A novel class of PTEN protein in Arabidopsis displays unusual phosphoinositide phosphatase activity and efficiently binds phosphatidic acid

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PTEN (phosphatase and tensin homologue deleted on chromosome ten) proteins are dual phosphatases with both protein and phosphoinositide phosphatase activity. They modulate signalling pathways controlling growth, metabolism and apoptosis in animals and are implied in several human diseases. In the present paper we describe a novel class of PTEN proteins in plants, termed PTEN2, which comprises the AtPTEN (Arabidopsis PTEN) 2a and AtPTEN2b proteins in Arabidopsis. Both display low in vitro tyrosine phosphatase activity. In addition, AtPTEN2a actively dephosphorylates in vitro the 3′-phosphate group of PI3P (phosphatidylinositol 3-phosphate), P(3,4)P2, (phosphatidylinositol 3,4-bisphosphate) and P(3,4,5)P3, (phosphatidylinositol 3,4,5-trisphosphate). In contrast with animal PTENs, P(3,4,5)P3 (phosphatidylinositol 3,4,5-trisphosphate) is a poor substrate. Site-directed mutagenesis of AtPTEN2a and molecular modelling of protein–phosphoinositide interactions indicated that substitutions at the PTEN2 core catalytic site of the Lys267 and Gly268 residues found in animals, which are critical for animal PTEN activity, by Met267 and Ala268 found in the eudicot PTEN2 are responsible for changes in substrate specificity. Remarkably, the AtPTEN2a protein also displays strong binding activity for PA (phosphatidic acid), a major lipid second messenger in plants. Promoter::GUS (β-glucuronidase) fusion, transcript and protein analyses further showed the transcriptional regulation of the ubiquitously expressed AtPTEN2a and AtPTEN2b by salt and osmotic stress. The results of the present study suggest a function for this novel class of plant PTEN proteins as an effector of lipid signalling in plants.

Key words: expression in planta, phosphatidic acid (PA) binding, phosphoinositide phosphatase, phylogenetics, site-directed mutagenesis.

INTRODUCTION

In eukaryotes, PIs (phosphoinositides) have emerged as second messengers in many signalling pathways, controlling important aspects of cell physiology including growth, metabolism, apoptosis, membrane dynamics, organelle identity and cytoskeletal rearrangements [1]. They are produced from the membrane lipid phosphatidylinositol and differ by the reversible phosphorylation of the inositol ring at its D3, D4 and D5 positions, giving rise to seven isomers with specific subcellular localizations and functions: PIP3 (phosphatidylinositol 3-phosphate), PIP4P (phosphatidylinositol 4-phosphate), PIP5P (phosphatidylinositol 5-phosphate), PIP3,4P2 (phosphatidylinositol 3,4-bisphosphate), PIP3,5P2 (phosphatidylinositol 3,5-bisphosphate), PIP4,5P2 (phosphatidylinositol 4,5-bisphosphate) and PIP3,4,5P3 (phosphatidylinositol 3,4,5-trisphosphate) [2]. PI signalling events are achieved by the binding of their negatively charged headgroup to specific proteins, modifying their subcellular localization and/or their activity.

Among the plethora of PI-metabolizing enzymes constituted by kinases, phospholipases and phosphatases, PTEN (phosphatase and tensin homologue deleted on chromosome 10; EC 3.1.3.67) has received massive attention since its discovery in 1997 for its function in human cancer (it is the most mutated gene after p53) [3]. PTEN harbours the specific catalytic motif (I/V)HCXAGXXR of protein tyrosine phosphatases and phospholipases and phosphatases, PTENs tumour suppressor activity has been attributed mainly to its role as a negative regulator of the PI3K (phosphatidylinositol 3-kinase)/Akt signalling pathway [4,5], which is a major growth and survival pathway in animals. In this pathway, PTEN antagonizes PI3K by removing the 3-phosphate from PIP3,4,5P3 [6], which is

Abbreviations used: BML, Bayesian maximum likelihood; DSP, dual-specificity phosphatase; DTT, dithiothreitol; GST, glutathione transferase; GUS, β-glucuronidase; LOI, lipid-of-interest; MP, maximum parsimony; MCMC, Metropolis-coupled Markov chain Monte Carlo; MS, Murashige and Skoog; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphoethanolamine; PI, phosphoinositide; PI3K, phosphatidylinositol 3-kinase; PI3P, phosphatidylinositol 3-phosphate; PI4P, phosphatidylinositol 4-phosphate; PI5P, phosphatidylinositol 5-phosphate; PI(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PI(3,5)P2, phosphatidylinositol 3,5-bisphosphate; PI(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; P(3′)-NPP, p-nitrophenyl phosphatase; PS, phosphatidylserine; PTEN, phosphatase and tensin homologue deleted on chromosome ten; AtPTEN, Arabidopsis PTEN; HsPTEN, human PTEN; RNAi, RNA interference; RT, reverse transcription; TBST, Tris-buffered saline plus Tween 20; TOR, target of rapamycin; YFP, yellow fluorescent protein.

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the activator of downstream effectors like the serine/threonine kinase Akt, a proto-oncogenic protein. In addition, a recent study revealed that PTEN protein phosphatase activity or PTEN interaction with protein partners may be crucial for several physiological processes, independently from its lipid phosphatase activity [7].

In plants, the first homologue of HsPTEN (human PTEN), AtPTEN (Arabidopsis PTEN) 1, has been characterized in Arabidopsis thaliana [8]. Like HsPTEN, the recombinant AtPTEN1 displays in vitro P(3,4,5)P₃ dephosphorylation activity. The RNAi (RNA interference) silencing of AtPTEN1, which is specifically expressed in pollen grains, leads to pollen cell death and plant sterility. However, the physiological relevance of this severe phenotype has not been established yet. It is unlikely to be due to P(3,4,5)P₃ dephosphorylation since neither this substrate nor the class I PISK responsible for its synthesis have been identified in plants. Although additional proteins with PTEN N-terminal phosphatase domains can be identified in plants, no characterization of their biochemical activity has been published to date. Advances in plant biology have triggered a renewed interest in plant PTENs. First, the crucial role played by lipid signalling in plant growth and development and responses to biotic and abiotic stress is now well established [9–14]. PTEN appears to be a likely effector of phospholipid signalling in plants, given its PI phosphatase activity in animals, which targets the D3 phosphate of the PI3P, P(3,4)P₂, and P(3,4,5)P₃ substrates [15,16]. Secondly, additional evidence of the function of PTEN domains has been obtained in plants, with the recent implication of such a domain in the apical localization of the moss For2 formin required for rapid elongation of actin filaments [17].

In the present paper we report the identification and characterization of new plant PTEN proteins from Arabidopsis containing PTEN phosphatase and C2 domains called AtPTEN2a and AtPTEN2b. We demonstrated the phospholipid activity of AtPTEN2a, which preferentially uses PI3P, in contrast with HsPTEN. We further showed that substitution of residues at the core catalytic site with those found in HsPTEN increased AtPTEN2a activity and modified its substrate specificities. Modelling of protein–PI interactions provided likely explanations for preferential substrate use. Promoter, transcript and protein analyses further indicated that AtPTEN2a and AtPTEN2b are differentially regulated in the plant in response to environmental constraints such as salinity and osmotic stress. Moreover, protein–lipid-binding experiments demonstrated that AtPTEN2a effectively binds the signalling lipid PA (phosphatidic acid). The results of the present study obtained in A. thaliana provide new insights into the evolution of PTEN genes from Arabidopsis and open the way for the study of their physiological function in planta.

**EXPERIMENTAL**

**Plant materials and growth conditions**

Seeds from A. thaliana (L.) Heynh. (ecotype Columbia-0) were surface-sterilized, stratified and either plated on 0.5× MS (Murashige and Skoog) medium supplemented with 0.8% agar and 1% (w/v) sucrose, or placed in 0.5× liquid MS medium supplemented with 2% (w/v) glucose and 20 mM Mes (pH 5.7) on a rotary shaker (120 rev/min). Seedlings (15-day-old) grown in liquid culture were osmotically stressed by transferring the plantlets to a new flask containing fresh medium supplemented with 0–200 mM NaCl or mannitol. For cultivation of adult plants, seedlings were transferred from the 0.5× MS solid medium to a soil-vermiculite mixture. For all experiments, the plants were cultivated at 22°C under a daily 16 h light period at 150 μmol of photons·m⁻²·s⁻¹.

**Sequence analysis and phylogeny**

PTEN sequences were recovered from several databases and mainly from sequenced genomes (database accession numbers are given in Supplementary Table S1 at http://www.BiochemJ.org/bj/441/bj4410161add.htm). TAIR (The Arabidopsis Information Resource; http://www.arabidopsis.org/) accession numbers for the Arabidopsis PTEN genes were At3g94000 (AtPTEN1), At3g19420 (AtPTEN2a) and At3g50110 (AtPTEN2b). Only the amino acid sequences of the highly conserved phosphatase domain (170–195 amino acids) were retained for analysis since the sequences of the C2 domain were too divergent. All sequences were edited and aligned manually using BioEdit [18]. The final alignment included six indels of 1–30 amino acids that were encoded as additional character states (absent, 0 and present, 1), regardless of their length. They were included in subsequent sequence analyses to exploit their phylogenetic information following the procedure of Barriel [19]. Phylogenetic relationships were estimated by MP (maximum parsimony) and BML (Bayesian maximum likelihood) methods. MP analysis was first conducted using PAUP* v4.0b10 [20]. The data matrix was analysed with the heuristic search and the default search options. Support of internal nodes was assessed using bootstrap analysis of 10000 replicates. BML analysis of the same dataset was performed using the MCMC (Metropolis-coupled Markov chain Monte Carlo) simulation program MrBayes v3.1 [21]. The indel events were included as a separate binary (restriction) data partition. Model parameters for each partition were estimated separately during the MCMC process. BML analyses were run for 5000000 generations with sampling every 100 generations to produce 50000 sampled trees. Trees sampled before stationarity was reached (burnin option = generation 5000) were discarded and a 50% posterior probability Bayesian consensus tree was generated from the remaining trees. The frequency of a clade in the consensus tree represents the posterior probabilities of that clade.

**Expression in bacteria and purification of recombinant proteins**

The Arabidopsis At3g19420 (AtPTEN2a) and At3g50110 (AtPTEN2b) genes were PCR-amplified from the RAFL09-38-D10 cDNA clone (RIKEN BioResource Center, Japan) and C105193 cDNA clone (The Arabidopsis Biological Resource Center, Ohio, U.S.A.) respectively. The primers used for amplification contained gene-specific sequences flanked by vector-specific sequences required for cloning into pET Eki/LIC expression vectors (Novagen; the primer sequences used are detailed in Supplementary Table S2 at http://www.BiochemJ.org/bj/441/bj4410161add.htm). The generated PCR fragments were purified, treated with T4 polymerase and ligated into pET Eki/LIC vectors following the manufacturer’s protocol. The full AtPTEN2a open reading frame was cloned in pET-30 Eki/LIC to produce an in-frame N-terminus His-tag fusion giving rise to pLiC30-AtPTEN2a. The AtPTEN2b ORF (open reading frame) was cloned in pET-41 Eki/LIC to produce in-frame N-terminus His- and GST (glutathione transferase)-tag fusions giving rise to pLiC41-AtPTEN2b. The site-directed mutagenesis (pLiC-AtPTEN2aC263S and pLiC-AtPTEN2aM267K/A268G) was performed using the QuikChange® kit (Stratagene) according to the manufacturer’s instructions. Pfu DNA polymerase (Stratagene) was used in all PCR reactions and all constructs were verified by sequencing. The constructs above were introduced into the BL21 (DE3) strain of Escherichia coli (Invitrogen) to
express the corresponding recombinant proteins. Transformed cells were cultured in 0.5 litres of Terrific broth medium containing 30 μg/ml kanamycin at 37 °C. When a D_600 value of 0.6 was reached, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 1 mM and incubation was continued for 4 h at 37 °C. Subsequent steps were carried out at 4 °C. After centrifugation (6000 g for 20 min), the cells were washed, resuspended in 15 ml of lysis buffer [50 mM Tris/HCl (pH 7.5), 1 mM DTT (dithiothreitol), 1 mM PMSF and 500 mM NaCl] containing 1 mg/ml lysozyme and EDTA-free protease inhibitors (Roche Applied Science) and incubated at 30 °C for 1 h. After sonication, the soluble fraction was obtained by centrifugation (20 000 g for 20 min at 4 °C). The supernatant was then loaded on to a 1 ml HisTrapTM HP column using an Äkta Purifier (GE Healthcare). After sample loading, the column was washed with lysis buffer containing 20 mM imidazole and the protein was eluted with the same buffer containing 250 mM imidazole. The eluted fraction was then diluted (1:4 dilution) in 20 mM Tris/HCl (pH 7.5), 1 mM DTT and 10% (v/v) glycerol. The protein was further purified by ion exchange using a MonoQ column HR 5/5 (GE Healthcare) equilibrated with the dilution buffer described above. The protein was eluted with a NaCl gradient ranging from 100 to 500 mM over 20 column volumes. Finally, purified proteins were frozen in liquid N2 and stored at −80 °C after adjusting the glycerol concentration up to 50%.

**Lipid phosphatase activity using Malachite Green assays**

PTEN hydrolysing activity towards the different PIs was determined *in vitro* by the Malachite Green-based assay that measures the released Pi [22]. Briefly, 2 μg of recombinant protein were incubated with 100 μM of one of the seven di/C8 PI lipids [PI3P, PI4P, PI5P, PI(3,4)P₂, PI(3,5)P₂, PI(4,5)P₂ or PI(3,4,5)P₃] (Echelon Biosciences) for 40 min (AtPTEN2a and AtPTEN2aM267K/A268G proteins) or 180 min (AtPTEN2b protein) at 30 °C in a final volume of 25 μl. The reaction buffer was 100 mM Tris/HCl (pH 8.0) containing 10 mM DTT. The reaction was stopped with 100 μl of Malachite Green reagent (Echelon Biosciences). The amount of phosphate released was determined by reading the absorbance at 660 nm and converted into a molar measure of released Pi by the Malachite Green-based assay that uses the standard curve. The amount of protein used and the duration of the assay were within the linear range of the reaction. For the reaction with PI3P, 8% of the substrate was consumed at the end of the reaction.

**Molecular modelling**

The different models of the phosphatase domain of *Arabidopsis* proteins have been built using the sequence alignment in Supplementary Figure S1 (at http://www.BiochemJ.org/bj/441/bj4410161add.htm) and the software Modeller 9 version 2 (http://salilab.org/modeller/) using the default options. In each case, 20 models were generated and the five lowest-energy conformers were kept for further investigation. The PI-binding assays were performed using InsightII software programs, mainly Discover and the CVFF (constant valence force field). Point mutations were built using the Homology Module of InsightII. The general protocol used throughout is the following: the starting point is the X-ray structure of HsPTEN (PDB code 1D5R) with PI(3,4,5)P₃ (PIP₃) docked as described by Lee et al. [15] by superimposing the phosphates of PIP₃ on the carboxylates of tartaric acid and then deleting the latter. PIP₃ was extracted from the X-ray structure of the pleckstrin homology domain of the kinase B/AKT (PDB code 1H10). The PIP₃ molecule was built from PIP₃, by deleting the corresponding phosphate groups. The first step was a multi-step minimization: (i) the backbone of the protein and the whole PIP₃ (or PI3P) are kept fixed and all side-chains are fully minimized; (ii) PIP₃ (or PI3P) is then released and full minimization is resumed until the RMSD (root mean square deviation) is <0.01. Then, the complex PIP₃ (or PI3P)–protein is submitted to a 100 ps molecular dynamics run at 1000 K (timestep = 1 fs) with the protein backbone fixed. The complex is fully minimized first with the protein backbone tethered with 200, 100 and 50 kcal/Å² (1 Å = 0.1 nm) constraints. Finally, the complex was submitted to a 200 ps molecular dynamics run at 500 K (timestep = 1 fs) with the protein backbone tethered with a 50 kcal/Å² constraint, and 400 conformers were stored. Following a cluster graph, the two or three best solutions are submitted to full minimization and are retained for further investigation. For the plant PTEN, the starting point is obtained by superimposition of the modelled structure of the unknown PTEs on the structure of HsPTEN with PIP₃ (or PI3P), then the PIP₃ (or PI3P) molecule is transferred between the two proteins and HsPTEN is deleted, leaving PIP₃ (or PI3P) docked within the studied protein.

**Promoter activity**

The 1.16 kbp sequence of the AtPTEN2a gene promoter and the 1.27 kbp sequence of the AtPTEN2b gene promoter were amplified from genomic DNA by using specific primers (Supplementary Table S2) and fused to a GUS (β-glucuronidase) reporter gene in the vector pGreenIK-GUSORF-Ternos (http://www.pgreen.ac.uk) between the KpnI and SalI sites. *A. thaliana* plants were transformed with Agrobacterium tumefaciens GV3101 carrying the constructs by using the ‘floral-dip’ method. GUS-activity staining was performed according to Sessions et al. [23].

**Western blotting**

Plant material (0.2 g) was homogenized and extracted in 0.1 ml of 50 mM Tris/HCl (pH 8.0), 1 mM EDTA, 0.1% SDS and 0.1% Triton X-100. The extracts were cleared by centrifugation at 13 000 g for 15 min at 20 °C and conserved at −80 °C. SDS/PAGE and Western blotting were performed using an ECL Western blotting Kit (GE Healthcare) according to the manufacturer’s protocol. The membranes were hybridized with a 1:5000 dilution of primary antibodies. Rabbit polyclonal antibodies against the FASTQNDSGVENTEK peptide for AtPTEN2a and the EPPKRDDPSANRSVT peptide of AtPTEN2b were from Eurogentec. Specificity and absence of cross-reactivity of the antibodies were further assessed by probing available pten2a and pten2b Arabidopsis insertional T-DNA mutants (results not shown).

**Lipid-binding assays**

For protein–lipid overlay, membrane strips pre-spotted with 100 pmol of lipids (Echelon Biosciences) were first blocked with 3% (w/v) fat-free BSA in TBST [Tris-buffered saline plus Tween 20; 10 mM Tris/HCl (pH 8.0), 150 mM NaCl and 0.1% Tween 20] and incubated with 0.5 μg/ml AtPTEN2a protein solution in the blocking buffer overnight at 4 °C. The strips were washed with TBST, and PTEN2a was subsequently revealed using immunological revelation as described above. To detect lipid binding in vesicles, lipid mixtures consisting of 45% PC (phosphatidylcholine), 45% PE (phosphoethanolamine) and 10% LOI (lipid-of-interest) were used to produce vesicles sized 200 nm using lipid extrusion, as described by Testerink et al. [24]. Dipalmitoyl PIPs were from Echelon Biosciences and all other...
lipids were obtained from Avanti Polar Lipids. Two total lipid concentrations were tested, 4 mM and 1 mM, corresponding to 200 μM and 50 μM of the LOI theoretically available for binding (with headgroups facing outside of the vesicle) respectively. AtPTEN2a protein (200 ng) was added to the vesicles and incubated for 1 h. Samples were centrifuged at 16000 g for 30 min at 22°C to spin down the vesicles, and the pellets were washed once and analysed by SDS/PAGE (10% gel) to detect protein bound to the vesicles. The gels were stained with colloidal Coomassie Brilliant Blue. The recombinant 2×FYVE domain tagged to GST–YFP (yellow fluorescent protein) [25] was used as a control for PI3P binding.

Transcript level analysis by real-time quantitative PCR

Total RNA was extracted from Arabidopsis tissues with the RNaseq plant mini kit (Qiagen). Purified RNA was treated with DNase I using the DNA-free kit (Ambion). First-strand cDNA was prepared from 1 μg of total RNA with the SuperScript RT II kit (Invitrogen) and oligo(dT)18 according to the manufacturer’s instructions. A 0.2 μl aliquot of the total reaction volume was used as a template in real-time RT (reverse transcription)-mediated PCR amplification. The PCR amplification was performed as described by Joubé et al. [26] with gene-specific primers (Supplementary Table S2). The relative abundance of ACT2, eIF-1α and eIF-4A-I in each sample was determined and used to normalize differences of total RNA amount according to the method described by Vanoesompele et al. [27].

RESULTS

Arabidopsis contains three PTEN-like genes

PTEN proteins are characterized by two domains: an N-terminal phosphatase domain with the catalytic core signature motif of protein tyrosine phosphatases (I/V)HCXAGXXR and a C-terminal C2 domain well-conserved among the metazoans which is critical for membrane binding [28,29]. Using HsPTEN and Drosophila PTEN protein as query sequences, we identified three PTEN homologues in the Arabidopsis genome and a higher number of proteins with PTEN domains, e.g. the For2 formin [17]. The AtPTEN1 protein has been biochemically characterized [8], whereas the two other PTEN proteins found in Arabidopsis, named AtPTEN2a and AtPTEN2b (corresponding to the At3g19420 and At3g50110 genes respectively) have not yet been studied (Figure 1A). The AtPTEN2a and AtPTEN2b genes encode putative proteins of 611 and 632 amino acids respectively, which are much larger than the HsPTEN and AtPTEN1 proteins (Figure 1B). All three Arabidopsis PTEN proteins present a phosphatase domain containing the catalytic protein tyrosine phosphatase motif (InterPro accession number IPR014019 Phosphatase_tensin-typ) showing 67–69% similarity with its human relative, and a C2 domain (InterPro accession number IPR014020 Tensin_phosphatase_C2-dom). The organization of these domains is conserved between human, Drosophila and Arabidopsis.

Phylogenetic analyses of PTEN

In order to obtain more insights into the functional relationships of PTEN proteins in plants, we performed phylogenetic analyses of all PTEN proteins found in plants. A BLAST homology search using the highly conserved phosphatase domain from HsPTEN (181 amino acids) allowed recovery of PTEN sequences from angiosperms and gymnosperms, which were compared with lycophytes (Selaginella moellendorffii), green algae (Chlamydomonas reinhardtii) and animal PTENs (Supplementary Table S1). The matrix used for phylogenetic analyses includes 182 characters, of which 30 were constant and 125 were phylogenetically informative. Parsimony analysis resulted in 96 equally most parsimonious trees of 555 steps, with a CI (consistency index) of 0.757 and a highest RI (retention index) of 0.866. The result of phylogenetic analysis using the Bayesian method is shown in Figure 2(A). The strict consensus tree, which resulted in similar tree topology, is presented in Supplementary Figure S2 at (http://www.BiochemJ.org/bj/441/bj4410161add.htm). Within these trees, the available sequences from animals are together, whereas those representing various lineages of tracheophytes (including angiosperms, gymnosperms and lycophytes) are divided into two distinct and strongly supported monophyletic groups (posterior probability of 1.0 and bootstrap support of 100%), hereafter named PTEN1 (including AtPTEN1) and PTEN2 (including AtPTEN2a and AtPTEN2b). Within the PTEN1 clade, one sequence of S. moellendorffii (lycophytes) is placed as sister to all available sequences from gymnosperms and part of the eudicot sequences. Within the PTEN2 clade, the other S. moellendorffii sequence is placed as sister to all remaining monocot and dicot sequences. The only sequence representing the green algae (C. reinhardtii) is more closely related to that of the PTEN2 than to that of the PTEN1 clade.

Interestingly, in comparison with the catalytic core motif HCKAGKGR found in animal PTENs, the translated sequences from the moss S. moellendorffii and monocots showed the typical PTEN signature, i.e. Cys124 and Arg130 (HsPTEN numbering) for catalysis and the two lysine residues Lys125 and Lys128 for lipid substrate-binding. Sequences from eudicots presented notable changes (Figure 2B). For the PTEN1 clade, Lys125 was replaced by a methionine residue and for the PTEN2 clade, Lys128 and Gly129 were substituted by methionine and alanine residues respectively.
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Figure 2  Phylogenetic analysis of the phosphatase domain of PTEN

(A) Posterior probability (>50%) Bayesian consensus tree based on the phosphatase domain of eukaryotic PTEN sequences. The tree was produced from $5 \times 10^6$ generations. Posterior probabilities are indicated above the branches. Major groups belonging to the plant and animal kingdoms are indicated. The sequence accessions are listed in Supplementary Table S1 (at http://www.BiochemJ.org/bj/441/bj4410161add.htm). Thale cress (A. thaliana), grape (Vitis vinifera), poplar (Populus trichocarpa), tomato (Solanum lycopersicum), cotton (Gossypium species, G. raimondii), wheat (Triticum aestivum), rice (Oryza sativa), sorghum (Sorghum propinuqum), sugarcane (Saccharum officinarum), white spruce (Picea glauca), pine (Pinus taeda), Japanese cedar (Cryptomeria japonica), spike moss (Selaginella moellendorfii), Chlamydomonas reinhardtii, zebrafish (Danio rerio), African frog (Xenopus laevis), mouse (Mus musculus), human (Homo sapiens) and worm (Caenorhabditis elegans). (B) Sequence alignment of the catalytic core for different clade representatives. The position of the amino acids is indicated. The amino acids involved in catalysis are indicated in bold. Grey boxes represent the main differences between the PTEN protein from animals and the PTEN2 protein from eudicots. •, position of the amino acids not determined because of incomplete an N-terminal sequence. *, conserved residues.

Considering the crucial role played by these amino acids in PTEN activity and substrate specificity [15], we hypothesized that such changes may alter the biochemical properties of plant PTEN2 and we therefore undertook the biochemical characterization of the eudicot PTEN2 from Arabidopsis.

Purification of recombinant Arabidopsis PTEN2 proteins from E. coli

HsPTEN was first described as a protein tyrosine phosphatase. It was later acknowledged as a preferential lipid phosphatase...
using phosphatidylinositol substrates such as \( \text{PI}(3,4,5)P_3 \) [6]. Both activities are conserved in AtPTEN1 [8]. To address the function of AtPTEN2a and AtPTEN2b, we expressed the recombinant proteins in *E. coli* as His-tag (AtPTEN2a) or His–GST tag (AtPTEN2b) fusion proteins. Initial attempts to purify the proteins using affinity columns were largely unsuccessful because large aggregates were formed with other proteins, especially for AtPTEN2b. Affinity chromatography followed by ion-exchange chromatography eventually succeeded in the purification of His–AtPTEN2a (and two mutated AtPTEN2a proteins obtained by site directed mutagenesis) and His–GST AtPTEN2b fusion proteins (Figure 3A). SDS/PAGE analysis of the purified proteins indicated a size of approximately 120 kDa for the AtPTEN2a and 130 kDa for AtPTEN2b proteins, whereas the calculated mass of their translated products is 72 kDa and 100 kDa respectively. This was attributed to unusual gel-migration patterns, possibly due to the structure of the AtPTEN2 protein domains, since MS analysis of the purified AtPTEN2a confirmed its PTEN identity (Supplementary Figure S3 at http://www.BiochemJ.org/bj/441/bj4410161add.htm).

**Substrate specificities of AtPTEN2 phosphatases**

Because the functional roles of PTEN are largely linked to their lipid phosphatase activity [4], we next investigated the phosphatase activity of AtPTEN2 towards various PI substrates as described in Merlot et al. [30]. Among monophosphorylated phosphatidylinositol isomers (PIPs), the recombinant AtPTEN2a enzyme efficiently removed phosphate from the D3 position of the inositol ring, less from the D4 position and not at all from the D5 position (Figure 3B). In the PIP2 isomers, the presence of a phosphate group in the D5 position led to a consistent reduction in AtPTEN2a lipid phosphatase activity. Strikingly, in contrast with animal PTEN [6] and with *Arabidopsis* AtPTEN1 [8], AtPTEN2a showed a very weak activity against \( \text{PI}(3,4,5)P_3 \) as substrate. The recombinant GST–AtPTEN2b protein displayed no detectable phospholipid activity, except a weak one towards PI3P. AtPTEN2b was therefore excluded from further detailed characterization.

We next analysed the impact of mutations on those amino acids that may play a role in PI dephosphorylation. As expected from previous studies on human PTEN [4], mutation of the catalytic Cys263 to a serine residue abolished AtPTEN2a activity independent of the substrate assayed (results not shown). In eudicot PTEN2, the Met267 residue replaces the lysine found in AtPTEN2a lipid phosphatase activity. Strikingly, in contrast with animal PTEN [6] and with *Arabidopsis* AtPTEN1 [8], AtPTEN2a showed a very weak activity against \( \text{PI}(3,4,5)P_3 \) as substrate. The recombinant GST–AtPTEN2b protein displayed no detectable phospholipid activity, except a weak one towards PI3P.

| A | SDS/PAGE gel electrophoresis of 3 μg of purified AtPTEN2a (lane 1), AtPTEN2a C263S (lane 2) and AtPTEN2a M267K/A268G (lane 3) and 7 μg of purified AtPTEN2b (lane 4). Staining was with Coomassie Brilliant Blue. Position of molecular markers (M, kDa) are indicated on the left-hand side. (B) Activity of recombinant AtPTEN2a and AtPTEN2b against various di-C8 PI substrates. The activity was measured by the amount of free Pi released from the substrate using a Malachite Green assay as detailed in the Experimental section. The absorbance at 660 nm was measured, and the released Pi was quantified by a standard curve in parallel in each experiment. Free Pi was measured in controls (buffer, substrate solutions and recombinant proteins alone) and subtracted for calculating AtPTEN2 activity. The AtPTEN2a C263S protein displayed no activity whatever the substrate tested. (C) Activity of recombinant AtPTEN2a M267K/A268G against various di-C8 PI substrates. The activity was measured as described in (B). Reactions in (B) and (C) were carried out in triplicate and results are means ± S.E.M. for two independent experiments.

![Figure 3 Biochemical characterization of PI phosphatase activity of recombinant AtPTEN2a and AtPTEN2b proteins](http://www.BiochemJ.org/bj/441/bj4410161add.htm)

**AIPTEK modelling studies of the phosphatase domain**

In order to provide additional information on the relationship between the *Arabidopsis* PTEN structure and function, different models of AtPTEN1, AtPTEN2a and AtPTEN2b proteins were built using the sequence alignment shown in Supplementary Figure S1 as described in Experimental section. In each case, 20 models were generated and the five lowest-energy conformers were kept for further investigation. Superimposition of the three models of AtPTEN on the X-ray structure of HsPTEN (PDB code 1D5R) revealed weak differences in the structure of the phosphatase domain (Figure 4A). AtPTEN2a

![Figure 3](http://www.BiochemJ.org/bj/441/bj4410161add.htm)
and AtPTEN2b differ mainly from AtPTEN1 and HsPTEN by a single unstructured lipophilic loop of eight amino acids (Figure 4A and Supplementary Figure S1). The two amino acid deletions in AtPTEN1 (Supplementary Figure S1) seem to have a minimal effect on the structure of the domain. MLPs (molecular lipophilicity potentials) were calculated at the atomic level for HsPTEN and the three models of AtPTEN1, 2a and 2b (see the Experimental section of the Supplementary online data at http://www.BiochemJ.org/bj/441/bj4410161add.htm). From this experiment, an increase in lipophilicity is clearly seen from AtPTEN1 (almost equivalent to HsPTEN) to AtPTEN2a and AtPTEN2b (Supplementary Figure S5 at http://www.BiochemJ.org/bj/441/bj4410161add.htm). This result corroborates perfectly the difficulties encountered during purification on affinity columns as stated above.

Docking of PI(3,4,5)P3 was explored from models of HsPTEN, AtPTEN2a and the AtPTEN2a mutant M267K/A268G (Figure 4B). The interactions observed were the following: (i) for HsPTEN P1 with Lys125 and His126, P3 with Lys128 and Arg130, P4 with Lys128, and P5 with Lys128 (no equivalent residue for HsPTEN Lys128 present in AtPTEN2a, the absence of lysine at position 267 leads to a reorientation of PI3P and to interaction with Lys303. AtPTEN2a M267K/A268G P1 with Lys264 and Lys269 (equivalent to Lys125 and Arg130 in HsPTEN), P3 with Arg269 (equivalent to Arg130 in HsPTEN), P4 with Lys303 and Lys267 (equivalent to HsPTEN Lys303), and P5 with Lys267 (equivalent to Lys128). In the AtPTEN2a M267K/A268G mutant, PI(3,4,5)P3 is engaged in the same interactions as in HsPTEN. On the other hand, in wild-type AtPTEN2a, the absence of lysine at position 267 leads to a reorientation of PI(3,4,5)P3 and to an interaction with Lys303. The same experiment has been done with PI3P in AtPTEN2a and AtPTEN2a M267K/A268G models. The AtPTEN2a protein displays interactions between P1 and Lys264 (one interaction), between P3 and Arg269 (three interactions), and between P3 and Lys264 (one interaction). The M267K/A268G AtPTEN2a mutant displays interactions between P1 and Lys264 (one interaction), between P3 and Arg269 (two interactions), between P3 and Lys267 (one interaction), between P3 and Lys264 (one interaction). This clear result states that Lys264 has replaced Lys303 for P1 and has replaced one of the interactions with Arg269. Moreover (and even if there are two different amino acids) the energy of the wild-type protein is largely lower than the mutant. The consequence of the mutation is a large displacement of PI3P within the active site as seen in Figure 4(C): the distance between the respective P3 is only 1.5 Å, but reaches 4.75 Å for the respective P1 which may easily explain the absence of reactivity for the M267K/A268G mutated AtPTEN2a.

**AtPTEN2a affinity for phospholipid membranes in vitro**

As PIs are membrane components, we investigated the ability of AtPTEN2a to be recruited to membranes. Surprisingly, protein–lipid overlay assays with commercial pre-spotted pure lipids revealed a strong binding to PA (Figure 5A), whereas no binding was visible for the other lipids. In order to investigate further this binding property, AtPTEN2a was incubated with liposomes containing a mixture of PC, PE and 10 % of LOI:PA, PS (phosphatidylserine), PI3P, PI4P or PI5P. The SDS/PAGE analysis of AtPTEN2a bound to vesicles confirmed the result obtained by protein–lipid overlay analysis with PA at both LOI concentrations (Figure 5B) and identified a weak but clear binding for PS, even visible at 50 μM LOI. No binding was obtained for liposomes (Figure 5C). The absence of reactivity for the M267K/A268G mutated AtPTEN2a is visible at 50 μM LOI:PA, PS and 10 % of LOI:PA, PS (phosphatidylserine), PI3P, PI4P or PI5P. The SDS/PAGE analysis of AtPTEN2a bound to vesicles confirmed the result obtained by protein–lipid overlay analysis with PA at both LOI concentrations (Figure 5B) and identified a weak but clear binding for PS, even visible at 50 μM LOI. No binding was obtained for liposomes.
Figure 5 Lipid-binding assays of recombinant AtPTEN2a

(A) Protein–lipid overlay assay for the detection of PTEN2a-interacting lipids. Strips included 100 pmoles of the following lipids: lyso-phosphatidic acid (L-PA); lyso-phosphocholine (L-PC); PI and phosphorylated derivatives PI3P, PI4P, PI5P, PI(3,4)P2, PI(3,5)P2, PI(4,5)P2 and PI(3,4,5)P3; PE; PS; PC; sphingosine-1-phosphate (S1P); and PA. After incubation of 0.5 μg/ml AtPTEN2a with the strips, the protein bound to the lipids was detected by immunological revelation. (B) AtPTEN2a liposome-binding assays. Lipid binding specificity of AtPTEN2a was determined by using 200 ng of AtPTEN2a recombinant protein and 200 nm-sized vesicles containing 45% PC, 45% PE and 10% LOI, as described in the Experimental section. After incubation of AtPTEN2a with the vesicles, they were recovered by centrifugation and protein bound was analysed by SDS/PAGE. The positive control of PI3P binding was 2xFYVE.

Expression patterns of APTEN2 genes in planta

To analyse the activity of AtPTEN2 promoters in planta, we produced A. thaliana plants stably transformed with AtPTEN2a and AtPTEN2b promoter::GUS transcriptional fusions. GUS staining of plant organs at several stages of development revealed that both genes were expressed in a wide range of plant organs (Figure 6A) and that activity of the AtPTEN2a promoter was always higher. For both genes, young tissues were more intensively stained as observed in seedlings, leaves, flowers and siliques. GUS staining results were consistent with transcript accumulation patterns obtained by semi-quantitative RT–PCR (Figure S6A) and with the Genevestigator database (https://www.genevestigator.com/gv/index.jsp). The latter further indicated that AtPTEN2a transcript abundance is 3–5-fold higher than that of AtPTEN2b independent of the plant tissue studied (Figure S6B). In contrast, AtPTEN1 transcript accumulation is very low except in reproductive tissues where it is restricted to pollen as described previously [8].

We next produced antibodies specific against either the AtPTEN2a or AtPTEN2b isoforms and analysed their protein expression patterns in plant tissues by Western blotting (Figure 6B). AtPTEN2a protein, like the AtPTEN2a transcript, was observed in all plant organs, but was not detectable any more in the mature leaves or siliques. Surprisingly, given its transcript accumulation pattern, the AtPTEN2b protein accumulated to higher levels in seedlings and in stem terminal nodes. Taken together, these results support a role for AtPTEN2 throughout plant development, whereas AtPTEN1 function would be more restricted to pollen. Discrepancies between promoter activity, transcript abundance and protein levels observed in several organs or physiological stages further suggest a tight post-transcriptional regulation of both AtPTEN2a and AtPTEN2b.

Since PI signalling is crucial for plant responses to stress [10,13], we next investigated whether AtPTEN2 expression was altered in response to various stresses including dehydration, salt (NaCl) and osmotic (mannitol) stresses. No significant changes were observed for 15-day-old plantlets submitted to 24 h of dehydration (results not shown), whereas only a weak response was observed to salinity (NaCl) stress (Figure 7A). Conversely, osmotic stress triggered by mannitol concentrations ranging from 100 to 200 mM considerably enhanced AtPTEN2a...
transcript abundance, and to a much lower level that of AtPTEN2b (Figure 7B). This was not followed by parallel protein accumulation since AtPTEN2a and AtPTEN2b protein levels remained stable in leaves and only showed a weak increase in roots after a 48 h exposure to mannitol at 150 mM (Figure 7C).

DISCUSSION

Animal PTENs are DSPs that can dephosphorylate both phosphotyrosine and phospho-serine/phospho-threonine residues within one substrate. In addition, animal PTENs have lipid phosphatase activity that targets the D3 phosphate from PIs. Two domains are known to play important roles in PTEN functions: the phosphatase domain and the C2 domain. The protein also contains a short N-terminal PI(4,5)P 2-binding domain which is crucial for the binding of PTEN to membrane lipids [31]. We have identified in the plant model Arabidopsis three PTEN proteins displaying the canonical PTEN phosphatase domain, which includes the catalytic core signature motif of protein tyrosine phosphatases, (I/V)HCXAGXXR, and the C2 domain (Figure 1B). Phylogenetic analyses of PTEN sequences from plant and animal kingdoms suggest a duplication event of the PTEN gene early during the evolutionary history of plants. As a consequence, two distinct monophyletic groups can be clearly identified in the vascular plants (Figure 2A). The PTEN1 clade comprises sequences from the angiosperms, gymnosperms and lycophytes. The Arabidopsis AtPTEN1 gene, which belongs to the PTEN1 clade, has been recently shown to display a PI(3,4,5)P 3-dephosphorylating activity and to strongly affect pollen viability when inactivated by RNAi silencing [8]. However, neither the PI(3,4,5)P 3, PI or the type I PI3K kinase which catalyses its formation from PI(4,5)P 2 have ever been identified in plants, thus questioning the mode of action of PTEN1 in plants. The PTEN2 clade includes sequences from the angiosperms (eudicots and monocots) and from lycophytes. That clade is evolutionarily distant from the PTEN1 clade and includes two genes in Arabidopsis: the AtPTEN2a and the AtPTEN2b genes. Like human PTEN, both AtPTEN2a and AtPTEN2b proteins are able to catalyse dephosphorylation of a synthetic protein phosphatase substrate (p-NPP) and of PIs (Figure 3 and Supplementary Figure S4), suggesting that other sequences that are present in this clade may have similar activities.

The phylogenetic pattern obtained for PTEN suggests that the duplication giving rise to the PTEN1 and the PTEN2 clades occurred at least before the divergence between lycophytes and spermaphytes (approximately 400 million years ago), and most likely earlier, before the origin of the green algae. In this case, the latter would have lost the PTEN1 gene. However, further investigations in other representatives of algae would be needed to test this hypothesis. Subsequently to this duplication event, the two PTEN paralogues evolved separately. Interestingly, the duplicated genes appear to have experienced different evolutionary pathways according to the plant lineage. Lycophytes and angiosperm eudicots retained the two genes, whereas angiosperm monocots (cereals and sugarcane) seem to have lost the PTEN1 gene. The current sequencing of additional plant and eukaryote genomes will precisely determine the origin and evolutionary dynamics of both genes in plants in the near future. In addition to the deletions observed in some lineages, subsequent duplications were observed in Arabidopsis and Populus trichocarpa by the occurrence of closely related PTEN2 sequences.

Most of the biological effects of animal PTEN are attributed to its lipid phosphatase activity that dephosphorylates the PI(3,4,5)P 3, and other D3 phosphate PIs [32]. Biochemical characterization of AtPTEN2 proteins shows their preferential use of 3-PIs as substrates (Figure 3B). However, PI(3,4,5)P 3 was found to be a very poor substrate for the AtPTEN2s, unlike animal PTEN and AtPTEN1. AtPTEN2a, which is by far the most active Arabidopsis PTEN2, dephosphorylates mostly PI3P, PI(3,4)P 3, and PI(3,5)P 3, and also has a weak activity towards PI4P, whereas the only AtPTEN2b substrate identified is PI3P. Detailed analysis of the amino acid residues at the active catalytic core of plant PTENs revealed mutations at crucial positions for the PTEN1 and PTEN2 sequences (corresponding to Lys 125 in HsPTEN for AtPTEN1 gene, which belongs to the PTEN1 clade, has been recently shown to display a PI(3,4,5)P 3-dephosphorylating activity and to strongly affect pollen viability when inactivated by RNAi silencing [8]. However, neither the PI(3,4,5)P 3, PI or the type I PI3K kinase which catalyses its formation from PI(4,5)P 2 have ever been identified in plants, thus questioning the mode of action of PTEN1 in plants. The PTEN2 clade includes sequences from the angiosperms (eudicots and monocots) and from lycophytes. That clade is evolutionarily distant from the PTEN1 clade and includes two genes in Arabidopsis: the AtPTEN2a and the AtPTEN2b genes. Like human PTEN, both AtPTEN2a and AtPTEN2b proteins are able to catalyse dephosphorylation of a synthetic protein phosphatase substrate (p-NPP) and of PIs (Figure 3 and Supplementary Figure S4), suggesting that other sequences that are present in this clade may have similar activities.

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All known PI isomers, with the exception of PI(3,4,5)P$_3$ and probably also PI(3,4)P$_2$, have been identified to occur in plants [13]. PI3P constitutes 5–15% of the plant PIs and is exclusively synthesized by the type III PI3K, which is the unique type of PI3K in plants, known as Vps34 (vacular protein sorting 34). Studies of Arabidopsis type III PI3K-deficient plants severely affected in both vegetative and reproductive development identified a role of PI3P in the response to salinity, as well as in endocytosis [33,34]. PI3P has also been found to be important for the control of stomatal movement [35]. PI(3,5)P$_2$ was found to be increased in response to osmotic stress [36], but its role is still unknown, similar to PI5P, which is probably a degradation product of PI(3,5)P$_2$. In Arabidopsis, whereas a number of proteins called SPTases (inositol polyphosphate 5-phosphatases) are able to dephosphorylate the D5 position of the inositol ring of PIs or soluble inositol polyphosphates [37], only PTEN and myotubulins are potentially able to dephosphorylate D3 PIs. Like their animal counterparts, which are involved through the PI3K/AKT signalling pathway in the regulation of a wide range of processes including cell proliferation and growth, cell metabolism and apoptosis [7], the PTEN2 proteins found in vascular plants are therefore likely to play a crucial role in the control of 3-phosphorylated PI signalling events and homeoeostasis.

PTEN activity can be further modulated in vivo by post-translational modifications [38], interactions with protein partners [39] and localization in the nucleus or in the cytoplasm, where it can shuttle between cytosol and plasma membrane [40]. Recruitment of animal PTEN to membrane requires a P(4,5)P$_2$-binding motif and phosphorylated residues in the C-terminal domain of the protein [31], which were not identified in plant PTEN2. The phosphatase domain of AtPTEN2 proteins differs mainly from HsPTEN and AtPTEN1 by a single unstructured lipophilic loop of 8 amino acids (Figure 4A), with no known functional significance. However, we identified in plant PTEN2 a C2 domain, also found in animal PTEN, which may help tether PTEN to the membrane [41]. Unexpectedly, we also discovered that AtPTEN2a was able to bind PA and, to a lesser extent, PS (Figures 5A and 5B). Interestingly, C2 domains from other proteins have been shown to bind PA, e.g. tomato P (phospholipase Dα) [42], and mammalian PKCs (protein kinase Cε), which has been shown to specifically respond to PA accumulation in vivo [43]. Whereas HsPTEN is known to bind PS [44], the binding of PA has not been reported for animal PTEN proteins. Both PS and PA are signalling lipids with various cellular and physiological effects in animals [45]. In plants, PS signalling properties have not yet been established. In contrast, PA has been implicated in a variety of processes including abiotic stress responses, pathogen defence and hormone signalling [14,46,47]. Little is known about how PA exerts its effects in plants. An increasing number of PA-interacting proteins have recently been isolated in plants [9,47,48]. Among these are several protein kinases and phosphatases with roles in stress and hormonal signalling pathways such as the Raf-1 kinase homologue CTR1, involved in ethylene signalling [24], and the ABI1 phosphatase involved in ABA (abscisic acid) signalling [49]. Both act as negative regulators in their respective pathways and are inhibited by PA. Interestingly, the plant 3-PDK1 (PI-dependent protein kinase-1), a component of the PI3K/Akt signalling pathway, which is negatively regulated by PTEN in animals, is activated by PA binding in plants [50]. In mammals, a major downstream target of this pathway, the TOR (target of rapamycin) kinase is also a target of PA, which further regulates the assembly of the mTOR (mammalian TOR) complexes [51]. Whether the PA-binding properties of AtPTEN2a modify its activity in vivo, e.g. through protein stabilization, membrane tethering, activation or inhibition of phosphatase activity, remains to be established.

In summary, our phylogenetical results strongly suggest that the PTEN gene has been duplicated in vascular plants (PTEN1 and PTEN2). When compared with HsPTEN, the eudicot PTEN2 proteins display changes in charged amino acids in the core catalytic site, which likely affect their biochemical properties. As a consequence, PI3P, one of the major PIs found in plants, is the preferential substrate of the Arabidopsis AtPTEN2a protein. In contrast, the preferred substrate of HsPTEN, PI(3,4,5)P$_3$, which has not been detected in vascular plants, is a very poor substrate for AtPTEN2a. The AtPTEN2a protein displays strong binding affinity for PA, a major lipid second messenger with roles in stress and hormonal signalling in plants. Taken together, these results suggest that the eudicot PTEN2 phosphatase has evolved to fulfill specific roles in lipid signalling. The possible regulatory role of PTEN2 in response to developmental and stress signals is consistent with the ubiquitous expression of Arabidopsis AtPTEN2 genes in the plant, especially in young organs, which undergo a high metabolic activity/growth rate. In addition, strong transcriptional regulation of AtPTEN2 genes in response to salt and osmotic stresses, and tight post-transcriptional regulation of AtPTEN2 protein levels, are observed. Elucidating the physiological function and signalling pathways in which PTEN2 is implicated in both eudicots and monocots constitutes a new major challenge in plant science.

**AUTHOR CONTRIBUTION**

Christophe Rothan, Anne Pribat and Véronique Germain designed the research, performed sequence analysis, purification and biochemical characterization of the PTEN proteins, and promoter activity and transcript analysis in planta. Rodnay Sormani conducted PTEN protein expression in planta and participated in promoter activity experiments. Mathieu Rousseau-Guilain performed the phylogeny analysis. Magdalena Jukkowska, Christa Testeirnik and Véronique Germain performed the lipid-binding assays. Jérôme Joublé conducted the expression analysis in stress conditions. Michel Castroviejo participated in purifying the PTEN proteins. Michel Laguerre performed the modelling studies of the phosphatase domain. Christophe Rothan wrote the paper, and Anne Pribat, Rodnay Sormani, Véronique Germain, Christa Testeirnik and Christian Meyer contributed to the writing.

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28特に、糖質や脂質などの代謝に重要な役割を果たす。更に、細胞の成長や分化、発生などの様々なプロセスで機能している。メンバーとして、PTENと呼ばれる一種のリン酸脂質分解酵素が重要である。これにより、細胞内での信号伝達が制御される。
SUPPLEMENTARY ONLINE DATA

A novel class of PTEN protein in Arabidopsis displays unusual phosphoinositide phosphatase activity and efficiently binds phosphatidic acid

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EXPERIMENTAL

MS analysis

Purified AtPTEN2a (1 μg) was subjected to SDS/PAGE electrophoresis and the protein band of interest was digested by trypsin as described by Pocaly et al. [1]. Peptides were analysed further by nano liquid chromatography coupled to a MS/MS (tandem MS) LTQ Orbitrap XL mass spectrometer (Thermo-Finnigan). Peptides were identified with SEQUEST through the Bioworks 3.3.1 interface (Thermo-Finnigan) against a subset of the UniProt database restricted to Arabidopsis thaliana entries (Uni-ProtKB, Release 2010.12, 52095 entries). Only peptides with Xcorr higher than 1.5 (single charge), 2.0 (double charge), 2.5 (triple charge) and 3 (four charges and more) were retained. In all cases, peptide p-value must be lower than 1.10 × 10⁻³ and ∆Cn had to be greater than 0.1. All protein identifications were based on a minimum of two peptide assignments. Under those conditions, no false positives were detected. Shared peptides are only counted for the protein that has overall the most matching peptides.

MLPs (molecular lipophilicity potentials)

MLPs were calculated at the atomic level with a homemade program originating from an idea of Audry et al. [2] and using an exponential function [3]. The fragmental atomic constants used were those of Broto et al. [4] and MLP maps were calculated via the MLPP program [5].

pNPP phosphatase activity

The hydrolysis of pNPP was assayed at 30°C with 10 μg of recombinant protein in the assay buffer containing 100 mM Tris/HCl (pH 7.0) and 10 mM DTT. The reaction was initiated by the addition of various concentrations of pNPP. After a 30 min incubation, the absorbance of the product pNP (p-nitrophenol) was measured at 405 nm and was converted into molar product using an extinction coefficient of 17 800 M⁻¹·cm⁻¹ [6–8]. Data were fitted to a hyperbolic curve with the MicroCal Origin 3.0 software.

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3 To whom correspondence should be addressed to (email veronique.germain@u-bordeaux1.fr)
Figure S1  Alignment used for homology building of the various vegetal PTEN proteins starting from the sequence and structure of HsPTEN (PDB code 1D5R)

The secondary structures are schematized in red above the corresponding sequences (α-helix and β-sheet only), the red asterisk shows the deletion present only in AtPTEN1 and the red L shows the insertion of the lipophilic loop in AtPTEN2a and AtPTEN2b.

Figure S2  Strict consensus tree of the 96 most-parsimonious trees obtained from phylogenetic analysis of the phosphatase domain of eukaryotic PTEN sequences

Bootstrap values were obtained from 10000 replicates. Numbers above the branches indicate bootstrap values, and only branches receiving bootstrap values greater than 50% are indicated. Major groups belonging to the plant and animal kingdoms are indicated.
Figure S3  Spectrometry analysis of the purified AtPTEN2a

Purified AtPTEN2a (1 μg) was submitted to MS/MS (tandem MS) analysis, according to the Experimental section of the Supplementary online data. The lines represent the different peptides obtained from the analysis. The sequences covered (55.81%) are presented in bold characters.

$\begin{align*}
1 & \text{MSSESPNLPA AAGTVPDNHP PPPVVTAAE AGSSDSPKGV ASRLSAAGIS} \\
51 & \text{NWAKNLKVPO PFASTQNDSG VENTEKSAPA KFTSGLGLIRL SPKSPQNTMDT} \\
101 & \text{TTGGTSSTE SSFQITITKQ LVDSKQNAVQ AVQKAKHARV SQNKRRQEG} \\
151 & \text{GFDDLLTYIT ENIAAMGFPA GDSSGGFSGY VEGFVRNQME EVINFLCTQR} \\
201 & \text{RGKYKVMZLC SEIRLyDVSLF EGKVAVFPPFD DHNCPPHLV TSFQSAYSW} \\
251 & \text{LKEDIENNHY VHCKASMRAT GLMICSLLLY LKKFTAECC MDFFYQKRQCV} \\
301 & \text{DGKGLVIQSFQ IRYVKYFIERI LTYFNGENQP GRRCMGRGFR LHRRCFYWIRP} \\
351 & \text{SITISDHNGV LPTIKKHTRT KDLSPEDFWF SAPKKGVMVF ALPGEPLTE} \\
401 & \text{LAGQDKLQFH DRQGDFYCWL NTMMENRVIL KITSELDGFD KRKLPSFGFM} \\
451 & \text{VEVVLADINATIPTNPSSET ASKTEETSAA NSSFVDSGSA SVPFGDKETE} \\
501 & \text{NPDKDDVSD NEGOSTGFTP TTSSASSQTF EAKSADETA VLLKATEKVS} \\
551 & \text{ISGNGSQQP VQCVTVSKGE ATEKPSGAVV NASSSSESEF KVMAADASVF} \\
601 & \text{SFGEDEDFES D}
\end{align*}$

Figure S4  AtPTEN2a phosphatase activity against pNPP

Results are means ± S.E.M. from two replicates. The $K_m$ and $V_{max}$ values are estimated by hyperbolic curve-fitting and are shown in the inset.
Figure S5  Lipophilicity potential maps of HsPTEN and the three models of APTEN1, APTEN2a and APTEN2b

General view for four proteins equivalent to a view from the membrane. Lipophilic lobes are in red, hydrophilic lobes are in bright blue and the philic/phobic interface is shown as a transparent golden surface.

Figure S6  Expression level of the three APTEN in the different A. thaliana organs

(A) Semi-quantitative RT–PCR analysis in different organs. RNA extraction and RT–PCR were performed as described in Experimental section of the main text. After 20 cycles, the PCR products were separated by agarose electrophoresis and revealed by ethidium bromide staining. (B) Expression levels obtained from the database Genevestigator (https://www.genevestigator.com/gv/index.jsp/).
REFERENCES


Table S1 Sequences used for phylogenetic analyses

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Table S2 Primers used for all cloning procedures and for real-time RT–PCR analyses

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A novel class of PTEN proteins in plants

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