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Distribution of glycosylinositol phosphoceramide-specific phospholipase D activity in plants

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Running title: GIPC-phospholipase D in plants

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Abbreviations: AGP, arabinogalactan protein; GIPC, glycosylinositol phosphoceramide; LPA, lysophosphatidic acid; MALDI-TOF MS, matrix assisted laser desorption/ionization time-of-flight mass spectrometry; PA, phosphatidic acid; PC, phosphatidylcholine; PC1P, phytoceramide-1-phosphate; PE, phosphatidylethanolamine; PI, phosphatidylinositol; SM, sphingomyelin; S1P, sphingosine-1-phosphate: THAP, 2,4,6-trihydroxyacetophenone; PLD, phospholipase D.

Enzyme(s): The enzyme mainly dealing with this manuscript is a kind of phospholipase D (E.C. No. 3.1.4.4). Partially purified enzyme does not hydrolyze glycerophospholipid, but hydrolyzes glycosylinositol phosphoceramide (GIPC). We abbreviated the enzyme activity to GIPC-PLD activity. Sources of this enzyme are plants.

Summary

Previously, we detected an unknown sphingophospholipid in cabbage leaves and identified it as phytoceramide-1-phosphate (PC1P). We also found an enzyme activity that produces PC1P by glycosylinositol phosphoceramide (GIPC)-specific hydrolysis in cabbage leaves. To characterize the GIPC-specific phospholipase D (GIPC-PLD) activity, we investigated distributions of GIPC-PLD activity in 25 tissues of 10 plants. In most plants, the GIPC-PLD activity was the highest in roots. Young leaves of cabbage and Welsh onion had higher activities than corresponding aged outer leaves. The GIPC-PLD activities in leaves, stems and roots of mung bean were higher in the sprouting stage than in more mature stages. We also examined distribution of substrate GIPC and product PC1P, and found that GIPC was ubiquitously distributed at 50-280 nmol/g (wet wt) in tissues of plants, whereas PC1P was detectable (3-60 nmol/g wet wt.) only in tissues showing considerable GIPC-PLD activity. These results suggest a possibility that GIPC-PLD activity is involved in plant growth.

Keywords: Glycosylinositol phosphoceramide/Phospholipase D/Plants/Sphingophospholipid

Introduction

Hydrolysis of membrane phospholipids is a cellular event that produces signaling molecules in both animal and plant cells. In plants, multiple phospholipase D (PLD) species (α, β, γ, δ, ζ) have been identified, and their involvements in various physiological processes, including responses to environmental stresses and plant hormones, have been demonstrated (*1, 2*). Glycerophospholipids such as phosphatidylcholine (PC) and phosphatidylinositol (PI) are exclusive substrates of these PLDs, and the product phosphatidic acid (PA) is a signaling lipid. However, to our knowledge plant PLDs that hydrolyzes sphingophospholipids has not been identified.

Previously, we detected an unknown sphingophospholipid in homogenates of cabbage leaves and identified it as phytoceramide-1-phosphate (PC1P) with α-hydroxy fatty acids as the *N*-acyl residues (*3*). This was the first report to show evidence of natural occurring C1P in plants. PC1P accounted for 5% of total phospholipids in homogenates of cabbage leaves. We also found an enzyme activity that produces PC1P by hydrolysis of the D position of glycosylinositol phosphoceramide (GIPC) in cabbage leaves (*3*). Importantly, a partially purified GIPC-PLD fraction from cabbage leaves did not hydrolyze phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and sphingomyelin (SM) at substantial levels. Conversely, PLD fractions prepared from cabbage leaves did not hydrolyze GIPC (*3*). Other properties, such as calcium requirements and pH dependency, were also distinct. These observations indicated the presence of an unidentified enzyme that specifically hydrolyzes GIPC to PC1P in cabbage leaves. Enzymes that hydrolyze the C position of inositolphosphoceramide (IPC), which produces ceramide and a phosphoinositol in yeast (*4*) and cryptococcal species (*5*), are called as "IPS-PLC" and "IPC-PLC", respectively. Thus, we suggest that "GIPC-PLD" is an appropriate name for the enzyme that produces ceramide-1-phosphate (C1P) and glycosylinositol from GIPC (Fig. 1).

GIPC is a predominant sphingophospholipid that is distributed widely in the plant kingdom (*6*). Markham et al. reported that GIPC is an abundant sphingolipid in *Arabidopsis*, tomato and soybean (*7*). In fact, GIPC, glycosylceramide and ceramide accounted for about 64%, 34% and 2%, respectively, of total sphingolipids in *Arabidopsis* (*7*). Several functions have been proposed for GIPC, such as a protein anchor (*8-11*) and precursor of signaling molecules (*12*). However, enzyme activity that releases the anchored proteins or signaling molecules from GIPC has not been shown yet.

It has been considered that C1P is produced by phosphorylation of ceramide in animals and in plants. Liang et al. (*13*) and Bi et al. (*14*) have cloned and characterized ceramide kinases of *Arabidopsis* and rice (*Oryza sativa*), respectively, and showed that exogenous C1P or a transgene of ceramide kinase rescues ceramide kinase-null mutants from apoptotic cell death. These results suggest that formation of C1P is associated with cell survival in plants. However, C1P levels have not been examined in various tissues of plants at different conditions. Furthermore, contributions of GIPC-PLD pathway to the levels of cellular C1P levels remain unknown.

To gain more insights on physiological roles of GIPC-PLD, we examined distributions of enzyme activity in various plant tissues over several growth stages. We also characterized the enzyme activity in *Arabidopsis thaliana* to confirm the presence of GIPC-PLD in plants. Distributions of substrate GIPC and product PC1P were also examined in several tissues of plants. Our results suggest the possibility that GIPC-PLD is involved in plant growth.

Materials and Methods

Materials

2,4,6-trihydroxy-acetophenone (THAP) was obtained from Sigma-Aldrich (St. Louis, MO). *N*-(Hexadecanoyl)-sphing-4-enine-1-phosphate (ceramide-1-phosphate; *d*18:1/16:0) and SM from bovine brain were purchased from Avanti Polar Lipids (Alabaster, AL). PC was prepared from lipid extracts of egg yolk using TLC. Phos-tag with monoisotopic Zn [^{68}Zn] was obtained from Wako Pure Chemical Industries (Osaka, Japan).

Plant material

Cabbage (*Brassica oleracea* L. var. capitata), broccoli (*Brassica oleracea* L. var. italica), radish (*Raphanus sativus* L), Japanese mustard spinach called *komatsuna* in Japan (*Brassica rapa* L), and carrot (*Daucus carota* L) were obtained from a local farmer. Cucumber (*Cucumis sativus* L), burdock (*Arctium lappa* L), and Welsh onion (*Allium fistulosum* L) were purchased from local markets. Seeds of mung bean (*Vigna radiata*) were obtained from a local nursery. Mung beans were grown using water culture in the dark at 23-28℃, and were

harvested on days 3-4 (stage 1, sprout) after seeding. They were also cultivated in soil with exposure to sunlight at around 23-28℃, and were harvested on days 7-14 (stage 2) and 14-28 (stage 3) after seeding. *Arabidopsis thaliana* (Columbia strain) was grown in soil with a 12-h light/12-h dark cycle at 23-28℃, and mature plants were used in experiments.

Isolation of PC1P

Plant tissues were cut into small pieces using a kitchen knife, added to an equal volume of water and ground in mortar with a pestle for 5 min at room temperature. Ground tissues were further homogenized using an ultradisperser (LK-21, Yamato Scientific, Tokyo Japan) for 5 min. Homogenates were then heated in boiling water for 3 min to inactivate lipolytic enzymes, and lipids were extracted using the Bligh and Dyer method (*15*) with acidification of the H_2O/MeOH phase as described previously (*3*). Extracted lipids were treated with 0.1 M KOH in 95% methanol at 65℃ for 15 min. After cooling, the alkali lysates were extracted by the Bligh and Dyer method after acidification with a small amount of 5 M HCl. Lipids were subjected to TLC and developed with chloroform-MeOH-28% ammonia (65:35:6, v/v/v). Silica gel corresponding to PC1P was scraped off the plates and extracted using the Bligh and Dyer method with acidification of the H_2O/MeOH phase. Isolated PC1P was then quantified by colorimetric method based on phosphomolybdenum-malachite green formation (*16*). An aliquot of the PC1P fraction was subjected to MALDI TOF-MS for structural confirmation.

Isolation of GIPC

Relatively high polar lipids of plants were extracted using the method reported by Markham *et al*. (*7*) with some modifications (*3*). First, plant tissues were heated in boiling water for 5 min to inactivate lipolytic enzymes. The boiled tissues were cut into small pieces with a kitchen knife, added to the lower layer of a mixed solvent consisting of isopropanol-hexane-water (55:20:25, v/v/v) (solvent A) and homogenized using an ultradisperser. After centrifugation of homogenates, supernatants were collected and evaporated to dryness. The extract was then treated with 40% methylamine-ethanol (5:7, v/v) at 50℃ for 1 h for mild alkali hydrolysis. After evaporation, alkaline lysates of the plant lipids were dissolved in small volumes of solvent A and were subjected to TLC using chloroform-MeOH-7% ammonia (45:35:10, v/v/v) as the developing solvent.

After spraying with primulin, the band corresponding to standard GIPC was identified under UV light. The silica gel was scraped off the plate, mixed with solvent A and centrifuged. GIPC was recovered from the supernatant, and its amount were determined using the colorimetric method described above. The structure of isolated GIPC was analyzed by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) in negative detection mode as described below.

Preparation of subcellular fractions of plant tissues

Frozen plant tissues were mashed, added to an equal amounts of ice-cold buffer (0.05 M Tris/HCl, pH 7.4) and homogenized using an ultradisperser for 5 min. Homogenates were then filtered through gauze, and sequentially centrifuged at 5,000 X *g* for 15 min, 13,000 X *g* for 30 min, and 100,000 X *g* for 60 min, at 4℃. Precipitate fractions were then suspended in the ice-cold buffer, and aliquots of each fraction were used for GIPC-PLD assays as described below. Protein contents were determined by a bicinchoninic acid protein assay (*17*).

GIPC-PLD assay

A typical assay mixture for the GIPC-PLD assay contained 48 nmol GIPC from cabbage, 0.05 ml of enzyme fraction (approximately 0.1 mg protein) and 1 mg sodium deoxycholate in 0.2 M Tris/HCl buffer (pH 7.4) in a total volume 0.7 ml. The reaction mixture was incubated at 30℃ with continuous stirring. After incubation for 5 min or 30 min, lipids in the reaction mixture were extracted using the method of Bligh and Dyer with acidification. The resulting PC1P was isolated by TLC for quantification.

MALDI-TOF MS

MALDI-TOF MS using Phos-tag was applied for the structural analyses of PC1Ps in several plant tissues as described previously (*18, 19*). Briefly, PC1P was dissolved in a small volume of methanol containing 0.1% aqueous ammonia. Ten μl of this solution was mixed with 5 μl of 0.1 mM ^{68}Zn Phos-tag solution and 2 μl of silica suspension (100 mg/ml in methanol). A small portion of the mixed solution (0.5 μl) was spotted on a sample plate. Then, it was layered with 0.5 μl THAP solution (10 mg/ml acetonitrile) on the plate. GIPCs prepared from *Arabidopsis* leaves, radish roots and Welsh onion white leaves were analyzed by MALDI-TOF

MS in negative detection mode. In this case, Phos-tag was not used, and THAP was used as the matrix. In both cases, the matrix/analyte cocrystal was analyzed by a Voyager DE STR (Applied Biosystems, Framingham, MA). The wavelength of the nitrogen-emitting laser, pressure in the ion chamber and accelerating voltage were 337 nm, 3.7 X 10^{-7} Torr, and 20 kV, respectively. To enhance the reproducibility, 256 single laser shots were averaged for each mass spectrum.

Statistical analysis

Statistical analyses of the difference between two means were performed using Student's *t*-test.

Results

Distribution of GIPC-PLD activity in plants

GIPC-PLD activities of various tissues of Brassicaceae (cabbage, broccoli, *Arabidopsis*, radish and Japanese mustard spinach), Fabaceae (mung bean), Apiaceae (carrot), Asteraceae (edible burdock) and Amaryllidaceae (Welsh onion) were examined (Fig. 2A). We found that GIPC-PLD activities of roots were higher than those of mature leaves. In cabbage, the white inner leaves had significantly higher activity than those of stems and green leaves on the outside of the ball. In broccoli, GIPC-PLD activity was detectable in stems, flowers and roots, but not in aged green leaves. Welsh onion is a non-Brassicaceous monocot. Similar to cabbage, young leaves located near the root had significantly higher GIPC-PLD activity than outer leaves of the topside. To know changes in the enzyme activity during tissue maturation, we examined enzyme activities of mung beans tissues at various growth stages. Results showed that GIPC-PLD activities of leaves, stems and roots at the aged stage (days 14-28 after seeding) were significantly lower than those at the sprouting stage (days 3-4 after seeding) of mung beans (Fig. 3).

Distribution of PC1P and GIPC in plants

GIPC was ubiquitously present at 50-280 nmol/g wet wt. in tissues of plants (Fig. 2C). Distribution patterns of the substrate GIPC did not reflect those of GIPC-PLD activity. In contrast, distribution patterns of the product PC1P reflected GIPC-PLD activity (Fig. 2B). These results indicated that GIPC-PLD activity determined the

PC1P levels in the homogenates of the tissues. PC1P accounted for 6.6% and 5.1% of total phospholipids in homogenates of cabbage roots and radish roots, respectively (Table 1). It was found that boiling the plant tissues before homogenization considerably decreased the PC1P levels. We confirmed that PC1P is not decomposed during short time boiling (data not shown). These results indicate that PC1P was formed enzymatically during homogenization of plant tissues.

Comparison of Molecular species of GIPC and PC1P in several plant tissues

Molecular species of PC1P and GIPC in *Arabidopsis* roots, radish roots and Welsh onion leaves were examined by MALDI-TOF MS. It has been reported that the polar head group of *Arabidopsis* GIPC is hexose-hexuronic acid-inositol phosphate and that its ceramide structure is a combination of phytosphingenine (*t*18:1 Δ-8 Z or E) and α-hydroxy fatty acids with chain lengths of C16, C22, C24 and C24:1 (*6, 7, 20, 21*). Consistent with the reports, peaks at *m/z* 1148.6, 1232.7, 1258.7 and 1260.7 in the mass spectrum of *Arabidopsis* GIPC (Fig. 4B) were assignable to 2 saccharide–GIPC (S2) with ceramide structures of *t*18:1/16:0h, *t*18:1/22:0h, *t*18:1/24:1h and *t*18:1/24:0h, respectively (Fig. 1). We found that molecular species composition of PC1P of *Arabidopsis* (Fig. 4A) was almost the same as that of *Arabidopsis* GIPC. Radish GIPC comprised molecular species similar to those in *Arabidopsis* except for the absence of *t*18:1/16:0h (S2) (Fig. 4D). The molecular species composition of radish GIPC was also similar to that of radish PC1P (Fig. 4C). These results indicated that PC1P in tissue homogenates was the hydrolytic product of GIPC. Welsh onion is a non-Brassicaceae monocot that contains substantial GIPC-PLD activity in its young leaves (Fig, 2A). Cacas et al. (*6*) showed that the major GIPC species in *Allium porrum* has three saccharides in its polar head group (hexose-hexosamine-hexuronic acid-inositol phosphate). Assuming that GIPC in Welsh onion has the same polar head structure as that in *Allium porrum*, peaks at *m/z* 1379.3, 1393.8, 1407.8 and 1421.8 were assignable to 3-saccharide-GIPC (S3) with ceramide structures of *t*18:1/21:0h, *t*18:1/22:0h, *t*18:1/23:0h, and *t*18:1/24:0h, respectively (Fig. 4F, Fig. 1). Although molecular species having fatty acids with odd number chains were abundantly observed in GIPC of Welsh onion, PC1Ps with odd number N-acyl chains were minor species in the plant.

Characterization of GIPC-PLD activity of Arabidopsis and mung beans

The obtain further evidence for presence of GIPC-specific PLD activity in plants, we characterized the enzyme activities in roots of *Arabidopsis* and mung beans in the sprouting stage. In *Arabidopsis*, total enzyme activities of the soluble fraction (100k sup) and membrane fractions (13k ppt, 100k ppt) were similar, whereas specific activity in the membrane fraction was higher than in the soluble fraction (Fig. 5A, B). Further characterization of the GIPC-PLD activity was conducted using the membrane fraction of *Arabidopsis* and GIPC prepared from cabbage leaves (Fig. 5C, D). A kinetic experiment revealed that the *Km* of the enzyme was 15 μM (Fig. 5C). We found that the addition of sodium deoxycholate or diethylether significantly enhanced the enzyme activity. The addition of EGTA significantly reduced enzyme activity compared to that in the presence of calcium (Fig. 5D), suggesting that GIPC-PLD requires calcium ion for its full activity. Under conditions in which GIPC was extensively hydrolyzed, sphingomyelin (SM) and PC were not hydrolyzed at substantial levels (Fig. 5E). We also examined the substrate specificity of GIPC-PLD activity of mung bean sprouts, and found that the GIPC-PLD activity of mung bean did not substantially hydrolyze SM or PC (Fig. 5F). A small amount of PA was formed from exogenous PC depending on preparations from both *Arabidopsis* and mung beans. Because the 13k pellet fraction contained PC-preferring PLD activity, PC-PLD activity may incompletely masked under calcium-depleted conditions.

Discussion

Because of its hydrophilic nature, usual methods for lipid extraction can not be applied for GIPC. The difficulty of extraction may have hampered structural, metabolic and physiological characterizations of this sphingophospholipid in plants. Recently, a method for extraction of GIPC was reported by Markham et al. (*7*). In this study, we extracted GIPC by the method, isolated it by TLC, and performed quantification by the established colorimetric method for measurement of phospholipid (*16*). The GIPC contents in leaves of *Arabidopsis* were similar to those reported by Markham et al. (*7*), and the average GIPC content in 16 tissues of nine plants was 120 nmol/g (wet wt.). Sugawara and Miyazawa (*22*) quantified monohexosyl ceramide, another predominant sphingolipid in plants, in 48 vegetables and fruits, and reported that an average monohexosyl ceramide content of these plants is 125 nmol/g (wet wt.). Thus, GIPC and monohexosyl ceramide are present in plant cells at similar levels.

Previously, we demonstrated the formation of PC1P during mashing and subsequent incubation of homogenates of cabbage leaves (*3*). In the present study, we showed that PC1P contents are very low in boiled tissues compared to those of fresh tissues from radish and cabbage. We also found that PC1P is detectable only in tissues that show higher GIPC-PLD activity. These results indicate that PC1P is not a constitutive component but a molecule transiently produced exclusively by GIPC-PLD activity. Because relative amounts of PC1P and GIPC are approximately 1:2 in cabbage roots, and 1:10 in *Arabidopsis* roots, it was calculated that 50% and 10% of GIPC is converted to PC1P during mashing of Brassicaceous roots. Currently, it is unclear why Brassicaceous plants show higher GIPC-PLD activity than other plants. A possible explanation is the structure of the substrate used for the assay. GIPC-PLD of a plant may preferably hydrolyze its own intrinsic GIPC.

In this study, we found that GIPC-PLD activity was high in the roots of most plants. Moreover, young inner leaves contained higher GIPC-PLD activity than mature outer leaves of the cabbage and Welsh onion. Because roots and inner leaves are growing tissues, GIPC-PLD may be involved in the growth of plants, such as cell expansion. The observation that GIPC-PLD activity was very low in matured stages of mung bean may support this hypothesis. Smith and Fry demonstrated that mannopyranosyl-glucuronopyranosyl-inositol accumulates in spent media of cell-suspension cultures of rose during periods of rapid cell growth (*12*). They hypothesized a signaling role for released mannopyranosyl-glucuronopyranosyl-inositol and suggested that GIPC may be a source. Results of present study suggest that GIPC-PLD is responsible enzyme for production of mannopyranosyl-glucuronopyranosyl-inositol during rapid cell growth (Fig. 6). It has been known that GIPC is condensed in lipid raft microdomains which are considered to be a platform for the signal transduction process in plants (*8*). Localization in lipid rafts is reasonable if GIPC serves as a source of signaling molecules.

GIPC has been known to serve as a glycosylphosphatidylinositol (GPI) anchor of a protein (*8*). Arabinogalactan protein (AGP) is a typical GPI-anchored protein containing a ceramide moiety as a hydrophobic residue (*23, 10*). Oxley and Bacic compared the native and secreted forms of AGP in *Pyrus communis* and found that the phosphoceramide moiety of native AGP was deleted in the secreted form. They considered that PLD activity is involved in the release of AGP (*10*). AGP is a hydroxyproline-rich glycoprotein that is thought to be involved in root growth, cell expansion and promotion (*24, 25*). GIPC-PLD in growing tissues may release AGP from the plasma membrane to extracellular destinations (Fig. 6). It has been shown

that mutants with altered sugar structure of GIPC show a severely dwarfed phenotype (*26*). It is worthwhile to examine the specificity of GIPC-PLD(s) toward the sugar structure of GIPC.

Our previous study (*3*) revealed that cabbage GIPC-PLD hydrolyzes GIPC specifically but not hydrolyzes SM, PC, PE or PI at substantial levels. We also reported that the cabbage GIPC-PLD require Ca^{2+} for its full activity under its optimum condition (pH 7.4 at 30 ℃). These results are consistent with those observed in *Arabidopsis* GIPC-PLD (Fig. 5). In addition, we found here that diethylether can enhance the enzyme activity. Because GIPC is soluble in water and insoluble in diethylether, the effect of diethylether is not attributable to enhancement of substrate dispersion, but direct effect on the enzyme. GIPC-PLD may be activated in a hydrophobic environment. GIPC-PLD is activated during homogenization as shown here and our previous results (*3*). Considering that the GIPC-PLD activity is distributed in both membrane and soluble fractions (Fig. 5A), GIPC-PLD in the cytosol or periplasmic space may be translocated to the plasma membrane in response to environmental changes, such as the influx of extracellular water, whereupon it produces PC1P by hydrolysis of GIPC or protein-anchoring GIPC (Fig. 6). To know whether GIPC-PLD in membrane fraction is peripherally-bound enzyme, we tried to release the enzyme by treatment of the membrane fraction with 0.1 M sodium carbonate. Results showed that GIPC-PLD didn't release from the membrane by the weak alkali treatment (data not shown), suggesting that GIPC-PLD rigidly binds to membranes not by electrostatic force but by hydrophobic force.

C1P that is produced by phosphorylation of ceramide has been associated with survival signaling in plant cells (*13*). It remains unknown whether PC1P produced by hydrolysis of GIPC plays similar roles in plant cells (Fig. 6). Further investigations are required to elucidate the mechanism of regulation and physiological roles of GIPC-PLD.

In conclusion, GIPC-PLD activity was higher in growing tissues, such as roots, young inner leaves and sprouts, than more aged tissues in plants. The substrate GIPC was distributed ubiquitously, whereas, the product PC1P was detectable only in tissues containing higher GIPC-PLD activity. GIPC-PLD activity may produce molecules that are required for plant growth.

Reference

1. Li, M., Hong, Y., and Wang, X. (2009) Phospholipase D- and phosphatidic acid-mediated signaling in plants. *Biochim. Biophys. Acta* **1791**, 927-935
2. Wang, X. (2004) Lipid signaling. *Curr. Opin. Plant Biol.* **7**, 329-336
3. 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
4. Sawai, H., Okamoto, Y., Luberto, C., Mao, C., Bielawska, A., Domae, N., and Hannun, Y.A. (2000) Identification of *ISC1* (*YER019w*) as inositol phosphosphingolipid phospholipase C in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **275**, 39793-39798
5. Henry, J., Guillotte, A., Luberto, C., and Poeta, M.D. (2011) Characterization of inositol phospho-sphingolipid-phospholipase C1 (Isc1) in *Cryptococcus neoformans* reveals uniquebiochemical features. *FEBS Lett.* **585**, 635-640
6. Cacas, J-L., Buré, C., Furt F., Maalouf J-P., Badoc, A., Cluzet, S., Schmitter, J-M., Antajan, E., and Mongrand, S. (2013) Biochemical survey of the polar head of plant glycosylinositolphosphoceramides unravels broad diversity. *Phytochem.* **96**, 191-200
7. Markham, J.E., Li, J., Cahoon, E.B., and Jaworski, J.G. (2006) Separation and identification of major plant sphingolipid classes from leaves. *J. Biol. Chem.* **281**, 22684-22694
8. Spassieva, S., and Hille, J. (2003) Plant sphingolipids today – are they still enigmatic? *Plant Biol.* **5**, 125-136
9. Morita, N., Nakazato, H., Okuyama, H., Kim, Y., and Thompson Jr, G.A. (1996) Evidence for a glycosylinositolphospholipid-anchored alkaline phosphatase in the aquatic plant *Spirodela oligorrhiza*. *Biochim. Biophys. Acta* **1290**, 53-62
10. 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. USA* **96**, 14246-14251
11. Voxeur, A., and Fry, S.C. (2014) Glycosylinositol phosphoceramides from *Rosa* cell cultures are boron-bridged in the plasma membrane and form complexes with rhamnogalacturonan II. *Plant J.* **79**, 139-149
12. Smith, C.K., and Fry, S.C. (1999) Biosynthetic origin and longevity in vivo of α-D-mannopyranosyl-(1→4)-α-D-glucuronopyranosyl-(1→2)-*myo*-inositol, an unusual extracellular oligosaccharide produced by cultured rose cells. *Planta* **210**, 150-156
13. Liang, H., Yao, N., Song, J.T., Luo, S., Lu, H., and Greenberg, J.T. (2003) Ceramides modulate programmed cell death in plants. *Genes and Dev.* **17**, 2636-2641
14. Bi, F-C., Zhang, Q-F., Liu, Z., Fang, C., Li, J., Su, J-B., Greenberg, J.T., Wang, H-B., and Yao,

N. (2011) A conserved cysteine motif is critical for rice ceramide kinase activity and function. *PLoS One* **6**, e18079

15. Bligh, E.G., and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911-917
16. Chalvardjian, A., and Rudnicki, E. (1970) Determination of lipid phosphorus in the nanomolar range. *Anal. Biochem.* **36**, 225-230
17. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, M.D., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76-85
18. Tanaka, T., Tsutsui, H., Hirano, K., Koike, T., Tokumura, A., and Satouchi, K. (2004) Quantitative analysis of lysophosphatidic acid by time-of-flight mass spectrometry using a phosphate capture molecule. *J. Lipid Res.* **45**, 2145-2150
19. Morishige, J., Urikura, M., Takagi, H., Hirano, K., Koike, T., Tanaka, T., and Satouchi, K. (2010) A clean-up technology for the simultaneous determination of lysophosphatidic acid and sphingosine-1-phosphate by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using a phosphate-capture molecule, Phos-tag. *Rapid Commun. Mass Spectrom.* **24**, 1075-1084
20. Buré, C., Cacas, J-L., Wang, F., Gaudin, K., Domergue, F., Mongrand, S., and Schmitter, J-M. (2011) Fast screening of highly glycosylated plant sphingolipids by tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **25**, 3131-3134
21. Blaas, N., and Humpf, H-U. (2013) Structural profiling and quantitation of glycosyl inositol phosphoceramides in plants with fourier transform mass spectrometry. *J. Agric. Food Chem.* **61**, 4257-4269
22. Sugawara, T., and Miyazawa, T. (1999) Separation and determination of glycolipids from edible plant sources by high-performance liquid chromatography and evaporative light-scattering detection. *Lipids* **34**, 1231-1237
23. Svetek, J., Yadav, M.P., and Nothnagel, E.A. (1999) Presence of a glycosylphosphatidylinositol lipid anchor on rose arabinogalactan proteins. *J. Biol. Chem.* **274**, 14724-14733
24. Ellis, M., Egelund, J., Schultz, C.J., and Bacic, A. (2010) Arabinogalactan-protein: Key regulators at the cell surface? *Plant Physiol.* **153**, 403-419
25. Showalter, A.M. (2001) Arabinogalactan-protein: structure, expression and function. *Cell. Mol. Life Sci.* **58**, 1399-1417
26. Mortimer, J.C., Yu, X., Albrecht, S., Sicilia, F., Huichalaf, M., Ampuero, D., Michaelson, L.V., Murphy, A.M., Matsunaga, T., Kurz, S., Stephens, E., Baldwin, T.C., Ishii, T., Napier, J.A., Weber, A.P.M., Handford, M.G., and Dupree, P. (2013) Abnormal glycosphingolipid

mannosylation triggers salicylic acid-mediated responses in Arabidopsis. *Plant Cell* **25**, 1881-1894

Table 1. Amounts of PC1P, phospholipids and percentage of PC1P in total phospholipids in raw and boiled roots of cabbage and radish. Lipids were extracted from raw or 5-min boiled tissues. PC1P was isolated from the lipid extracts by TLC. Amounts of phospholipid and PC1P were determined based on their lipid phosphorus contents. Values are presented as mean ± S.D. from three to four independent experiments.

Plants	PC1P	Total phospholipids	PC1P
	nmol/g wet wt.	nmol/g wet. wt	% of total phospholipid
Cabbage roots			
raw	64 ± 17	1680 ± 630	6.6 ± 3.0
boiled	14 ± 2.0	1700 ± 170	0.8 ± 0.1
Radish roots			
raw	30 ± 13	540 ± 250	5.1 ± 0.4
boiled	3.7 ± 0.9	470 ± 280	1.8 ± 1.0

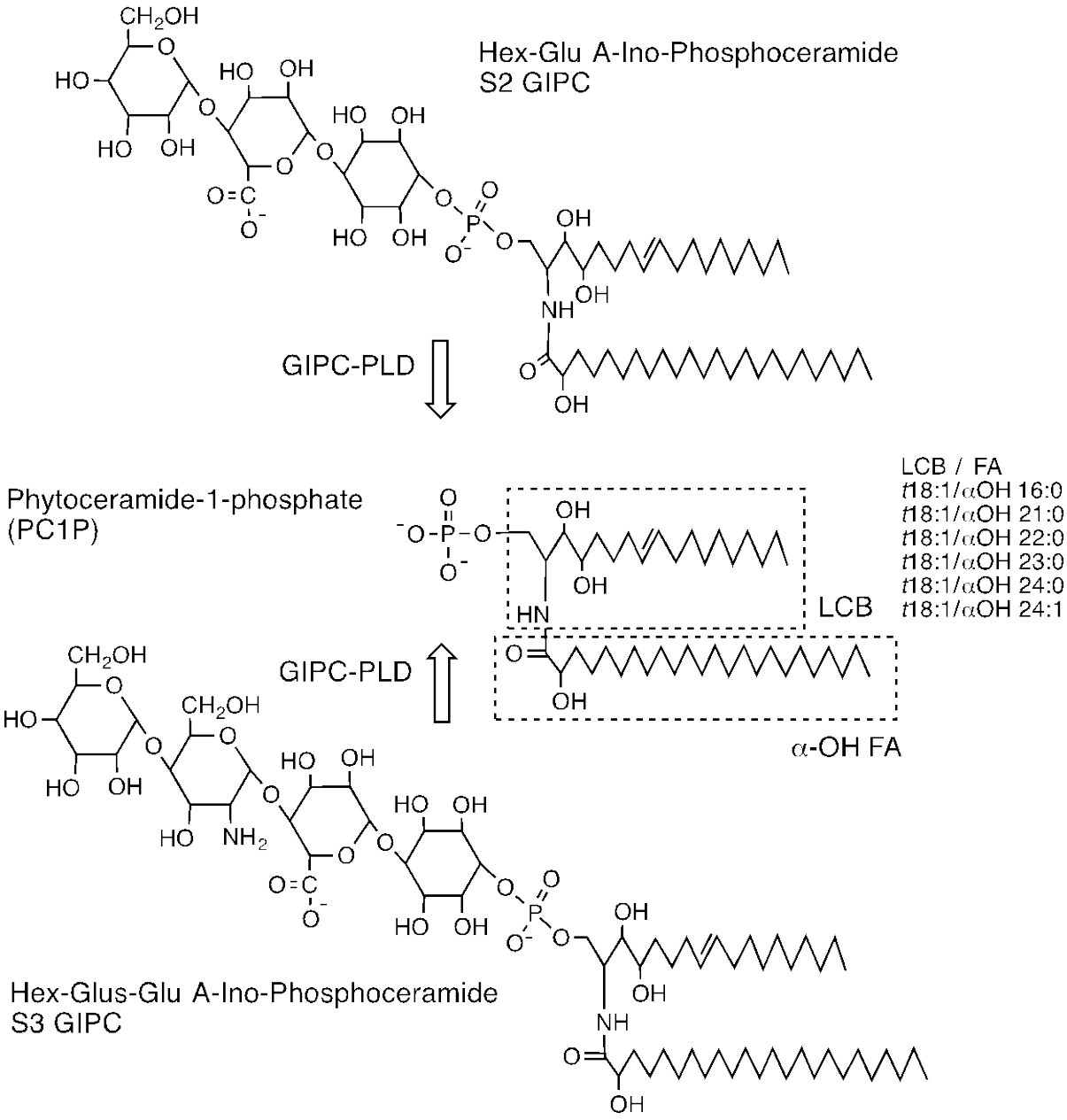
Hex-Glu A-Ino-Phosphoceramide
S2 GIPC
GIPC-PLD
Phytoceramide-1-phosphate
(PC1P)
LCB / FA
t18:1/αOH 16:0
t18:1/αOH 21:0
t18:1/αOH 22:0
t18:1/αOH 23:0
t18:1/αOH 24:0
t18:1/αOH 24:1
LCB
GIPC-PLD
α-OH FA
Hex-Glus-Glu A-Ino-Phosphoceramide
S3 GIPC

Fig. 1

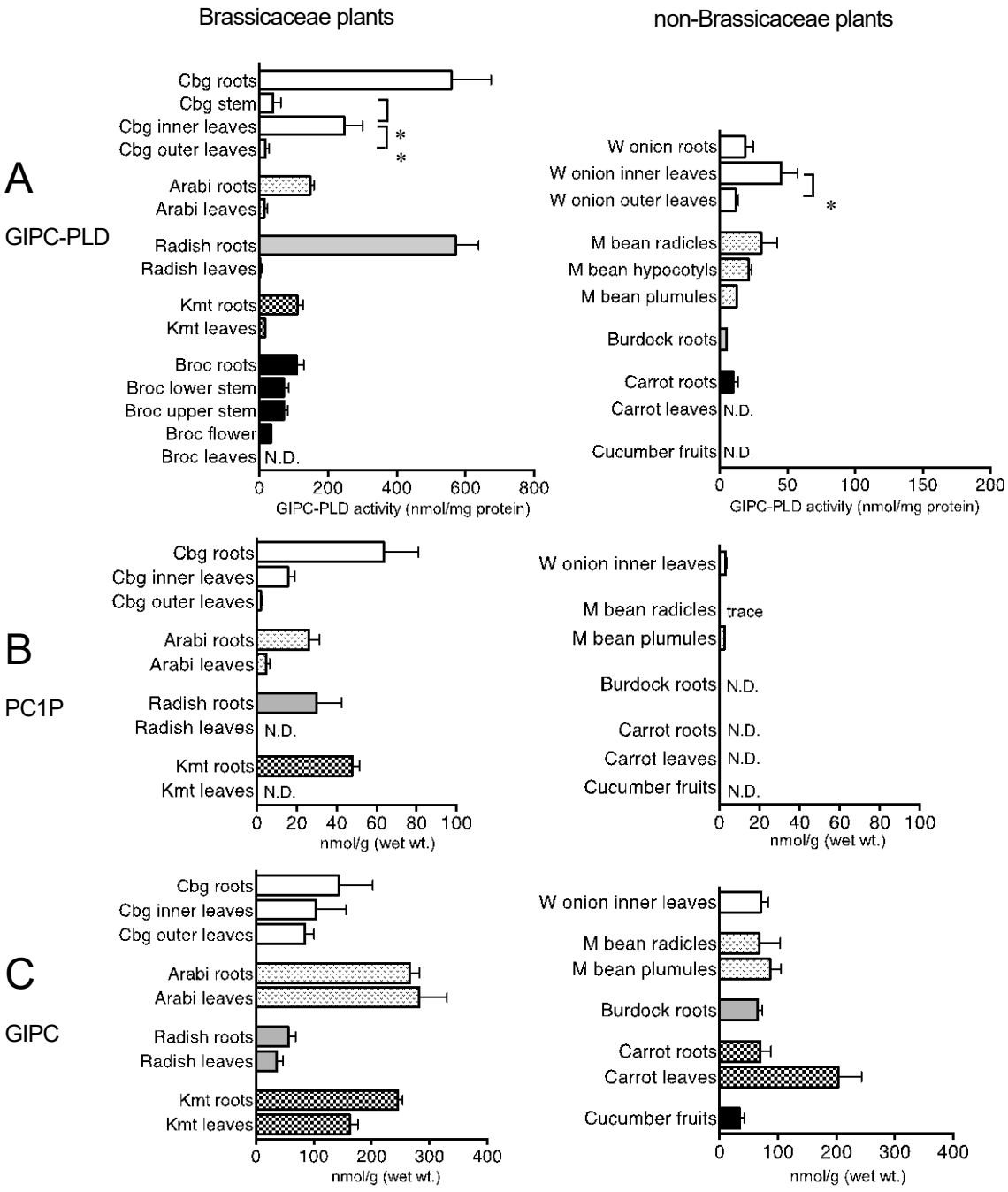
Brassicaceae plants
non-Brassicaceae plants
A
GIPC-PLD
Cbg roots
Cbg stem
Cbg inner leaves
Cbg outer leaves
Arabi roots
Arabi leaves
Radish roots
Radish leaves
Kmt roots
Kmt leaves
Broc roots
Broc lower stem
Broc upper stem
Broc flower
Broc leaves N.D.
0 200 400 600 800
GIPC-PLD activity (nmol/mg protein)
W onion roots
W onion inner leaves
W onion outer leaves
M bean radicles
M bean hypocotyls
M bean plumules
Burdock roots
Carrot roots
Carrot leaves N.D.
Cucumber fruits N.D.
0 50 100 150 200
GIPC-PLD activity (nmol/mg protein)
B
PC1P
Cbg roots
Cbg inner leaves
Cbg outer leaves
Arabi roots
Arabi leaves
Radish roots
Radish leaves N.D.
Kmt roots
Kmt leaves N.D.
0 20 40 60 80 100
nmol/g (wet wt.)
W onion inner leaves
M bean radicles trace
M bean plumules
Burdock roots N.D.
Carrot roots N.D.
Carrot leaves N.D.
Cucumber fruits N.D.
0 20 40 60 80 100
nmol/g (wet wt.)
C
GIPC
Cbg roots
Cbg inner leaves
Cbg outer leaves
Arabi roots
Arabi leaves
Radish roots
Radish leaves
Kmt roots
Kmt leaves
0 100 200 300 400
nmol/g (wet wt.)
W onion inner leaves
M bean radicles
M bean plumules
Burdock roots
Carrot roots
Carrot leaves
Cucumber fruits
0 100 200 300 400
nmol/g (wet wt.)

Fig. 2

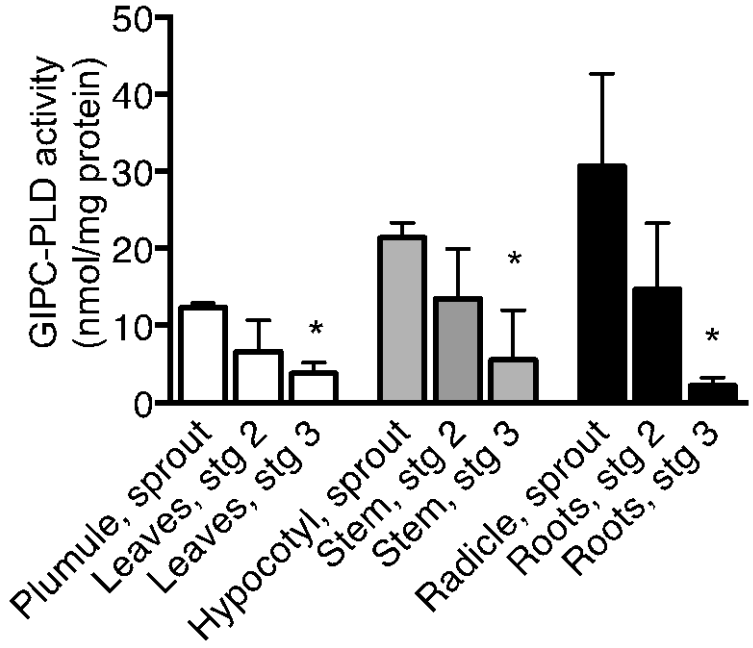
GIPC-PLD activity
(nmol/mg protein)
50
40
30
20
10
0
*
*
*
Plumule, sprout
Leaves, stg 2
Leaves, stg 3
Hypocotyl, sprout
Stem, stg 2
Stem, stg 3
Radicle, sprout
Roots, stg 2
Roots, stg 3

Fig. 3

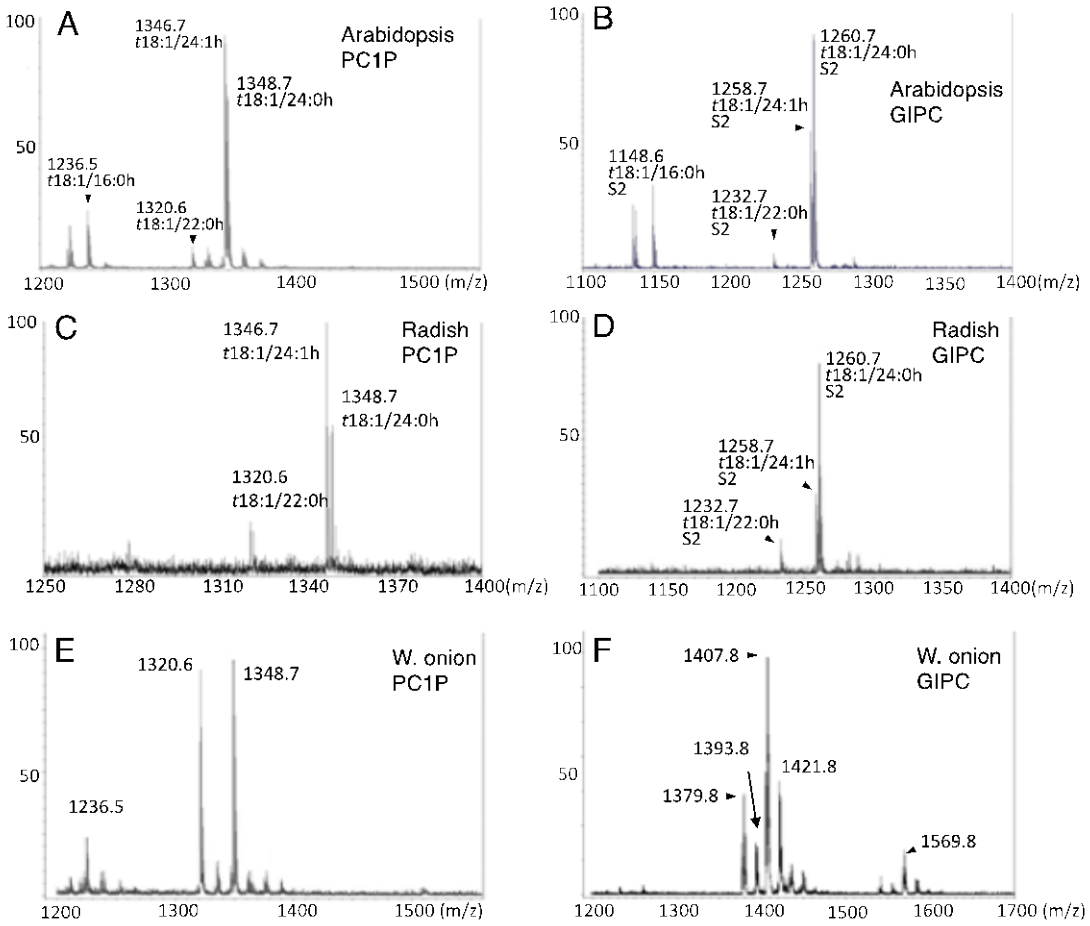
A
Arabidopsis PC1P
1346.7 t18:1/24:1h
1348.7 t18:1/24:0h
1236.5 t18:1/16:0h
1320.6 t18:1/22:0h
B
Arabidopsis GIPC
1260.7 t18:1/24:0h S2
1258.7 t18:1/24:1h S2
1148.6 t18:1/16:0h S2
1232.7 t18:1/22:0h S2
C
Radish PC1P
1346.7 t18:1/24:1h
1348.7 t18:1/24:0h
1320.6 t18:1/22:0h
D
Radish GIPC
1260.7 t18:1/24:0h S2
1258.7 t18:1/24:1h S2
1232.7 t18:1/22:0h S2
E
W. onion PC1P
1320.6
1348.7
1236.5
F
W. onion GIPC
1407.8
1393.8
1421.8
1379.8
1569.8

Fig. 4

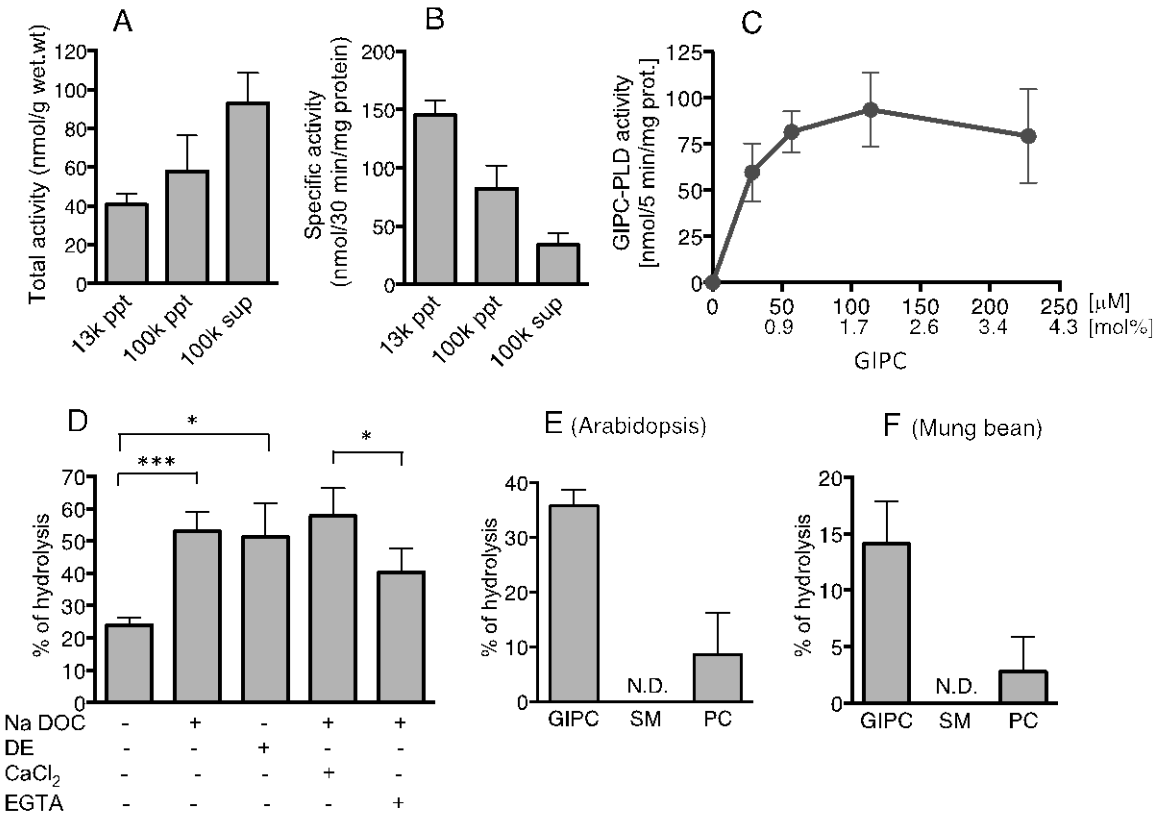
A
Total activity (nmol/g wet.wt)
120
100
80
60
40
20
0
13k ppt
100k ppt
100k sup
B
Specific activity
(nmol/30 min/mg protein)
200
150
100
50
0
13k ppt
100k ppt
100k sup
C
GIPC-PLD activity
[nmol/5 min/mg prot.]
125
100
75
50
25
0
0
50
100
150
200
250
[μM]
0.9
1.7
2.6
3.4
4.3
[mol%]
GIPC
D
*
***
*
% of hydrolysis
70
60
50
40
30
20
10
0
Na DOC - + - + +
DE - - + - -
CaCl2 - - - + -
EGTA - - - - +
E (Arabidopsis)
% of hydrolysis
40
30
20
10
0
N.D.
GIPC
SM
PC
F (Mung bean)
% of hydrolysis
20
15
10
5
0
N.D.
GIPC
SM
PC

Fig. 5

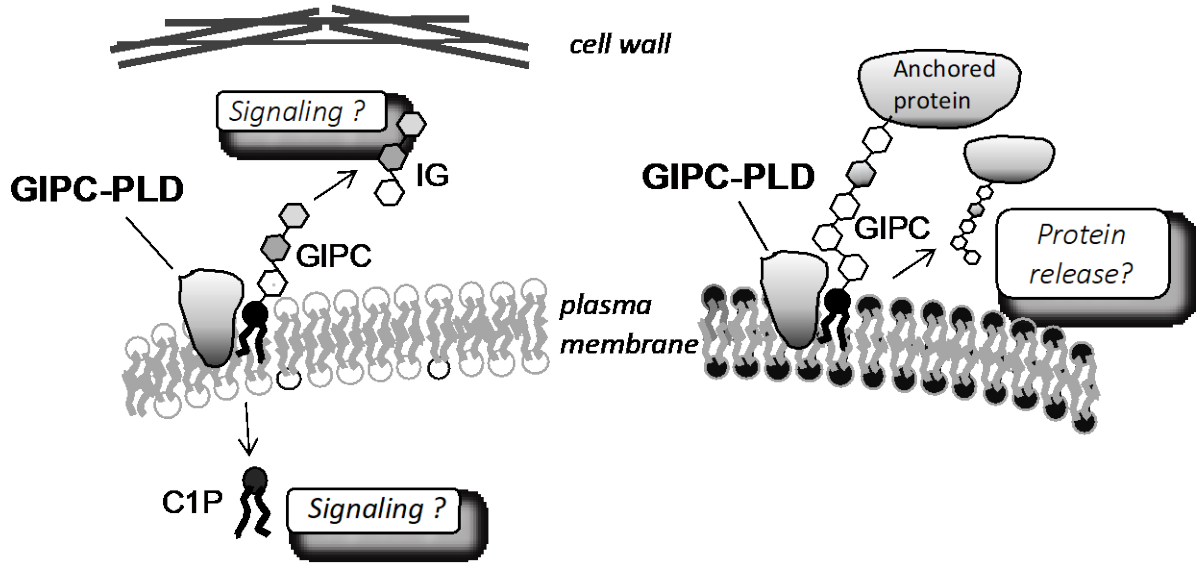
cell wall
Signaling ?
IG
GIPC-PLD
GIPC
plasma
membrane
C1P
Signaling ?
Anchored
protein
GIPC-PLD
GIPC
Protein
release?

Fig. 6

Legend to figures

Figure 1 Formation of PC1P from GIPC-PLD activity

Hexose-Glucuronic acid-Inositolphosphoceramide is a GIPC having 2 sugars (GIPC S2). Hexose-Glucosamine-Glucuronic acid- Inositolphosphoceramide is a GIPC having 3 sugars (GIPC S3). They are reported to exist as major GIPC species in *Arabidopsis thaliana* and *Allitum porrum,* respectively, as described in the text. Hydrophobic part of GIPC is a ceramide composed of a 1,3,4-trihydroxy analogue of long chain base (LCB) (phytosphingosine, *t*18:0 or dehydrophytosphingosine, *t*18:1) and an α-hydroxy fatty acid (α-OH FA). The hydrolysis of D position of GIPC produces phytoceramide-1-phosphate (PC1P) and glycosylinositol.

Figure 2. GIPC-PLD activity (A), amounts of PC1P (B) and GIPC (C) in cabbages (Cbg), *Arabidopsis* (Arabi) radishes, Japanese mustard spinach (Kmt), broccoli (Broc), mung beans (M bean), carrots, burdocks, cucumbers and Welsh onions (W onion).

(A) The 13k pellet fraction of homogenates of plant tissues was used as the enzyme source. GIPC was incubated with the pellet suspension for 30 min at 30℃. The resulting PC1P was isolated by TLC, and its lipid phosphorus content was determined. Values are presented as mean ± S.D. from three to four independent experiments. *Significantly different from each other ($P<0.05$). (B, C) PC1P and GIPC were isolated from the lipid extracts of plant tissues by TLC, and their lipid phosphorus was determined. Values are presented as mean ± S.D. from three to four independent experiments. N.D.: not detectable.

Figure 3. GIPC-PLD activity of mung beans at various developmental stages

Mung beans were grown at 23-28℃, and harvested on days 3-4 (stage 1, sprout), 7-14 (stage 2) and 14-28 (stage 3) after seeding. The 13k pellet fraction of homogenates of each plant tissue was used as the enzyme source in the GIPC-PLD assay. Data are presented as mean ± S.D. from three to four independent experiments. *Significantly different from the value at sprouting stage ($P<0.05$).

Figure 4. MALDI-TOF MS of PC1P and GIPC of *Arabidopsis* roots (A, B), radish roots (C, D) and

Welsh onion white leaves (E, F)

The isolated PC1P from plant tissues were analyzed by MALDI-TOF MS with Phos-tag. The isolated GIPC from the plant tissues was analyzed by MALDI-TOF MS at negative ion detection mode. Structure of GIPC and PC1P of *Arabidopsis* roots and radish roots were assigned according to other investigator's reports and our previous study. Because structural information on GIPC of Welsh onion has not accumulated yet, only masses of detected ions were shown. Possible assignments were described in the text.

Figure 5. Characterization of GIPC-PLD activity of *Arabidopsis* roots and substrate preference of GIPC-PLD activity of mung bean sprouts

(A, B) Homogenates of *Arabidopsis* roots were fractionated by sequential centrifugation, and the resulting fractions (13,000 x g pellet, 100,000 x g pellet and its supernatant) were used for GIPC-PLD assays. Enzyme activity is presented as total activity (A) and specific activity (B). (C) The 13k pellet fraction was incubated with different amounts of GIPC for 5 min at 30℃ in the presence of fixed amount of sodium deoxycholate for determination of *Km* value of GIPC-PLD activity. (D) GIPC-PLD assays were conducted in the presence or absence of 10 mM calcium, 1 mM EGTA, 1 mg of sodium deoxycholate (NaDOC) and 1 ml of diethylether (DE). (E, F) The 13k pellet fractions of homogenates of *Arabidopsis* roots (E), and mung bean sprouts (F) were used as the enzyme source. The pellet suspension was incubated with each substrate phospholipid for 30 min at 30℃. Resulting PLD products (PC1P or PA) were isolated by TLC, and determined its lipid phosphorus content. Data are presented as mean ± S.D. from three independent experiments. N.D.: not detectable.

Figure 6. Hypothetical roles of GIPC-PLD in plants

Left, GIPC-PLD produces glycosylinositol (GI) and creamed-1-phosphate (C1P) as cellular signaling molecules by cleavage of D position of GIPC. Right, GIPC-PLD releases anchored protein, such as arabinogalactan protein, by cleavage of D position of GIPC.

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Conflict of interest

None declared.