The plant non-specific phospholipase C gene family. Novel competitors in lipid signalling

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**Abstract**

Non-specific phospholipases C (NPCs) were discovered as a novel type of plant phospholipid-cleaving enzyme homologous to bacterial phosphatidylcholine-specific phospholipases C and responsible for lipid conversion during phosphate-limiting conditions. The six-gene family was established in Arabidopsis, and growing evidence suggests the involvement of two articles NPCs in biotic and abiotic stress responses as well as phytohormone actions. In addition, the diacylglycerol produced via NPCs is postulated to participate in membrane remodelling, general lipid metabolism and cross-talk with other phospholipid signalling systems in plants. This review summarises information concerning this new plant protein family and focuses on its sequence analysis, biochemical properties, cellular and tissue distribution and physiological functions. Possible modes of action are also discussed.

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1. Introduction

Phospholipases (Fig. 1) are now well recognised as key components of the regulatory systems of cellular growth and development in living organisms. They give rise to an array of second messenger molecules and lipid derivatives and are implicated in both metabolism and intracellular signalling. Recent research progress has made it possible to convincingly reveal an important role for phospholipases in mediation of stress responses directed to provide acclimation to ever-changing environmental conditions.

Phospholipases C (PLC) are able to cleave membrane phospholipids, facilitating release of water-soluble phosphorylated head-groups from hydrophobic diacylglycerols (DAG). PLCs in living systems can generally be divided into phosphatidylinositol-specific phospholipases C (PI-PLC) and phosphatidylcholine-specific phospholipases C (PC-PLC) according to substrate specificity range. The role of specific phosphatidylinositol 4,5-bisphosphate (PIP$_2$) cleaving PI-PLCs in cell metabolism regulation is fairly well studied in various organisms. Thus, multidomain animal PI-PLCs are G-protein activated enzymes that are notably responsible for intracellular calcium level regulation and protein kinase C (PKC) activation [1]. Despite a lack of identified inositol 1,4,5-trisphosphate receptors in plants [2], PI-PLCs are unquestionably implicated in the regulation of growth, development and stress responses in Arabidopsis and other plant species [3]. In turn, bacterial PI-PLCs belong to secreted pathogenicity factors that typically confer virulence [4].

PC-PLCs, in plants also known as non-specific PLCs (NPC), are characterised by broader substrate ranges that include abundant phosphatidylcholine (PC) (discovered in 1847 by a French chemist Theodore Nicolas Gobley as “lecithin” molecules), while their role in cell signalling and regulation in general remains far less understood. PC-hydrolysing phospholipases were first discovered under the name “lecithinase C” in bacteria [5] and later established as important bacterially secreted pathogenicity factors performing host membrane lysis and defence signalling interference [6]. PC-PLC activity was also identified in fungi [7] and was acknowledged to be an essential source of phospholipid-derived signal molecules in animal cells [8,9]. It was demonstrated that transient increases in DAG production occur in cellular membranes in response to various stimuli [10,11], whereas attributed PC-PLC activity appears to be involved in a number of intracellular regulatory events (see section 3). However, the current lack of molecular and genetic characterisation of PC-PLCs in animals hampers the progress of research.

Although putative PC-PLC activity in plants was observed as early as 1955 [12], PC-PLC functions in plants have long remained vague and their role elusive. Some supporting clues for PC-hydrolysing PLC occurrence were identified in plant organs and tissues such as peanut seeds [13], rice grains [14], tomatoes [15] and others [16]. However, this data lacked sufficient enzymatic characterisation [17]. Eventually, in 2002, fluorescently labelled PC was shown to be directly cleaved to produce DAG in parsley and tobacco cells, suggesting the presence of NPC (PC-PLC) as a novel type of phospholipase in plants with putative signalling functionality [18]. Later, six NPC genes were identified in the Arabidopsis genome based on sequence similarities with bacterial PC-PLCs [19]; nine NPC genes were identified in soybean plants [20]. DAG production via PC hydrolysis was also observed in Petunia hybrida, suggesting the omnipresence of NPCs in the plant kingdom [21]. A subsequent increase in research interest in dissection of NPC’s roles in plants over the recent years has promoted the identification of NPC

Fig. 1. Phospholipase C- and phospholipase D-dependent signalling in plants. A schematic diagram depicts contemporary model of metabolism regulation carried out by plant cell phospholipases. Various augmenting signalling pathways are shown, demonstrating synergistic interactions between phospholipases and lipid second messenger molecules in excitation of cell responses. PIP$_2$, phosphatidylinositol 4,5-bisphosphate; PC, phosphatidylcholine; NPC, non-specific phospholipase C; PI-PLC, phosphatidylinositol-specific phospholipase C; PLD, phospholipase D; P chol, phosphocholine; DAG, diacylglycerol; PA, phosphatic acid; DGPP, diacylglycerol pyrophosphate; IP$_1$/IP$_2$, inositol 1,4,5-trisphosphate/inositol hexakisphosphate; PKC, protein kinase C; DGK, diacylglycerol kinase; PAP, phosphatic acid phosphatase; PAK, phosphatic acid kinase; DGPPP, diacylglycerol pyrophosphate phosphatase; ROS, reactive oxygen species; PAB, PA-binding proteins.
involvement in regulation of diverse cellular processes including root development and brassinosteroid hormone signalling [22], AI stress signalling [23], abscisic acid (ABA) sensing, and tolerance to hypersomotic and salt stresses [24,25]. However, the NPC3 protein of Arabidopsis was shown to perform lypoxygenase activity (LPA) phasatase activity, producing monacoyglycerol (MAG) rather than DAG, which may hint at a possible multivalent functionlity for NPC3s [26]. Taken together, this evidence denotes NPCs in plant species and suggests their putative role in plant metabolism regulation.

2. Bacterial phosphatidylycholine-specific phospholipase C

Since the discovery of PC-PLC specific activity in Clostridium perfringens (formerly known as C. welchi) toxin in 1941 [5], various PC-PLC genes and corresponding enzymes have been identified in Gram-positive bacteria, including Bacillus cereus, Listeria monocytogenes, Clostridium perfringens species [6] and, more recently, in Gram-negative bacteria, such as Pseudomonas species [27], Burkholderia pseudomallei [28], Legionella pneumophila [29]. All identified bacterial PC-PLCs fall into two distinct groups of sequentially unrelated enzymes.

PC-PLCs from the first group are predominantly found in Gram-positive bacteria and were identified as potent toxins with haemolytic properties related to the Clostridium perfringens α-toxin. Such toxic PC-PLCs represent single polypeptide enzymes that require zinc ions for activation and are reversibly inactivated by EDTA or α-phenantrone metal chelators [6]. Several toxic PC-PLCs have been shown to also require Ca²⁺ or Mg²⁺ ions for their activation [30]. Some genes encoding this zinc-metallophospholipase C have been isolated and characterised [31]. The molecular mass of the enzymes lies within the range of 28–34 kDa [6]. Amino acid composition of these PC-PLCs revealed the presence of a signal sequence denoting protein recruitment into secretory systems. Toxic bacterial PC-PLCs have wide-ranging substrate specificity that, apart from phosphatidylcholine, includes phosphatidylethanolamine (PE), phosphatidylserine (PS), sphingomyelin and other lipids [6]. The molecular structure of the well-studied C. perfringens PC-PLC toxin (α-toxin) revealed a two-domain protein. The N-terminal domain contains the phospholipase C active site, which also incorporates zinc ions. The C-terminal C2-like PLAT (Polycystin-1, Lipoygenase, Alpha-Toxin) domain was found to be similar to lipid binding domains in eukaryotes [32] and appears to be responsible for binding membrane phospholipids in a calcium-dependent manner [33]. Interestingly, the N-terminal domain of α-toxin retained PC-PLC activity when expressed in Escherichia coli, but lacked haemolytic and sphingomyelinase activities that are supposedly granted by a lipoygenase-like C-terminal domain [34]. Toxic bacterial PC-PLCs have been extensively studied as pathogenicity factors responsible for host membrane lysis. In addition, PC-PLCs can interfere with eukaryotic cellular signalling and take control of host immune responses [35]. For instance, secreted PC-PLC from C. perfringens plays a role in the aggregation of blood platelets [36] and was shown to inhibit defensive superoxide generation in human polymorphonuclear leukocytes by interacting with membrane components of NADPH oxidase [37]. However, some PC-PLC from Gram-positive bacteria can have functions unrelated to those of typical toxic enzymes. Secreted PC-PLC from B. cereus is involved in the defense mechanism of bacteria to phagocytosis [38]. Furthermore, some bacterial PC-PLCs may be used as vaccines against diseases such as gangrene [36].

The second group of PC-PLCs is primarily produced by Gram-negative bacteria. These enzymes do not contain zinc ions and typically lack haemolytic properties. Their molecular weight differs several-fold from that of the toxic enzymes. For example, the M₉₀ of Pseudomonas cepacia PC-PLC is 72 kDa [39]. Non-toxic PC-PLCs have not responded to in vitro treatment with bivalent ions [40], and EDTA chelator was reported to significantly stimulate the activity of PC-PLC from L. pneumophila [29]. The PlcHR phospholipase C found in Pseudomonas species was also shown to be Ca²⁺-independent [40]. Interestingly, Gram-negative Pseudomonas aeruginosa produced several types of PC-PLCs [41] that differ in substrate specificity and toxic properties. Thus, PLC-H identified in P. aeruginosa was haemolytic [42] and possessed sphingomyelin synthase activity [43].

It is apparent that PC-PLCs from Gram-positive and Gram-negative bacteria have evolved independently. Intriguingly, non-toxic bacterial PC-PLCs, including PlcB from Mycobacterium tuberculosis and PlcH from Ralstonia solanacearum clearly show a resemblance to putative plant NPCs [19], indicating not only a common evolutionary history but also putative cell functions unrelated to straightforward membrane lysis. Some data also indicate that NPC may belong to a superfamily of phosphatase/phospholipase proteins related to the acid phosphatase AcpA from Francisella tularensis [44].

At the same time, bacteria apparently contain several evolutionary divergent types of PC-PLC, such as NaCl-stimulated enzyme purified from a marine streptomycete [45], or membrane-bound PC-PLC protein with a broad pH optimum identified in Ureaplasma urealyticum, which lacks cell walls [46].

Alongside additional data available on PC-PLC pathogenic properties, it is surprising that to date little is known about the physiological functions of non-toxic PC-PLCs. They are thought to be involved in lipid remodelling during phosphate-limiting conditions, as has been shown for the intracellular PC-PLC of Sinorhizobium meliloti [47]. Regulation of some PC-PLC genes by exogenous phosphate levels also supports this hypothesis [6].

3. Animal phosphatidylycholine-specific phospholipase C

PC hydrolysis is now recognised as a constitutive element of cell signalling and metabolism regulation in animals [8,9], while PC-cleaving PLC appears to be a key agent in the generation of second messengers [48,49].

Animal PC-PLC genes apparently show no sequence similarities with other known PC-PLCs in diverse living organisms, which would explain why no animal PC-PLC genes have yet been cloned or characterised. However, considerable effort has been invested into PC-PLC functional characterisation, and it is now evident that PC-PLC is directly involved in the control of cell proliferation and differentiation, as well as a number of other cell functions [50,51].

Until now, PC-PLC activity has been detected in multiple animal sources including canine myocardium [52], acrosomes of bull seminal plasma [53], human vascular endothelial cells [54] and chicken blastodic [55]. Interestingly, cytosolic 66 kDa PC-PLC identified using antibacterial PC-PLC antibody in mouse NIH-3T3 fibroblasts [56] and NIH-3T3 H-ras transformants [57] was rapidly translocated to the plasma membrane upon mitogen-mediated cell receptor stimulation. A similar pattern was observed with PC-PLC activity predominantly localised to the plasma membrane in oncogenically transformed human epithelial ovarian cells [58] and NK cytolytic cells, where PC-PLC may contribute to lysis of target cells [59,60].

To date, the regulatory role of PC-PLC has been largely attributed to DAG production. In animals, DAG can typically arise via PC or PI₃ hydrolysis, or by phosphatidic acid (PA) dephosphorylation. DAG has multivalent functionality that includes alteration of membrane curvature and properties, orchestration of lipid metabolism, and participation in lipid-mediated signalling [61,62]. Among the most important features of DAG is its ability to bind to the C1 domain [63] and facilitate the activation of PKC– an
enzyme known for its immensely wide cellular functionality [64,65]. DAG-binding C1 domains have also been identified in a number of other proteins with diverse regulatory functions. For instance, a DAG-mimicking phorbol ester induced a PKC-independent exocytosis in rat PC12 cells mediated by a RasGR3 protein that contains a C1 domain [66].

Initial reports indicated that PC-derived DAG may be ineffective for PKC activation [67], but it was later shown that B. cereus derived PC-PLC not PI-PLC, inhibited the formation of cAMP by adenylate cyclase, which is negatively regulated by PKC in Swiss 3T3 fibroblasts [68]. In addition, the regulatory function of melatonin hormone in rat retinal ganglion cells was mediated by PKC activated exclusively via PC-PLC action [69]. A specific role for PC-PLC in the regulation of leukemic myeloid cell proliferation via MAP kinase cascade stimulation and NF-κB activation has also been reported to be PKC-dependent [70]. It is now thought that PC-derived DAG is responsible for activation of PKC isoforms dependent solely on DAG, namely PKC-δ, PKC-ζ, PKC-η and PKC-θ [71]. Alternatively, PC-PLC may provide a sustained DAG source that can assist in the activation of conventional type PKCs that depend both on DAG and Ca^{2+} ions (PKC-α, PKC-β, PKC-βII, PKC-γ) and are first initiated by DAG produced via short-lived PIP_2 hydrolysis [72].

DAG produced by PC-PLC may also be phosphorylated to PA by diacylglycerol kinases (DGK) [73,74] and act as a contributor to PA-dependent cell metabolism regulation in addition to phospholipase D (PLD) [75]. The reverse dephosphorylation of PA may also play a regulatory role. Thus, PA-derived DAG has been shown to be important for vesicle and tubule formation in the Golgi apparatus of HeLa cells [76].

In animals, the regulatory role of phosphocholine, which is concurrently produced alongside DAG during PC hydrolysis by PC-PLC, has also been discussed. Phosphocholine is involved in many long-term cellular responses such as activation of proliferation/differentiation and cell transformation promotion [77]. Moreover, phosphocholine induces DNA synthesis and mitogenesis in mouse fibroblasts [78] and acts as a specific receptor molecule for perforin in lymphocytes [79]. It is interesting that in animal cells, choline kinases are also responsible for phosphocholine production through choline phosphorylation in the presence of ATP and magnesium [80]. Choline kinases have been shown to be involved in cell division and growth regulation [80], and play significant role in regulation of both normal human mammary epithelial cell proliferation and breast tumour progression [81].

Several other putative functions of PC-PLCs affecting animal cell metabolism are now being considered. Understanding of the specificity of PC-PLC activity and the role of phospholipid-derived signalling substances in animal cells is impeded by a lack of PC-PLC sequence and structural information.

4. Plant non-specific phospholipase C (NPC)

4.1. In silico analysis of NPC gene family in plants

Initial reports on plant NPCs suggested that they were not related to any other known plant phospholipase family. Previously, multiple sequence alignments of Arabidopsis NPCs with PLC from M. tuberculosis revealed three conserved regions unrelated to known domains of plant phospholipases [19]. Although the NPC family clearly arose early in evolution, bacterial, plant and invertebrate lineages have each developed distinct NPC sequence features. Genomes of higher plants typically contain several genes coding for putative NPCs proteins consisting of 510–540 amino acid residues. NPC proteins contain a central phosphoesterase domain (Fig. 2) that is typically present in enzymes with esterase activity, such as NPCs and acid phosphatases. No motifs known from other plant lipid signalling proteins (i.e., C2, XY, EF, PH, PX, ENTH, FYVE domains) are present. Generally, plant NPCs show a high level of similarity, especially in the phosphoesterase domain (Fig. 2). Close comparison with Gram-negative bacterial PC-PLCs reveals four motifs with invariant residues that are likely crucial for the PC-PLC catalytic activity (Figs. 2 and 4). The majority of plant NPCs
Fig. 3. Phylogenetic analysis of plant NPC. Multiple alignment of NPC sequences obtained from the NCBI database, Doe Joint Genome Institute and Genoscope-Centre National de Séquençage, was created using Mafft v6.238b in L-INS-I mode [208] and then edited with Jalview [209]. The conserved blocks were concatenated to give a final matrix with 428 positions from 47 NPC-like sequences that were then subjected to phylogenetic analysis. A phylogenetic tree was constructed using maximum likelihood method from NPC amino acid sequences using PhyML (WAG model + I) [210]. Numbers at nodes represent values of neighbour-joining bootstrap support obtained using aLRT (approximate likelihood-ratio test) method. Circles at the nodes represent 100% support, and missing values indicate support below 75%. Branches collapsed where the support was below 50%. Non-plant PC-PLC homologues were used as a phylogenetic tree root. The scale bar represents 0.2 substitutions/site.
contain a putative signal peptide at the N-terminus that is followed by a short variable region and a highly conserved domain containing invariable ENRSFDxxxG motifs at the beginning of the phosphoesterase domain. The putative signal peptide is missing in Arabidopsis NPC3, NPC4 and NPC5. Two other invariable motifs, TxPNR and DExxGxxDHV, are found in the middle of the domain followed by a GxRVPxxxxxP region that closes the phosphoesterase domain. Interestingly, the 50–100 amino acids at the C-terminus form the most divergent part of NPC sequences, with distinct lengths and sequence conservation among NPC subfamilies. This may be the part of the molecule responsible for the functional differences of various NPC isoforms through facilitating interactions with other proteins or defining protein localization.

To gain insight into the evolution of plant NPCs and determine whether NPC diversity seen in Arabidopsis is conserved across plants, we performed a phylogenetic analysis of NPC protein sequences from several evolutionarily distinct plant species (Fig. 3). In addition to dicot species like poplar (Populus trichocarpa), grapevine (Vitis vinifera) and soya (Glycine max), we also analysed the genomes of the monocotyledons rice (Oryza sativa) and sorghum (Sorghum bicolor)—representing gymnosperms—and moss Physcomitrella patens and lycophyte Selaginella moellendorffii—representing evolutionarily ancient plants. Phylogenetic analysis of plant NPC sequences suggested that the common ancestor of all seed plants already had at least one NPC1-, NPC2- and NPC6-like gene. Interestingly, the NPC3–5 subfamily was not identified in spruce (and other available gymnosperm sequences, data not shown). As no complete gymnosperm genome sequence is available and spruce NPC analysis was based on EST sequences, one cannot yet exclude the presence of this.

Fig. 4. 3D structure of plant NPC. 3D model of plant NPC structure was predicted using AtNPC2 sequence as a template. Final model was designed based on published structures of acid phosphatase from bacteria F. tularensis (PDB code 2DG1, [44]) and human estrone sulphatase (PDB code 1P49, [211]) using MODELLER 9v7 software [212]. Seventy generated structures were then evaluated with Prosa (https://prosa.services.came.sbg.ac.at/prosa.php) and WhatIf (http://swift.cmbi.ru.nl/servers/html/index.html) algorithms, and the best model is shown. The pair of images is rotated 90° around y-axis. Side chains are shown for putative active site residues (orange) and a structure-stabilising ion pair (cyan). Electrostatic potential was mapped onto AtNPC2 ranging from −5 (red) to +5 (blue) k_BT/ε. See the main text for details.
subfamily in gymnosperms. Alternatively, it is possible that NPC3–5 subfamily is present only in angiosperms and emerged after the separation of gymnosperm and angiosperm ancestors, possibly from NPC2 clade (as indicated by phylogeny). Surprisingly, Physcomitrella and Selaginella were found to contain only NPC1-like genes. Two evolutionary simple explanations are possible: either the common ancestor of land plants contained NPC1, NPC2 and NPC6 subfamilies and NPC2 with NPC6 gradually disappeared during evolution towards lycophytes and bryophytes, or the NPC1 subfamily is the ancestral NPC, which subsequently gave rise to all other NPC types (this scenario is not supported by the phylogenetic analysis). Both Selaginella and Physcomitrella NPCs further multiplied independently after the separation of mosses, lycophenes, and seed plants. Rapid diversification of NPCs is evident from the evolution of angiosperm orthologs, where multiple duplications within all NPC subfamilies are frequently observed. In contrast, no NPCs were found in green algae, suggesting that the NPC family may have been lost throughout algae evolution.

### 4.1.1. 3D model of plant NPC

Although no experimental three-dimensional structure of a plant NPC is available, the homology of plant PC-PLCs with the acid phosphatase (AcpA) family enabled us to construct a 3D model for Arabidopsis NPC employing recently published structure of AcpA from the bacterium _F. tularensis_ [44]. Analysis of the AtNPC2 3D model (Fig. 4) showed that, similarly to the bacterial AcpA, the backbone of plant PC-PLC is formed by a beta sheet (composed of 7 beta structures) embedded inside the protein and surrounded by several alpha helices (6 in PtAcpA, 7 in plant NPCs). Importantly, the majority of amino acid residues forming the active site of the bacterial AcpA are conserved, including the residues that bind a still-undefined metal cation. This is also true for the ion pair of aspartate-arginine, which probably acts as a stabiliser of the active site topology. Altogether, this evidence suggests that for eukaryotic PC-PLCs, the bacterial PC-PLCs and homologous acid phosphatases, the common ping-pong reaction mechanism, with an intermediate in which the substrate phosphate group is covalently bound to nucleophilic amino acid from the active site, is likely to be maintained. By contrast, none of the four cysteines, which form two disulphide bridges stabilising the AcpA molecule, are present in plant NPCs. The predicted model of AtNPC2 also indicates that the variable C-terminal domain forms a “cap” that covers part of the molecule from the proposed active centre and thus does not participate directly to the catalysis. This finding is consistent with the sequence analysis of the NPC proteins. Interestingly, the active site of plant NPCs forms a relatively strongly negatively charged pocket that may be involved in phospholipid substrate binding (Fig. 4).

### 4.2. Biochemical properties of NPC

NPC3, NPC4 and NPC5 from *Arabidopsis thaliana* have been cloned and partly characterised biochemically (Table 1). Recombinant NPC4 expressed in _E. coli_ showed activity towards PC and PE, while the ability to cleave PA and PIP2 substrates was negligible [19]. NPC4 also demonstrated moderate hydrolytic activity towards PS and hydrolyses phosphatidylglycerol to a small extent [25]. NPC4 activity was slightly elevated in the presence of 2 mM EGTA chelator [19].

Recombinant NPC5 protein expressed in _E. coli_ was able to cleave PC and PE to produce DAG. However, the hydrolytic activity of NPC5 was more than 40-fold lower than that of NPC4 [82].

Purified recombinant NPC3 protein demonstrated specific LPA phosphatase activity, resulting in MAG production [26]. LPA phosphatases are known as important regulatory enzymes in animals [83], but their importance in plants has not yet been ascertained. The enzyme was marginally stimulated by low concentrations of the non-ionic detergent Triton X-100, but treatment with CHAPS or NP40 detergents resulted in inhibition of substrate conversion. LPA phosphatase activity was also reduced in the presence of lysophosphatidylcholine (LPC) or lysophosphatidylethanolamine (LPE), but it was not affected by PA, PC, MAG or DAG. Phosphatase activity of NPC3 did not require the presence of Ca²⁺ or other bivalent ions and was independent of fatty acid variations in the LPA substrate. NPC3 was also unresponsive to treatment with non-specific sulfur o-vanadate and sodium fluoride phosphatase inhibitors. The authors [26] additionally showed that the purified enzyme did not hydrolyse sphingosine-1-phosphate, diacylglycerol pyrophosphate, glycerol-3-phosphate, LPC or LPE. More importantly, NPC3 lacked the ability to hydrolyse PC, PE or PS [26], indicating that the NPC3 gene may be paralogous to other plant NPC genes that code for phospholipase C-type enzymes.

The studies of plant NPC activity in vitro revealed that in microsomal fractions prepared from BY-2 cells, PC hydrolysis was inhibited by Al at high concentrations (>100 μM AlCl₃) [23].

Specific inhibitors serve as important tools for revealing the role of the studied proteins. No specific inhibitor has been described for NPCs, although both animal PC-PLC-directed inhibitors [84,85] and bacterial PC-PLC-directed [86] inhibitors have been identified.

Tricycloadecan-9-yl-xanthogenate, commonly known as D609, has been widely studied as a specific inhibitor of PC-PLC activity in animals, exhibiting a variety of biological effects including antiviral, antitumoural, and anti-inflammatory influence [87]. However, it is also a potent antioxidant [88] and inhibitor of group IV cystolic phospholipase A₂ [89]. This indicates that at least some observed functions may be attributed to side effects of the influence of D609 on PC-PLC activity. Interestingly, D609 also inhibits

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**Table 1**

Biochemical properties, physiological properties and expression patterns of NPC, characteristic of identified NPC-coding genes and corresponding to gene products in _A. thaliana_. Putative roles for NPCs to particular cell reactions are also provided. Data were obtained by in silico analysis and from mentioned sources. aa, amino acid; ABA, abscisic acid; BL, 24-epibrassinolide; LPA, lysophosphatidic acid; NPC, non-specific phospholipase C; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

<table>
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<tr>
<th>Name</th>
<th>Gene locus</th>
<th>Cloned number of aa</th>
<th>Calculated Mₙ (kDa)b</th>
<th>Substrate</th>
<th>Predominant tissue expression</th>
<th>Activation/stimulus expression</th>
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<td>60.7</td>
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<tr>
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<td>520</td>
<td>57.9</td>
<td>a Mitochondria, plastidsb</td>
<td>Siliques, roots</td>
<td>Phosphate deficiency</td>
<td>[22,25,82]</td>
</tr>
</tbody>
</table>

a Experimically unidentified.

b Based on sequence analysis and prediction programmes (The Arabidopsis Information Resource (TAIR), www.arabidopsis.org; Cell eFP browser, http://bar.utoronto.ca/cell_efp/cgi-bin/cell_efp.cgi).
some bacterial PC-PLC enzymes, indicating that animal and bacterial PC-PLC may share some structural similarities [90]. For instance, PC-PLC from Gram-negative Pseudomonas fluorescens reacted to animal D609 PC-PLC inhibitor treatment [27]. NPC activity in BY-2 cells was not altered after treatment with 20 M of D609 animal PC-PLC inhibitor [23], indicating that plant NPC may possess regulatory properties different from those of animal or bacterial PC-PLC.

Taking into consideration the identified sequence similarities between plant NPC and bacterial PC-PLC, TDTMA-type (tetracyclithymethylammonium bromide) inhibitors of bacterial enzyme may also be effective in repressing NPC activity [86].

4.3. Intracellular distribution

Localisation data may provide additional clues into the functional role of NPC in plant cells. In terms of subcellular localisation, plant phospholipases can be typically divided into cytosolic and membrane-bound enzymes [17,91]. NPC activity has been experimentally detected in the cellular membranes of stamens and pistils of Petunia hybrida [21] and the plasma membrane fraction prepared from BY-2 suspension cell cultures [23]. A similar pattern was observed in oat roots, where a phospholipase that reacted with NPC4-raised antibody was found in the plasma membrane [92]. Analysis of subcellular fractions from Arabidopsis leaves using polyclonal anti-NPC4 antibody revealed that NPC4 is located in the microsomal membrane fraction and not the plastids [19]. Further analysis demonstrated that NPC4 is localised specifically to the plasma membrane [19], as confirmed by analysis of the NPC4-GFP construct [82] (Fig. 5). NPC3 was experimentally identified to be present in the tonoplast, as shown by one-dimensional SDS–PAGE analysis of a tonoplast-enriched fraction followed by nano-LC MS/MS [93]. Unfortunately, the determinants for NPC4 and NPC3 membrane localisation are currently unknown and may include palmitoylation, myristoylation, GPI-anchoring among other factors.

NPC5-GFP constructs localised to the cytosol in Arabidopsis [82]. Because phospholipases convert hydrophobic substrates, it is possible that cytosolic phospholipases are translocated to membranes upon stimulation, as shown for PLDα in Arabidopsis [94].

The subcellular distribution of other NPCs is uncertain but has been predicted to localise to endomembranes despite a lack of identified transmembrane domains (Cell eFP Browser, http://bar.utoronto.ca; http://www.cbs.dtu.dk/services/TMHMM).

4.4. Tissue distribution

Identification of the levels and spatial properties of NPC expression in tissues and organs of Arabidopsis may provide an effective means to further disclose their role in plant growth and development. NPC genes are characterised by changeable level of expression, with a somewhat pronounced intra-organismal expression pattern. Average signal percentiles of the corresponding mRNAs in single channel arrays range from 25 for NPC4 to 71.4 for NPC1 and 77.2 for NPC6 (The Arabidopsis Information Resource (TAIR), www.arabidopsis.org), consistent with the differentially expressed potential of other phospholipases known to be implicated in cell signalling, including PI-PLC (percentile ranges ≈42–90) and PLD (percentile ranges ≈26–94). More importantly, generally low expression potential may suggest an inducible expression and putative signalling role for NPC2 (51.6), NPC3 (51.5), NPC4 and NPC5 (24.8), which have been experimentally defined for NPC3 and NPC4 [22,24,25]. Peters et al. have provided a tissue-specific analysis of NPCs expression in Arabidopsis [25]. Experiments exploiting a β-glucuronidase (GUS) reporter system demonstrated that PnNPC3::GUS and PnNPC4::GUS constructs localised to root tips, cotyledons and outer leaf margins. Additionally, in floral organs, PnNPC3::GUS and PnNPC5::GUS constructs were expressed predominantly in pollen sac tissues of young anthers [22]. In another study, PnNPC6::GUS constructs were expressed in ageing leaves with low levels of expression in roots, stems, siliques and flowers [95]. GUS staining additionally allowed determination of NPC2 gene expression patterns, which were localised to the meristic zone, elongation zone and vascular tissue of maturative zone of root tips and to developing leaves, with observed deterioration with leaf maturation [96] (Fig. 6A).

Analysis of array data (Arabidopsis eFP Browser, http://bar.utoronto.ca; Genevestigator, www.genevestigator.com) allowed the identification of significantly amplified expression of all NPC genes in developing seeds, notably in chalazal endosperm and seed coat – regions known to be among primary lipid storage and metabolism sites (Fig. 6B and C) [97]. Elevated expression of NPC5 was also detected in roots, floral organs, siliques and leaves at various stages of development. In addition, expression of NPC1 was increased in root columella cells, stem epidermis, xylem and cork, as well as in guard cells and trichomes. Expression of NPC1 was low in germinating and mature pollen. NPC2 was upregulated in siliques and during early stages of seed development in the meristem of developing embryos and had relatively low expression in roots and leaves of A. thaliana. Schematic representation of identified and putative sites of NPC expression in Arabidopsis cells are depicted by corresponding coloured circles. Main cell organelles and compartments are indicated. Intracellular expression of AtNPC4, AtNPC5 and AtNPC3 was observed in plasma membrane, cytosol and tonoplast, respectively [19,82,93]. Expression patterns of AtNPC1, AtNPC2 and AtNPC6 remain uncertain. However, signal peptide sequences identified in above-mentioned NPCs and in silico sequence analysis have provided clues for AtNPC1 and AtNPC2 localisation to endomembranes, while AtNPC6 was predicted to localise to plastids and mitochondria. NPC, non-specific phospholipase C. Results were obtained experimentally.
NPC tissue expression were provided by quantitative PCR and GUS staining [22,25,95,96]. The expression levels of individual NPC genes are compared within the whole plant; circle size represents relative gene abundance. However, the combined quantities of transcripts of all NPC genes are not comparable in this figure. Analyses of Arabidopsis Affymetrix 22 K array data for NPC expression in tissues of *Arabidopsis* (B) and throughout ontogeny (C) were performed via Genevestigator (http://www.genevestigator.com). Developmental stages of *Arabidopsis* are abbreviated as follows: G, germinated seed; S, seedling; yR, young rosette; dR, developed rosette; B, bolting; yF, young flower; dF, developed flower; FS, flowers and siliques; S, siliques. NPC, non-specific phospholipase C.

### 4.5. Physiological function of NPC

NPC is relevant to various plant metabolic responses to both environmental and organisinal stimuli. Long-term changes in NPC activity and gene expression were observed under biotic and abiotic stresses, phosphate deficiency and certain hormone treatments (auxins, cytokinins, ABA). In contrast, rapid changes in NPC activity and DAG production were observed in plant cells in response to AI, brassinosteroids and elicitors. These responses occur faster than de novo protein synthesis under the control of fast promoters, which take approximately 30–45 min [98]. Thus, changes in NPC activity can be attributed to stimulation of presynthesised enzymes by posttranslational modification and/or intracellular translocation. Therefore, there is a physiological role for NPC in plant cells, and NPC should be added to the list of phospholipases involved in signal transduction.

#### 4.5.1. Phosphate deficiency

During phosphate shortages, organic phosphate can be mobilised from membrane phospholipids, which incorporate up to a third of total phosphorus in plant cells. NPCs are able to facilitate such mobilisation by separating the phosphate-containing head groups from phosphatidylcholine or other phospholipids. Concurrently produced DAG can serve as a precursor to production of substituting sulpholipids [99] and glycolipids [100], allowing retention of membrane integrity and other crucial properties. In 2003, it was shown that under phosphate deprivation, transient increases in PC content in *Arabidopsis* followed rapid PC decline accompanied by DAG accumulation, suggesting NPC activation [101]. Later, the NPC4 gene was strongly expressed in *Arabidopsis* plants experiencing lack of phosphate [19]. NPC4 T-DNA insertion mutant lines in similar conditions demonstrated a significant reduction in NPC activity. Based on these results, Nakamura et al. [19] postulated that PC hydrolysis by NPC4 plays an important role in securing supplies of inorganic phosphate and DAG from membrane-localised phospholipids. Liberation of phosphate serves cellular metabolism, while DAG contributes to galactolipid synthesis, for example digalactosyldiacylglycerol (DGDG). However, the galactolipid composition of the npc4 mutants was unchanged, leaving the role of NPC4 questionable [19].

Thus, NPC5 also appears to be essential for normal growth and galactolipid accumulation during phosphate deficiency [82]. NPC3 expression was increased and T-DNA mutant npc5 displayed reduced DGDG accumulation during phosphate deficiency. Analysis of acyl chains in DGDG molecules revealed that NPC5 affects mainly eukaryotic-type galactolipid synthesis that takes place outside of plastids [82]. Another study suggested specific activation of the NPC5 gene in roots of *Arabidopsis* induced by P deficiency. 

### Expression pattern of *Arabidopsis*

Arabidopsis, NPC3 was amply expressed in endodermis, phloem pole and phloem companion cells in root elongational and matura-

![Fig. 6. Expression pattern of *Arabidopsis* NPC genes in planta. Diagrams show that colour-coded NPC genes are expressed divergently in *Arabidopsis*. Experimental data (A) for NPC tissue expression were provided by quantitative PCR and GUS staining [22,25,95,96]. The expression levels of individual NPC genes are compared within the whole plant; circle size represents relative gene abundance. However, the combined quantities of transcripts of all NPC genes are not comparable in this figure. Analyses of Arabidopsis Affymetrix 22 K array data for NPC expression in tissues of *Arabidopsis* (B) and throughout ontogeny (C) were performed via Genevestigator (http://www.genevestigator.com). Developmental stages of *Arabidopsis* are abbreviated as follows: G, germinated seed; S, seedling; yR, young rosette; dR, developed rosette; B, bolting; yF, young flower; dF, developed flower; FS, flowers and siliques; S, siliques. NPC, non-specific phospholipase C.](image-url)
4.5.2. Role in abiotic stress

Phospholipid signalling plays a role in the activation of defence reactions in osmotic and salt stress conditions. Rapid accumulation of PC-derived DAG was observed in plasma membranes of Dunaliella salina following hypo-osmotic shock [106]. The expression level of NPC4 increased 12-fold in the roots of Arabidopsis after 3 to 6 h of 100 mM NaCl treatment [24]. The authors also demonstrated that DAG production via NPC activity increased in a time- and dose-dependent manner in salt-stressed Arabidopsis seedlings. More importantly, unlike WT plants, npc4 knockout mutants were characterised by a reduced germination rate when sown on media containing 150 mM NaCl [24]. In another study [25], npc4-7 plants were also shown to have reduced germination and overall viability under salt and drought stress conditions. Unlike WT plants, mutants overexpressing NPC4 were characterised by a higher germination level and maintained a greater root length and dry weight under both salt stress and hyperosmosis [25]. Based on experimental data, both groups of authors suggested that NPC4 participates in triggering plant salt stress responses likely via ABA-dependent mechanisms (for details, see section 4.5.4.).

Aluminium toxicity can cause rapid inhibition of root growth mediated by depolarisation of the plasma membranes [107,108], disruption of ion fluxes [109], and calcium homeostasis [110] as well as by affecting the cytoskeleton [111]. Certain phospholipases and lipid intermediates are known to perform signalling or metabolic roles during Al stress [112,113]. By studying the metabolic pathways involved in the formation and degradation of DAG, it has been shown that decreases in DAG accumulation in Al-treated tobacco BY-2 cells depend on NPC activity [23]. The authors demonstrated that a reduction in DAG production was not due to a decrease in cell viability and that exogenously applied DAG was able to restore Al-inhibited growth of tobacco pollen tubes, indicating an essential role for DAG during Al stress [23].

The expression level of NPC3 was increased 14.6-fold after 2 h in Arabidopsis seedlings subjected to 37 °C heat stress [114], suggesting a role for NPCs in thermotolerance. Gene expression arrays also provided clues into the behaviour of NPC1, NPC3 and NPC4[5] in response to drought stress, heat stress, cold stress, hypoxia and osmotic stress responses—conditions that may require membrane readjustment and stress signalling performed by NPCs. Additionally, NPC1 reacted to chemical-induced genotoxic and oxidative stress. Expression of some NPCs was also altered following changes in the plant illumination regime, indicating putative allocation to control of photosynthetic membranes. Conversely, NPC2 did not significantly react to abiotic stress, while expression of NPC6 was inhibited during osmotic and cold stress conditions (Arabidopsis eFP Browser, http://bar.utoronto.ca; Genevestigator, http://www.genevestigator.com).

4.5.3. Role in biotic stress

The role of phospholipases in plant defence reactions has been demonstrated repeatedly [115,116], giving evidence for a prominent role for lipid second messengers. The first evidence of possible NPC function in signal transduction during plant defence responses was provided by Scherer et al. [18] who studied the effect of glycoprotein elicitors from Phytophthora sojae and cryptogein elicitors isolated from Phytophthora cryptogea in cell lines of parsley (Petroselinum crispum) and tobacco (Nicotiana benthamiana), respectively. Adding synthetic PC-bearing fluorescent tags on both fatty acid residues (bis-BODIPY-PC) to the cell cultures demonstrated a rapid decline in fluorescently labelled DAG production, suggesting inhibition of NPC activity. A decrease in DAG level also occurred after treatment with mastoparan, a peptide capable of G-proteins activation. The observed level of fluorescently labelled PA was insignificant, indicating that the fluorescent DAG originated predominantly through direct action of NPC and not due to activation of PLD and PAP [18]. By contrast, the expression of the gene homologous to AtNPC5 was increased 4-fold in Citrus sinensis plants following infection with Candidatus Liberibacter asiaticus [117].

DNA microarray data available on the web (Arabidopsis eFP Browser, http://bar.utoronto.ca; Genevestigator, www.genevestigator.com) demonstrate that the NPC gene family, notably NPC6, is predominantly downregulated following inoculation with various strains of Pseudomonas syringae and Pseudomonas parasitica, representing both compatible and incompatible plant-pathogen interactions. NPC6 was also significantly repressed after flg22 treatment. However, NPC3 and NPC4 did demonstrate a positive response to Botrytis cinerea, Colavolinomyces orontii, Pseudomonas syringae and Phytophthora infestans treatment. In addition, NPC1 and NPC4 reacted to bacteria-deriveld elicitors, namely flg22 and HrpZ, suggesting a probable bivalent function for NPCs during stress responses and the ability to perceive dissimilar elicitors. Interestingly, NPC3 expression was elevated during Bemisia tabaci whitefly infection and after methyl jasmonate (MeJA) treatment in Arabidopsis ler and penta mutants, suggesting the possibility that NPC3 is involved in defence reactions against insect pests.

The putative role of NPC in defence responses may be attributed to DAG production, its conversion to PA via DGK, or other lipid second messengers. Synthetic DAG was able to activate defensive 6-methoxymellein phytoalexin production in carrot cells [118]. DAG and PA also control the generation of reactive oxygen species (ROS) and the induction of elicitor-responsive genes in rice cells [119]. Treatment with N,N,N′,N′′-tetrachloroethyldithanolase and flg22 elicitors promoted accumulation of PA in tomato cell culture that was not associated with PLD activation [120], giving evidence for the role of DAG/DGK in specific plant defence reactions. DGK-assisted DAG transformation may supplement PA production, which was shown to directly regulate defensive ROS generation by NADPH oxidase in Arabidopsis [121] and to be an early step in plant response to wounding [122]. Contribution of DAG/DGK to PA production was also observed during the reaction of suspension-cultured alfalfa cells to chitotetraose and xylanase elicitors [123], the early NO-dependent PA production in tomato cells elicited with xylanase [124] and the avirulent interaction of tobacco cells transformed with the Cladosporium fulvum Avr gene product [125]. Moreover, expression of the rice defence-responsive Os-BID1K DGK gene in tobacco has increased its resistance to tobacco mosaic virus and Phytophthora parasitica var. nicotianae [126], suggesting a function for DGK in lipid signalling pathways.

Putative inclusion of plant NPC and other DAG production/conversion enzymes in defence signalling to elicited cells and probable roles in other signalling pathways have been postulated. Moreover, it is becoming evident that NPC falls into a dualistic pattern common for other stress-responsive elements, demonstrating...
repression or activation depending on the nature of the stimulus and its spatial and temporal characteristics.

4.5.4. Role in hormonal regulation

The functions of plant hormones are mediated by a number of signalling agents including phospholipases C and D. At the same time, the direct actions of the novel NPCs on hormonal regulation remain unclear. NPC activity was rapidly induced in a BY-2 cell culture upon treatment with 24-epibrassinolide (BL) in situ [22]. The authors also found that npc3 and npc4 knockout mutants of Arabidopsis exhibited impaired sensitivity to BL and altered expression of TCH4 and LRX2 BL-responsive genes, in addition to reduced primary root length and lateral root number. Sensitivity to 1-ABA auxin was sustained in NPC-deficient plants, but expression of IAA19 and IAA20 genes involved in auxin signalling was decreased in npc3. In addition, auxins, cytokinins and brassinosteroids affected the expression level of NPCs as assessed by semiquantitative PCR [22]. The NPC4 transcript level increased after 3 h of zeatin treatment and 24 h of brassinolide treatment. However, only minor changes in the expression levels of the other NPC genes were observed [22]. The expression of PnPC2:GUS and PnPC3:GUS constructs after treatment with 1-NAI or brassinolide was intensified in root tips, cotyledons, leaf margins and floral organs, similar to the expression of the synthetic DRR3:GUS auxin reporter [127], which functionally controlled auxins, brassinosteroids and NPC that is important for root cell division and expansion. The expression pattern of the PnPC2:GUS construct correlated with auxin-rich root zones. However, an npc2 mutant was not aberrant in root formation density and length when treated with IAA [96].

A role for NPC in ABA-mediated responses in Arabidopsis has also been disclosed. It was shown that expression of NPC4 was induced by ABA treatment, but not by salicylic acid (SA) or MeJA treatment [95]. More importantly, npc4 mutant plants that accumulated higher levels of ABA in seeds were less sensitive to ABA treatment, resulting in abnormal germination, growth and stoma movement [25]. Expression levels of ABA-responsive genes (ABI2, RAB18, PP2CA, OST1, RD29A, ERA1 and SOT12) were also significantly altered in npc4 mutants under ABA treatment and under salt stress treatment that concurrently evoke ABA signalling [24,25]. NPC4-overexpressing Arabidopsis plants demonstrated increased sensitivity to ABA inhibition of seed germination, accompanied by induced salt and osmotic stress tolerance, giving strong evidence for a role for NPC in ABA-mediated plant stress responses. Interestingly, npc4 mutants accumulated lower DAG levels in leaves under normal growth conditions and under ABA treatment, although the composition of DAG molecular species, membrane phospholipids and galactolipids was unchanged. The phenotype of ABA-insensitive npc4 mutants was restored by application of both synthetic DAG and PA. Moreover, when DAG was applied with diacylglycerol kinase inhibitor 1 in the presence of ABA, DAG was no longer able to fully restore the phenotype. This evidence suggests that NPC-derived DAG production influences ABA signalling and is also important in DAG/PA conversion. Based on these results [25], the authors proposed a working model for the function of NPC4 and derived DAG in mediating the response of Arabidopsis to ABA. Under normal conditions, NPC4 contributes to the basal production of DAG that promotes stoma opening. Under stress conditions, ABA levels increase and consequently induce NPC4 to produce DAG. Stress-induced DAG is phosphorylated by DGK to PA, which promotes ABA-promoted stoma closure.

DNA microarray data (Arabidopsis eFP Browser http://bar.utoronto.ca; Genevestigator www.genevestigator.com) indicate that brassinolide evokes inhibition of NPC1 and NPC4 expression accompanied by activation of NPC6. ABA treatment resulted in a somewhat reversed pattern, with down-regulation of NPC6 expression and up-regulation of NPC3/NPC4 expression in Arabidopsis. Under specific conditions, NPC4 expression was also positively responsive to ABA, MeJA, BL or SA treatment. NPC1 and NPC6 were also activated under the influence of zeatin.

Growing evidence indicates a response from NPC to hormonal treatment. Localisation of some NPC isoforms in the plasma membrane suggests a role for NPC in early stages of hormonal perception. In addition, expression patterns of NPC matching sites of intensive growth and hormone accumulation suggest that NPC participation in intracellular amplification of hormonal signals is essential both for normal growth and stress reactions.

4.6. Possible mode of action

The regulatory role of NPC in plants is mediated via diacylglycerol production [128]. Diacylglycerol molecular species consist of two fatty acid moieties bonded to a glycerol by ester linkages to form highly non-polar acylglycerols conferring distinct properties as lipid intermediates, membrane components and second messengers. Impaired DAG generation or turnover has severe effects on development and growth in most living organisms [129–133]. In recent years, great progress in the understanding of DAG metabolism in human cells has been made at the molecular level. Deregulation of DAG metabolism has been linked to the pathophysiology of several human diseases including cancer, diabetes, immune system disorders and Alzheimer’s disease, as well as the disruption of organ development and cell growth [62]. It is currently thought that the role of DAG in plant cells is restrained to participation in lipid turnover and endomembrane remodelling. However, emerging research data indicate a complementary role for DAG in cell regulation and signalling.

4.6.1. Lipid metabolism

In plants, NPC is responsible for the production of both DAG and phosphocholine that enter cell metabolic pathways. While the known metabolic role of phosphocholine is rather trivial in plants, DAG is known as a key intermediate in generic lipid metabolism (Fig. 7). In plant cells, DAG can arise either metabolically from glycerol-3-phosphate or through plasma membrane lipid cleavage by lipase enzymes [62]. Due to its hydrophobicity, transiently generated DAG typically remains bound to cellular membranes where numerous supplementary enzymatic systems are employed to precisely control its turnover [61]. Depending on its origin and cellular localisation, the fatty acid composition of DAG may vary and form distinct C18/C16 and C18/C18 molecular species. DAG may give rise to diverse lipid species that, in turn, are of crucial importance for orchestration of cell metabolism and physiological reactions. In plants, DAG predominantly functions as a precursor of glycerolipid metabolism. However, diverse cellular DAG pools also participate in the synthesis of membrane glycolipids, sulfolipids and other lipid types, as well as in biosynthesis of storage triacylglycerols. In the ER, DAG acts as a substrate for both PC and PE synthesis; while in the plastid envelope, DAG is converted into monogalactosyldiacylglycerol (MGDG) and sulfolipids (for review see [134,135]). DAG can also be converted in two steps to cydinediphosphate-diacylglycerol, which is important for photosynthetic processes and biosynthesis of phosphatidylinositol, phosphatidylglycerol and cardiolipin [136]. In the capacity of DAG-derived products, galactolipids are essential for maintaining plastid thylakoid membrane composition and the direction of photosynthesis [137], while sulphoquinovosyldiacylglycerol (SDQG) sulfolipids are specifically implicated in photosystem II regulation and stabilisation [138]. MGDG also appears to be important during pollen germination and pollen tube growth, as well as under phosphate-limiting conditions [139].

There is direct evidence that NPC affects DAG production and the balance of other lipids in plants. Specifically, npc4 mutants
accumulate less DAG in leaves [25]. The production of DAG was also reduced in npc4 mutants during phosphate-limiting conditions in Arabidopsis [19], while such plants accumulated fewer MGDG and DGDG glycolipids and marginally more SQDG. However, the overall profile of DAG molecular species and the content of other lipids did not significantly change in npc4 under normal growth conditions [25]. In vitro activity of NPC5 was considerably lower than for NPC4; however, NPC5 seems to be responsible for mainstream production of DGDG in leaves during phosphate deprivation [82] (for details see section 4.5.1). Galactolipid content and levels of main membrane phospholipids were unchanged in npc5, although some changes were observed in the fatty acid composition of galactolipids [82].

Interestingly, some DAG-derived lipid species appear to be implicated in plant hormone perception and stress tolerance. Thus, Arabidopsis mutants deficient in DGDG synthase that utilises DAG as a substrate showed impaired thermotolerance [140]. Arabidopsis plants lacking diacylglycerol acyltransferase showed increased sensitivity to ABA and osmotic stress during germination [141]. DAG-derived SQDG lipids appear to be important for plant salt stress acclimation [142]. DAG can also be converted to PA by DGK and participate in jasmonic acid biosynthesis via lipase activity [143].

From the other hand, phosphocholine produced by NPC may give rise to free choline and contribute to biosynthesis of the osmoprotectants glyceine betaine [144] and choline-O-sulphate [145].

4.6.2. Signal transduction

The signalling role of DAG is not obvious in plants and is now being discussed [146]. So far, no DAG-responsive proteins have been identified in plant cells [147]. Munnik and Meijer [148,149] presented evidence that DAG, a product of PIP2 hydrolysis, is rapidly phosphorylated to PA, which plays an active role in plant signalling processes. The question remains as to how important the contribution of NPC is to the formation of these signalling molecules alongside metabolic DAG biosynthesis and DAG production via PI-PLC and PLD/PAP. It also appears that in some plant systems, DAG is likely to act as a signalling molecule per se. Thus, synthetic DAGs were shown to elicit ion flux in protoplasts of patch-clamped guard cells of Vicia faba and promote the opening of intact stomata of Commelina communis, which may again point to their possible recruitment to ABA signalling [150]. Additionally, Atippp2–2 knockout mutants of Arabidopsis with depressed lipid phosphate phosphatase activity, responsible for DAG production via PA dephosphorylation, demonstrated impaired ABA sensitivity accompanied by excess PA accumulation [151]. However, the activity of DGK, which is responsible for reverse DAG—PA conversion, was also reported to be elevated under ABA treatment in the same study, suggesting an interconnection of lipid messengers. DAG was also able to mediate membrane polarisation via endocytic recycling required for tobacco pollen tube polar growth [152] and is implicated in the perception of light irradiation that is important for circadian clock functioning and leaflet movements of Samanea saman plants [153]. Indirect evidence exists for the involvement of DAG species in the regulation of cell-cycle progression in stamen hair cells of Tradescantia virginiana [154]. Such data indicate that DAG may act as a signalling molecule in plant cells, but the molecular nature of such signalling cascades awaits elucidation. It is important to mention that PI-PLC-derived DAG and NPC-derived DAG are likely to differ in fatty acid composition.
and may have different roles in plant metabolism regulation [155].

Esters of phorbol—plant-derived tetracyclic diterpenoid compounds—have long been known as DAG-mimicking molecules that, in animals, are able to excite distinct intracellular responses [156]. More than 30 different mammalian proteins, including protein kinase C [157] and D [158], have been shown to bind both DAG and phorbol esters. Both have also shown functional similarity in regulation of cell growth and differentiation, tumour progression and other processes [159]. In plants, the commonly used DAG substitute phorbol 12-myristate 13-acetate (PMA) was involved in ROS-dependent regulation of tobacco defence hsr203) defense gene expression [160], intensified 6-methoxymellitin phytalexin production in carrot cells [118] and had a flowering-promoting effect in Lemma aquinodicalis and Lemma gibba plants [161]. Additionally, PMA elicited transient activation of a 45-kDa protein with properties of wound-induced MAP kinase in tobacco cell suspension cultures [162], and both PMA and DAG in suspended Rubus cells affected the activity of lamarinase, an enzyme that can lyse fungal cells and induce oligosaccharide production important for signalling in pathogenic invasion [163]. PMA also induced benzophenanthridine alkaloid accumulation in cell suspension cultures of Sanguinaria canadensis [164] and mediated the hypersensitivive response in lemon seedlings by inducing phenylalanine ammonia-lyase activity and synthesis of scopone [165]. Sato et al. [166] showed that the A. thaliana K⁺/KAT1 channel implicated in stomata functioning was inhibited by PMA when expressed in frog oocytes. Thus, PMA/DAG is likely involved in biotic/abiotic stress responses either directly or through the activation of plant protein kinases or PKC-analogous proteins.

Indeed, PKC is the principal target of DAG regulation in animal cells [167]. PKC enzymes belong to a ubiquitous serine/threonine kinase family, which are strongly activated by DAG molecules owing to the presence of C1 DAG/phospholipid-binding sites in the N-terminal regulatory domain.

There is no direct evidence for the existence of plant protein kinases that incorporate C1 domains. However, putative DAG-binding sites rich in cysteines and histidines were predicted in numerous A. thaliana proteins, including 104 (Protein kinase C-like, phorbol ester/diacylglycerol binding domain - IPR002219), 224 (C1-like domain - IPR011424), and 208 (DC1 – plant-specific “divergent C1” domain - IPR004146) (InterPro database, http://www.ebi.ac.uk/interpro, Pokotylo, unpublished results). Among them, DGKs and proteins containing zinc-finger and PHD-finger (Plant Home Domain) motifs were found, suggesting putative regulatory functions via binding to DNA, RNA, or other proteins. Intriguingly, C1-like domain-containing histone-lysine N-methyltransferase ATX1 of Arabidopsis functions both in ABA-dependent and ABA-independent osmotic stress response [168]. However, the majority of the putative DAG-binding proteins remain uncharacterised.

In addition to Arabidopsis, DAG-binding domains were also identified in stress-responsive NtDC1A and NtDC1B proteins from tobacco [169] and TaChp protein from wheat [170]. DAG-binding sites were found in, for example, nucleotide sequences of histone methyltransferases from barley and P. patens and in putative nucleoredoxin from Ricinus communis (European Nucleotide Archive, http://www.ebi.ac.uk/ena, Pokotylo, unpublished results), giving evidence for a role in gene regulation and signalling.

A multiplicity of functions has been ascribed to DAG-binding PKC in animal cells. It is established that PKCs are implicated in intracellular signalling and regulation of immune responses, receptor desensitisation, and transcription, as well as cell proliferation and differentiation (for review see [71,167]). At the same time, no PKC sequence homologies were detected in Arabidopsis or other plants, although a number of identified plant kinases exhibit a striking resemblance to animal PKC in their structural or biochemical properties. Thus, cloned plant transcripts of bean PVPK-1, and rice G11A-encoding protein kinases were characterised by their relationship to the catalytic domain of PKC but differed in the structure of their regulatory domains [171], and the phospholipid-activated protein kinase of Amaranthus tricolor cross-reacted with antiseraum raised against the regulatory sub-unit of PKC from bovine brain [172,173]. Similarly, PKC-like enzymes from Marchantia polymorpha thalli interacted with PKC-specific fluoroscent tags and reacted to spermine treatment during programmed cell death [174].

The activity of PKC-type enzymes involved in nitrate reductase gene expression in maize was regulated by Ca²⁺ and PMA, phopholipids or synthetic analogues of diacylglycerol [175]. Earlier, the same authors showed that PKC-like kinase activity in maize was stimulated by PMA in the presence of PS and calcium and was precipitated by animal PKC antibodies [176]. Purified protein kinase from Brassica campestris showed typical characteristics of conventional PKC while responding to Ca²⁺, diacylglycerol, phopholipids or phorbol esters [177], and PMA treatment stimulated PKC-like protein kinase in Brassica juncea [178]. The latter enzyme was purified to homogeneity and characterised as calcium-dependent and oleylacylglycerol- and PMA-activated. Calcium-dependent protein kinase from rice membranes was able to phosphorylate MARCKS (myristoylated alanine-rich C-kinase substrate) peptide, a highly specific substrate for animal PKC [179]. In wheat cells, protein kinase activity was stimulated by concurrent treatment with phospholipids and PMA/synthetic DAGs [180]. In this regard, attention is drawn towards the disclosure of a possible role of plant PKC-like proteins in cell regulation. A functional homolog of mammalian PKC was shown to mediate defence responses in the elicitor-induced potato plants [181]. Pea DNA topoisomerase I was activated after phosphorylation either by animal-derived PKC or by endogenous plant PKC-like phorbol-ester-binding enzyme localised in nuclei [182]. Moreover, treatment with animal PKC in vitro promoted the activity of stress-inducible pea DNA helicase 47 [183]. Treatment with the NPC-15437 specific inhibitor of animal PKC repressed calcium-dependent intracellular responses induced by ergosterol and cryptogeen elicitors in tobacco suspension cells [184]. PKC-like enzyme, alongside PI-PLC, was also implicated in anthraquione formation in Rubia tinctorum under chitosan treatment, while PMA alone was capable of mimicking the effects of the chitosan elicitor [185].

Independently, the regulatory role of NPC-derived DAG can be mediated through conversion to PA via the action of DGK. PA is currently known as an irreplaceable signalling agent in all living organisms, while the number of PA-binding regulatory proteins/protein kinases are crucially involved in the regulation of plant metabolism (for review see [128]). In turn, DGKs belong to a conserved lipid kinase family responsible for ATP-mediated DAG phosphorylation [155]. DGKs are ubiquitously found in eukaryotes, where the role of DGK is attributed to attenuation of DAG levels and potentiation of PA production, thus interconnecting lipid metabolism with signalling [186]. DGKs identified in plant systems are thought to localise to cell membranes [187] and are generally classified into three phylogenetic groups based on the presence of functional domains [155,188]. Interestingly, alternatively spliced variants of tomato LeCBDGK genes revealed a calmodulin-binding DGK [189]. However, the general functions and properties of intracellular regulation of plant DGKs remain largely unclear. In animals, PIP₂ is considered to be a precursor of DAG production involved in DGK-assisted conversion and activation of PKC enzymes that has so far been elusive in plants. However, PIP₂ levels in plants are as much as 100-fold lower than in mammalian cells [190]. Thus, DAG molecules involved in DGK-assisted turnover may arise from other sources, including NPC-mediated PC hydrolysis and phosphatidylinositol 4-phosphate cleavage by PI-PLC.
It is now established that in plants DGKs may provide intracellular amplification of PA signalling, either supplementing or substituting PA molecular species generated by PLD. The importance of DGK-mediated conversion of DAG to PA in ABA signalling was clearly shown [25]. DGK were shown to be responsible for PA production in cold-stressed Arabidopsis cells [191]. Moreover, some data suggest that the PA pool originating from DAG functions within the initial signalling phase of biphasic PA accumulation in stimulus-triggered cells [124]. Similarly to animal systems, where DGKs are often regarded as DAG signal modulators rather than as mediators of PA signalling [192], DGK in plants may possess complementary intracellular functionality not attributed to PA formation while modulating DAG, thereby implicating it in both signalling and lipid biosynthesis. It was shown that overexpression of the rice DGK gene OsBDIK1 activated under elicitor treatment enhanced disease resistance in transgenic tobacco [126]. Treatment with R59022 DGK inhibitor increased phytoalexin accumulation induced by fungal elicitor in pea epicotyl tissues [193]. R59022 also reduced root elongation and depressed growth of Arabidopsis seedlings [133], suggesting a role for DGKs in both stress responses and developmental processes in plants. Thus, while both DAG, originated from PC cleavage by NPC, and PA are considered to be important lipid-derived second messengers, DGKs appear to be among the main regulatory elements that provide coordination of their functions.

Plant NPCs may also affect cell function via protein–protein interactions independently of their primary enzymatic activity. NPC3 was shown to interact with PIN4 auxin efflux carrier protein (Interactomics Web, http://interactomics2.stanford.edu) which is involved in auxin transport to developing roots [194], although the significance of this interaction remains to be elucidated. The particular signalling role of phosphocholine produced either metabolically or via PC cleavage is currently neglected in plants. Nevertheless, the phosphocholine moiety has been shown to be important in the excitation of defence responses in cultured rice cells by glycosyl inositol-phosphoceramides from the phytopathogenic fungus Trichoderma viride [195]. Choline kinases have also been significantly activated in salt-stressed [196] and heat-stressed Arabidopsis [197].

4.6.3. Membrane remodelling

DGK species are principal membrane constituents that have a role in membrane biogenesis, remodelling and behaviour. Currently, DAG is known to have a major function as a multivalent modulator of various membrane-related cellular activities such as vesicular transport, endocytosis, secretion, and others [61,62,135,198,199].

Plants are typically characterised by somewhat increased membrane DAG content compared that with in animals, although its overall level remains low in the membranes. Depending on the stimulus, the membranes of animal cells contain approximately 0.2 to 1.4 nmol of DAG per 100 nmol of phospholipids (PL) [200–202]. DAG content measured in the alga Dunaliella salina was 4.2 nM per 100 nM PL [106] and in the tree Samanea saman varied from 3.5 nM/100 nM PL in unstimulated tissues to 4.2 nM/100 nM PL following exposure to light [153], suggesting relatively high importance for membrane stabilisation in plants.

In animals, DAG levels directly affect cytoskeletal reorganisation, membrane trafficking and exocytosis [147]. Local DAG accumulation can perturb phospholipid planar bilayers, promote the formation of non-lamellar membrane phases and influence membrane curvature [61,62]. Szule et al. [203] have shown that dioleoylglycerol induced reverse hexagonal (H₃) phase transition and development of negative curvature in PC monolayers. Additionally, DAG was shown to promote lamellar to non-lamellar transitions in pure PE and PC:PE:cholesterol mixtures [204]. Such transitions are essential for membrane fusion and fission processes. Thus, the activity of bacterial PC-PLC facilitates fusion of PC/PS/cholesterol containing vesicles [205], while 1,2-isomers of diacylglycerol, but not 1,3-isomers, increase calcium-induced fusion of PS vesicles in vitro [206], implicating NPC in various cell physiological activities including exocytosis, endocytosis, membrane build-up and cell division.

DAG produced by PI-PLC [152] and possibly by NPC [23] presumably undergoes endocytic recycling in membranes important for tobacco pollen tube growth. The role of NPC and DAG in cell growth can be demonstrated in pollen tubes. Under physiological conditions, pollen tube growth requires the transport of large quantities of material for the rapidly emerging membrane, and an accumulation of DAG was indeed observed [152]. By contrast, in the presence of Al³⁺, DAG production was reduced and pollen tube growth was inhibited [23].

In membranes, DAG can modify lipid-protein interactions and alter exposure of surface membrane receptors [62]. Interestingly, it was recently reviewed that DAG can modify activity of the very PLC enzymes that grant its production [207]. Asymmetrical DAG distribution in the membrane may additionally facilitate formation of membrane domains and functional lipid rafts [198].

5. Conclusions and perspective

Here, we present collective evidence for plant NPC enzymes as novel agents involved in the control of cell physiological functions. An NPC regulatory role implemented via DAG production affects metabolic regulation, biotic and abiotic stress responses and hormone sensing. The available data provide support for including NPC gene family as key agents involved in cell signalling alongside other plant phospholipase enzymes. Despite the significance of DAG in cell signalling, the regulatory role of DAG produced by NPC may also be uncoupled from DGK and be accomplished via other putative DAG responsive elements in plant cells. However, further studies are required for elucidation of the molecular properties of NPC action, the implication of NPC in as yet unexplored growth and developmental processes, and the putative interaction of NPC with other signalling systems in plant cells.

Much progress has been made in the general understanding of NPC functioning. A wealth of information on the expression of the NPC gene family in different plant species is now available, but much remains to be elucidated about the nature of the extended role of NPC in cell regulation and signalling. There is also a large void in the understanding of the molecular properties of NPC action. These and similar questions will most likely be answered using novel experimental approaches, including advanced genetic manipulations and biophysical methods for studying membrane-associated processes.

We should also mention a possible future use for NPC as a target of genetically altered plants resistant to disease or stress. Initial evidence has been already demonstrated for the role of NPC in resistance to salt stress.

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