Nonspecific phospholipase C3 of radish has phospholipase D activity toward glycosylinositol phosphoceramide

Rumana Yesmin Hasi[1], Toshiki Ishikawa[2], Keigo Sunagawa[1], Yoshimichi Takai[1], Hanif Ali[1], Junji Hayashi[1], Ryushi Kawakami[1], Keizo Yuasa[3], Mutsumi Aihara[1], Kaori Kanemaru[1], Hiroyuki Imai[4], and Tamotsu Tanaka[1]*

[1]Graduate School of Technology, Industrial and Social Sciences, Tokushima University, Tokushima 770-8513, Japan

[2]Graduate School of Science and Engineering, Saitama University, Saitama 338-8570, Japan

[3]Graduate School of Science and Engineering, Setsunan University, Neyagawa 572-8508, Japan

[4]Graduate School of Natural Science, Konan University, Kobe 658-8501 Japan

*Correspondence
T. Tanaka,
Graduate School of Technology, Industrial and Social Sciences, Tokushima University, Tokushima 770-8513, Japan
Tel: +81 88 656 7249
E-mail: tanaka.tamotsu@tokushima-u.ac.jp

Abbreviations

BSA, bovine serum albumin; DAG, diacylglycerol; DE, diethyl ether; GIPC, glycosylinositol phosphoceramide; GMT1, GIPC-mannosyl-transferase 1; InoGly, inositol glycan; IPS, inositol phosphorylceramide synthase; IPUT1, inositol phosphorylceramide glucoronosyl transferase 1, LPA, lysophosphatidic acid; NaDOC, sodium deoxycholate; NPC3, nonspecific phospholipase C3; PA, phosphatidic acid: PC1P, phytoceramide 1-phosphate; PCer, phytoceramide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PLC, phospholipase C; PLD, phospholipase D; SphM, sphingomyelin; THAP, 2,4,6-trihydroxy-acetophenone.

Abstract

Previously, we found an enzyme activity that produces phytoceramide 1-phosphate (PC1P) by hydrolysis of D position of glycosylinositol phosphoceramide (GIPC) in cabbage and called its activity as GIPC-phospholipase D (PLD). Here, we purified GIPC-PLD by sequential chromatography from radish roots. Peptide mass fingerprinting analysis revealed that the potential candidate for GIPC-PLD protein was nonspecific phospholipase C3 (NPC3), which has not been characterized as PLD. The recombinant NPC3 protein obtained by heterologous expression system with *E. coli* produced PC1P from GIPC, and showed essentially the same enzymatic properties as those we characterized as GIPC-PLD in cabbage, radish, and *A. thaliana.* From these results, we concluded that NPC3 is one of the enzymes that degrade GIPC, a major sphingolipid in plants.

Keywords

Glycosylinositol phosphoceramide, phospholipase D, phytoceramide 1-phosphate, nonspecific phospholipase C3, *Raphanus sativus*.

Introduction

Sphingolipids are one of the major components of the plasma membranes of animal and plant cells. The most abundant sphingophospholipid in plants is glycosylinositol phosphoceramide (GIPC), which consists of a ceramide backbone linked to an inositol glycan unit via a phosphodiester bond [1–4]. The structural identification of GIPC was attained in 1958 [5]. Since then, the biosynthesis and physiological roles of GIPC have been studied. An enzyme involved in GIPC biosynthesis, inositol phosphorylceramide synthase (IPS), was identified and cloned by Brompley et al. in 2003 [6,7]. Recent investigations have identified and characterized inositol phosphorylceramide glucoronosyl transferase 1 (IPUT1) [8,9] and GIPC-mannosyl-transferase 1 (GMT1) [10]. The physiological role of GIPC in plants was elucidated in the 2000s using gene silencing techniques. These include cell wall anchoring [11], protein anchoring [12,13], and salt sensors for the Ca^{2+} influx channel [9,14], in addition to the architectural role as raft domain of the plasma membrane [15,16].

In 2013, we found an uncharacterized sphingolipid in cabbage leaves and identified it as phytoceramide 1-phosphate (PC1P). We also found that PC1P was generated by the cleavage at the D position of GIPC by GIPC-specific hydrolyzing activity (Fig. 1) [17]. We called the enzymatic activity as GIPC-PLD and characterized its substrate specificity, pH dependency, the effect of cofactors, distribution of plant parts, and transphosphatidylation activity [17–19].

Here, we describe that the GIPC-PLD activity in radish is responsible for the enzymatic activity of non-specific phospholipase C3 (NPC3). The recombinant NPC3 protein of radish, produced by a heterologous expression system with *E. coli*, showed essentially the same enzymatic nature as the previously characterized GIPC-PLD in cabbage, radish, and *A. thaliana* [17–19].

Materials and Methods

Materials

Sphingomyelin (SphM) from chicken egg, phosphatidylcholine (PC) from soybean, and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (PE) were purchased from Avanti Polar Lipids (Alabaster, AL). Phosphatidylinositol (PI) and GIPC was isolated from cabbage lipids by TLC (Merck, Darmstadt, Germany), and confirmed their structures by MALDI-TOF/MS as previously described in [17,20]. Cabbage (*Brassica oleracea L. var. capitata*) and radish (*Raphanus sativus*) were obtained from the local market. The TOYOPEARL Giga-Cap Q-650 and Butyl-650 were purchased from Tosoh Corporation (Tokyo, Japan). The protein assay kits and gels used for SDS-PAGE were obtained from Integral Corporation (Tokushima, Japan) and Bio-Rad Laboratories, Inc. (Hercules, CA), respectively. The silver staining kit and protein molecular weight maker were purchased from Atto Co. Ltd. (Tokyo, Japan). 2,4,6-Trihydroxy-acetophenone (THAP) was obtained from Sigma-Aldrich (St. Louis, MO). The phos-tag with Zn [^{68}Zn] was obtained from Wako Pure Chemical Industries (Osaka, Japan). All organic solvents used in this study were reagent grade and obtained from Nacalai Tesque Inc. (Tokyo, Japan).

Preparation of GIPC-PLD from radish

GIPC-PLD was extracted from radish roots. Four hundred grams of radish root were disrupted with a juicer mixer grinder and filtered with gauze. The resulting juice was mixed with an appropriate amount of cold 200 mM Tris-HCl buffer (pH 7.5) to reach the final concentration of 50 mM, and centrifuged at 13,000 × *g* for 30 min at 4 °C. Ammonium sulphate was added to the supernatant with continuous stirring to reach 60% saturation. The mixture was centrifuged at 12,000 × *g* for 20 min at 4 °C, and the precipitate was stored at – 80 °C until further use.

Strong anion-exchange chromatography with Giga-Cap Q-650 stepwise

Column chromatography was performed at 4 °C. Protein amounts were determined using the Bradford method with the bovine serum albumin (BSA) as the standard. The 60% ammonium sulfate precipitate was suspended in a small amount of 10 mM Tris-HCl buffer (pH 7.5) and dialyzed in the same buffer at 4 °C for 24 h. After centrifugation at 12,000 × *g* for 10 min at 4 °C, the supernatant was subjected to Giga-Cap column chromatography that was equilibrated with the same buffer used for dialysis. The enzymes were eluted with 10 mM Tris-HCl buffer (pH 7.5)

containing 0, 50, 100, 150, 200, 250, and 500 mM NaCl. The eluates were collected at a rate of 4 mL/fraction. GIPC-PLD activity and the amounts of protein in each fraction were determined as described below. The typical experimental conditions were as follows: loaded amount of protein (86 mg), column volume (4 mL), the volume of elution buffer (20 mL/each NaCl concentration), and flow rate (0.5 $mL.min^{-1}$).

Hydrophobic chromatography with Butyl-650

Active fractions from Giga-Cap Q-650 column chromatography were combined and dialyzed against 10 mM Tris-HCl buffer (pH 7.5). The dialysate was dissolved in 20% saturation level of ammonium sulphate in the same buffer and loaded onto a Butyl column that is pre-equilibrated with 20% ammonium sulphate in the same buffer. The proteins were eluted with a linear gradient of decreasing concentration of ammonium sulphate (20–0%) in the same buffer (pH 7.5). The eluates were collected at a rate of 2 mL/fraction. Typical experimental conditions were as follows: loaded amount of protein (10 mg), column volume (2 mL), the volume of buffer for elution (20 mL), and flow rate (0.5 $mL.min^{-1}$).

Strong anion-exchange chromatography with Giga-Cap Q-650 linear

Active fractions of Butyl-650 column chromatography were combined and dialyzed against 100 mM NaCl containing 10 mM Tris-HCl buffer (pH 8.5). The dialyzed sample was subjected to a Giga-Cap column pre-equilibrated with the same buffer used for dialysis. The proteins were eluted with a linear gradient of 100–500 mM NaCl in the equilibration buffer. The eluates were collected at a flow rate of 0.4 mL/fraction. Typical experimental conditions were as follows: loaded amount of protein (0.71 mg), column volume (0.8 mL), elution of each NaCl-containing buffer (10 mL), and flow rate (0.1 $mL.min^{-1}$).

GIPC-PLD assay

The GIPC-PLD assay was conducted as previously described [18,19]. GIPC-containing crude lipid extract was prepared from cabbage using the lower layer of the solvent consisting of isopropanol: hexane: water (55:20:25, v/v), according to the method described in [20,21], and used for the assay. The assay mixture consisted of GIPC-containing cabbage lipids (GIPC: 26 nmol), 0.05–0.1 mL enzyme fraction, and 2 mg of sodium deoxycholate (NaDOC) (4.6 mM) in 50 mM

Tris-HCl buffer (pH 7.5) with a total volume of 1 mL. The reaction mixture was incubated at 30 °C for 30 min with continuous stirring. Following the inactivation of the enzyme at 80 °C for 10 min, the lipids were extracted by the Bligh and Dyer method [22] and subjected to a TLC plate developed with chloroform: methanol: 28% NH_3 (60:35:8, v/v). The PC1P band was visualized under UV light after primulin spray. The pixel intensity of the digital image of PC1P band on the TLC plate was obtained by Image J software. The amount of PC1P was determined based on its value relative to that of the standard PC1P band [23].

SDS-PAGE

SDS-PAGE was carried out on a slab gel of a 10% acrylamide. Samples of 0.015 mL (0.6 to 1 μg of protein) were mixed with an equal volume of 50 mM dithiothreitol containing 2X Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA), and heated at 80 °C for 20 min. Electrophoresis was conducted at a constant current of 200 V/15 mA in a running buffer containing 0.1% SDS. The gel was stained with silver staining kits according to the manufacturer's instructions.

N-terminal amino acid sequence

The candidate of GIPC-PLD protein band (50 kDa protein) was excised from the gel, digested with trypsin, and subjected to nano-LC/MS/MS analysis according to standard protocol. The analysis was performed by Japan Proteomics [Sendai, Japan].

Cloning, sequencing, and expression of NPC3 gene from radish

The radish cDNA containing the full-length coding region of NPC3 was chemically synthesized by Eurofins Genomics (Tokyo, Japan). The NPC3 cDNA was cloned into the pBAD-DEST49 vector with a His-tag at the C-terminal and a His-patched thioredoxin (HP-Trx)-tag at the N-terminal and transformed into *E. coli* strain TOP10 according to the manufacturer's instructions (Thermo Fisher Scientific, MA). As the expression of recombinant NPC3 can cause severe toxicity in *E. coli* under normal culture conditions, the induction was conducted at 12 °C with 0.2% arabinose after growth at the exponential phase in the presence of 0.1% glucose. Cells were resuspended in Tris-buffered saline (pH 8.0) containing 20 mM imidazole and 1 $mg.mL^{-1}$ lysozyme and disrupted by ultrasonication. Cell debris was removed by centrifugation, and the

recombinant NPC3 protein was purified using Ni-NTA agarose according to the manufacturer's protocol. Purified protein was desalted and subjected to 10% (w/v) SDS-PAGE, and the gel was stained using Coomassie brilliant blue.

Enzyme assay with recombinant NPC3

The recombinant enzyme (0.025 mL) was incubated with 26 nmol of each substrate and 2 mg of NaDOC (4.6 mM) in 50 mM Tris-HCl buffer (pH 7.5) with a final volume of 1 mL under above indicated condition. The lipids were recovered by the Bligh and Dyer method, and separated by TLC using chloroform: methanol: 28% NH_3 (60:35:8, v/v) for PC1P and PA, and chloroform: methanol: 28% NH_3 (85:15:1, v/v) for PCer as developing solvents. The PLD or PLC products were quantified using Image J software as described in the GIPC-PLD assay section. To determine the optimum pH, the recombinant enzyme assay was performed using 100 mM sodium acetate buffer (pH 4.5–5.5), 100 mM potassium phosphate buffer (pH 6.7–7.5), and 100 mM Tris-HCl buffer (pH 7.5–8.5). The following compounds were added to evaluate the effect on recombinant enzyme activity: 2 mg of NaDOC (4.6 mM), 0.5 mL of diethyl ether (DE), 10 mM of $CaCl_2$, and 1 mM of EGTA.

Transphosphatidylation assay

The recombinant enzyme (0.025 mL) was incubated with purified GIPC (52 nmol) from cabbage in the presence of various alcohols (20% by volume) in a total volume of 1 mL under the standard experimental conditions as described above. The resultant products were separated by TLC with chloroform: methanol: 28% NH_3 (60:35:8, v/v) and quantified using Image J software as described above.

MALDI-TOF MS

The structure of PC1P formed by recombinant NPC3 was analyzed using MALDI-TOF MS with phos-tag, as previously described [17,18]. An aliquot of PC1P was dissolved in 100 µL of methanol containing 0.3% ammonia. The resulting solution (10 µL) was mixed with 5 µL of 0.1 mM ^{68}Zn phos-tag solution, and a small portion (0.5 µL) of this mixture was spotted on the MALDI sample plate. Immediately, 0.5 µL of 2,4,6-trihydroxyacetophenone (THAP) solution (10 $mg.mL^{-1}$ in acetonitrile) was layered on the mixture as the matrix solution. After drying the sample plate

for a few minutes, the matrix/analyte was subjected to MALDI-TOF MS (Bruker Daltonics, Bremen, Germany) in the positive-ion detection mode.

Statistical analysis

All results were expressed as mean ± SD. The significant differences between the two means were calculated using Student's t-test. *$p < 0.05$ and ***$p < 0.005$ were defined as statistically significant.

Results

Purification and identification of GIPC-PLD

We attempted to purify GIPC-PLD protein from radish roots. The types of chromatography used were weak anion-exchange (DEAE-Cellulose), strong anion-exchange (Giga-Cap Q-650), hydrophobic chromatography (Butyl-650, phenyl-650), gel-filtrate (Superdex 200 HR 10/300 column, GE Healthcare), and affinity chromatography (Ceramic Hydroxy apatite). Typical results of the attempt were shown in Fig. 2A–C. Proteins in the 13,000 × *g* supernatant fraction of the radish root were separated by three steps column chromatography. Firstly, GIPC-PLD activity was eluted at 200 mM NaCl fractions in the stepwise Giga-Cap column chromatography (Fig. 2A). The active fractions were combined and subjected to Butyl column chromatography. The GIPC-PLD activity was eluted at 10–5% ammonium sulphate fractions (Fig. 2B). The active fractions were combined and subjected to linear gradient NaCl Giga-Cap column chromatography. The GIPC-PLD activity was eluted at fractions 10.5–12.5 (Fig. 2C). Among these, the fraction 11.5 showed the highest activity with the specific activity of 16,667 nmol.mg^{-1} protein, which was 120-fold higher than that of the 13,000 × *g* supernatant (Table 1). Fractions 10–13.5 were applied to SDS-PAGE and stained. As shown in Fig. 2D, several bands were detected in the gel. The protein band corresponding to 50 kDa (indicated with the red arrow) were seemed to be consistent with the elution profile of the GIPC-PLD activity. This notion was supported by our other experimental results of GIPC-PLD purification which had been conducted with separation modes other than anion-exchange and hydrophobicity (data not shown).

Peptide mass fingerprinting analysis (Mascot analysis) was performed for the isolated 50 kDa band. Results showed that the probable candidate for this 50 kDa protein in the radish was a protein named NPC3 (non-specific phospholipase C3, LOC108863372) based on the radish (*Raphanus sativus*) genome database search. This probability was supported by the fact that peptides detected in Mascot analysis covered 42% of the whole sequences of NPC3 (red letter in Fig. 2E), and that the probability-based score for NPC3 as the candidate was 600 (Fig. 2E).

Expression and kinetic characterization of the recombinant NPC3

Chemically synthesized cDNA of the radish NPC3 gene was introduced into the pBAD-DEST49 vector and transfected into *E. coli* as described in the Materials and Methods. Most of the tested bacterial expression systems failed to produce the active enzyme protein, except for the

arabinose-inducible HP-Trx tagging system. The recombinant protein was extracted from vector-transfected *E. coli*, purified using Ni-NTA column chromatography, and subjected to SDS-PAGE. As shown in Fig. 3, two bands, located around 60−75 kDa, were detected in the final fraction. Possible assignments of these proteins were recombinant NPC3 with HP-Trx and His6 (A), and NPC3 with His6 (B) with a molecular weight of 74.1 and 61.3 kDa, respectively (Fig. 3).

GIPC-PLD assay was performed with this recombinant NPC3 fraction. We found that PC1P was formed from GIPC with NPC3-concentration dependent manner (Fig. 4A, B) and substrate-dependent fashion (Fig. 4C, E). PCer was not formed at any concentrations of NPC3 (Fig. 4B) or GIPC (Fig. 4D, E), indicating that recombinant NPC3 possesses GIPC-PLD activity but not GIPC-PLC activity. After quantification of PC1P using Image J software, we found that PC1P was formed in a saturable fashion at higher GIPC concentration (Fig. 4E). We confirmed the structure of PC1P by MALDI-TOF MS with phos-tag, which binds to compounds having phosphate monoester [17]. We found that the molecular species of PC1P formed were *t*18:1/h16:0, *t*18:1/h22:0, *t*18:1/h24:1, *t*18:1/h24:0 as ceramide backbone (Fig. 4G). The relative abundance of these molecular species, deduced from intensities of the peaks, was essentially the same as those of GIPC from cabbage used as substrate [17,18]. The K_m and V_{max} values of the recombinant NPC3 activity towards GIPC, as calculated from the Lineweaver-Burk plot (Fig. 4F), were 162 μM and 75 nmol.min^{-1}, respectively. This K_m value of NPC3 for GIPC-PLD activity was similar to that obtained in our previous study with partially purified GIPC-PLD activity from cabbage (110 μM) [17].

Substrate specificity of NPC3

The substrate specificity of the recombinant NPC3 was also examined (Fig. 5). We found that both GIPC and PI were good substrates for the recombinant NPC3. Detectable hydrolysates were not found in the reaction with SphM, PC, and PE. These results were in good agreement with our previous results on partially purified GIPC-PLD activity from cabbage except for results with PI [17,18]. When the reaction was performed with PI, the hydrolysate was exclusively phosphatidic acid (PA) but not diacylglycerol (DAG) (data not shown), indicating that NPC3 shows PLD but not phospholipase C (PLC) activity.

pH dependency, cofactor requirement, and transphosphatidylation activity of recombinant NPC3

The optimum pH of the recombinant NPC3 was found to be in the neutral range (Fig. 6A). We found that the recombinant enzyme activity was stimulated by the addition of NaDOC and DE, whereas Ca^{2+} or EGTA did not affect the enzyme activity (Fig. 6B). These results are consistent with the characteristics of partially purified GIPC-PLD activity in cabbage and *Arabidopsis* [17,18].

The transphosphatidylation activity of recombinant NPC3 was characterized. As shown in Fig. 6C, the transphosphatidylation products were detected on TLC when 20% (v/v) of methanol, ethanol, 1-propanol, 1-butanol, or 2-methyl-1-propanol were present in the reaction mixture of GIPC-PLD assay performed with recombinant NPC3. Whereas transphosphatidylation could not be observed against 1-pentanol, 2-propanol, 2-butanol, choline, glycerol, and inositol. After quantitative analysis of these products, we found that this recombinant enzyme prefers primary alcohols below chain length C4, whereas secondary alcohols or alcohol with bulky moiety could not serve as acceptors for the transphosphatidylation of the recombinant enzyme (Fig. 6D). These results are comparable to those of our previous studies on the activity of partially purified GIPC-PLD activity from cabbage [19].

Discussion

Previously, we discovered an uncharacterized sphingolipid that was produced in response to the homogenization of young cabbage leaves and identified it to be PC1P [17]. We also found an enzyme activity that hydrolyzes the D position of GIPC to produce PC1P in cabbage leaves. This enzymatic activity was considerably high. Indeed, almost all GIPC disappeared and the resulting PC1P increase to account for 10% of the total phospholipids in the homogenates of the young cabbage leaves [18]. In this study, we purified GIPC-PLD from radish roots, and found that the GIPC-PLD activity was mediated by the enzymatic activity of NPC3. This conclusion is supported by following enzymatic characteristics. 1) Recombinant NPC3 hydrolyzed the D position of GIPC with a K_m value of 162 μM. This value is close to that of our previous results for GIPC-PLD from cabbage (110 μM) [17]. 2) The pH dependency of this recombinant protein was neutral, consistent with our earlier results [17]. 3) The PLD activity of the recombinant protein was enhanced in the presence of detergent (NaDOC) or organic solvent (DE), whereas divalent cations were not essential. These characteristics are also compatible with those of our previous study on cabbage enzyme [18]. 4) The preferable substrate for transphosphatidylation reaction of the recombinant NPC3 was found to be short chain primary alcohols, whereas secondary or relatively bulky alcohols were not. This substrate preference was essentially the same as that observed in our earlier study of the transphosphatidylation reaction of GIPC-PLD from cabbage [19].

The substrate specificity of recombinant NPC3 toward phospholipids was somewhat different from that we described in our previous reports, in which cabbage GIPC-PLD did not hydrolyze PI [17]. Here, we found that PI was also a good substrate for recombinant NPC3. During purification, we noticed that GIPC-PLD activity from both cabbage and radish could hydrolyze PI as well as GIPC, indicating that substrate specificity of recombinant NPC3 was essentially the same as that of GIPC-PLD in the plants. The enzymatic assay performed in our previous report with PI as the substrate must not have been adequately performed [17]. Probably, the amount of PA produced was underestimated.

A number of PLDs from many organisms have been cloned and sequenced. Many of them have a consensus amino acid sequence at the catalytic domain, called the HKD domain (HxKxxxxD). It is also known that HKD-type PLD performs the transphosphatidylation reaction in the presence of a low concentration of alcohols (0.1–2% in the reaction mixture) [24]. The amino acid sequences of NPC3 from Brassica plants have HxKxD and HxKD at positions 264–268, and

447–450, respectively (Fig. 2E). Although these sequences do not exactly coincide with the HxKxxxxD motif, they may work as catalytic domains for the cleavage of inositol-phosphate bonds.

NPC3 has been categorized as a member of the PLC family [25,26]. However, as far as we know, there are no reports showing PLC activity in NPC3 protein. Here, we found that recombinant NPC3 from radish shows PLD activity but no detectable PLC activity toward any of the phospholipids tested. Reddy et al. have reported that recombinant NPC3 of *A. thaliana* shows lysophosphatidic acid (LPA) phosphatase activity [27]. However, we could not observe LPA phosphatase activity in recombinant NPC3 from radish in our assay system [data not shown]. Radish has two homologous NPC3 genes (named NPC3a, identified in this study, and homologous NPC3b); and NPC3a showed 96.1%, sequence similarity with NPC3 from *A. thaliana* (Fig. 7, Table 2). This small difference in the protein structure may explain the difference in the catalytic properties of NPC3 from radish and *A. thaliana*. In this regard, an examination of whether *A. thaliana* NPC3 has PLD activity is required.

The *Arabidopsis* NPC3 knockout mutant was generated by Wimalasekera et al. [28]. They found that AtNPC3 was implicated with brassinolide-induced root growth. Indeed, gene expression of AtNPC3 has been shown to be higher in roots [28,29]. This is compatible with our previous results showing that GIPC-PLD activity was higher in roots of many plants, such as radish, cabbage, *A. thaliana*, Japanese mustard spinach, and broccoli [18].

In this study, we found that the recombinant NPC3 could hydrolyze GIPC to produce PC1P. This protein could also act on PI to form PA. Several researchers have hypothesized that these hydrolysates of phospholipids may act as second messengers in plant development [13,30,31]. The products of NPC3 might have such functions in plants. Considering the location of GIPC-PLD activity in growing tissues such as roots, young leaves, and sprouts, in plants [18], it may be involved in the proliferation of plant cells by transducing extracellular stimuli via intracellular messengers.

Six types of NPC genes (NPC1 to NPC6) have been identified in the *Arabidopsis thaliana* genome [25,26,32]. Among these, NPC3 and NPC4 are the most closely related. Indeed, the amino acid similarities between them are 69.3% (67.4% identity between NPC3 and NPC5, and 52.8% between NPC3 and NPC6) (https://www.uniprot.org/uniprotkb/A0A6J0P8G3/entry, Table 2) [33]. Recently, Yang et al. have reported that *Arabidopsis* NPC4 exhibited GIPC-PLC activity. They

also showed evidence that NPC4 implicates with phosphate metabolism during phosphate-deficient condition [34]. They hypothesized that converting GIPC to glucosylceramide via the GIPC-PLC activity of NPC4 is operating for supplying phosphate. It would be interesting to examine whether other NPC family members also have catalytic activity toward sphingolipids.

In conclusion, we identified that the gene encodes GIPC-PLD activity, which was characterized in Brassica plants, was NPC3. However, the physiological functions of GIPC-PLD remain unclarified. Recombinant NPC3 may help to elucidate the regulatory mechanism of GIPC-PLD and to develop its selective inhibitors. Future studies will contribute to a better understanding of the physiological significance of GIPC hydrolysis in plants.

Acknowledgements

This work was supported by grants-in-aid from the Ministry of Education, Science, Sports, and Culture of Japan (19K05863 to T.T.), Kobayashi Foundation (to T. T.), Research grant of Research Institute of Konan University (to H.I and T.T).

Authors contribution

Rumana Yesmin Hasi: Conceptualization, Investigation, Data curation, Writing original draft and editing.

Toshiki Ishikawa: Conceptualization, Investigation, Data curation, Writing - review & editing.

Keigo Sunagawa: Investigation.

Yoshimichi Takai: Investigation.

Hanif Ali: Investigation, Writing - review & editing.

Junji Hayashi: Investigation, Resources, Writing - review & editing.

Ryushi Kawakami: Resources, Writing - review & editing.

Keizo Yuasa: Resources, Methodology.

Mutsumi Aihara: Investigation, Writing - review & editing

Kaori Kanemaru: Resources, Investigation, Writing – review & editing

Hiroyuki Imai: Conceptualization, Funding acquisition.

Tamotsu Tanaka: Conceptualization, Project administration, Resources, Funding acquisition, Data curation, supervision, Writing – review & editing

References

1. Gronnier J, Germain V, Gouguet P, Cacas JL and Mongrand S (2016) GIPC: Glycosyl Inositol Phospho Ceramides, the major sphingolipids on earth. *Plant Signal Behav* **11**, e1152438.
2. Buré C, Cacas JL, Mongrand S and Schmitter JM (2014) Characterization of glycosyl inositol phosphoryl ceramides from plants and fungi by mass spectrometry. *Anal Bioanal Chem* **406**, 995–1010.
3. Cacas JL, Buré C, Grosjean K, Gerbeau-Pissot P, Lherminier J, Rombouts Y, Maes E, Bossard C, Gronnier J, Furt F, Fouillen L, Germain V, Bayer E, Cluzet S, Robert F, Schmitter JM, Deleu M, Lins L, Simon-Plas F and Mongrand S (2016) Revisiting Plant Plasma Membrane Lipids in Tobacco: A Focus on Sphingolipids. *Plant Physiol* **170**, 367–384.
4. Ishikawa T, Ito Y and Kawai-Yamada M (2016) Molecular characterization and targeted quantitative profiling of the sphingolipidome in rice. *Plant J* **88**, 681–693.
5. Carter HE, Gigg RH, Law JH, Nakayama T and Weber E (1958) Biochemistry of the Sphingolipides. *Biol Chem J* **233**, 1309–1314.
6. Bromley PE, Li YO, Murphy SM, Sumner CM and Lynch DV (2003) Complex sphingolipid synthesis in plants: characterization of inositolphosphorylceramide synthase activity in bean microsomes. *Arch Biochem Biophys* **417**, 219–226.
7. Wang W, Yang X, Tangchaiburana S, Ndeh R, Markham JE, Tsegaye Y, Dunn TM, Wang GL, Bellizzi M, Parsons JF, Morrissey D, Bravo JE, Lynch DV and Xiao S (2008) An inositolphosphorylceramide synthase is involved in regulation of plant programmed cell death associated with defense in *Arabidopsis*. *Plant Cell* **20**, 3163–3179.
8. Rennie EA, Ebert B, Miles GP, Cahoon RE, Christiansen KM, Stonebloom S, Khatab H, Twell D, Petzold CJ, Adams PD et al. (2014) Identification of a sphingolipid a-glucuronosyltransferase that is essential for pollen function in *Arabidopsis*. *Plant Cell* **26**, 3314–3325.
9. Liu C, Zhu M, and Sun J. (2022) Overexpression of an Inositol Phosphorylceramide Glucuronosyltransferase Gene IbIPUT1 Inhibits Na^+ Uptake in Sweet Potato Roots. *Genes* **13**, 1140.
10. Fang L, Ishikawa T, Rennie EA, Murawska GM, Lao J, Yan J, Tsai AY-L, Baidoo EE, Xu J and Keasling JD (2016) Loss of inositol phosphorylceramide sphingolipid mannosylation

induces plant immune responses and reduces cellulose content in *Arabidopsis*. *Plant Cell* **28**, 2991–3004.

11. Voxeur A and Fry SC (2014) Glycosylinositol phosphorylceramides from Rosa cell cultures are boron-bridged in the plasma membrane and form complexes with rhamnogalacturonan II. *Plant J* **79**, 139–149.
12. Morita N, Nakazato H, Okuyama H, Kim Y and Thompson GA Jr (1996) Evidence for a glycosylinositolphospholipid-anchored alkaline phosphatase in the aquatic plant Spirodela oligorrhiza. *Biochim Biophys Acta* **1290**, 53–62.
13. Oxley D and Bacic A (1999) Structure of the glycosylphosphatidylinositol anchor of an arabinogalactan protein from *Pyrus communis* suspension-cultured cells. *Proc Natl Acad Sci U S A* **96**, 25.
14. Jiang Z, Zhou X, Tao M, Yuan F, Liu L, Wu F, Wu X, Xiang Y, Niu Y and Liu F et al. (2019) Plant cell- surface GIPC sphingolipids sense salt to trigger Ca^{2+} influx. *Nature* **572**, 341–346.
15. Cassim AM, Gouguet P, Gronnier J, Laurent N, Germain V, Grison M, Boutté Y, Gerbeau-Pissot P, Simon-Plas F and Mongrand S (2019) Plant lipids: Key players of plasma membrane organization and function. *Prog Lipid Res* **73**, 1–27.
16. Cassim AM, Navon Y, Gao Y, Decossas M, Fouillen L, Grelard A, Nagano M, Lambert O, Bahammou D and Van Delft P et al. (2020) Purification, characterization and influence on membrane properties of the plant-specific sphingolipids GIPC Running title: Plant sphingolipids GIPCs. *BioRxiv*. https://doi.org/10. 1101/2020.10.01.313304.
17. Tanaka T, Kida T, Imai H, Morishige J, Yamashita R, Matsuoka H, Uozumi S, Satouchi K, Nagano M and Tokumura A (2013) Identification of a sphingolipid-specific phospholipase D activity associated with the generation of phytoceramide 1-phosphate in cabbage leaves. *FEBS J* **280**, 3797–3809.
18. Kida T, Itoh A, Kimura A, Matsuoka H, Imai H, Kogure K, Tokumura A and Tanaka T (2017) Distribution of glycosylinositol phosphoceramide-specific phospholipase D activity in plants. *Biochem J* **161**, 87–195.
19. Hasi RY, Miyagi M, Morito K, Ishikawa T, Kawai-Yamada M, Imai H, Fukuta T, Kogure K, Kanemaru K, Hayashi J, Kawakami R and Tanaka T (2019) Glycosylinositol phosphoceramide-specific phospholipase D activity catalyzes transphosphatidylation. *Biochem J* **166**, 441–448.

20. Hasi RY, Majima D, Morito K, Ali H, Kogure K, Nanjundan M, Hayashi J, Kawakami R, Kanemaru K and Tanaka T (2020) Isolation of glycosylinositol phosphoceramide and phytoceramide 1- phosphate in plants and their chemical stabilities. *Chrom*. *B* **1152**, 122213.
21. Markham JE, Li J, Cahoon EB and Jaworski JG (2006) Separation and Identification of Major Plant Sphingolipid Classes from Leaves. Biol. Chem J 281, 22684–22694.
22. Bligh EG & Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Biochem Physiol J* **37**, 911–917
23. Tanaka T, Kassai A, Ohmoto M, Morito K, Kashiwada Y, Takaishi Y, Urikura M, Morishige J, Satouchi K and Tokumura A (2012) Quantification of Phosphatidic Acid in Foodstuffs Using a Thin-Layer-Chromatography-Imaging Technique. *Agric. Food Chem J* **16**, 4156–4161.
24. Selvy PE, Lavieri RR, Landsley CW and Brown HA (2011) Phospholipase D: Enzymology, Functionality, and Chemical Modulation. *Chem. Rev* **111**, 6064–6119.
25. Nakamura Y and Nago AH (2021) Non-specific phospholipase C (NPC): an emerging class of phospholipase C in plant growth and development. *Plant Res. J* **133,** 489–497**.**
26. Sagar S and Singh A (2021) Emerging role of phospholipase C mediated lipid signaling in abiotic stress tolerance and development in plants. *Plant Cell Rep* **40**, 2123–2133.
27. Ready VS, Rao D.K V and Rajasekharan R (2010) Functional characterization of lysophosphatidic acid phosphatase from *Arabidopsis thaliana*. *Biochim. Biophys. Acta* **1801**, 455–461.
28. Wimalasekeraa R, Pejcharb P, Holka A, Martinecb J and Scherer GFE (2010) Plant Phosphatidylcholine-Hydrolyzing Phospholipases C NPC3 and NPC4 with Roles in Root Development and Brassinolide Signaling in *Arabidopsis thaliana*. *Molecular Plant* **3**, 610–625.
29. Peters C, Li M, Narasimhan R, Roth M, Welti R and Wang X (2010) Nonspecific Phospholipase C NPC4 Promotes Responses to Abscisic Acid and Tolerance to Hyperosmotic Stress in *Arabidopsis*. *Plant Cell* **22**, 2642–2659.
30. Smith CK and Fry SC (1999) Biosynthetic origin and longevity in vivo of œ-D-mannopyranosyl-(1→4)-œ-D-glucuronopyranosyl-(1→2)-myo-inositol, an unusual extracellular oligosaccharide produced by cultured rose cells. *Planta* **210**, 150–156.
31. Hong, Y, Devaiah, SP, Bahn SC, Thamasandra, BN, Li M, Welti R, and Wang X (2009) Phospholipase D and phosphatidic acid enhance *Arabidopsis* nitrogen signaling and growth. *Plant J* **58**, 376–387.

32. Pokotylo I, Pejchar P, Potocký M, Kocourková D, Krčková Z, Ruelland E, Kravets V and Martinec J (2013) The plant non-specific phospholipase C gene family. Novel competitors in lipid signaling. *Prog Lipid Res* **52**, 62–79.
33. Nakamura Y, Awai K, Masuda T, Yoshioka Y, Takamiya K and Ohta H (2005) A novel phosphatidylcholine-hydrolyzing phospholipase C induced by phosphate starvation in *Arabidopsis*. *Biol Chem J* **280**, 7469–7476
34. Yang B, Li M, Phillips A, Li L, Ali U, Li Q, Lu S, Hong Y, Wang X and Guo L (2021) Nonspecific phospholipase C4 hydrolyzes phosphosphingolipids and sustains plant root growth during phosphate deficiency. *Plant Cell* **33**, 766–780.

Table 1. Purification table of GIPC-PLD from radish roots.

Purification steps	Protein Contents (mg)	Total Activity (nmol.30 min^{-1})	Yield (%)	Specific Activity (nmol.mg^{-1} protein.30 min^{-1})	Purification (Fold)
Cytosolic crude (13000 × *g* supernatant)	252	35,000	100	139	1.00
60% ammonium sulphate precipitate	172	28,000	80	163	1.2
Giga-Cap (I)	10	20,302	58	2,030	15
Butyl column	0.71	3,284	9	4,621	33
Giga-Cap (II)	0.03	500	1.4	16,667	120

Table 2. Amino acid sequence similarities between *Raphanus sativus* (Rs) and *Arabidopsis thaliana* (At).

Name	Amino acid sequence similarity
RsNPC3a – RsNPC3b	99.9%
RsNPC3a – RsNPC4	63.2%
RsNPC3a – AtNPC3	96.1%
AtNPC3 – AtNPC4	69.3%
RsNPC4 – AtNPC4	89%

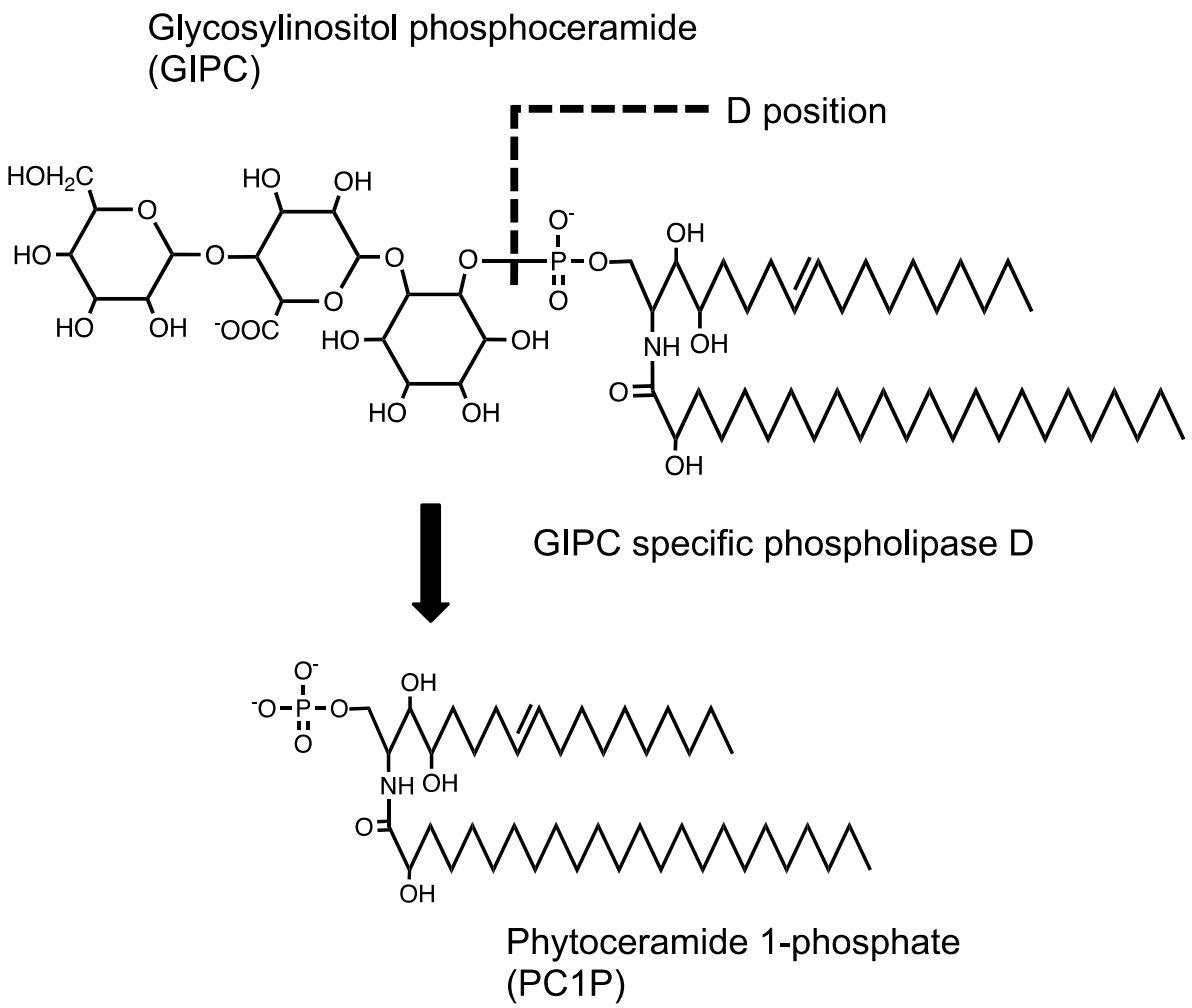
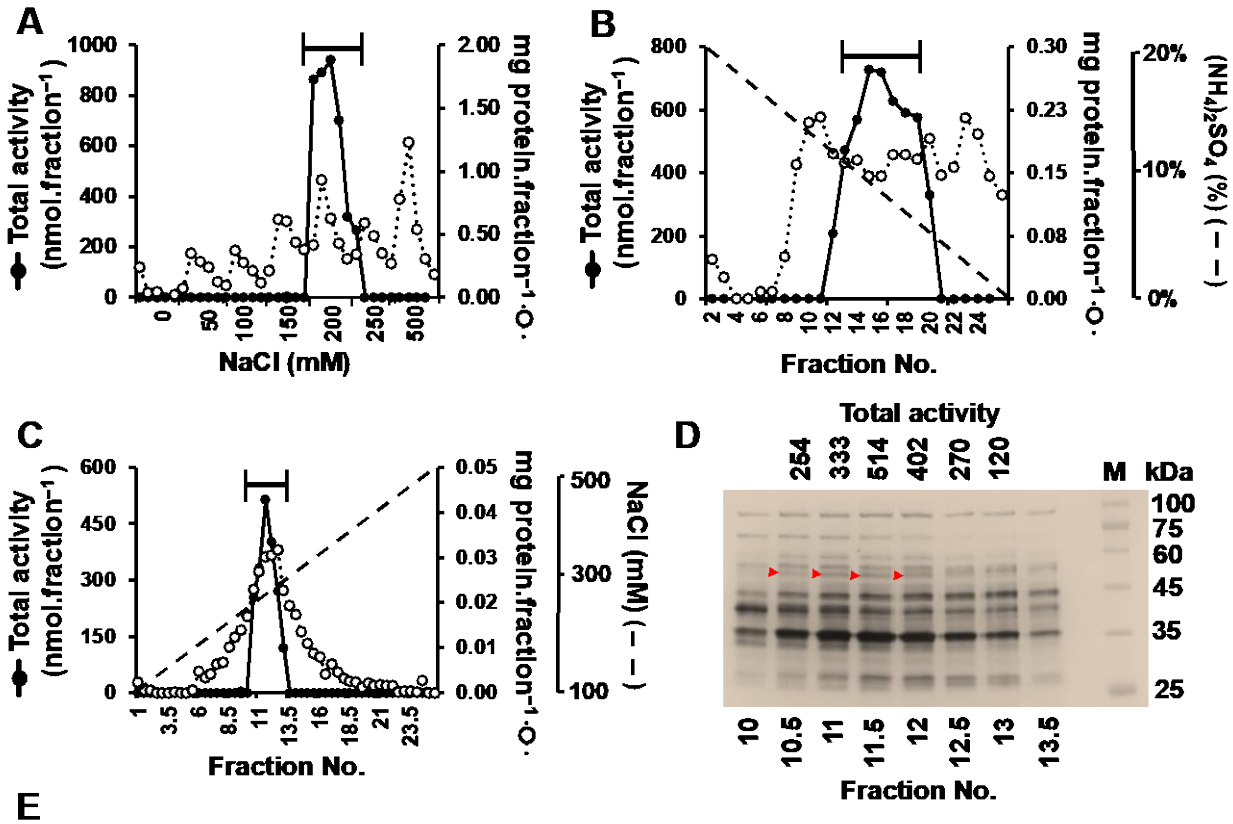


Fig. 1. Formation of PC1P from GIPC-PLD activity.



E

```
  1 MVEETSSGGS SSASPIRTIV VLVQENRSFD HMLGWFKELN PEIDGVSESE
 51 PRSNPVSTSD PNSAQVFFGK ESQNIDPDPG HSFQAIYEQV FGKPFSDESP
101 YPAPTMNGFV QNAEAITPGM SEKVVMQGFP PERLPVFKEL VQEFAVCDRW
151 FSSLPSSTQP NRLYVHSATS NGAFSNDTNT LVRGFPQKTV FESLEESGFT
201 FGIYYQSFPN CLFYRNMRKL KYVDNFHQYQ LSFKRHCKEG KLPNYVVIEP
251 RYFKILSAPA NDDHPKNDVA EGQNLVKEIY EALRASPQWN EILFVVVYDE
301 HGGYYDHVPT PVTGVPNPDG LVGGPPFNFK FDRLGVRVPA LLISPWIEPG
351 TVLHEPNGPE PTSQFEHSSI PATLKKIFNL KSFLTKRDEW AGTFDAVINR
401 TSPRTDCPVT LPELPRARAI GTQEEDEDLT DFQIELIQAA AVIRGDHIKD
451 IYPLKLVDNM KVSDAAKYVE EAFTKFYGES KKAKEVGRDE HEIVDLSHGT
501 TRHSSPKSFI QKFFSCLICD N
```

Fig. 2. Purification and identification of GIPC-PLD.

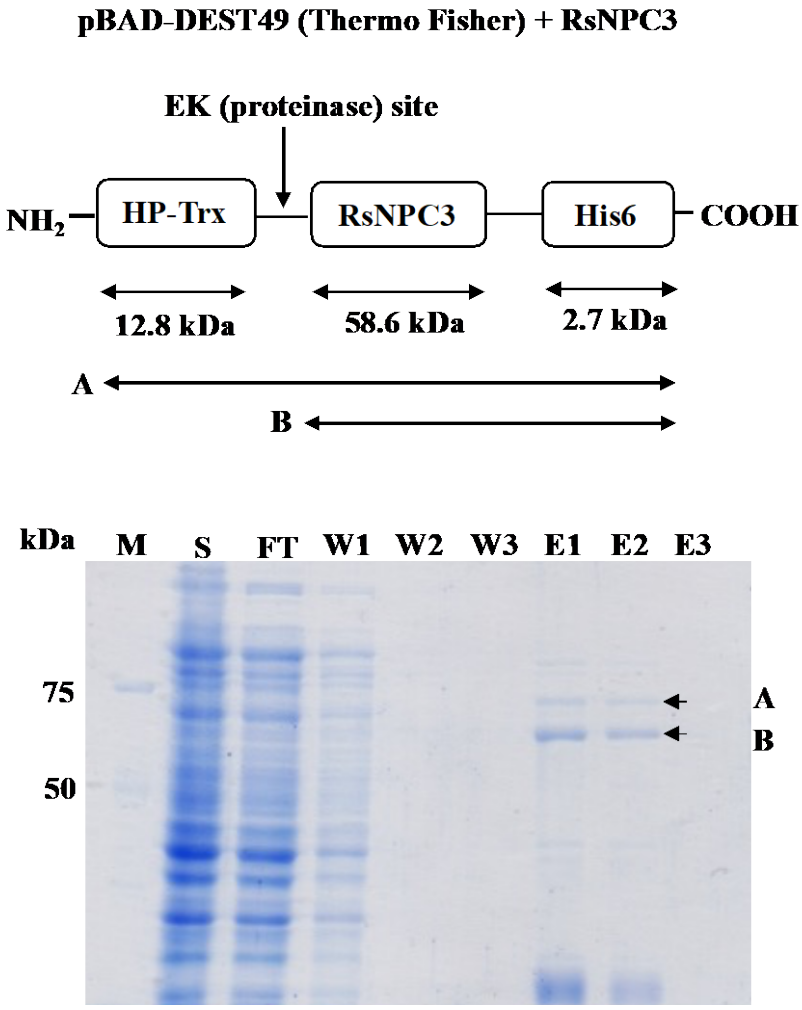


Fig. 3. Cloning, expression, and purification of the NPC3 gene.

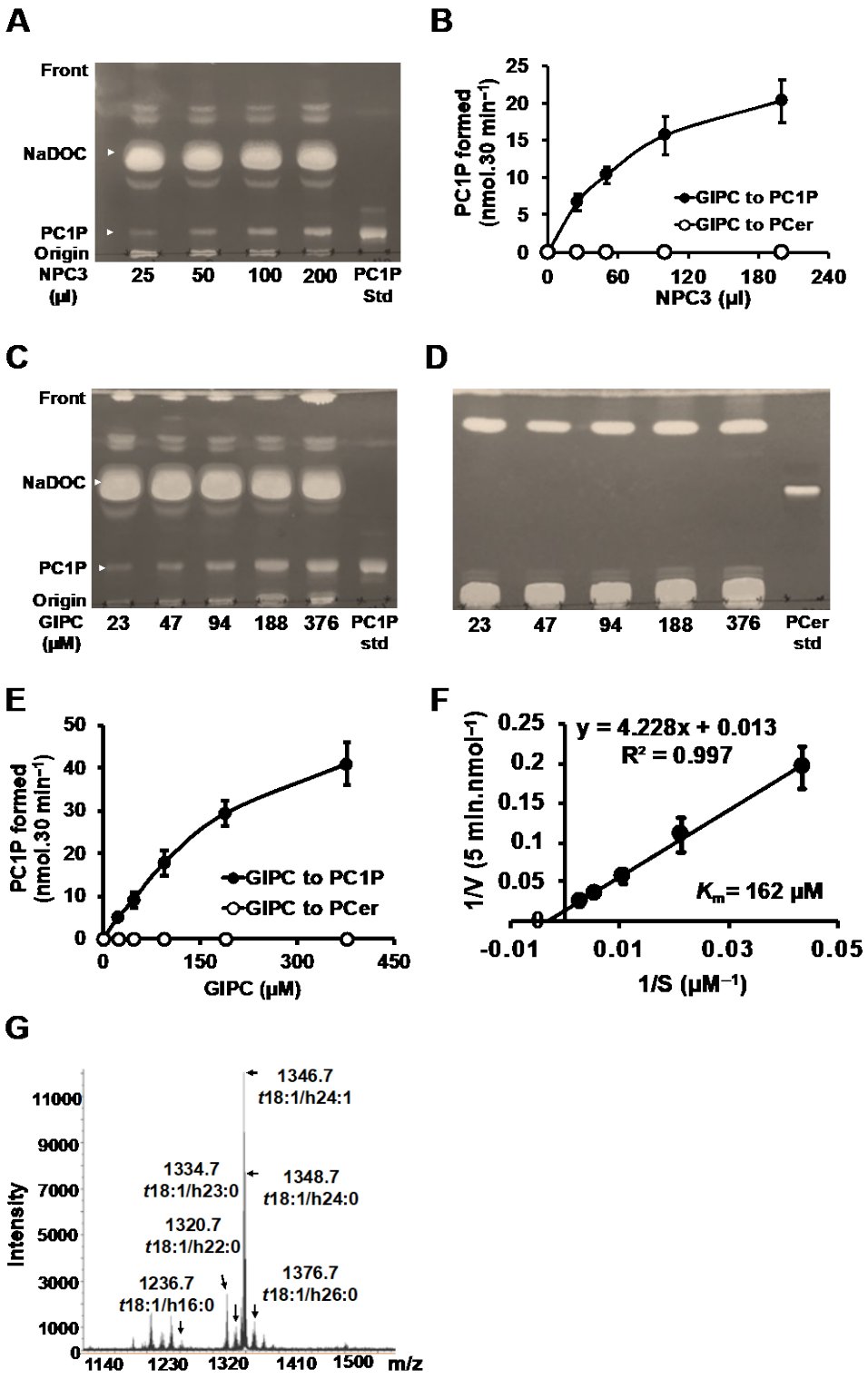


Fig. 4. Formation of PC1P but not PCer by recombinant NPC3 expressed in *E. coli* at various concentrations of recombinant NPC3 and GIPC.

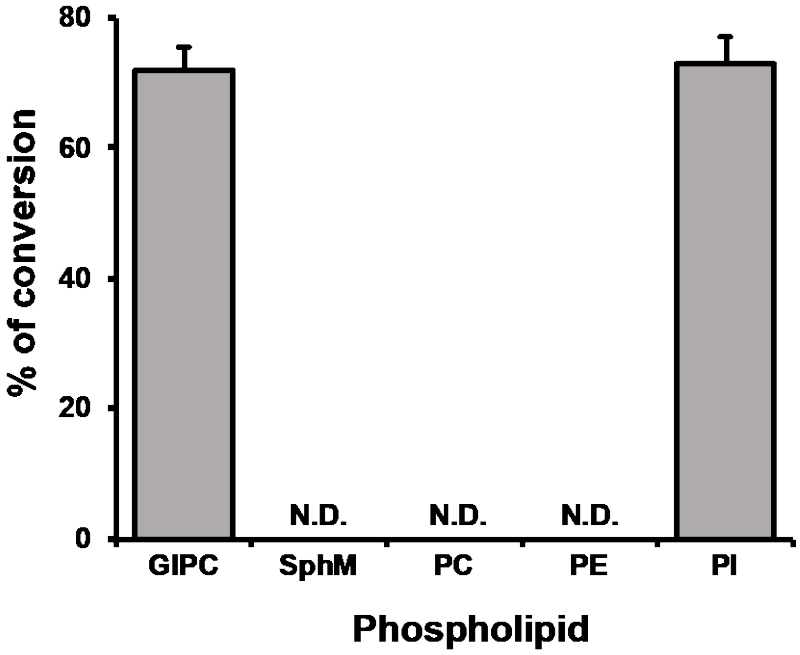


Fig. 5. Substrate preference of recombinant NPC3 on phospholipids.

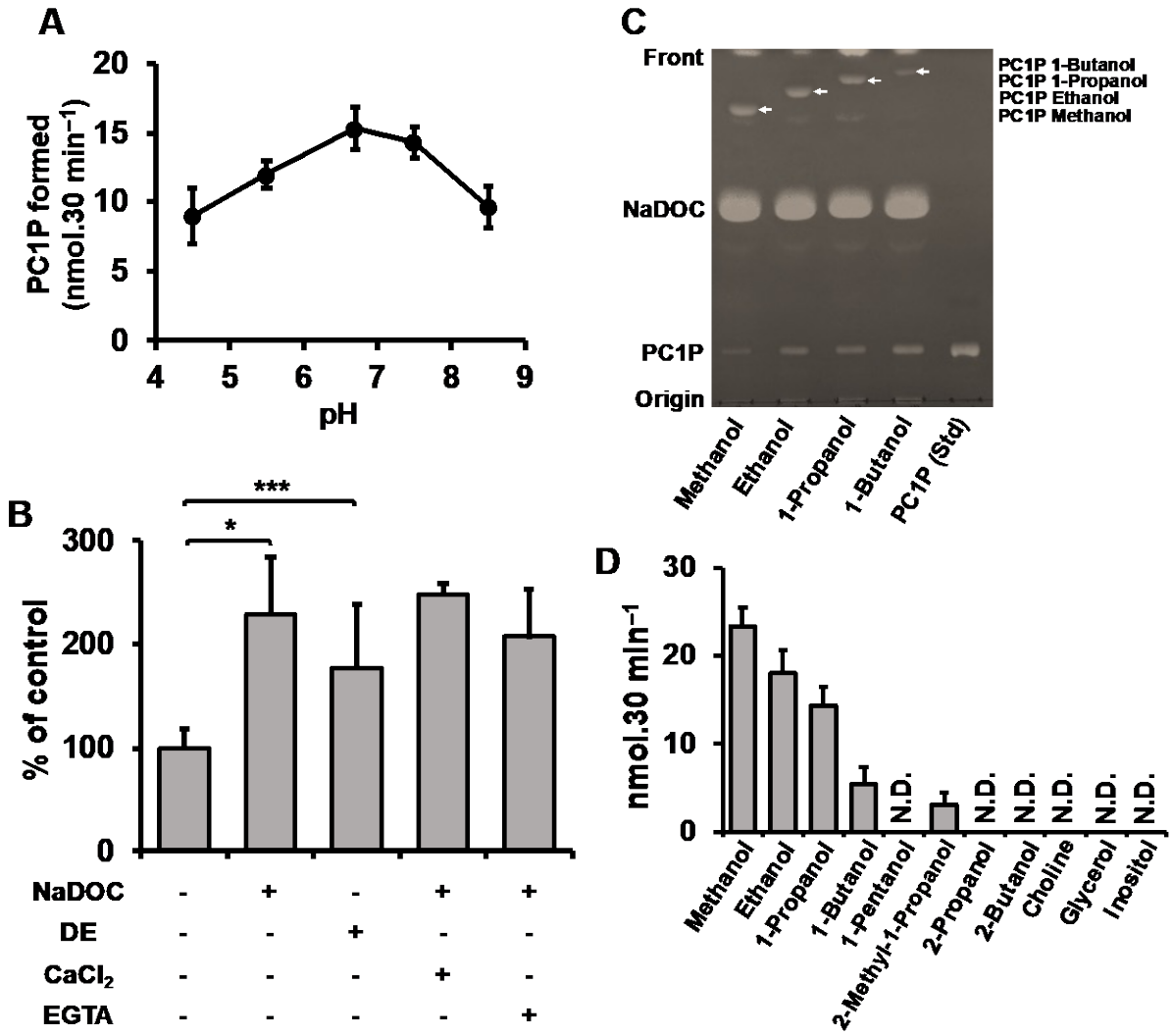


Fig. 6. Biochemical characterization of *E. coli* expressing NPC3.

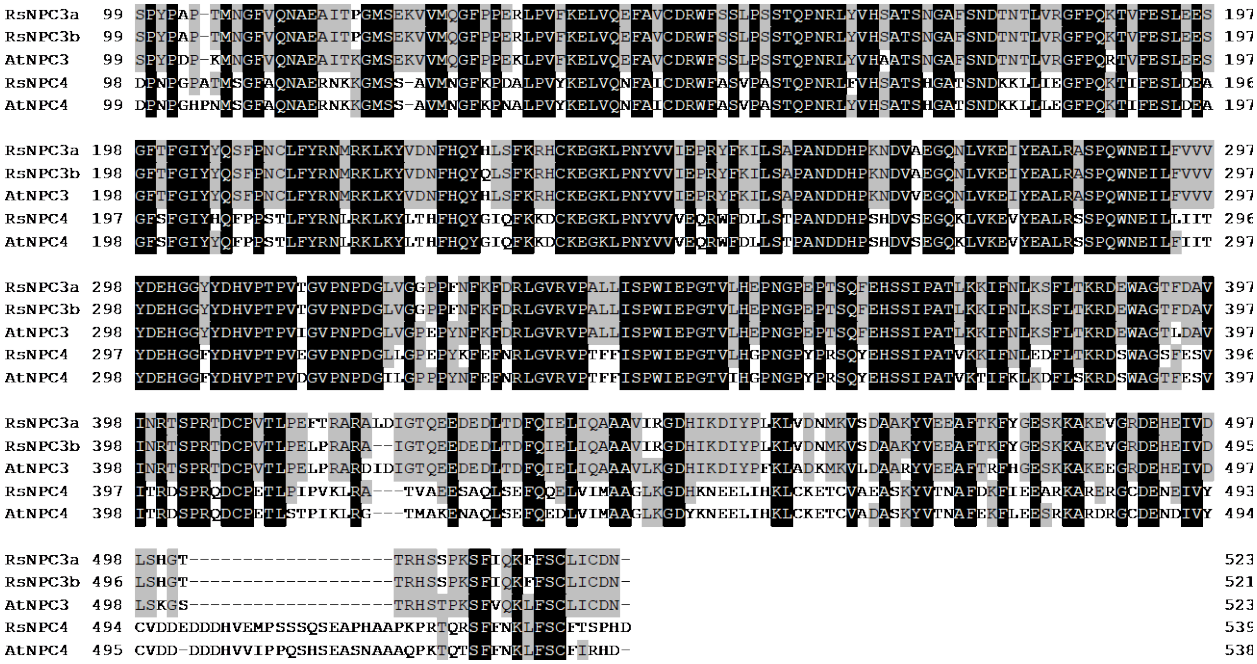


Fig. 7. Alignment of the deduced amino acid sequences of NPC3 and NPC4 from *Raphanus sativus* (Rs) and *Arabidopsis thaliana* (At) (Genetyx software, GENETYX Corporation, Tokyo, Japan).

Figure legends

Fig. 1. Formation of PC1P from GIPC-PLD activity.

Fig. 2. Purification and identification of GIPC-PLD.
Elution profile of GIPC-PLD activity on (A) stepwise Giga-Cap column, (B) Butyl column and (C) Linear Giga-Cap column. GIPC-PLD activity (nmol.fraction^{-1}, -●-), protein amounts (mg.fraction^{-1}, -○-), and $(NH_4)_2SO_4$ (%) or NaCl (mM) concentration (- - -). (D) The active fraction from linear Giga-Cap was concentrated and applied to SDS-PAGE, followed by silver staining. (E) Identification of GIPC-PLD peptides using peptide mass fingerprinting analysis. M indicates the protein marker.

Fig. 3. Cloning, expression, and purification of the NPC3 gene.
The NPC3 cDNA was cloned into the pBAD-DEST49 vector with His6 and HP-Trx and transfected into *E. coli*. The sample was purified by Ni-NTA agarose. The purified protein was separated by 10% SDS–PAGE and stained with Coomassie Brilliant Blue. S, supernatant; FT, flow-through; W1–3, washing fraction 1–3; E1–3, Elution fraction 1–3. M indicates the protein marker.

Fig. 4. Formation of PC1P but not PCer by recombinant NPC3 expressed in *E. coli* at various concentrations of recombinant NPC3 and GIPC.
(A) Twenty-six nmol of GIPC was treated with increasing concentrations of NPC3 in the presence of NaDOC (pH 7.5). Lipids were recovered from the reaction mixture followed by TLC as described in the materials and methods section. (B) PC1P was quantified by Image J software. (C, D) Increasing concentrations of GIPC were treated with recombinant NPC3 in the presence of NaDOC at pH 7.5. The resulting lipids recovered from the reaction mixture were separated by TLC as mentioned in materials and methods. (E, F) The amounts of PC1P and PCer were quantified by Image J software. The data obtained in experiment C were analyzed by Lineweaver -Burk plot. (G) The PC1P isolated by TLC was analyzed by MALDI-TOF/MS.

Fig. 5. Substrate preference of recombinant NPC3 on phospholipids.
The substrates employed were GIPC from cabbage, sphingomyelin (SphM) from chicken egg, phosphatidylcholine (PC) from soybean, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (PE) (synthetic) and phosphatidylinositol (PI) from cabbage. Values are shown as the means ± SD from three independent experiments. N.D. denotes not detected.

Fig. 6. Biochemical characterization of *E. coli* expressing NPC3.

(A) GIPC-PLD assay was conducted with recombinant NPC3 at indicated pH values in the presence of NaDOC (2 mg). The buffers utilized were 100 mM sodium acetate (pH 4.5–5.5), 100 mM potassium phosphate (pH 6.7–7.5), and 100 mM Tris-HCl (pH 7.5–8.5). Values are presented as means ± SD from three independent experiments. (B) GIPC-PLD assay was performed with recombinant NPC3 in the presence or absence of 2 mg of NaDOC, 0.5 mL of DE, 10 mM of $CaCl_2$ and 1 mM of EGTA. (C) GIPC (52 nmol) was treated with recombinant NPC3 in the presence of 20% various alcohols. Lipids extracted from the reaction mixture were separated by TLC. (D) The resulting products were quantified by Image J software. Values from three independent experiments are represented as mean ± SD. Values with $*p < 0.05$ and $***p < 0.005$ were considered statistically significant. N.D. denotes not detected.

Fig. 7. Alignment of the deduced amino acid sequences of NPC3 and NPC4 from *Raphanus sativus* (Rs) and *Arabidopsis thaliana* (At) (Genetyx software, GENETYX Corporation, Tokyo, Japan).