Plant PIP_2-dependent phospholipase D activity is regulated by phosphorylation

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Abstract Phospholipase D (PLD) forms the major family of phospholipases that was first discovered and cloned in plants. In this report we have shown, for the first time, that C2 phosphatidylinositol-4,5-bisphosphate (PIP_2)-dependent PLD(s) from 5 day hypocotyls of *Brassica oleracea* associated with plasma membrane is covalently modified-phosphorylated. Pre-incubation of the plasma membrane fraction with acid phosphatase resulted in concentration-dependent inhibition of PIP_2-dependent PLD activity. Using matrix-assisted laser desorption/ionization time of flight mass spectrometry of tryptic in-gel digests, the BoPLD$\gamma_{1,2}$ isoform was identified. Comparing the spectra of the proteins obtained from the plasma membrane fractions treated and non-treated with acid phosphatase, three peptides differing in the mass of the phosphate group (80 Da) were revealed: TMQMMYQTIYK, EVADGTVSVYNSPR and KASKSRGLGK which possess five potential Ser/Thr phosphorylation sites. Our findings suggest that a phosphorylation/dephosphorylation mechanism may be involved in the regulation of plant PIP_2-dependent PLDγ activity.

Key words: Phospholipase D; Phosphorylation; Cytoskeleton; *Brassica oleracea*

1. Introduction

Phospholipase D (PLD), which was identified as a ubiquitous microbial, plant and animal enzyme, plays an important role in numerous cellular processes. One of the two products of its catalytic action, phosphatidic acid, acts as a second messenger and can be further converted to other messenger molecules, which activate various downstream signalling events [1–3]. In plants, PLD has been proposed to participate in cellular events such as abscisic acid signalling, response to various biotic and abiotic stress cues, including water stress, wounding and pathogen attack [4]. Recently Gardiner et al. [5] reported that the microtubule-associated protein in *Arabidopsis* is a membrane-localized phosphatidylinositol-4,5-bisphosphate (PIP_2)-dependent PLD and Munnik et al. [6] speculate on the role of plant PLDs in vesicular trafficking. The biochemical properties, domain structures, and genomic organization of plant PLDs are much more diverse than those of other organisms. The PLD enzyme family can be divided into distinct types according to sequence homology, biochemical properties, and their dependence on cofactors [7–9]. Out of 12 members of the AtPLD family only four members of C2-PLD have been characterized at the protein level. PLDα (PIP_2-independent PLD), the prevalent form of the enzyme, which has been extensively studied in many plant species, shows high in vitro activity at neutral pH and millimolar levels of Ca^{2+}. Two additional forms, AtPLDβ and AtPLDγ, are active at neutral pH and micromolar levels of Ca^{2+}, and require PIP_2 and high concentrations of phosphatidylethanolamine [10,11]. Finally, AtPLDδ is stimulated by oleic acid [12]. The distinct biochemical properties of various PLDs might predetermine their different regulation and functions. Several factors have been reported to stimulate plant PLD activity including Ca^{2+}, polyphosphoinositides, G proteins, *N*-acylethanolamines, free fatty acids and membrane lipids [1,4], but nothing is known about the regulatory role of protein modification.

Reversible phosphorylation of target proteins by protein kinases and phosphatase is a universal mechanism. However, only relatively recently has the extent of the involvement of such mechanisms in regulatory processes begun to be appreciated in plant systems. A number of protein kinases and protein phosphatases have been characterized at the gene and protein level in plants, although the target proteins for only relatively few of them have been identified [13]. Phosphorylation plays an important role in the regulation of animal PLDs [14–19] and thus the question arises whether the same mechanism is also functioning in plants.

The work described here concerns the possible covalent modification-phosphorylation of plant PLD and its essential role in the regulation of enzymatic activity.

2. Materials and methods

2.1. Plant material

Mature seeds (*Brassica oleracea* var. *capitata*) were germinated at 30°C for 12 h in the light, then 12 h at 10°C in the dark. Subsequently seedlings were grown in continuous light at 25°C. For the experiments, 5 day hypocotyls were separated from other parts of plants.

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Abbreviations: PLD, phospholipase D; PIP_2, phosphatidylinositol-4,5-bisphosphate; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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2.2. Plasma membrane purification

Enriched plasma membrane fraction was obtained from hypocotyls of seedlings by partitioning microsomes in an aqueous dextran-polyethylene glycol two-phase system [20]. The purified plasma membrane fraction was resuspended in homogenization buffer and used immediately or stored at −70°C.

2.3. Protein content

Protein concentration was determined according to Bradford [21], using bovine serum albumin (BSA) as a standard.

2.4. Preparation of rabbit polyclonal anti-peptide antibody (anti-PLDγ)

The 13 amino acid C-terminal peptide corresponding to residues 838–851 of AtPLDγ was synthesized on a polylysine tree and used as an antigen to raise antibody in rabbits.

2.5. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), non-denaturing PAGE

Denaturing electrophoresis was performed in 10% SDS polyacrylamide mini gels (5% stacking, 0.75 mm thick) with a constant voltage of 180 V. The proteins were dissolved in SDS gel loading buffer and boiled for 3 min. Non-denaturing electrophoresis was performed in 8% polyacrylamide mini gels (3.5% stacking, 0.75 mm thick) with a constant voltage of 150 V. The proteins were dissolved in gel loading buffer containing 1% Triton X-100.

2.6. Western blot analysis

After SDS–PAGE and non-denaturing PAGE, proteins were transferred onto nitrocellulose membranes by electroblotting. The transfer was completed in 1 h at a constant voltage of 50 V (transfer buffer: Tris–glycine/methanol, pH 8.3). After blocking (1 h) in TTBS buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl and 0.05% Tween 20) containing 5% (w/v) non-fat dry milk powder or 2% solution of BSA, the membranes were incubated for 1 h with rabbit polyclonal anti-PLDγ serum or rabbit polyclonal anti-P-Tyr and/or anti-P-Thr/Ser/Tyr antibodies (Promega, USA, diluted with blocking solution in a ratio 1:1000). Finally, the membrane was incubated for 1 h with goat anti-rabbit IgG labelled with alkaline phosphatase. Detection was performed using an amplified biotin streptavidin Bio-Rad kit according to the manufacturer's instructions. All the immunoblot procedures were carried out at room temperature.

2.7. In vitro PLD activity assay

PIP_2-dependent PLD (PLDβ/γ) activity was determined radiometrically with [methyl-^{14}C]phosphatidylcholine as substrate and 100 μM Ca^{2+}, as described earlier [20]. For identification of PLD activity after native PAGE, the nitrocellulose membrane with transferred proteins was cut into 2 mm strips. Each cut was dipped into 200 μl of incubation mixture [20] and after 30 min incubation at 30°C, the reaction was stopped by addition of 1 ml of a chloroform/methanol mixture (2:1, v/v) and 100 μl of 2 M KCl. Radioactivity was measured in the water phase (200 μl aliquots were mixed with 3 ml of scintillation solution).

2.8. Matrix-assisted laser desorption ionization/time of flight (MALDI-TOF) mass spectrometry

The fraction of the plasma membrane containing the PLD protein was separated in 8% gel by SDS–PAGE (see above). The gel was washed with water (two times for 10 min) and then the band of interest was excised and cut into 1 mm cubes. For in-gel digestion of proteins with trypsin, a method adapted from Shevchenko et al. [22] was used. Extracts (tryptic digest) were pooled, vacuum dried and purified on $ZipTip_{C18}$ column (Millipore). 1 μl of the tryptic digest was mixed with 3 μl of matrix solution prepared as follows: 10 mg of 2-hydroxy-5-methoxybenzoic acid (Sigma) was dissolved in 1 ml of acetonitrile/0.1% trifluoroacetic acid (1:2, v/v). The mixture was spotted on a MALDI target plate. Peptide mixture for external calibration was purchased from Bruker, Germany. A BIFLEX IV mass spectrometer (Bruker, Germany) with reflector was used for analyses. Spectra of peptide mixtures were recorded in reflector mode at an accelerating voltage of 19 kV at a laser wavelength of 337 nm. Peptide mass maps were searched against theoretically derived maps from proteins in the non-redundant protein database (NCBI) using the ProFound online program (www.proteometrics.com). Theoretical digests were obtained through the online MS-Digest program http://us.expasy.org/tools/peptide-mass.html.

2.9. In silico prediction and analysis of B. oleracea *PLDγ*

For searches of *B. oleracea* genome data we used the BLAST toolkit at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST; [23]). Non-redundant, GSS, EST and trace file databases were employed for the searches. Searches were done in parallel with the low complexity filter on and off, respectively. Other parameters were kept default. Preliminary *B. oleracea* sequence data were also obtained from the Institute for Genomic Research website (http://www.tigr.org/tdb/e2k1/bog1/). The final check of these databases was done on April 23, 2003. Shotgun reads were assembled using the CAP3 program (http://bio.ifom-firc.it/ASSEMBLY/assemble.html) and exon–intron structures were predicted manually. Multiple alignments were constructed using MACAW [24] and ClustalX with PAM 120 or Blosum 30 protein alignment matrices, respectively. The alignments were edited manually with the assistance of GENEDOC (Free Software Foundation).

3. Results and discussion

3.1. Biochemical identification of the phosphorylated PIP_2-dependent form of PLD associated with plasma membrane

Previously, we have demonstrated that different forms (PIP_2-dependent and/or PIP_2-independent) of *Brassica napus* PLDs are distributed in cytosol and associated with plasma membrane vesicles and their distribution between these two fractions changes during plant development [20]. It was also shown that one or more forms of plasma membrane-bound PLDs are possibly phosphorylated [25]. To confirm these preliminary findings and to identify the form(s) undergoing the modification, the approach of comparing enzyme activities versus immunoblot analysis was adopted. The plasma membrane fraction prepared from hypocotyls of *B. oleracea* was subjected to native PAGE and transferred to nitrocellulose. When the cuts of the nitrocellulose membrane after native PAGE were subjected to PLD assay, PIP_2-dependent activity was predominantly detected in the band with mobility $R_f = 0.13$ (Fig. 1). Furthermore, Western blot analysis with anti-PLDγ antibody showed immunoreactivity for the band of the same relative mobility (Fig. 1). The immunoblot with specific anti-P-Tyr and anti-P-Ser/Thr/Tyr antibodies (Fig. 1) revealed immunoreactive bands with the same mobility ($R_f = 0.13$), suggesting that this membrane-associated PIP_2-dependent form of PLD (PLDγ) could be phosphorylated.

3.2. Dephosphorylation of PIP_2-dependent PLD decreases its catalytic activity

The fact that the activity of animal PLD isoforms is modulated by phosphorylation of Tyr [15–18] and Ser/Thr [26] of the enzyme led us to speculate whether the same mechanism is also operating in plant PLDs. To solve this problem, the plasma membrane fraction (10 μg of total proteins) was preincubated with increasing concentrations of the wheat acid phosphatase (Sigma P3627) for 15 min at 37°C prior to in vitro PIP_2-dependent PLD activity assay. As shown in Table 1, this preincubation significantly decreased the plasma membrane PIP_2-dependent PLD activity in a concentration-dependent manner to approximately 20% of the original activity. In contrast, acid phosphatase preparation inactivated by boiling (for 10 min) did not reduce PIP_2-dependent PLD activity. Furthermore, the PIP_2-dependent PLD activity in the band with mobility $R_f = 0.13$ (Fig. 1) after native PAGE was decreased in a typical experiment to approximately 40% of the

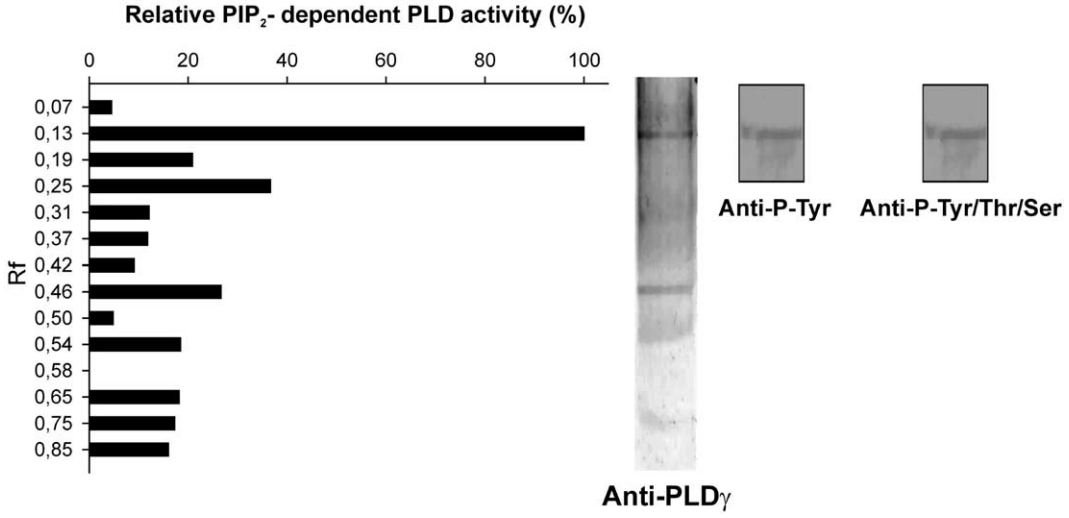

Fig. 1. Identification of PIP_2-dependent BoPLD associated with the plasma membrane. The plasma membrane fraction was separated by native PAGE (8%). Proteins were transferred to nitrocellulose membranes by electroblotting and analyzed by measurement of hydrolytic activity of PLD. PIP_2-dependent activity was measured as described in Section 2. Immunoblot analysis using antibody against PIP_2-dependent PLD (anti-PLDγ, 1:1000 dilution) and antibody against anti-P-Tyr or anti-P-Thr/Tyr/Ser (1:1000 dilution), PLD antibody complexes were visualized by staining the alkaline phosphatase activity (Bio-Rad kit).

original activity in the samples treated with phosphatase prior to electrophoresis. The decrease of PIP_2-dependent PLD activity was not caused by phosphatase cleavage of phospholipid substrate as no activity of acid wheat phosphatase on the nitrocellulose membrane was detected (results not shown).

The presented results reveal that phosphorylation could modulate PIP_2-dependent PLD activity in plants and thus be important for the regulation of this enzyme. A question remains for future investigation, whether the phosphorylation/dephosphorylation itself alters the activity of PLD on the plasma membrane or causes an interaction with another partner and thus modulates the activity of the enzyme.

3.3. *Identification of phosphorylation sites in plasma membrane-associated PLDγ*

To find the possible phosphorylation sites of PLDγ, the plasma membrane fraction was treated with acid wheat phosphatase (dephosphorylated form) or non-treated (phosphorylated form) prior to electrophoresis to remove or preserve presumed phosphate groups, respectively. After SDS–PAGE, the phosphorylated and dephosphorylated forms of PLD protein were detected by Western blotting with anti-PLDγ serum

Table 1
Effect of acid phosphatase from wheat germ on activity of plasma membrane-associated PIP_2-dependent PLD

Phosphatase (U)	PIP_2-dependent PLD activity (nmol/min/mg protein)	
	Without treatment	Boiling
0	1.36 ± 0.15	1.36 ± 0.15
0.05	1.02 ± 0.24	0.90 ± 0.40
0.25	0.25 ± 0.17	1.00 ± 0.23

Plasma membrane fractions (10 μg of total proteins) were preincubated with increasing concentrations of the acid phosphatase from wheat germ (0–0.25 U) for 15 min at 37°C and in vitro PIP_2-dependent activity was measured as described in Section 2. Acid phosphatase was not treated or treated by boiling. Values are means ± S.E.M. of two independent experiments, activity was measured in duplicate in each experiment.

(data not shown) and bands were excised from the gel and their tryptic digest was analyzed by MALDI-TOF mass spectrometry. The masses of peptide fragments observed in the mass spectra of PLD digests of phosphorylated and dephosphorylated forms were compared with those calculated for theoretical tryptic cleavage of the partially deduced sequence of BoPLDγ$_{1,2}$ and AtPLDγ$_{1,2,3}$. Although the sequence of the PLDγ homologue from *B. oleracea* has not been reported so far, ongoing genome sequencing projects allowed us to explore the diversity of PLD in *B. oleracea*. Blast similarity searches revealed the presence of putative *Brassica* gene orthologues to all members of the PLD family in *Arabidopsis*. At least two distinct PLDγs were identified and partial coding sequences assembled from individual shotgun reads (Fig. 2). The deduced partial amino acid sequence of PLDγ1 protein from *B. oleracea* was 90%, 80% and 85% identical to those of *Arabidopsis* PLDγ$_{1,2,3}$, respectively, which may indicate that *Arabidopsis* PLDγ$_1$ is the closest orthologue of BoPLDγ1. On the other hand, no direct orthologue within the PLDγ cluster can be attributed to BoPLDγ2, as it shares 78–79% identity with all PLDγ proteins from *Arabidopsis*. Using MALDI-TOF mass spectrometry 16 peptide fragments of PLDγ corresponding to deduced sequences of BoPLDγ$_1$ and seven fragments corresponding to BoPLDγ$_2$ were identified (bold letters in Fig. 2). Identified peptides represent 19% coverage of the BoPLDγ$_1$ partial sequence and 12% of BoPLDγ$_2$. Additionally 12 peptides corresponding to AtPLDγ$_{1,2,3}$ theoretical tryptic digests were also found in the mass spectra (data not shown), which supports the identification of BoPLDγ$_{1,2}$.

In the mass spectrum of the dephosphorylated form of PLD, high peaks at *m/z* values of 1437.70, 1493.60 and 1031.48 were found. In contrast, in the phosphorylated form of PLD these three peaks were missing but new lower peaks at *m/z* values of 1517.46, 1573.36 and 1111.24 appeared; the mass shift 79.76 is equal to that of the phosphate group. This observation is in agreement with the findings of Liao et al. [27] showing that the desorption/ionization efficiencies

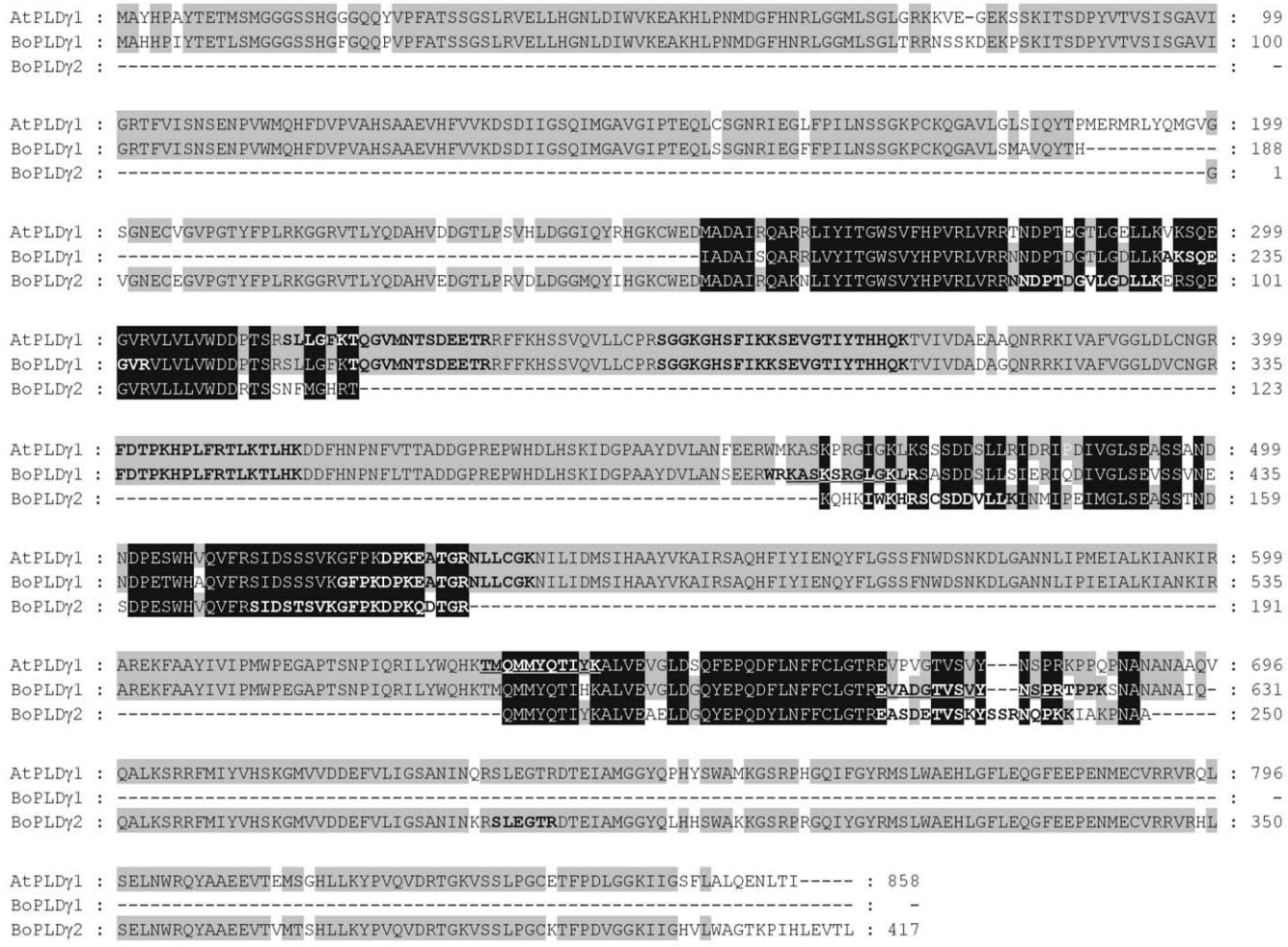

Fig. 2. Multiple alignment of predicted BoPLDγ. Partial sequences of BoPLDγ1 and BoPLDγ2 were aligned with AtPLDγ1 using ClustalX. Regions identified by MALDI-TOF mass spectrometry are shown in bold and matching phosphopeptides are underlined. Black and gray shading indicate conserved protein residues.

for phosphopeptides in MALDI-TOF mass spectroscopy are approximately an order of magnitude lower than for the non-phosphorylated forms.

Sequence KASKSRGLGK (underlined in Fig. 2) corresponding to the peak at *m/z* 1031.48 contains two serine residues. Output scores of prediction of protein kinase consensus sites using the NETPhos 2 program (http://www.cbs.dtu.dk/services/NETPhos) were 0.993 and 0.976 respectively. This sequence is localized in a region that was recently shown to confer PIP_2 binding in animal and certain plant PLDs [30,31]. Therefore we can hypothesize that reversible phosphorylation of this region affects PIP_2 binding and thus regulates PLD activity.

Peptide TMQMMYQTIYK (underlined in Fig. 2) corresponding to the peak at *m/z* 1437.70 contains two tyrosine and two threonine residues with output scores 0.903 for the second Thr while the other Tyr and Thr residues were below the 0.5 scoring value. Thus we can suppose that most probably the second threonine residue of this peptide could be phosphorylated. Sequence TMQMMYQTIY(H)K is localized in the region corresponding to that of mammalian PLD which binds β-actin and the KASKSRGLGK motif lies in close proximity to the N-terminal part of this domain [28]. Interestingly, Kusner and colleagues [29] demonstrated that monomeric G-actin inhibits and F-actin filaments enhance PLD activity. This phenomenon is evolutionarily conserved showing the same effect for bacterial, mammalian and plant PLDs. Based on our preliminary finding we can speculate that phosphorylation of serine/threonine residues in these sequences could be involved in interaction of PLDγ with phalloidin-stabilized F-actin filaments. It is therefore tempting to speculate that these motifs could be regulatory phosphorylation sites.

Sequence EVADGTVSVYNSPR (underlined in Fig. 2) corresponding to the peak at *m/z* 1493.60 contains one threonine residue, one tyrosine residue and two serine residues. Output scores of prediction were 0.939 and 0.974 for Ser, other Tyr and Thr residues were below the 0.5 scoring value and thus we can conclude that in this peptide most probably the Ser residue is phosphorylated. It should be noted that this motif lies next to the second catalytic HKD motif, its potential functional significance remains to be established.

Mass spectrometry analysis of tryptic digests of plasma membrane-associated PLDγ revealed for the first time possible regulatory phosphorylation sites of this isoform, but further investigation including e.g. site-directed mutagenesis would be necessary.

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References

[1] Wang, X. (2002) Curr. Opin. Plant Biol. 5, 408–414.
[2] Munnik, T. (2001) Trends Plant Sci. 6, 227–233.
[3] Munnik, T., de Vrije, T., Irvine, R.F. and Musgrave, A. (1996) J. Biol. Chem. 271, 15708–15715.
[4] Meijer, H.J.G. and Munnik, T. (2003) Annu. Rev. Plant Biol. 54, 10.1–10.42.
[5] Gardiner, J.C., Harper, J.D.I., Weerakoon, N.D., Collings, D.A., Ritchie, S., Gilroy, S., Cyr, R.J. and Marc, J. (2001) Plant Cell 13, 2143–2158.
[6] Munnik, T. and Musgrave, A. (2001) Sci. STKE, PE 42.
[7] Wang, X. (2000) Prog. Lipid Res. 39, 109–149.
[8] Wang, X. (2001) Annu. Rev. Plant Physiol. Plant Mol. Biol. 52, 211–231.
[9] Pappan, K. and Wang, X. (1999) Biochim. Biophys. Acta 1439, 151–166.
[10] Pappan, K., Austin-Brown, S., Chapman, K.D. and Wang, X. (1998) Arch. Biochem. Biophys. 353, 131–140.
[11] Lu, F., Zheng, S. and Wang, X. (1999) Plant Physiol. 119, 1371–1378.
[12] Wang, C. and Wang, X. (2001) Plant Physiol. 127, 1102–1112.
[13] Stone, J.M. and Walker, J.C. (1995) Plant Physiol. 108, 451–457.
[14] Lopez, I., Burns, D.J. and Lambeth, D.J. (1995) J. Biol. Chem. 270, 19465–19472.
[15] Houle, M.G. and Bourgoin, S. (1999) Biochim. Biophys. Acta 1439, 135–150.
[16] Min, D.S., Kim, E.G. and Exton, J.H. (1998) J. Biol. Chem. 273, 29986–29994.
[17] Exton, J.H. (2002) FEBS Lett. 531, 58–61.
[18] Mehta, S., Maglio, J., Kobayashi, M.S., Sipple, A.M. and Horwitz, J. (2003) Biochim. Biophys. Acta 5603, 1–9.
[19] Kim, Y., Han, J.M., Han, B.R., Lee, K.A., Kim, J.H., Lee, B.D., Jang, I.H., Suh, P.G. and Ryu, S.H. (2000) J. Biol. Chem. 275, 13621–13627.
[20] Novotná, Z., Martinec, J., Profotová, B., Žďárová, Š., Kader, J.C. and Valentová, O. (2003) J. Exp. Bot. 54, 691–698.
[21] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[22] Shevchenko, A., Wilm, M., Vorm, O. and Mann, M. (1996) Anal. Chem. 68, 850–858.
[23] Altschul, S.F., Madden, T.L. and Schaffer, A.A. (1997) Nucleic Acids Res. 25, 3389–3402.
[24] Schuler, G.D., Altschul, S.F. and Lipman, D.J. (1991) Proteins 9, 180–190.
[25] Novotná, Z., Valentová, O., Martinec, J., Feltl, T. and Nokhrina, K. (2000) Biochem. Soc. Trans. 28, 817–818.
[26] Watanabe, H. and Kanaho, Y. (2000) Biochim. Biophys. Acta 1495, 121–124.
[27] Liao, P.-C., Leykam, J., Andrews, P.C., Gage, D.A. and Allison, J. (1994) Anal. Biochem. 219, 9–20.
[28] Lee, S., Park, J.B., Kim, J.H., Kim, Y., Shin, K.J., Lee, J.S., Ha, S.H., Suh, P.G. and Ryu, S.H. (2001) J. Biol. Chem. 276, 3028252–3028260.
[29] Kusner, D.J., Barton, J.A., Quin, Ch., Wang, X. and Iyer, S.S. (2003) Arch. Biochem. Biophys. 412, 231–241.
[30] Sciorra, V.A., Rudge, S.A., Prestwich, G.D., Frohman, M.A., Engebrecht, J. and Morris, A.J. (1999) EMBO J. 18, 5911–5921.
[31] Zheng, L., Shan, J., Krishnamoorthi, R. and Wang, X. (2002) Biochemistry 41, 4546–4553.