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# Plant non-specific phospholipase C gene family. Novel competitors in lipid signalling

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## Keywords

Plant nonspecific phospholipase C, phosphatidylcholine-specific phospholipase C, diacylglycerol, cell signaling, metabolism regulation

## Abbreviations

ABA, abscisic acid; BL, 24-epibrassinolide; BY-2, Bright Yellow 2; BODIPY, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene; D609, tricyclodecan-9-yl-xanthogenate; DAG, diacylglycerol; DGDG, digalactosyldiacylglycerol; DGK, diacylglycerol kinase; EDTA, ethylenediaminetetraacetic acid; GUS,  $\beta$ -glucuronidase; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; MAG, monoacylglycerol; DGDG, digalactosyldiacylglycerol; MeJA, methyl jasmonate; NPC, non-specific phospholipase C; PA, phosphatidic acid; PAP, phosphatidic acid phosphatase; PC, phosphatidylcholine; PC-PLC, phosphatidylcholine-specific phospholipase C; PE,

phosphatidylethanolamine; PIP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; PI-PLC, phosphatidylinositol-specific phospholipase C; PKC, protein kinase C; PL, phospholipid, PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; PS, phosphatidylserine; ROS, reactive oxygen species; SA, salicylic acid; SQDG, sulfoquinovosyldiacylglycerol; TDTMA, tetradecyltrimethylammonium bromide

## Abstract

Non-specific phospholipases C (NPCs) were discovered as a novel type of plant phospholipid cleaving enzymes homologous to bacterial phosphatidylcholine-specific phospholipases C and responsible for lipid conversion during phosphorous-limiting conditions. Six-gene family was established in *Arabidopsis* and since then evidence is growing indicating involvement of NPCs to both biotic and abiotic stress responses as well as to phytohormones action. In addition, diacylglycerol produced via NPCs was postulated to participate in membrane remodelling, general lipid metabolism and accomplish cross-talk with other parts of known phospholipid signalling systems in plants. This review summarises most topical information concerning this new plant protein family and focuses on its sequence analysis, biochemical properties, cellular and tissue distribution and physiological functions. Possible mode of action is also discussed.

## 1. Introduction

Phospholipases (Fig. 1) are now well recognized to be among the key components of cell growth and development regulation systems in living organisms. They give rise to an array of second messenger molecules and lipid derivatives implicated in both metabolism and intracellular signalling. Recent research progress has made it possible to convincingly reveal an important role of 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 headgroup from hydrophobic diacylglycerol (DAG). PLCs in living systems can be generally divided into phosphatidylinositol-specific phospholipases C (PI-PLC) and phosphatidylcholine-specific phospholipases C (PC-PLC) according to substrate specificity range. Implication of specific phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) cleaving PI-PLCs to cell metabolism regulation is fairly well studied in different organisms. Thus, multidomain animal PI-PLCs are G-protein activated enzymes responsible notably for intracellular calcium level regulation and protein kinase C (PKC) activation [1]. Despite lack of identified inositol 1,4,5-trisphosphate receptor in plants [2], PI-PLCs are unquestionably implicated to regulation of growth, development and stress responses in *Arabidopsis* and in other plant species [3]. In turn, bacterial PI-PLCs belong to secreted pathogenicity factors typically conferring virulence [4].

PC-PLCs, in plants also known as non-specific PLCs (NPC), are characterized by broad substrate range that include abundant phosphatidylcholine (PC) (discovered in 1847 as 'lecithin' molecules), while their role in cell signalling and regulation in general remains far less disclosed. PC-hydrolysing phospholipases were first discovered under the name "lecithinase C" in bacteria [5] and later established as important bacterial 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 increase in DAG production occurs in cellular membranes in response to various stimuli [10-11] whereas attributed PC-PLC activity appears to be implicated in a number of intracellular regulatory events (see Chapter 3). However, present-day lack of molecular and genetic characterization of PC-PLCs in animals hampers the research progress.

Though putative PC-PLC activity in plants was observed as early as 1955 [12], their recognition in plants have long remained vague and their role stayed elusive. Some supporting clues for PC-hydrolysing PLC occurrence were identified in various plant organs and tissues such as peanut seeds [13], rice grains [14], tomatoes [15] and others [16]. However, obtained data lacked sufficient characterization of the enzyme [17]. Eventually, in 2002 fluorescently labelled PC was shown to be directly cleaved to produce DAG in parsley and tobacco cells suggesting a 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 *Arabidopsis* genome based on shared sequence similarities with bacterial PC-PLCs [19] and nine NPC genes were identified in soybean plants [20]. DAG production via PC hydrolysis was also observed in *Petunia hybrida* giving a suggestion regarding omnipresent occurrence of NPCs in plant kingdom [21]. Subsequent increase of researchers interest in dissection of NPC's roles in plants over the recent years have promoted the identification of NPC involvement in regulation of diverse cellular processes including root development and brassinosteroid hormone signalling [22], A1 stress signalling [23], abscisic acid (ABA) sensing and tolerance to hyperosmotic and salt stresses [24-25]. On the other hand, NPC3 protein of *Arabidopsis* was shown to perform lysophosphatidic acid (LPA) phosphatase activity providing monoacylglycerol (MAG) rather than DAG production that may hint at a possible multivalent functionality of NPCs [26].

Taken together evidences denote occurrence of NPC in plant species and their putative role in plant metabolism regulation.

## 2. Bacterial phosphatidylcholine-specific phospholipase C

Since early discovery in 1941 of PC-PLC specific activity in *Clostridium welchii* toxin [5], various PC-PLC genes and corresponding enzymes were identified in both Gram-positive e.g. *Bacillus cereus*, *Listeria monocytogenes*, *Clostridium* species and, more recently, Gram-negative bacteria e.g. *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 to be potent toxins with haemolytic properties related to *Clostridium perfringens*  $\alpha$ -toxin. Such toxic PC-PLCs represent single polypeptide enzymes that require zinc ions for activation and which are reversibly inactivated by EDTA or *o*-phenanthroline metal chelators. Several toxic PC-PLCs have been shown to also require  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  ions for their activation [30]. Some genes encoding this zinc-metallophospholipases C have been isolated and characterized [31]. The molecular mass of the enzymes lies within the range of 28 to 34 kDa [6]. Amino acid composition of these PC-PLCs revealed the presence of signal sequence denoting protein recruitment into secretory systems. Toxic bacterial PC-PLCs have broad range substrate specificity that apart from phosphatidylcholine includes phosphatidylethanolamine (PE), phosphatidylserine (PS), sphingomyelin and other lipids [6]. The molecular structure of the well-studied *Clostridium perfringens* toxic PC-PLC ( $\alpha$ -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, Lipoxygenase, Alpha-Toxin) domain was found to be similar to lipid binding domains found in eukaryotes [32] and appears to be responsible for binding membrane phospholipids in a calcium-dependent manner [33]. Interestingly, N-terminal domain of  $\alpha$ -toxin sufficiently retained PC-PLC activity when expressed in *Escherichia coli*, however it lacked haemolytic and sphingomyelinase activities that are supposedly granted by lipoxygenase-like C-terminal domain [34]. For a long time 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 over host immune responses [35]. For instance, secreted PC-PLC from *Clostridium perfringens* has a role in aggregation of blood platelets [36] and PC-PLC from *Clostridium welchii* was shown to inhibit defensive superoxide generation in human polymorphonuclear leukocytes by interacting with membrane component of the NADPH oxidase [37]. However, it was shown that some PC-PLC from Gram-positive bacteria can have functions unrelated to those of typical toxic enzymes. Thus secreted PC-PLC from *Bacillus cereus* is involved in defence mechanism of bacteria to phagocytosis [38]. Furthermore, some, bacterial PC-PLC may also be used as vaccines against diseases such as gangrene [36].

The second group of PC-PLCs is primarily produced by Gram-negative bacteria. Such enzymes do not contain zinc ions and typically do usually not possess hemolytic properties. Their identified molecular weight differs from that of the toxic ones several fold as well. For example,  $M_w$  of *Pseudomonas cepacia* PC-PLC is 78 kDa. Non-toxic PC-PLCs have not responded to *in vitro* treatment with bivalent ions [39] and EDTA chelator was reported to significantly stimulate the activity of PC-PLC from *Legionella pneumophila* [29]. PlcHR<sub>2</sub> phospholipase C found in *Pseudomonas* species was also shown to be  $\text{Ca}^{2+}$ -independent [39]. Interestingly, Gram-negative *Pseudomonas aeruginosa* produced several types of PC-PLC [40] that differ in substrate specificity and toxic properties. Thus PLC-H identified in *Pseudomonas aeruginosa* was hemolytic [41] and possessed sphingomyelin synthase activity [42].

It is apparent that PC-PLCs from Gram-positive and Gram-negative bacteria have evolved independently and, intriguingly, non-toxic bacterial PC-PLCs, e.g. PlcB from *Mycobacterium tuberculosis* and PlcN from *Ralstonia solanacearum* [19] clearly show resemblance to putative plant NPCs indicating not only a common evolutionary descent but also putative cell functions unrelated to straightforward membrane lysis. Some data also indicate that NPC may be also attributed to a superfamily of phosphatase/phospholipase proteins related to acid phosphatase AcpA form *Francisella tularensis* [43].

At the same time bacteria apparently contain several evolutionary divergent types of PC-PLC such as NaCl-stimulated enzyme purified from a marine streptomycete [44], or membrane bound PC-PLC protein with a broad pH optimum identified in cell-wall lacking *Ureaplasma urealyticum* [45].

Alongside with data available on PC-PLC pathogenic properties it is surprising that to date little is known about physiological functions of non-toxic PC-PLCs. They thought to be involved in lipid remodelling during phosphorous-limiting conditions, as shown for intracellular PC-PLC of *Sinorhizobium meliloti* [46]. Regulation of some PC-PLC genes by exogenous phosphate levels also supports this hypothesis [6].

### 3. Animal phosphatidylcholine-specific phospholipase C

PC hydrolysis is now long recognized as a constitutive element of cell signalling and metabolism regulation in animals [8-9] while PC-cleaving PLC is appearing to be a key agent in second messenger molecules generation [47-48].

Animal PC-PLC genes probably show no sequence similarities with other known PC-PLCs in diverse living organisms. That is why no animal PC-PLC genes have been cloned or characterized as of present day. Yet, considerable effort has been invested into PC-PLC functional characterization and it is now become evident that PC-PLC is directly involved into control of cell proliferation and differentiation as well as to number of other cell functions.

Up to now PC-PLC activity was detected in multiple animal sources such as canine myocardium [49], acrosomes of bull seminal plasma [50], human vascular endothelial cells [51] and chicken blastodisc [52]. Interestingly, cytosolic 66 kDa PC-PLC identified using antibacterial PC-PLC antibody in mouse NIH-3T3 fibroblasts [53] and in NIH-3T3 H-ras transformants [54] was rapidly translocated to plasma membrane upon mitogen-mediated cell receptor stimulation. Similar pattern with PC-PLC activity predominantly localized to plasma membrane was observed in oncogenically transformed human epithelial ovarian cells [55] and NK cytolytic cells where PC-PLC may contribute to lysis of target cells [56-57].

To date, regulatory role of PC-PLC has been largely attributed to DAG production. In animals, DAG can typically arise via PC or PIP<sub>2</sub> hydrolysis or by phosphatidic acid (PA) dephosphorylation. DAG has multivalent functionality that includes alteration of membranes curvature and properties, orchestration of lipid metabolism and participation in lipid-mediated signalling [58-59]. Among the most important features of DAG is its ability to bind to C1 domain [60] and facilitate PKC activation – an enzyme known for its immensely wide cellular functionality [61-62]. DAG-binding C1 domain has been also identified in number of other proteins with variegated regulatory functions. For instance, DAG-mimicking phorbol ester induced a PKC-independent exocytosis in rat PC12 cells mediated by RasGRP3 protein that contains a C1 domain [63].

Initial reports indicated that PC-derived DAG may be ineffective for PKC activation [64]. Yet it was later shown that *Bacillus cereus* derived PC-PLC, but not PI-PLC, inhibited the formation of cAMP by adenylate cyclase, negatively regulated by PKC in Swiss 3T3 fibroblasts [65]. In addition, regulatory function of melatonin hormone in rat retinal ganglion cells was mediated by PKC activated exclusively via PC-PLC action [66]. A specific implication of PC-PLC to regulation of leukemic myeloid cell proliferation via MAP kinase cascade stimulation and NF-κB activation has been reported to be also PKC-dependent [67]. It is now thought, that PC-derived DAG can be responsible for activation of PKC isoforms dependent solely on DAG, namely PKC-δ, PKC-ε, PKC-η and PKC-θ. From the other hand, PC-PLC may provide a sustained DAG source that can assist in activation of conventional type PKCs dependent both on DAG and Ca<sup>2+</sup> ions (PKC-α, PKC-β, PKC-βII, PKC-γ) firstly initiated by DAG produced via short-lived PIP<sub>2</sub> hydrolysis [68].

It is also considered, that DAG produced by PC-PLC may be phosphorylated to PA by diacylglycerol kinases (DGK) [69-70] and act as a contributor to PA-dependent cell metabolism regulation in addition to phospholipase D (PLD) [71]. 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 Golgi apparatus of HeLa cells [72].

In animals, the regulatory role of phosphocholine, which is concurrently produced alongside with DAG during PC hydrolysis by PC-PLC, is also discussed. Phosphocholine was found to be involved in many long-term cellular responses such as activation of proliferation/differentiation and cell transformation promotion [73]. Moreover, phosphocholine was shown to induce DNA synthesis and mitogenesis in mouse fibroblasts [74] and acted as a specific receptor molecule for perforin in lymphocytes [75]. It is interesting to mention, that in animal cells choline kinases are also responsible for phosphocholine production through choline phosphorylation in the presence of ATP and magnesium. They have been shown to be implicated in cell division and growth regulation [76], and play significant role in regulation of both normal human mammary epithelial cell proliferation and breast tumour progression [77].

Number of other putative functions of PC-PLCs affecting animal cell metabolism are now being considered. Despite, understanding of the specificity of PC-PLC activity and role of phospholipid-derived signalling substances in animal cells is impeded by the 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

The initial reports on plant NPCs suggested that they are not related to any other known plant phospholipase family. Previously, multiple alignment of *Arabidopsis* NPCs with PLC from *Mycobacterium tuberculosis* revealed three conserved regions unrelated to known domains in plant phospholipases [19]. Although the NPC family clearly arose early in evolution, bacterial, plant and invertebrate lineages have each developed distinct features in NPC sequences. Genomes of higher plants typically contain several genes coding for putative NPCs proteins consisting of 510 - 540 amino

acid residues. NPC proteins contain central phosphoesterase domain (Fig. 2), which is typically present in enzymes with esterase activity like NPCs and acid phosphatases. No other motifs known from other plant lipid signalling proteins (i.e. C2, XY, EF, PH, PX, ENTH, FYVE domains) are present. Generally plant NPCs show high level of similarity, especially in the phosphoesterase domain (Fig. 2). Close comparison with Gram-negative bacterial PC-PLCs reveals four motifs with invariable residues, suggesting that these are probably crucial for the PC-PLC catalytic activity (Fig. 2, 4). Majority of plant NPCs contains a putative signal peptide at the very N-terminus that is followed by short variable region and highly conserved domain containing invariable ENRSFDxxxG motif in the beginning of phosphoesterase domain. The putative signal peptide is missing in *Arabidopsis* NPC3, NPC4 and NPC5. Other two invariable motifs, TxPNR and DExxGxxDHV are found in the middle of the domain followed by GxRVPxxxxxP region that closes the phosphoesterase domain. Interestingly, last 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. Tentatively, this may be a part of the molecule responsible for functional differences of various NPC isoforms through facilitating interactions with other proteins or defining protein localization.

In order to get first insight into evolution of plant NPCs and to determine whether NPC diversity seen in *Arabidopsis* is conserved across plants, we performed 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 analyzed genomes of monocotyledons rice (*Oryza sativa*) and sorghum (*Sorghum bicolor*), sitka spruce (*Picea sitchensis*) representing gymnosperms and moss *Physcomitrella patens* and lycophyte *Selaginella moellendorffii* representing evolutionarily ancient plants. Phylogenetic analysis of plant NPC sequences suggested that common ancestor of all seed plants had already at least one NPC1-, NPC2- and NPC6-like gene. Interestingly, NPC3-5 subfamily was not identified in spruce (and other available gymnosperms 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 subfamily in gymnosperms. Alternatively, it is possible that NPC3-5 subfamily is present only in angiosperms and emerged after separation of gymnosperm and angiosperm ancestor, possibly from NPC2 clade (as indicated by phylogeny). Surprisingly, *Physcomitrella* and *Selaginella* were found to contain only NPC1-like genes. Two evolutionary simplest explanations are possible: either the common ancestor of land plants contained already NPC1, NPC2 and NPC6 subfamilies and NPC2 with NPC6 gradually disappeared during evolution towards lycophytes and brychophytes, or NPC1 subfamily is the ancestral NPC, which subsequently given rise to all other NPC types (however this scenario is not supported by the phylogenetic analysis). Both *Selaginella* and *Physcomitrella* NPCs further multiplied independently after the separation of mosses, lycophytes, and seed plants. Rapid diversification of NPCs is evident also from the evolution of angiosperm orthologs, where multiple duplications within all NPC subfamilies are frequently observed. In contrast, no NPC were found in green algae suggesting that NPC family may have been lost throughout algae evolution.

#### 4.1.1. 3D model of plant NPC

Although no experimental three-dimensional structure of plant NPC is available, homology of plant NPCs with the acid phosphatase (AcpA) family enabled us to construct a 3D model for *Arabidopsis* NPC, employing recently published structure of AcpA from bacteria *Francisella tularensis* [43]. Analysis of the AtNPC2 3D model (Fig. 4) showed that, similarly to the bacterial AcpA, the backbone of plant PC-PLC is formed by beta sheet (composed of 7 beta structures), which are embedded inside the protein and surrounded by several alpha helices (6 in FtAcpA, 7 in plant NPCs). Importantly, the majority of amino acid residues forming the active site of bacterial AcpA are conserved, including the residues that bind yet unidentified metal cation. This is true also for the ion pair of aspartate-arginine, which probably acts as a stabilizer of active site topology. Altogether this suggests that for eukaryotic PC-PLCs, the bacterial PC-PLCs and homologous acid phosphatases, the common ping-pong reaction mechanism, with the existence of an intermediate with the substrate phosphate group covalently bound to nucleophilic amino acid from the active site, is likely to be maintained. On the other hand, none of the four cysteines, that form two disulfide bridges stabilizing 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 outlying from the proposed active center and thus does not participate directly to the catalysis. This is consistent with the sequential analysis of NPC proteins. Interestingly, active site of plant NPCs forms relatively strongly negatively charged pocket, which could be involved in phospholipid substrate binding (Fig. 4).

#### 4.2. Biochemical properties of NPC

Up to now, NPC3, NPC4 and NPC5 from *Arabidopsis thaliana* were cloned and partly biochemically characterized (Table 1). Recombinant NPC4 expressed in *Escherichia coli* showed activity towards PC and PE while the

ability to cleave PA and PIP<sub>2</sub> substrates was negligible [19]. NPC4 also demonstrated moderate hydrolysing activity towards PS and to a little degree could hydrolyse phosphatidylglycerol [25]. NPC4 activity was slightly elevated in the presence of 2 mM EGTA chelator [19].

Recombinant NPC5 protein expressed in *Escherichia coli* was able to cleave PC and PE producing DAG. However, hydrolysing activity of NPC5 was more than 40-fold lower comparing to NPC4 [78].

The purified recombinant NPC3 protein demonstrated specific LPA phosphatase activity resulting in MAG production [26]. LPA phosphatases are known to be among important regulatory enzymes in animals [79], however their occurrence in plants have not been ascertained up to this point. The enzyme was marginally stimulated by low concentrations of Triton X-100 non-ionic detergent, 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). However, it was not affected by PA, PC, MAG or DAG. Phosphatase activity of NPC3 did not require the presence of Ca<sup>2+</sup> or other bivalent ions and was independent on fatty acid variations in LPA substrate. NPC3 was also unresponsive to treatment with sodium *o*-vanadate and sodium fluoride non-specific phosphatase inhibitors. The authors have additionally shown that purified enzyme did not hydrolyse sphingosine-1-phosphate, diacylglycerol pyrophosphate, glycerol-3-phosphate, LPC, LPE or PA. More importantly, NPC3 was shown to lack ability to hydrolyse PC, PE or PS, indicating that NPC3 gene may be paralogous to other plant NPC genes coding phospholipase C-type enzymes.

The studies of plant NPC activity *in vitro* have allowed to reveal that in microsomal fraction prepared from BY-2 cells PC hydrolysis was inhibited by Al at high AlCl<sub>3</sub> concentrations (≥ 100 μM) [23].

Specific inhibitors serve as important tools for revealing role of studied proteins. As of today, no specific inhibitor was described for NPCs. However, both animal PC-PLC-directed [80-81] and bacterial PC-PLC-directed [82] inhibitors were identified.

Tricyclodecan-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, antitumour, and anti-inflammatory influence. However, it has been shown to be a potent antioxidant [83] and inhibitor of group IV cytosolic phospholipase A<sub>2</sub> [84]. Thus, at least some of observed functions may be attributed to the by-effects of D609 influence on PC-PLC activity. Interestingly, D609 also inhibits some bacterial PC-PLC enzymes indicating that animal and bacterial PC-PLC may share some structural similarities [85]. For instance, PC-PLC from Gram-negative *Pseudomonas fluorescens* reacted to animal D609 PC-PLC inhibitor treatment [27]. On the other part, NPC activity in BY-2 cells was not altered after treatment with 20 μM of D609 animal PC-PLC inhibitor [23], indicating that plant NPC may possess regulatory properties different from those of animal or bacteria PC-PLC.

Taking into consideration identified sequence similarities between plant NPC and bacterial PC-PLC, TDTMA-type (tetradecyltrimethylammonium bromide) inhibitor of bacterial enzyme may also be effective in repressing NPC activity [82].

**Table 1**

Biochemical properties, physiological properties and expression pattern of NPC. Given here is characteristic of identified NPC-coding genes and corresponding gene products in *Arabidopsis thaliana*. Putative implication of NPCs to particular cell reactions is also provided. Data was 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.

| Name        | Gene locus | Cloned | Number of aa | Calculated M <sub>w</sub> (kDa)* | Substrate   | Localization            | Predominant tissue expression | Activation/stimulus expression   | Reference        |
|-------------|------------|--------|--------------|----------------------------------|-------------|-------------------------|-------------------------------|--|------------------|
| <i>NPC1</i> | At1g07230  | -      | 533          | 60.0                             | ?           | Endomembranes, unclear* | Siliques                      | ?  | [25]             |
| <i>NPC2</i> | At2g26870  | -      | 514          | 57.6                             | ?           | Endomembranes*          | Siliques, roots               | ?  | [25]             |
| <i>NPC3</i> | At3g03520  | +      | 523          | 59.1                             | LPA         | Tonoplast               | Leaves, roots                 | BL signalling, auxin, cytokinine   | [22, 25, 26, 86] |
| <i>NPC4</i> | At3g03530  | +      | 538          | 60.7                             | PC, PE (PS) | Plasma membrane         | Old leaves, roots             | Phosphorous deficiency, ABA, salt stress, BL signalling, auxin, cytokinine | [19, 22, 24, 25] |
| <i>NPC5</i> | At3g03540  | +      | 521          | 59.0                             | PC, PE      | Cytosol                 | Inflorescences                | Phosphorous deficiency   | [22, 25, 76]     |
| <i>NPC6</i> | At3g48610  | -      | 520          | 57.9                             | ?           | Mitochondria, plastids* | Siliques, roots               | ?  | [25]             |

? Experimentally unidentified, \* Based on sequence analysis and prediction programmes (The *Arabidopsis* Information Resource (TAIR), [www.arabidopsis.org](http://www.arabidopsis.org)), Cell eFP browser, [http://bar.utoronto.ca/cell\\_efp/cgi-bin/cell\\_efp.cgi](http://bar.utoronto.ca/cell_efp/cgi-bin/cell_efp.cgi)).



#### 4.3. Intracellular distribution

Localization data may provide additional clues for the disclosure of functional role of NPC in plant cells. In terms of subcellular localization plant phospholipases can be typically divided to cytosolic and membrane-bound enzymes [17, 86].

NPC activity has been experimentally detected in cell membranes of stamens and pistils of *Petunia hybrida* [21] and plasma membrane fraction prepared from BY-2 suspension cell culture [23]. The similar pattern was observed in oat root where a phospholipase that reacted with NPC4-raised antibody was found in plasma membrane [87]. Analysis of subcellular fractions from *Arabidopsis* leaves using polyclonal anti-NPC4 antibody has revealed that NPC4 is located in microsomal membrane fraction and not to the plastids [19]. Further analysis demonstrated that NPC4 is localized specifically to plasma membrane [19] confirmed by analysis of NPC4-GFP construct [78] (Fig. 5). NPC3 in turn was experimentally identified to be present in tonoplast as shown by one-dimensional SDS-PAGE analysis of a tonoplast-enriched fraction followed by nano-LC MS/MS [88]. Unfortunately, the determinants for NPC4 and NPC3 membrane localization are currently unknown and may include palmitoylation, myristoylation, GPI-anchoring etc.

NPC5-GFP construct was found to be localized to cytosol in *Arabidopsis* [78]. Because phospholipases are performing conversion of hydrophobic substrates it is possible to assume that cytosolic phospholipases are translocated to membranes upon stimulation as shown for PLD $\alpha$  in *Arabidopsis* [89].

The cellular distribution of other NPCs is currently uncertain and was predicted to be localized presumably to endomembranes in spite of lack of identified transmembrane domains (<http://bar.utoronto.ca/>; <http://www.cbs.dtu.dk/services/TMHMM/>).

#### 4.4. Tissue distribution

Identification of level and spatial properties of NPC expression in tissues and organs of *Arabidopsis* may provide effective means of further disclosure of their role in plant growth and development. NPC genes can be characterized by changeable level of expression with somewhat pronounced intra-organismal expression pattern. Average signal percentiles of the corresponding mRNAs in single channel arrays were ranging from around 25 for NPC4 to 71.4 for NPC1 and 77.2 for NPC6 (The *Arabidopsis* Information Resource (TAIR), [www.arabidopsis.org](http://www.arabidopsis.org)). Such data is consistent with differentiated expression potential of other phospholipases known to be implicated to cell signalling including PI-PLC (percentile ranges  $\approx$ 42-90) and PLD (percentile ranges  $\approx$ 26-94). More importantly, low generalized expression potential may suggest an inducible expression and putative signalling role for NPC2 (51.6) NPC3 (51.5), NPC4 and NPC5 (24.8) which was 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  $\beta$ -glucuronidase (GUS) reporter system also allowed demonstrating  $P_{NPC3}:GUS$  and  $P_{NPC4}:GUS$  constructs localisation to root tips, cotyledons and outer margins of leaves. Additionally, in floral organs  $P_{NPC3}:GUS$  and  $P_{NPC4}:GUS$  constructs were expressed predominantly in pollen sac tissues of young anthers [22]. In another study  $P_{NPC4}:GUS$  constructs were expressed in aging leaves with low level of expression found in roots, stems, siliques and flowers [90]. GUS staining additionally allowed to determine NPC2 gene expression pattern to be localised to meristematic zone, elongation zone and vascular tissue of maturation zone of root tips and to developing leaves with observed deterioration towards leaves maturation [91] (Fig. 6A).

Analysis of array data allowed identifying significantly amplified expression of all NPC genes in developing seeds, notably in chalazal endosperm and seed coat – sections known to be among the main lipid storage and metabolism sites (Fig. 6B,C). Elevated expression of NPCs was also detected in roots, floral organs, siliques and leaves at different 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. On the contrary, expression of NPC1 was low in germinating and mature pollen. NPC2 was upregulated in siliques and during early stages of seed development in meristem of developing embryo and had relatively low expression in roots and leaves of *Arabidopsis*. NPC3 was amply expressed in endodermis, phloem pole and phloem companion cells in root elongational and maturational zones, root stela as well as in cork, cambium and xylem of stem. NPC3 expression was also relatively active in dry seeds and early germinating seeds. NPC4 can be additionally distinct by expression in root xylem and in developing floral organs. NPC4 also has low expression level in germinating pollen. NPC6 expression has seemingly uniform distribution across organs of *Arabidopsis*. However it was notably higher in seedling hypocotyls and lower in seedling roots and germinating pollen. Interestingly, NPC6 was expressed significantly higher in young leaves than in old leaves of *Arabidopsis* and was intensively expressed in shoot apical meristem, leaf primordial and floral organs (*Arabidopsis* eFP Browser, <http://bar.utoronto.ca/>; Genevestigator, [www.genevestigator.com](http://www.genevestigator.com)). It is important to mention that NPC5 and NPC4 expression data is indistinguishable in *Arabidopsis* ATH1 22k genome array. However, because expression level of NPC5

was estimated to be significantly lower from that of *NPC4* in majority of *Arabidopsis* tissues, obtained results may for some extent be applicable for *NPC4* [25].

Proteomics data analysis has confirmed that NPC proteins, excluding NPC5, are present in roots of *Arabidopsis*. NPC1, NPC2 and NPC6 were also found in leaves, NPC3 and NPC4 in seeds and NPC 1 and NPC2 in floral organs of *Arabidopsis* plants. NPC5 protein was presented in leaves subjected to mannitol induced drought stress (<http://www.ebi.ac.uk/pride/>).

#### 4.5. Physiological function of NPC

As of today NPC implication to various responses of plant metabolism both to environmental and organismal stimuli has been reported. Long-term changes in NPC activity and gene expression were observed under biotic and abiotic stresses, phosphorous deficiency, certain hormone treatment (auxins, cytokinins, ABA) and throughout membrane remodelling. In contrast, rapid changes of 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 control of quick promoter, which takes about 30-45 minutes [92]. Thus changes in NPC activity can be attributed to stimulation of presynthesized enzyme caused by posttranslational modification and/or intracellular translocation. This allows to claim a physiological role of NPC in plant cells and to include NPC to the list of phospholipases involved in signal transduction.

##### 4.5.1. Phosphate deficiency

Upon phosphorous shortages, organic phosphate can be mobilized from membrane phospholipids that incorporate up to a third of total phosphate in plant cells. It was assumed that NPCs are able to facilitate such mobilization by disjoining phosphorous containing head group from phosphatidylcholine or other phospholipids. Concurrently produced DAG can serve as a precursor to production of substituting sulfolipids [93] and glycolipids [94] which allow to retain membranes integrity and crucial properties. In 2003 it was shown, that under phosphate deprivation transient increase in PC content in *Arabidopsis* was followed by its rapid decline accompanied by DAG accumulation, suggesting NPC activation [95]. Later, characterized NPC4 gene was strongly expressed in *Arabidopsis* plants experiencing lack of phosphate [19]. NPC4 T-DNA insertion mutant lines in the similar conditions demonstrated a significant reduction in NPC activity. Based on these results, authors believe that PC hydrolysis by NPC4 plays an important role in securing supplies of inorganic phosphate and DAG from membrane-localized phospholipids. Liberation of phosphate would then serve in cellular metabolism, while DAG contributes to galactolipids synthesis, e.g. digalactosyldiacylglycerol (DGDG). However, galactolipid composition of the *NPC4* mutants was not changed which left the role of NPC4 questionable.

Thus, NPC5 also appears to be essential for normal growth and galactolipids accumulation during phosphate deficiency [78]. *NPC5* expression was increased and T-DNA mutant *npc5* had 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 [78]. Another study suggested specific activation of NPC5 gene in roots of *Arabidopsis* induced by P<sub>i</sub> deficiency [96]. Interestingly, while expression levels of NPC4/5 genes were significantly induced during phosphate starvation, recovery of phosphorous uptake resulted in rapid inhibition of their transcription [97]. In addition, activation of NPC activity during phosphorous-limiting conditions was impaired in mutants defect in IAA14 and ARF7/19 transcription factors, suggesting implication of auxin-mediated signalling pathways [98]. Besides, *npc3* knockout mutant demonstrated weak impairment of lateral root growth induced by phosphate deficiency [22]. It is important to notice that phospholipids conversion to phosphate-lacking lipids can be also accomplished by combined action of PLD and phosphatidate phosphatase (PAP) [99]. Thus, antibody raised against C-terminus of NPC4 interacted with a protein exhibiting PLD-like activity which was induced in phosphate-limited oat [87]. Nevertheless, plant NPCs are unquestionably appearing among key enzymes responsible for lipid remodelling during phosphorous-limited conditions.

##### 4.5.2. Role in abiotic stresses

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 membrane in *Dunaliella salina* following hypoosmotic shock [107]. It was additionally shown that expression level of *NPC4* was 12-fold increased in roots of *Arabidopsis* after 3 to 6 hours of 100 mM NaCl salt treatment. Concurrently authors 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 characterized by reduced germination rate when sown on media containing 150 mM NaCl [24]. In another study [25] *npc4* plants were also shown to have reduced overall viability under salt and drought stress conditions. Unlike, comparing to WT plants NPC4-overexpressing mutants were characterised by higher germination level and maintained higher root length and dry weight both under salt stress and hyperosmosis. Based on

experimental data, both groups of authors suggested that NPC4 participate in triggering of plant salt stress responses likely via ABA-dependent mechanisms. (for details see also chapter 4.5.4.).

Aluminium toxicity can cause rapid inhibition of root growth conditioned by depolarization of the plasma membranes [100-101], disruption of ion fluxes [102], calcium homeostasis [103] and by affecting the cytoskeleton [104]. Certain phospholipases and lipid intermediates are known to hold signalling or metabolic role during Al stress [105-106]. By studying the metabolic pathways involved in the formation and degradation of DAG it has been shown that decrease in DAG accumulation in Al-treated tobacco BY-2 cells is dependent on NPC activity [23]. Authors demonstrated that reduction in DAG production was not due to cell viability decrease 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.

In turn, expression level of *NPC3* was 14.6 fold increased after 2 h in *Arabidopsis* seedlings subjected to 37 °C heat stress [108], suggesting putative NPC implication to thermotolerance. From the other hand gene expression arrays also provided clues for implication of *NPC1*, *NPC3* and *NPC4(5)* to drought stress, heat stress, cold stress, hypoxia and osmotic stress responses – conditions that may require membrane readjustment and stress signalling performed by NPCs. *NPC1* additionally reacted to chemical-induced genotoxic and oxidative stress. Expression of some NPCs was also remodelled following changes in plants illumination regime indicating putative allocation to control of photosynthetic membranes. On the contrary, *NPC2* did not significantly react to abiotic stresses while expression of *NPC6* was inhibited during osmotic and cold stress conditions (*Arabidopsis* eFP Browser, <http://bar.utoronto.ca>; Geneinvestigator, [www.geneinvestigator.com](http://www.geneinvestigator.com)).

#### 4.5.3. Role in biotic stresses

An implication of phospholipases in plant defence reactions has been demonstrated repeatedly [109-110], giving evidence for a prominent role for lipid second messengers. The first evidence about the possible involvement of NPC in signal transduction during plant defence responses was provided by Scherer et al. [18] who studied the effect of glycoprotein elicitor from *Phytophthora sojae* and cryptogein elicitor isolated from *Phytophthora cryptogea* in cell lines of parsley (*Petroselinum crispum*) and tobacco (*Nicotiana benthamiana*), respectively. The addition of synthetic PC bearing fluorescent tags on both fatty acid residues (bis-BODIPY-PC) to the cells allowed demonstrating a rapid decline in fluorescently labelled DAG production, suggesting inhibition of NPC activity. The 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, specifying that a fluorescent DAG originated predominantly by direct action of NPC and not due to activation of PLD and PAP. On the other hand, expression gene homologous to *AtNPC5* was 4-fold increased in *Citrus sinensis* plant following the infection with *Candidatus Liberibacter asiaticus* [111].

Web available DNA microarrays data (*Arabidopsis* eFP Browser, <http://bar.utoronto.ca>; Geneinvestigator, [www.geneinvestigator.com](http://www.geneinvestigator.com)) demonstrated that NPC gene family, notably *NPC6*, was predominantly downregulated following inoculation with varied 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* demonstrated some positive responsiveness to *Botrytis cinera*, *Golovinomyces orontii*, *Pseudomonas syringae* and *Phytophthora infestans* treatment. In addition, *NPC1* and *NPC4* reacted to bacteria-derived elicitors, namely flg22 and HprZ, suggesting a probable bivalent functionality for NPCs during stress responses and perception of dissimilar elicitors. Interestingly, *NPC3* expression was elevated during *Bemisia tabaci* whitefly infection and after methyl jasmonate (MeJA) treatment in *Arabidopsis ler* and *penta* mutants providing possibility for *NPC3* implication to defense reactions against insect pests.

The putative role of NPC in defence responses may either be attributed to DAG production or to its conversion to PA via DGK or to other lipid second messengers. In support, synthetic DAG was able to activate defensive 6-methoxymellein phytoalexin production in carrot cells [112]. DAG, alongside with PA, were also implicated to control of reactive oxygen species (ROS) generation and induction of elicitor-responsive genes in rice cells [113]. On the other part, treatment with *N,N',N'',N'''*-tetraacetylchitotrehalose and flg22 elicitors promoted increase in PA accumulation in tomato cell culture that was not associated with PLD activation [114], giving evidence for DAG/DGK implication to specific plant defence reactions. DGK-assisted DAG transformation may supplement PA production that was shown to directly regulate defensive ROS generation by NADPH oxidase in *Arabidopsis* [115] and be an early step in plant response to wounding [116]. Contribution of DAG/DGK to PA production was also observed in reaction of suspension-cultured alfalfa cells to chitotetraose and xylanase elicitors [117], during early NO-dependent PA production in tomato cells elicited with xylanase [118] and throughout avirulent interaction of transformed tobacco cells with *Cladosporium fulvum* Avr gene product [119]. Moreover, expression of rice defence-responsive *OsBIDK1* DGK gene in tobacco has increased their resistance to tobacco mosaic virus and *Phytophthora parasitica* var. *nicotianae* [120], suggesting credible implication of DGK to functioning of lipid signalling pathways.

Thereby, putative inclusion of plant NPC, as well as other DAG production/conversion enzymes, to defence signalling in elicited cells and probable implication to other signalling pathways has been postulated. Moreover, it is becoming evident that NPC fall into the similar dualistic pattern common for other stress-responsive elements demonstrating repression or activation depending on stimulus nature and its spatial and temporal characteristics.

#### 4.5.4. Role in hormonal regulation

Functions of plant hormones are mediated by a number of signalling agents including phospholipases C and D. At the same time, direct implementation of novel NPC to hormonal regulation remains vague. It was shown that NPC activity was rapidly induced in BY-2 cell culture upon treatment with 24-epibrassinolide (BL) *in situ* [22]. Authors also found, that *npc3* and *npc4* knockout mutants of *Arabidopsis* demonstrated impaired sensitivity to BL and altered expression of *TCH4* and *LRX2* BL-responsive genes accompanied by reduced primary root length and lateral root number. Sensitivity to 1-NAA auxin was sustained in NPC-deficient plants, however expression of *IAA19* and *IAA20* genes implicated to auxin signalling was decreased in *npc3*. In addition, auxins, cytokinins and brassinosteroids affected expression level of *NPCs* assessed by semiquantitative PCR. Thus, *NPC4* transcript level was increased after 3 h of zeatin treatment and 24 h of brassinolide treatment. However, only minor changes in expression levels of other NPC genes were observed [22]. From the other hand, expression of *P<sub>NPC3</sub>:GUS* and *P<sub>NPC4</sub>:GUS* constructs after treatment with 1-NAA or brassinolide was intensified in root tips, cotyledons, leaf margins and floral organs - similarly to expression of synthetic *DR5:GUS* auxin reporter [121], suggesting functional connection of auxins, brassinosteroids and NPC important for root cells division and expansion. Expression pattern of *P<sub>NPC2</sub>:GUS* construct also correlated with auxin-rich root zones. However, *npc2* mutant was not aberrant in root formation density and length when treated with IAA [91].

Implication of NPC to ABA-mediated responses in *Arabidopsis* has also been disclosed now. It was shown that expression of *NPC4* was induced by ABA treatment, but not by salicylic acid (SA) or MeJA treatment [90]. 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 stomata movement [25]. Expression levels of ABA responsive genes (*ABI1*, *ABI2*, *RAB18*, *PP2CA*, *OST1*, *RD29B*, *ERA1* and *SOT12*) were also significantly altered in *npc4* mutants under salt stress and ABA-treatment [24-25]. *NPC4*-overexpressing *Arabidopsis* plants were demonstrating increased sensitivity to ABA inhibition of seed germination accompanied by induced salt and osmotic stress tolerance, giving strong evidence for implication of NPC to plant stress responses. Interestingly, *npc4* mutants accumulated lower DAG levels in leaves under normal growth conditions and under ABA-treatment though the composition of DAG molecular species, membrane phospholipids and galactolipids was unchanged. It was also shown that phenotype of ABA-insensitive *npc4* mutants was restored by application of both synthetic DAG and PA. Moreover, when DAG was applied together with diacylglycerol kinase inhibitor 1 in the presence of ABA, DAG was no longer able to fully restore the phenotype. These gives evidence not only for implication of NPC-derived DAG production to ABA signalling but point at importance of DAG/PA conversion. Based on all results in this study [25], authors proposed a working model for the function of *NPC4* and derived DAG in mediating *Arabidopsis* response to ABA. In normal conditions, *NPC4* contributes to the basal production of DAG that promotes stomatal opening. In stress condition, ABA level is increased which consequently induce *NPC4* to produce DAG. The stress-induced DAG is phosphorylated by DGK to PA that promotes ABA-promoted stomatal closure.

DNA microarray data (*Arabidopsis* eFP Browser <http://bar.utoronto.ca>; Genevestigator [www.genevestigator.com](http://www.genevestigator.com)) indicates that brassinolide evoke inhibition of *NPC1* and *NPC4* expression accompanied by activation of *NPC6*. ABA treatment resulted in somewhat reversed pattern with downregulation of *NPC6* expression and upregulation of *NPC3/NPC4* expression in *Arabidopsis*. In specific conditions *NPC4* expression was also positively responsive to ABA, MeJA, BL or SA treatment. *NPC1* and *NPC6* were additionally activated under influence of zeatin.

Thereby, growing evidence indicate NPC responsiveness to hormonal treatment. Localization of some NPC isoforms in plasma membrane gives chance for possible NPC role in early stages of hormonal perception. In addition, expression pattern of *NPC* matching sites of intensive growth processes and hormone accumulation suggests NPC participation in intracellular amplification of hormonal signal essential both for growth programme and stress reactions.

#### 4.6. Possible mode of action

NPC regulatory role in plants is acknowledged to be mediated via diacylglycerol production [124]. 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 [125-129]. In recent years, great progress has been made in the understanding of DAG metabolism in human cells 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 to disruption of organ development and cell growth [59]. It is currently thought that role of DAG in plant cells is restrained to participation in lipid turnover and endomembrane remodelling. However, emerging research data indicate a complementary implication of DAG to cell regulation and signalling.

##### 4.6.1. Lipid metabolism

In plants, NPC is responsible for production of both DAG and phosphocholine that may enter cell metabolic pathways. While known metabolic role of phosphocholine is rather trivial in plants, DAG is known as one of the key intermediates in generic lipid metabolism (Fig. 7). In plant cells DAG can arise either metabolically from glycerol-3-phosphate or through membrane polar lipid cleavage by lipase enzymes. 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. Depending on the origin and cellular localization, 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 precursor of glycerolipid metabolism. However, diverse cellular DAG pools also participate in 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 and sulfolipids (for review see [130-131]). DAG can be also two-step converted to cytidinediphosphate-diacylglycerol important for photosynthetic processes and biosynthesis of phosphatidylinositol, phosphatidylglycerol and cardiolipin [132]. In the capacity of DAG-derived products, galactolipids are important for maintaining of plastid thylakoid membranes composition and photosynthesis direction [133], while sulfoquinovosyl diacylglycerol sulfolipids are specifically implicated in photosystem II regulation and stabilization [134]. Monogalactosyldiacylglycerol also appears to be important during pollen germination and pollen tube growth and under phosphorous-limiting conditions [135].

There are direct evidences showing that NPC affect DAG production and balance of other lipids in plants. It was demonstrated that *npc4* mutants accumulate less DAG in leaves [25]. Production of DAG was also reduced in *npc4* mutant during phosphorous limiting conditions in arabidopsis [19] while such plants accumulated less MGDG and DGDG glycolipids and marginally more SQDG. However the overall profile of DAG molecular species and content of other lipids was not significantly changed in *npc4* under normal growth conditions [25]. *In vitro* activity of NPC5 was considerably smaller comparing to NPC4, despite NPC5 seem to be responsible for mainstream production of DGDG in leaves during phosphate deprivation [78] (for details see chapter 4.5.1). Galactolipid content and levels of main membrane phospholipids were not changed in *npc5* though some changes in fatty acid composition of galactolipids were observed [78].

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 utilizes DAG as a substrate demonstrated impaired thermotolerance [136], *Arabidopsis* plants lacking diacylglycerol acyltransferase showed increased sensitivity to ABA and osmotic stress during germination [137] and DAG-derived sulfoquinovosyl diacylglycerol lipids supposedly are important for plant salt stress acclimation [138]. DAG can also be converted to PA by DGK and participate in jasmonic acid biosynthesis via diacylglycerol lipase activity.

From the other hand, phosphocholine produced by NPC may give rise to free choline and potentially participate in biosynthesis of glycine betaine [139] and choline-*O*-sulfate osmoprotectants [140].

#### 4.6.2. Signal transduction

The signalling role of DAG is not obvious in plants and is now being discussed [141]. So far no DAG-responsive proteins have been identified in plant cells [142]. Munnik and Meijer [143-144] presented evidences that DAG as a product of PIP<sub>2</sub> hydrolysis is rapidly phosphorylated to PA, which plays active role in plant signalling processes. The question remains how important is the contribution of NPC to the formation of these signalling molecules alongside with metabolic DAG biosynthesis and DAG production via PI-PLC and PLD/PAP. It also appears that in some plant systems DAG is likely to acts as signalling molecule *per se*. Thus, synthetic DAGs were shown to excite ion fluxes in protoplasts of patch-clamped guard cell of *Vicia faba* and promote opening of intact stomata of *Commelina communis* that may point at their possible recruitment to ABA signalling [145]. Additionally, *Atlpp2-2* knockout mutant of *Arabidopsis* with depressed lipid phosphate phosphatase activity, responsible for DAG production via PA dephosphorylation, demonstrated impaired ABA sensitivity accompanied by excess PA accumulation [146]. However activity of DGK, responsible for reverse DAG→PA conversion, was also elevated under ABA treatment, suggesting an interconnection of lipid messengers. DAG was also able to mediate membrane polarization via endocytic recycling required for tobacco pollen tube polar growth [147] and hypothesized to be implicated to light irradiation perception important for circadian clock functioning and leaflet movements of *Samanea saman* plants [148]. Indirect evidence has also been given for involvement of DAG species in cell cycle progression regulation in stamen hair cells of *Tradescantia virginiana* [149]. Such data indicate that DAG may act as a signalling molecule in plant cells, but molecular nature of such signalling cascade is awaiting elucidation. Is it important to mention that PI-PLC-derived DAG and NPC-derived DAG are likely to be dissimilar in fatty acid composition and may have different roles in plant metabolism regulation [150].

Esters of phorbol – plant-derived tetracyclic diterpenoid compounds – are long known as DAG mimicking molecules that in animals are able to excite distinct intracellular responses [151]. Now, more than 30 different mammalian proteins including protein kinase C [152] and D [153] were shown to bind both DAG and phorbol esters.

Both, DAG and phorbol esters have shown also functional similarity in regulation of cell growth and differentiation, tumour progression etc. [154]. In plants, commonly used DAG substitute phorbol 12-myristate 13-acetate (PMA) was involved to ROS-dependent regulation of tobacco defence *hsr203J* defence gene expression [155], and intensified 6-methoxymellein phytoalexin production in carrot cells [112]. Additionally, PMA elicited transient activation of a 45-kDa protein with properties of wound-induced MAP kinase in tobacco cell suspension cultures [156] and both PMA and DAG in suspended *Rubus* cells affected activity of laminarinase – an enzymes which can lyse fungal cells and implicated to oligosaccharides production important for signalling during pathogenic invasion [157]. PMA also induced benzophenanthridine alkaloid accumulation in cell suspension cultures of *Sanguinaria canadensis* [158] and mediated hypersensitive response in lemon seedlings by inducing phenylalanine ammonia-lyase activity and synthesis of scoparone [159]. Sato et al. [160] have shown that *Arabidopsis thaliana* K<sup>+</sup> KAT1 channel implicated to stomata functioning was inhibited by PMA when expressed in frog oocytes. Thus, probable implication of PMA/DAG to biotic/abiotic stress responses either directly or through activation of plant protein kinases or PKC-analogous proteins can be postulated.

Indeed, PKC is the principal target of DAG regulation in animal cells [161]. PKC enzymes belong to a ubiquitous serine/threonine kinase family strongly activated by DAG molecules owing to C1 DAG/phospholipid-binding sites presence in N-terminal regulatory domain.

There are no direct evidences for existence of plant protein kinases that incorporate C1 domain. However, putative DAG-binding sites rich in cysteines and histidines were predicted in as many as 104 (Protein kinase C-like, phorbol ester/diacylglycerol binding domain - IPR002219), 224 (C1-like domain - IPR011424), 208 (DC1 – plant specific “divergent C1” domain - IPR004146) proteins of *Arabidopsis thaliana* (European Bioinformatics Institute, InterPro database, <http://www.ebi.ac.uk/interpro/>, Pokotylo, unpublished results). Among them - DGKs and proteins containing zinc finger and PHD finger (Plant Homeo 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 [162]. However, majority of the putative DAG-binding proteins remain to be characterised.

Apart from *Arabidopsis*, DAG-binding domains were also identified in stress-responsive NtDC1A and NtDC1B proteins from tobacco [163] and TaCHP protein from wheat [164]. DAG-binding sites were found in e.g. nucleotide sequences of histone methyltransferases from barley and *Physcomitrella patens* moss and in putative nucleoredoxin from *Ricinus communis* (European Bioinformatics Institute, European Nucleotide Archive, <http://www.ebi.ac.uk/ena/>, Pokotylo, unpublished results) giving evidence for putative implication to 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 desensitization, transcription as well as cellular cell proliferation and differentiation (for review see [161, 165]). At the same time, no PKC sequence homologues were detected in *Arabidopsis* or other plants, though number of identified plant kinases in their structure or biochemical properties show high resemblance to animal PKC. Cloned plant transcripts of bean PVPK-1 and rice G11A encoding protein kinases were characterized by relation to catalytic domain of PKC but had differences in regulatory domains structure [166] and phospholipid-activated protein kinase of *Amaranthus tricolor* cross-reacted with antiserum raised against regulatory sub-unit of PKC from bovine brain [167-168]. Similarly, PKC-like enzyme in *Marchantia polymorpha* thalli interacted with PKC-specific fluorescent tags and reacted to spermine treatment during programmed cell death [169]. Activity of PKC-type enzyme, involved in nitrate reductase gene expression in maize, was regulated by Ca<sup>2+</sup> and PMA, phospholipids or synthetic analogues of diacylglycerol [170]. Earlier, same authors have shown that PKC-like kinase activity in maize was stimulated by PMA in the presence of PS and calcium and that it was precipitated by animal PKC antibodies [171]. Purified protein kinase from *Brassica campestris* showed typical characteristics of the conventional type PKC while responding to Ca<sup>2+</sup>, diacylglycerol, phospholipids or phorbol esters [172] and PMA treatment stimulated PKC-like protein kinase in *Brassica juncea* [173]. The latter enzyme was purified to homogeneity and characterized to be calcium-dependent and oleylacetyl-glycerol- and PMA-activated. Calcium-dependent protein kinase from rice membranes was able to phosphorylate MARCKS peptide, a highly specific substrate for animal PKC [174]. In wheat cells, protein kinase activity was stimulated by concurrent treatment with phospholipids and PMA/synthetic DAGs [175]. In this regard, attention is drawn towards the disclosure of 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 [176]. Pea DNA topoisomerase I was activated after phosphorylation either by animal-derived PKC or endogenous plant PKC-like phorbol-ester bounding enzyme localized in nucleus [177]. Moreover, treatment with animal PKC *in vitro* was also demonstrated to promote activity of stress inducible pea DNA helicase 47 [178]. Treatment with NPC-15437 specific inhibitor of animal PKC repressed calcium-dependent intracellular responses induced by ergosterol and cryptogein elicitors in tobacco suspension cells [179]. PKC-like enzyme alongside with PI-PLC was also implicated to anthraquinone formation in *Rubia tinctorum* under chitosan treatment, while PMA alone was capable of mimicking the effects of chitosan elicitor [180].

Independently, regulatory role of NPC-derived DAG can be mediated through conversion to PA via DGK action. PA is currently known as an irreplaceable signalling agent in all living organisms while number of PA-binding regulatory

proteins/protein kinases are crucially involved to metabolism regulation in plants (for review see [124]). In turn, DGKs belong to a conserved lipid kinase family responsible for ATP-mediated DAG phosphorylation. DGKs are ubiquitously found in eukaryotes where role of DGK is attributed to attenuation of DAG levels as well as potentiation of PA molecules production thus interconnecting lipid metabolism with signalling [181]. DGKs identified in plant systems are thought to localize to cell membranes [182] and generally classified into three phylogenetic groups based on presence of functional domains [150, 183]. Interestingly, alternatively spliced variant of tomato LeCBDGK gene revealed a calmodulin-binding DGK [184]. Yet in general functions and properties of intracellular regulation of plant DGKs largely remain unclear. In animals, PIP<sub>2</sub> is considered to be precursor of DAG production implicated in DGK-assisted conversion and activation of PKC enzyme, so far elusive in plants. However, PIP<sub>2</sub> levels in plants are up to 100-fold lower than in mammalian cells [185]. Thus, DAG molecules implicated to DGK assisted turnover may arise from other sources including NPC mediated PC hydrolysis as well as phosphatidylinositol 4-phosphate cleavage by PI-PLC.

It is now established, that in plants DGKs may serve to provide an intracellular amplification of PA signalling either supplementing or substituting PA molecular species generated by PLD. In support, importance of DGK mediated conversion of DAG to PA in ABA signalling was clearly shown [25]. Also, DGK were shown to be responsible for PA production in cold stressed *Arabidopsis* cells [186]. Moreover, some data suggests that PA pool originating from DAG functions within initial signalling phase during biphasic PA accumulation in stimulus-triggered cells [118]. In accordance with animal systems, where DGKs often regarded as DAG signal modulator rather than mediators of PA signalling, DGK in plants may possess complementary intracellular functionality not attributed to PA formation while modulating DAG implication to both signalling and lipid biosynthesis. It was shown that overexpression of a rice DGK gene *OsBIDK1* activated under elicitor treatment enhanced disease resistance in transgenic tobacco [120]. And R59022 DGK inhibitor treatment increased phytoalexin accumulation induced by fungal elicitor in pea epicotyl tissues [187]. R59022 also reduced root elongation and depressed growth of *Arabidopsis* seedlings [129], suggesting DGKs implication to both stress responses and developmental processes in plants. Thereby, while both DAG, originated from PC cleavage by NPC, and PA are considered to be important lipid-derived second messengers, DGKs are appearing to be amidst the main regulatory elements providing coordination of their functions.

Plant NPCs may also affect cell functions via protein-protein interactions independently of their primary enzymatic activity. Despite insufficient data available, NPC3 was shown to interact with PIN4 auxin efflux carrier protein (<http://interactomics2.stanford.edu/>) implicated to auxin transport in developing roots [188] though significance of such interaction remains to be elucidated.

The particular signalling role of phosphocholine produced either metabolically or via PC cleavage is currently neglected in plants. Still, phosphocholine-moiety was shown to be important in for excitation of defense responses in cultured rice cells by glycosyl inositol-phosphoceramides from phytopathogenic fungi *Trichoderma viride* [189]. Also choline kinases were significantly activated in salt stressed [190] and, possibly, heat stressed *Arabidopsis* [191].

#### 4.6.3. Membrane remodelling

DAG species, being one of principal membrane constituents, were established to have a role in its biogenesis, remodelling and behaviour. Up to now, DAG was shown to have a major function as a multivalent modulator implicated to various membrane-related cellular activities such as vesicular transport, endocytosis, secretion etc. [58-59, 131, 192-193].

Plants are typically characterized by somewhat increased membrane DAG content comparing to the animals, despite its overall level remains low in the membranes. Depending on the stimulation membranes of animal cells contain approximately 0.2 to 1.4 nmol of DAG per 100 nmol of phospholipids (PL) [194-196]. At the same time measured DAG content in *Dunaliella salina* was 4.2 nM per 100 nM PL [107] and in *Samanea saman* varied from 3.5 nM/100 nM PL in unstimulated tissues to 4.2 nM/100 nM PL following exposure to light [148] that may suggest elevated importance for membrane stabilisation.

In animals, DAG levels directly affect cytoskeletal reorganization, membrane trafficking and exocytosis [142]. Local DAG accumulation can perturb phospholipid planar bilayers and promote the formation of non-lamellar membrane phases and influence membrane curvature. Szule et al. [197] have shown that dioleoylglycerol induced reverse hexagonal (H<sub>II</sub>) 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 in PC:PE:cholesterol mixtures [198]. Such transitions are essential for membrane fusion and fission processes. Thus, activity of bacterial PC-PLC facilitated fusion of PC/PS/cholesterol containing vesicles [199], while 1,2-isomers of diacylglycerol, but not 1,3-isomers, increased calcium-induced fusion of PS vesicles *in vitro* [200], indicating a possibility for NPC implication to various cell physiological activities including exocytosis, endocytosis, membrane build-up and cell division.

DAG produced by PI-PLC [147] and possibly by NPC [23] presumably undergoes endocytic recycling in membranes important for tobacco pollen tubes growth. The role of NPC and DAG in cell growth can be explained. Under physiological conditions pollen tube growth requires a transport of large quantities of material for the rapidly emerging

membrane and increased amount of DAG was observed. By contrast, in the presence of  $Al^{3+}$  level of 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 [59]. Interestingly, it was recently reviewed that DAG can modify activity of the very PLC enzymes that grant its production [201]. Asymmetrical DAG distribution may additionally facilitate formation of membrane domains and functional lipid rafts [192]. It is possible, that changes in DAG production upon stimulus treatment could represent a non-specific defence response against pathogens and cold stress conditions in the form of membrane fortification or rigidisation.

## 5. Conclusions and perspective

Here we present collective evidences for plant NPC enzymes as a novel agents implicated in control of cell physiological functions. NPC regulatory role implemented via DAG production affects metabolism regulation, biotic and abiotic stress responses and hormone sensing. Available data give support for NPC gene family inclusion to key agents implicated in cell signalling alongside with other plant phospholipase enzymes. Despite arising significance of DGK in cell signalling it appears that regulatory role of DAG produced by NPC may also be uncoupled from DGK and be accomplished via other putative DAG responsive elements in plant cell. However, further studies are required for elucidation of molecular properties of NPC action, NPC implication to other unidentified growth and developmental processes and NPC putative interaction with other signalling systems in plant cells.

Much progress has been made in general understanding of NPC functioning and wealth of information on the NPC gene family expression in different plant species is now available. Yet much remains to be elucidated on the nature of the extended NPC role in cell regulation and signalling. There is also a large void in understanding 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 a membrane-associated processes.

We also should not omit the possible future use of NPC as a target of genetically altered plants resistant to diseases or stresses. First hints have been already demonstrated for salt stress [25].

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## Figure legends

**Figure 1.** Phospholipase C and phospholipase D dependent signalling in plants. A schematic diagram depicts the contemporary model of metabolism regulation carried out by chosen phospholipases in plant cells. 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; Pchol, phosphocholine; DAG, diacylglycerol; PA, phosphatidic acid; DGPP, diacylglycerol pyrophosphate;  $IP_3/IP_6$ , inositol 1,4,5-trisphosphate/inositol hexakisphosphate; PKC, protein kinase C; DGK, diacylglycerol kinase; PAP, phosphatidic acid phosphatase; PAK, phosphatidic acid kinase; DGPPP, diacylglycerol pyrophosphate phosphatase; ROS, reactive oxygen species; PAB, PA-binding proteins.

**Figure 2.** Schematic alignment of Arabidopsis NPCs with known PC-PLCs from *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* [193]. The schema is based on an excerpt from large multiple alignment that contained 83 plant, bacterial, fungal and protist sequences and was constructed using program Mafft [194]. The presence of putative N-terminal signal peptide, phosphoesterase domain (Pfam entry PF04185), domain of unknown function 756 (Pfam entry PF05506), conserved regions, putative active site residues and conserved active site-stabilising ion pair is indicated. Various shades of blue indicate conserved aminoacid residues (light blue – less conserved, deep blue – most conserved). At, *Arabidopsis thaliana*; Pa, *Pseudomonas aeruginosa*, NPC, non-specific phospholipase C, plcN, non-haemolytic phospholipase C. See text for more details.

**Figure 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 program in L-INS-I mode [202] and then edited with Jalview program [203]. The conserved blocks were concatenated, giving a final matrix with 428 positions from 47 NPC-like sequences, which were then subjected to phylogenetic analysis.



Phylogenetic tree was constructed using maximum likelihood method from NPC amino acid sequences using PhyML program (WAG model+ $\Gamma$ +I) [204]. Numbers at nodes represent values of neighbor-joining bootstrap support obtained using aLRT (approximate likelihood-ratio test) method. The circles at the nodes represent 100% support and missing values indicate support below 75%. Branches were collapsed where the support was below 50%. Non-plant PC-PLC homologues were used as a root of phylogenetic tree. The scale bar represents 0.2 substitutions/site. Species abbreviations: At, *Arabidopsis thaliana*; Gm, *Glycine max*; Os, *Oryza sativa*; Pp, *Physcomitrella patens*; Ps, *Picea sitchensis*; Pt, *Populus trichocarpa*; Sb, *Sorghum bicolor*; Sm, *Selaginella moellendorffii*; Vv, *Vitis vinifera*; NPC, non-specific phospholipase C.

**Figure 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 the published structures of acid phosphatase from bacteria *Francisella tularensis* (PDB code 2DG1, [43]) and human estrone sulfatase (PDB code 1P49, [205]) using MODELLER 9v7 software [206]. 70 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 by 90° around y-axis. Side chains are shown for putative active site residues (orange) and structure-stabilizing ion pair (cyan). Electrostatic potential was mapped on the structure of AtNPC2 ranging from -5 (red) to +5 (blue) kBT/ec. See the main text for details.

**Figure 5.** NPC intracellular distribution pattern in *Arabidopsis thaliana*. Schematic representation of identified and putative sites of NPC expression in *Arabidopsis* cells are depicted by corresponding colour 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, 78, 88]. Expression patterns of AtNPC1, AtNPC2 and AtNPC6 are currently uncertain. However, signal peptide sequences identified in above mentioned NPCs and *in silico* sequence analysis have provided clues for AtNPC1 and AtNPC2 localization to endomembranes while AtNPC6 was predicted to localize to plastids and mitochondria. NPC, non-specific phospholipase C, \* Results are obtained experimentally.

**Figure 6.** Expression pattern of *Arabidopsis thaliana* 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 by GUS staining [22, 25, 90-91]. The expression levels of individual NPC genes are compared within whole plant and circle size represents relative gene abundance. However, combined quantity of transcripts of all NPC genes is not comparable in this figure. Analysis of *Arabidopsis* Affymetrix 22K array data for NPC expression in tissues of *Arabidopsis* (B) and throughout ontogeny (C) were performed via Genevestigator ([www.genevestigator.com](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.

**Figure 7.** Possible mode of NPC action in plant cells. Several putative regulatory pathways are shown for plant NPCs including cell signalling, implication to lipid metabolism and impact on cell membranes and membrane-associated processes. NPC can enzymatically give rise to both DAG and PChol. The cellular role of the latter is called into question. Dissimilarly, DAG has a number of potential ascribed functions in plant cells. DAG can affect cell signalling either directly by binding to proteins/PK or via conversion to PA. DAG as a substrate can provide means to various lipid species biosynthesis including those required for stress adaptation. Finally, as a highly hydrophobic molecule DAG can significantly affect properties of cell membranes as sites of crucial cell activities. Non-enzymatic functions of NPC are also considered and include putative protein-protein interactions that may affect activity of either target or both interaction proteins. DAG, diacylglycerol; DGK, diacylglycerol kinase; NPC, non-specific phospholipase C; PA, phosphatidic acid; PC, phosphatidylcholine; Pchol, phosphocholine; PK, protein kinase.

Fig. 1

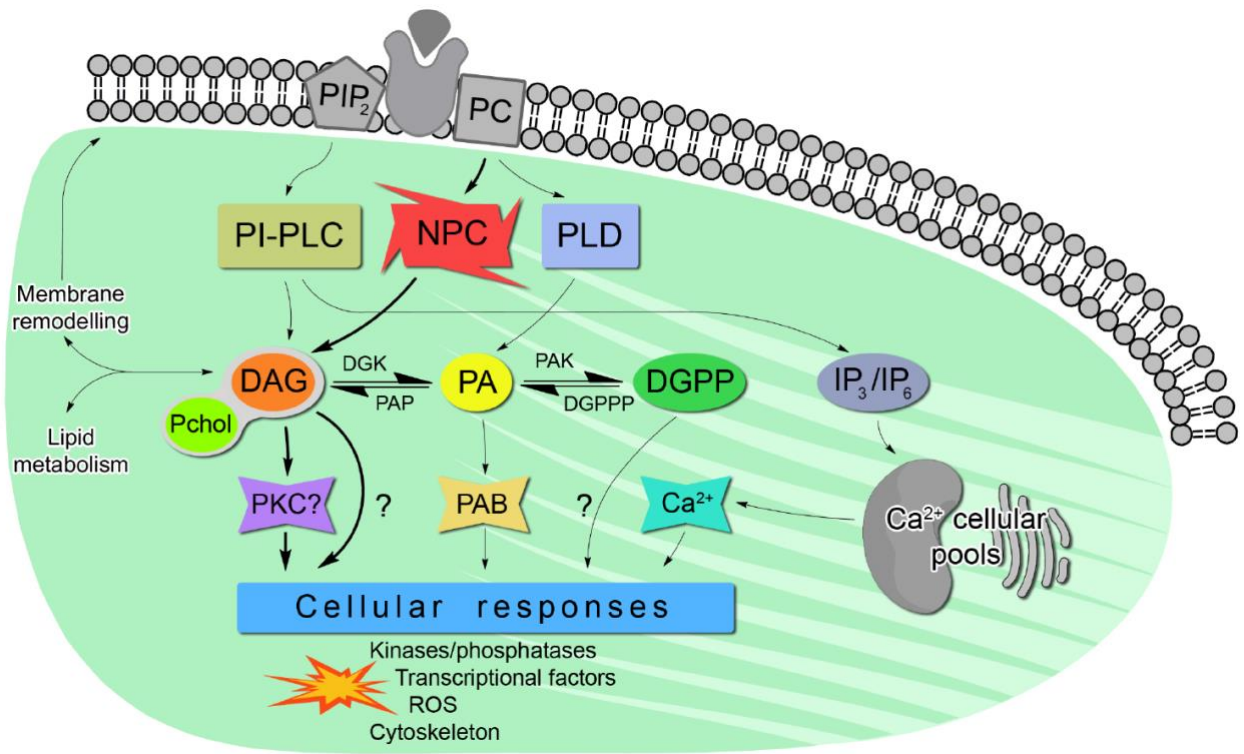


Fig. 2

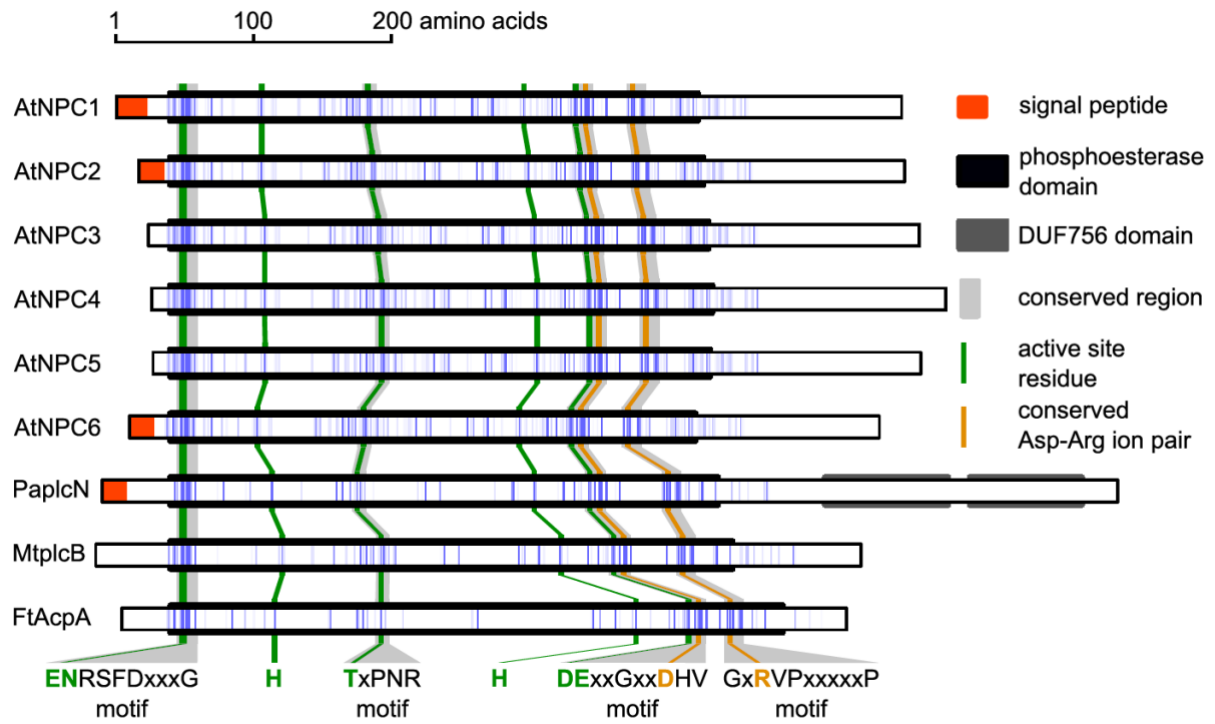


Fig. 3

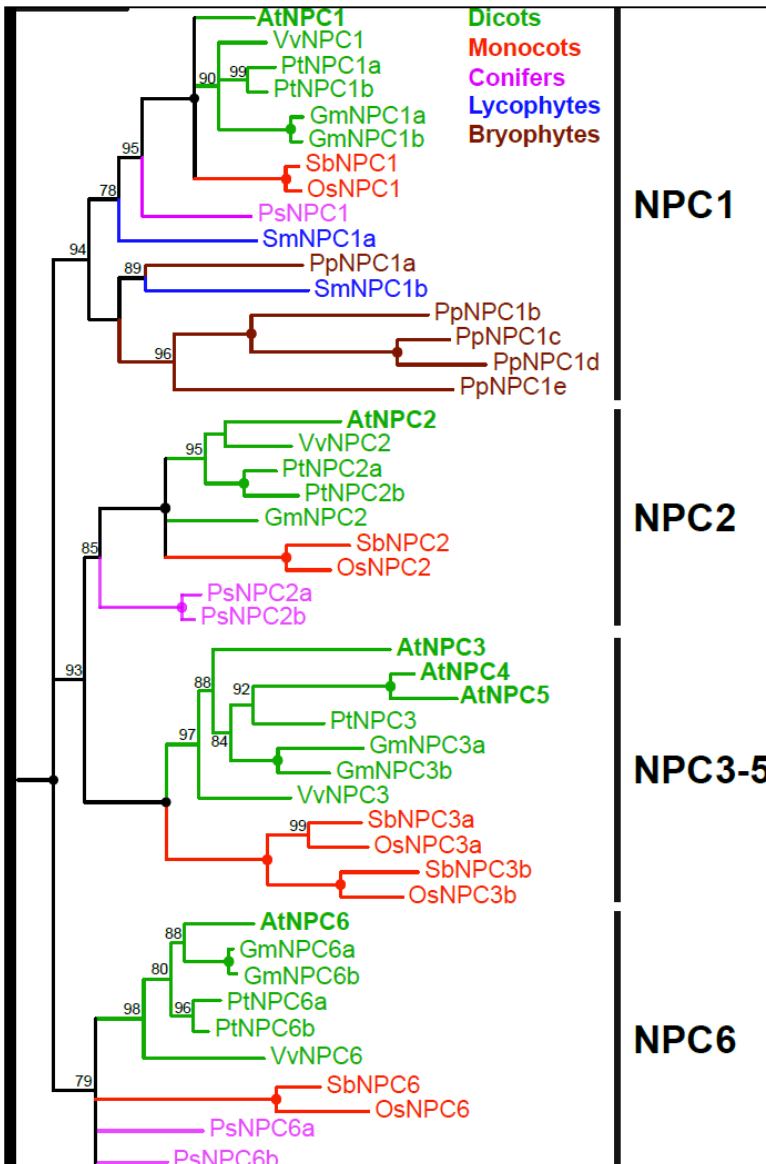


Fig. 4

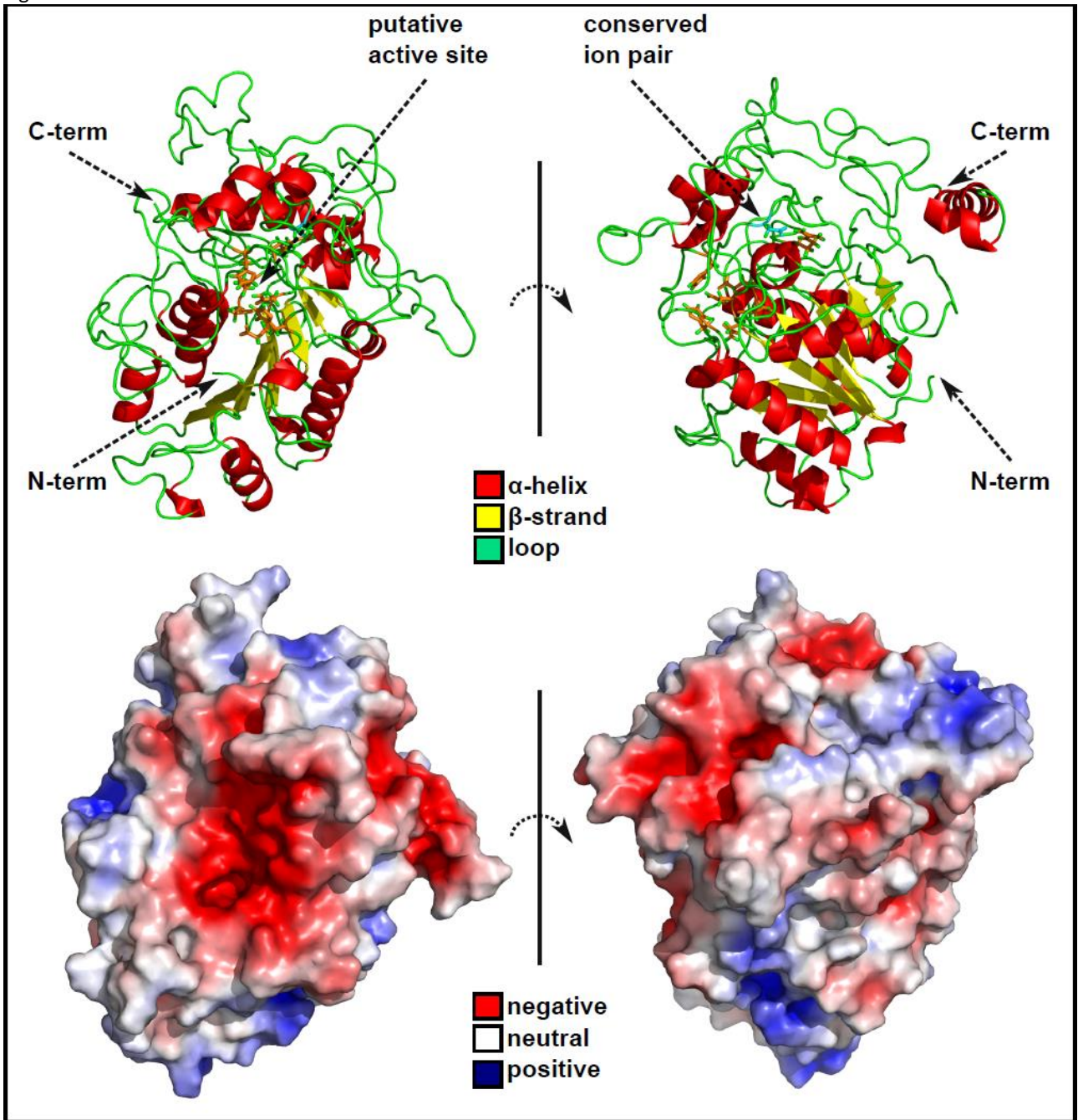


Fig. 5

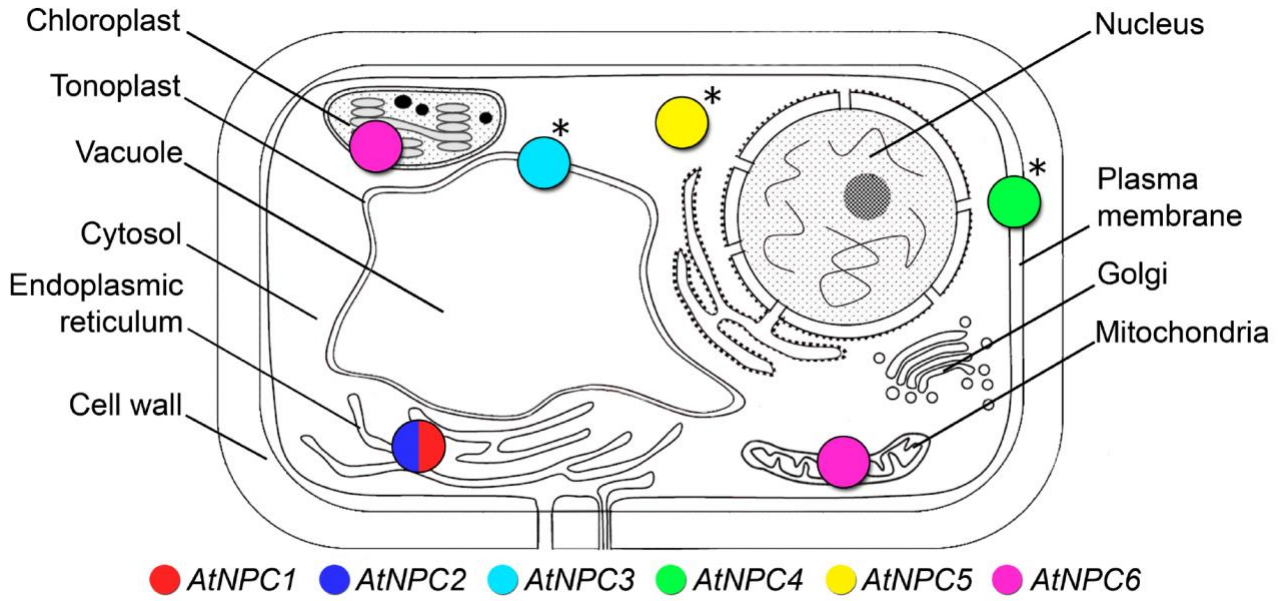


Fig. 6

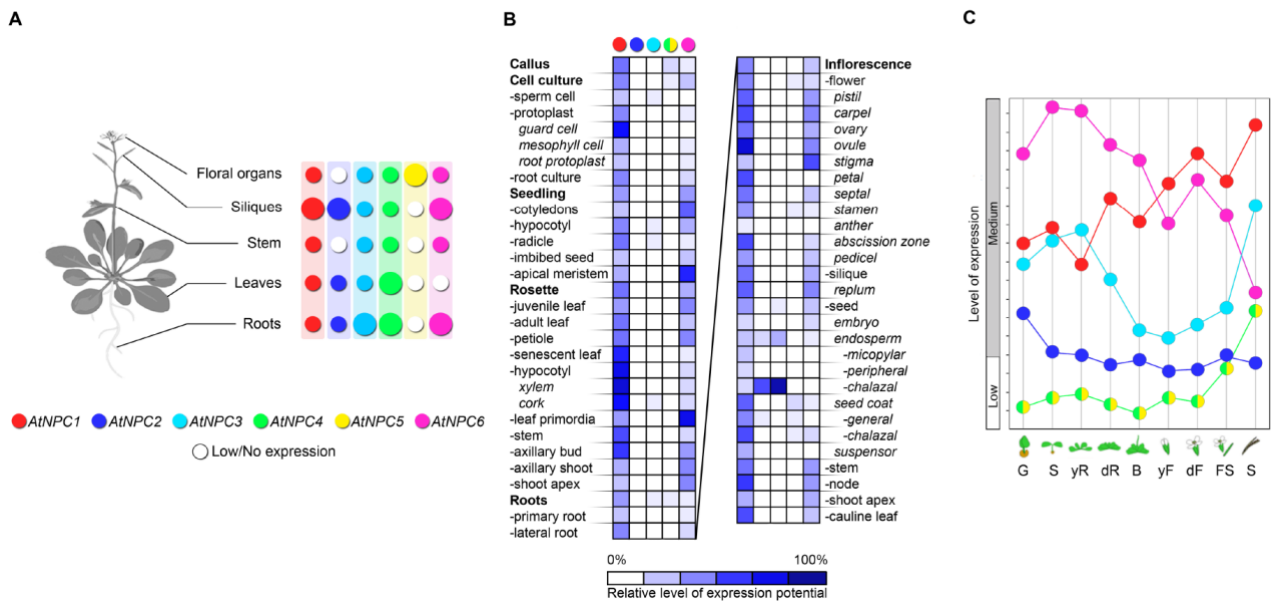


Fig. 7

