the potent glycosylation sites are really glycosylated, although the major protein was not detected in the supernatant of culture broth of *Pichia* containing only pPICZα C vector without PNGase-Le insert, supporting the 65 kDa protein may be rPNGase-Le (Fig. 5B, lane C).

Effects of pH, temperature and metal ions

The effects of pH and temperature on the activity of the recombinant PNGase-Le were determined using the dansylated glycopeptides prepared from human transferrin as a substrate. As shown in Fig. 6A, the recombinant enzyme showed a maximum relative activity at approximately pH 4.5 and the activity declined rapidly after pH 6.0. The RP-HPLC profiles of the PNGase activity of the recombinant enzyme at various pHs (pH 4.5, 5.5 and 6.5) are shown in Fig. 6B. At pH 4.5, almost all the substrates (S) were converted into products (P), whereas at pH 6.5, no substrate was hydrolysed. This result is consistent with the optimum pH values of other PNGases isolated from plant cells (*1*, *4–5*, *7*) as well as fungal PNGase-At (*35*). Most PNGases of plant origin have optimum pH in the acidic region, suggesting that this deglycosylation enzyme seems to occur and function in acidic organelle or region, such as vacuole (protein body) or cell wall. The optimum temperature for the activity of the PNGase-Le was found to be approximately 40°C (Fig. 6C). We also examined the effects of 8 metals ions, namely Mn^{2+}, Fe^{2+}, Mg^{2+}, Co^{2+}, Ca^{2+},

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PNGase-Le  MAASSSIFLVYFLLPLFSTATLHSTSSLYRSQLITQQESSPKNATPTTFFEVTKPIKLPK 60
PNGase-A   -----------------EPTPLHDTP-------------------PTVFFEVTKPIEVPK 24
                            ..:.**.*.                   **.********::**

PNGase-Le  TKPFSHLILEHDFGSTYRKPPILANYTPPFNCPSQKFSKIVLEWRATCKGRQFDRIFGVW 120
PNGase-A   TKPCSQLILQHDFAYTYGQAPVFANYTPPSDCPSQTFSTIVLEWKATCRRRQFDRIFGVW 84
           *** *:***:***. ** :.*::****** :****.**.*****:***: **********

PNGase-Le  VSGVEIFRSCTAEPTKNGIFWTVKKDITRYSSLLMKNQIFAVYLGNIVDSTYTGVYHVEI 180
PNGase-A   LGGVEILRSCTAEPRPNGIVWTVEKDITRYYSLLKSNQTLAVYLGNLIDKTYTGIYHVNI 144
           :.****:*******  ***.***:****** *** .** :******::*.****:***:*

PNGase-Le  FVHFYPAKVRLG-----------GFDSGADLIVPISRNMHLNDGLWFEIENSTDVQSKDF 229
PNGase-A   SLHFYPAKEKLNSFQQKLDNLASGYHSWADLILPISRNLPLNDGLWFEVQNSNDTELKEF 204
            :****** :*.           *:.* ****:*****: ********::**.*.: *:*

PNGase-Le  KIPPNVYRAVLEVYVSFHENDEFWNGNPPNEYISSNNLS-IAGNGAFREVVVSLDEMVVG 288
PNGase-A   KIPQNAYRAVLEVYVSFHENDEFWYSNLPNEYIAANNLSGTPGNGPFREVVVSLDGEVVG 264
           *** *.****************** .* *****::****  .***.*********  ***

PNGase-Le  VVWPFTVIYTGGVNPLFWRPISGIGSFDLPSYDIEITPLLGKILDGNSHKISFGVTDALN 348
PNGase-A   AVWPFTVIFTGGINPLLWRPITAIGSFDLPTYDIEITPFLGKILDGKSHKFGFNVTNALN 324
           .*******:***:***:****:.*******:*******:*******:***:.*.**:***

PNGase-Le  VWYVDANLHLWLDGKSKKTEGKLLRYSLLPLSLSVLTNFTGFDGSFITNASRSITLTGMV 408
PNGase-A   VWYVDANLHLWLDKQSVTKTEGKLSKHSSLPLVVSLVSDFKGLNGTFLTRTSRSVSSTGWV 384
           ************* :*.****** ::* *** :*::::*.*::*:*:*.:***:: ** *

PNGase-Le  KSSYGTITTKSSQSLSYSNHMVMGNEGNLQIVDQIIEFNDTVYATTPSSYVHSLESFKKY 468
PNGase-A   KSSYGNITTRSIQDFYYSNSMVLGKDGNMQIVNQKIIFNDSVYINLPSSYVHSLTSHKTF 444
           *****.***:* *.: *** **:*::**:***:* * ***:** . ******** *.*.:

PNGase-Le  LLKLYSDNVDQGNQSYTSISNLTLGLDDKRVKGSKYGSSVSSVNNLQNAQGYMIVKGHLV 528
PNGase-A   PLYLYTDFLGQGNGTYLLITNVDLGFIE---KKSGLGFSNSSLRNLRSAEGNMVVKNNLV 501
            * **:* :.*** :*  *:*: **: :   * *  * * **:.**:.*:* *:**.:**

PNGase-Le  VKGLGSTQQVYKHNDDSCCYSRNISSSNYTILYDKVSNSCSNSTWSHWPLRIDKKRSLPG 588
PNGase-A   VSGLESTQQIYRYDGGKFCYFRNISSSNYTILYDKVGSKCNKKSLSNLDFVLSRLWPFGA 561
           *.** ****:*:::... ** ***************...*.:.: *:  : ::  .: .

PNGase-Le  ----------
PNGase-A   RMNFAGLRFT 571
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Fig. 3 Comparison of deduced amino acid sequence of PNGase-Le (GenBank accession number FJ804752) with that of almond PNGase-A. The asterisks (*) indicate sequence identity. The three most conserved regions are enclosed in the box.

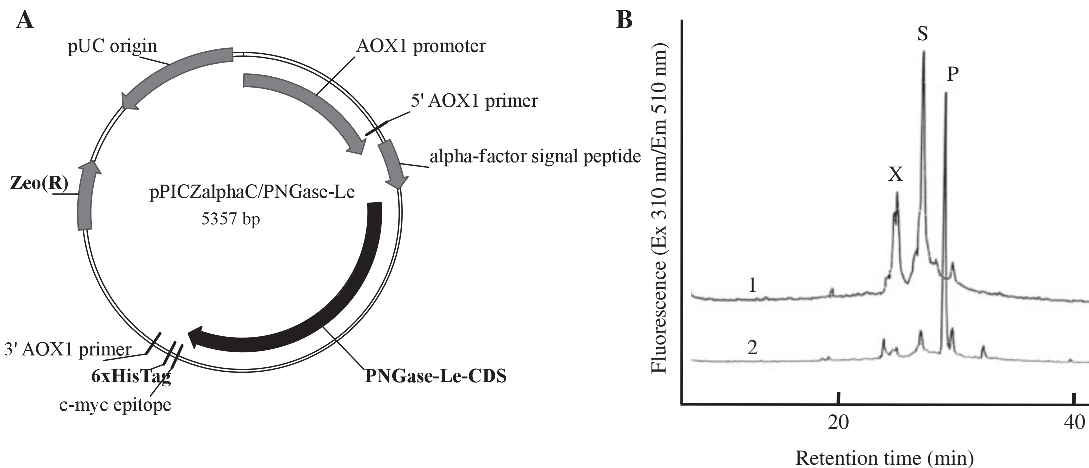

Fig. 4 Construction of expression plasmid containing the putative PNGase-Le gene and activity assay of recombinant PNGase-Le. (A) Structure of expression plasmid vector, pPICZαC/PNGase-Le. (B) RP-HPLC of $Man_3Gal_2GlcNAc_4NeuAc_2$-dansylated peptide incubated with rPNGase-Le. Denatured and native rPNGase-Le treated glycopeptides were analyzed using the ODS column, and the peptides were eluted as described in the text. 1 and 2, Treated with denatured and native recombinant PNGase-Le, respectively. S and P indicate the elution positions of substrate and deglycosylated peptide (product) after reaction, respectively. X, unidentified peak (see text).

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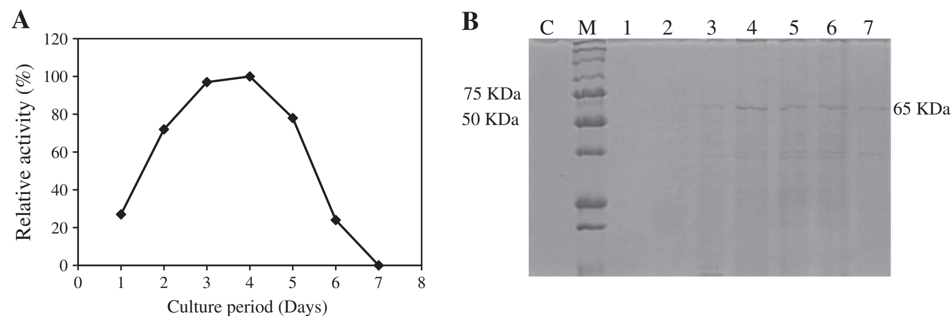

Fig. 5 Heterologous expression of soluble PNGase-Le in *Pichia pastoris*. (A) Expression analysis on 12.5% SDS-PAGE. Fifty microliters of supernatant was concentrated to approximately10 µl, which was mixed with 10 µl of sample buffer, and the entire sample was used for the SDS-PAGE analysis. Lane C, Pichia containing only pPICZα C vector without PNGase-Le insert as control; lane M, marker protein; lanes 1–7, number of days for optimization of expression. (B) Relative PNGase activity (%) analysis against fluorescence-labeled human transferrin glycopeptide as substrate. The detailed procedure of the activity analysis is described in the text.

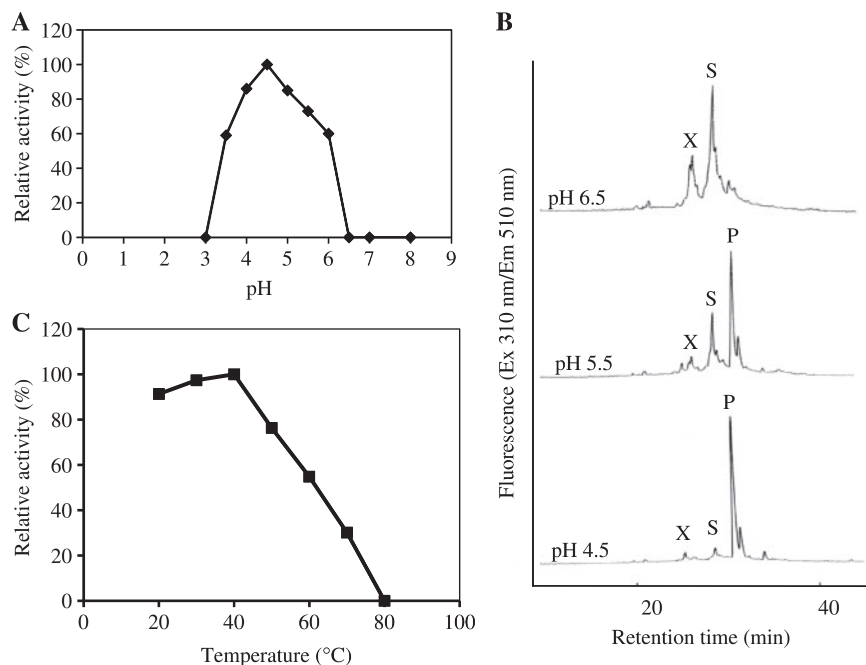

Fig. 6 Effects of pH and temperature on PNGase-Le activity. (A) The optimum pH was determined after incubation in buffers of various pHs (pHs 3–5, 0.1 M citrate buffer; pHs 5.5–6.5, 0.1 M MES buffer; pHs 7–8, 0.1 M HEPES buffer) containing 5 mM EDTA and 5 mM PMSF at 37°C for 14 h. (B) Comparison of RP-HPLC profiles for various pHs (pHs, 4.5, 5.5 and 6.5). S and P indicate the elution positions of substrate and deglycosylated product after reaction, respectively. X indicates unidentified peak. (C) The optimum temperature was examined after incubation in 0.1 M citrate buffer, pH 4.5 containing 5 mM EDTA and 5 mM PMSF at 37°C for 7 h.

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Fe^{3+}, Zn^{2+}, Cu^{2+} and EDTA at 10 mM concentration on the activity of the recombinant PNGase-Le. Like other PNGases such as PNGase-A, PNGase-F and L-929 PNGase (*1*, *36*, *37*), the recombinant PNGase-Le did not require metal ions for its maximum activity. Fe^{3+}, Cu^{2+} and Zn^{2+}, which are known as inhibitors of various PNGases, (*1*, *7*, *8*, *11*, *37*) did not cause a significant loss of activity of rPNGase-Le, although Cu^{2+} decreased the activity (data not shown).

Phylogenetic analysis of PNGase-Le

Cytosolic PNGases share a highly conserved ‘core’ sequence of about 300 amino acids. In higher eukaryotes, they possess both the N- and C-terminal extended domains (*11*) whereas in lower eukaryotes, a short peptide extension without any similarity with other sequence is present. It is yet unclear whether acidic PNGases possess such a core sequence like that of cytosolic PNGases. To shed light on the property and evolutionary position of PNGase-Le, a neighbor-joining phylogenetic tree was constructed (Fig. 7). The analyzed PNGases were phylogenetically clearly separated into two families: plants and fungi. Blastp search using the amino acid sequence of PNGase-Le could not show any homologous genes in other organisms such as bacteria and animals, suggesting that the acidic PNGase gene is conserved in plants and fungi. Moreover, the plant acidic PNGase has no significant sequence homology with the cytosolic PNGase of any organism reported so far. Therefore, the plant acidic PNGase is completely different from

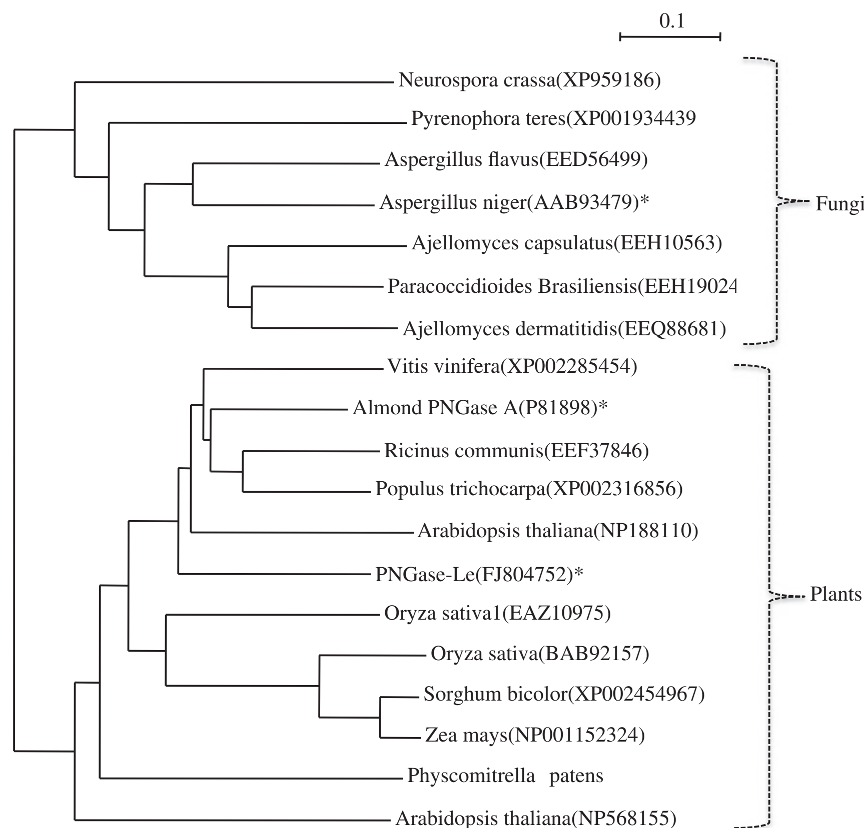

Fig. 7 Neighbor-joining phylogenetic tree analysis of deduced amino acid sequences of acidic PNGase homologues from 19 organisms found in NCBI/Blastp search. Multiple sequence alignment was performed using ClustalX (2.0.6) program. Most of the homologues except that indicated by an asterisk (*) are the predicted/putative PNGase gene of various organisms with Genbank accession number. The analyzed acidic PNGases are clearly separated into two families: plants and fungi. The indicated scale represents 0.1 amino acid substitutions per site.

the ubiquitous cytosolic PNGase and is involved in a different de-*N*-glycosylation mechanism associated with plant growth and development.

In conclusion, we have succeeded in expressing the acidic tomato PNGase (PNGase-Le) gene in *Pichia pastoris*. The PNGase activity of the recombinant PNGase-Le was confirmed using human complex-type N-linked glycopeptides or glycoproteins (transferrin glycopeptides) as a substrate. Although, at this stage, the subcellular localization of PNGase-Le is unclear, the optimum activity at acidic pH suggested that it could reside in the vacuoles (protein bodies) and/or the weak acidic cell-wall region. To elucidate the physiological function of PNGase-Le, the construction of transgenic tomato plants in which the PNGase-Le gene suppressed or over-expressed is in progress. Therefore, this study is the first step toward elucidating the physiological function of acidic PNGase in plants.

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Conflict of interest

None declared.

References

1. Takahashi, N. and Nishibe, H. (1978) Some characteristics of a new glycopeptidase acting on aspartylglycosylamine linkages. *J. Biochem.* **84**, 1467–1473
2. Altmann, F., Paschinger, K., Dalik, T., and Vorauer, K. (1998) Characterization of peptide-*N*4-(*N*-acetyl-β-glucosaminyl) asparagine amidase A and its *N*-glycans. *Eur. J. Biochem.* **252**, 118–123
3. Plummer, T.H. Jr, Elder, J.H., Alexander, S., Phelan, A.W., and Tarentino, A.L. (1984) Demonstration of peptide: *N*-glycosidase F activity in endo *N*-acety1-β-D-glucosaminidase F preparations. *J. Biol. Chem.* **259**, 10700–10704
4. Lhernould, S., Karamanos, Y., Lerouge, P., and Morvan, H. (1995) Characterization of the peptide-*N*4-(*N*-acetylglucosaminyl) asparagine amidase (PNGase Se) from *Silene alba* Cells. *Glycoconjugate J.* **12**, 94–98
5. Berger, S., Menudier, A., Julien, R., and Karamanos, Y. (1995) Endo-*N*-acetyl-β-D-glucosaminidase and peptide-*N*4-(*N*-acetylglucosaminyl) asparagine amidase activities during germination of *Raphanus sativus*. *Phytochemistry* **39**, 481–487
6. Chang, T., Kuo, M.-C., Khoo, K.-H., Inoue, S., and Inoue, Y. (2000) Developmentally regulated expression of a peptide:*N*-glycanase during germination of rice seeds (*Oryza sativa*) and its purification and characterization. *J. Biol. Chem.* **275(1)**, 129–134
7. Kimura, Y. and Ohno, A. (1998) A new peptide-*N*4-(acetyl-β-glucosaminyl)asparagine amidase from soybean (*Glycime max*) seeds: purification and substrate specificities. *Biosci. Biotech. Biochem.* **62(2)**, 412–418

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8. Barsomian, G.D., Johnson, T.L., Borowski, M., Denman, J., Ollington, J.F., Hirani, S., McNeilly, D.S., and Rasmussen, J.R. (1990) Cloning and expression of peptide-*N*4-(*N*-acetyl-beta-D-glucosaminyl)asparagine amidase F in *Escherichia coli*. *J. Biol. Chem.* **265 (12)**, 6967–6972
9. Suzuki, T., Park, H., and Lennarz, W.J. (2002) Cytoplasmic peptide:*N*-glycanase (PNGase) in eukaryotic cells: occurrence, primary structure, and potential functions. *FASEB J.* **16**, 635–641
10. Lee, J.H., Choi, J.M., Lee, C., Yi, K.J., and Cho, Y. (2005) Structure of a peptide:*N*-glycanase-Rad23 complex: insight into the deglycosylation for denatured glycoproteins. *Proc. Natl Acad. Sci. USA* **102**, 9144–9149
11. Zhao, G., Zhou, X., Wang, L., Li, G., Kisker, C., Lennarz, W.J., and Schindelin, H. (2006) Structure of the mouse peptide *N*-glycanase-HR23 complex suggests co-evolution of the endoplasmic reticulum-associated degradation and DNA repair pathways. *J. Biol. Chem.* **281**, 13751–13761
12. Priem, B., Gitt, R., Bush, C.A., and Gross, K.C. (1993) Structure of ten free *N*-glycans in ripening tomato fruit. *Plant Physiol.* **102**, 445–158
13. Faugeron, C., Lhernould, S., Maes, E., Lerouge, P., Strecker, G., and Morvan, H. (1997) Tomato plant leaves also contain unconjugated *N*-glycans. *Plant Physiol. Biochem.* **35**, 73–79
14. Faugeron, C., Lhemould, S., Lemoine, J., Costa, G., and Morvan, H. (1999) Identification of unconjugated *N*-glycans in strawberry plants. *Plant Physiol. Biochem.* **35**, 891–895
15. Kimura, Y. and Ueyama, T. (1999) Structural analysis of free *N*-glycans in bamboo *(Phyllostachys heterocycla)* shoots. *Sci. Rep. Fac. Agric. Okayama Univ.* **88**, 19–24
16. Kimura, Y., Takagi, S., and Shiraishi, T. (1997) Occurrence of free *N*-glycans in pea *(Pisum sativum* L) seedlings. *Biosci. Biotechnol. Biochem.* **61**, 924–926
17. Kimura, Y. and Kitahara, E. (2000) Structural analysis of free *N*-glycans occurring in soybean seedlings and dry seeds. *Biosci. Biotechnol. Biochem.* **64**, 2109–2120
18. Kimura, Y. and Matsuo, S. (2000) Free *N*-glycans already occur at an early stage of seed development. *J. Biochem.* **127**, 1013–1019
19. Kimura, Y., Matsuo, S., Tsurusaki, S., Kimura, M., Hara-Nishimura, I., and Nishimura, M. (2002) Subcellular localization of endo-β-*N*-acetylglucosaminidase and high-mannose type free *N*-glycans in plant cell. *Biochem. Biophys. Acta* **1570**, 38–46
20. Nakamura, K., Inoue, M., Yoshiie, T., Hosoi, K., and Kimura, Y. (2008) Changes in structural features of free *N*-glycan and endoglycosidase activity during tomato fruit ripening. *Biosci. Biotechnol. Biochem.* **72(11)**, 2936–2945
21. Priem, B. and Gross, K.C. (1992) Mannosyl- and xylosyl-conaining glycans promote tomato *(Lycopersicon escuientum* Mill.) fruit ripening. *Plant Physiol.* **98**, 399–401
22. Yunovitz, H. and Gross, K.C. (1994) Delay of tomato fruit ripening by an oligosaccharide *N*-glycan Interaction with IAA, galactose and lectins. *Physiol. Plant* **90**, 152–156
23. Kimura, Y., Matsuo, S., and Takagi, S. (1998) Enzymatic properties of a *Ginkgo bioloba* endo-β-*N*-acetylglucosaminidase and *N*-glycan structures of storage glycoprotein. *Biosci. Biotechnol. Biochem.* **62**, 253–261
24. Lhernould, S., Karamanose, Y., Priem, B., and Morvan, H. (1994) Carbon starvation increases endoglycosidase activities and production of 'unconjugated *N*-glycans' in *Silene alba* cell-suspension cultures. *Plant Physiol* **106**, 779–784
25. Berger, S., Menudier, A., Julien, R., and Karamanos, Y. (1995) Do de-N-glycosylation enzymes have an important role in plant cells? *Biochimie* **77**, 751–760
26. Maeda, M. and Kimura, Y. (2005) Functional future and gene expression of plant endo-β-*N*-acetylglucosaminidase. *Glycoconjugate J.* **22**, 310
27. Kimura, Y. (2007) Structural and functional features of plant glycoprotein glycans in *Comprehensive Glycoscience* (Kamerling J.P., ed.) Vol. 3, pp. 67–78, Elsevier, Amsterdam
28. Nakamura, K., Inoue, M., Maeda, M., Hosoi, K., and Kimura, Y. (2007) Plant endo-β-*N*-acetylglucosaminidase: gene, distribution, and changes in enzyme activity and gene expression during fruit ripening. *Glycoconjugate J.* **24**, 350
29. Nakamura, K., Inoue, M., Maeda, M., Nakano, R., Hosoi, K., Fujiyama, K., and Kimura, Y. (2009) Molecular cloning and gene expression analysis of tomato endo-β-*N*-acetylglucosaminidase, endoglycosidase involved in the production of high-mannose type free *N*-glycans during tomato fruit ripening. *Biosci. Biotechnol. Biochem.* **73**, 461–464
30. Wan, C.Y. and Wilkins, T.A. (1994) A modified hot borate method significantly enhances the yield of high-quality RNA from cotton (*Gossypium hirsutum* L.). *Anal. Biochem.* **223**, 7–12
31. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
32. Rost, B., Yachdav, G., and Liu, J. (2004) The Predict Protein Server. *Nucleic Acids Res.* **32** (Web Server issue), W321–W326
33. Dereeper, A., Guignon.V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.F., Guindon, S., Lefort.V., Lescot, M., Claverie, J.M., and Gascuel, O. (2008) Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res.* **36** (Web Server issue), W465–9
34. Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, M.I., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., and Higgins, D.G. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* **23**, 2947–2948
35. Ftouhi-Paquin, N., Hauer, C.R., Stack, R.F., Tarentino, A.L., and Plummer, T.H. Jr. (1997) Molecular cloning, primary structure, and properties of a new glycoamidase from fungus *Aspergillus tubigensis*. *J. Biol. Chem.* **272**, 22960–22965
36. Tarentino, A.L., Quinones, G., Trumble, A., Changchien, L.M., Duceman, B., Maley, F., and Plummer, T.H. Jr. (1990) Molecular cloning and amino acid sequence of peptide-N4-(*N*-acetyl-β-D-glucosaminyl) asparagine amidase from *Flavobacterium meningosepticum*. *J. Biol. Chem.* **265**, 6961–6966
37. Suzuki, T., Seko, A., Kitajima, K., Inoue, Y., and Inoue, S. (1994a) Purification and enzymatic properties of peptide: *N*-glycanase (PNGase) from C3H mouse-derived L-929 fibroblast cells. Possible widespread occurrence of post-translational remodification of proteins by *N*-deglycosylation. *J. Biol. Chem.* **269**, 17611–17618

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