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Review

Phosphatidic acid signaling and function in nuclei

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ABSTRACT

Membrane lipidomes are dynamic and their changes generate lipid mediators affecting various biological processes. Phosphatidic acid (PA) has emerged as an important class of lipid mediators involved in a wide range of cellular and physiological responses in plants, animals, and microbes. The regulatory functions of PA have been studied primarily outside the nuclei, but an increasing number of recent studies indicates that some of the PA effects result from its action in nuclei. PA levels in nuclei are dynamic in response to stimuli. Changes in nuclear PA levels can result from activities of enzymes associated with nuclei and/or from movements of PA generated extranuclearly. PA has also been found to interact with proteins involved in nuclear functions, such as transcription factors and proteins undergoing nuclear translocation in response to stimuli. The nuclear action of PA affects various aspects of plant growth, development, and response to stress and environmental changes.

1. Introduction

Membrane lipids are not only structural backbones for cellular and intracellular compartmentalization, but also rich sources for generating cellular mediators in response to various stimuli. Phosphatidic acid (PA), which is a central intermediate in glycerolipid metabolism (Fig. 1), has emerged as an important class of lipid mediators in various cellular and physiological processes [1,2,3,4,5,6]. PA affects plant growth, development, reproduction, and responses to abiotic and biotic challenges (reviewed in [7,8,9,3,10,6]). In animal systems, PA is involved in multiple pathophysiological processes, such as inflammation, malignant transformation, neurodegenerative disorders, and infection (reviewed in [11,12,13]). In microbes, PA regulates various processes, such as mediating lipid metabolism and cellular response to nutrient availability [14,15,16].

The regulatory functions of PA and its mode of action have been studied primarily outside the nuclei. However, recent studies indicate that some of the PA effects are mediated through its action in nuclei [17,18,19,20,21,22,23]. Here, we will review evidence on the production and detection of nuclear PA, PA interaction with proteins that function in nuclei, and cellular and physiological processes affected by nuclear PA, as well as current knowledge gaps in the rapidly progressing research field.

2. Production of PA associated with nuclei

PA is a minor component of membrane lipids, with the simplest head group, phosphate without any modification (Fig. 1). The cellular level of PA is highly dynamic, changing rapidly and transiently in plant response to stress, such as wounding, stress hormones, dehydration, salt, cold/freezing, and pathogen attack [24,25,26,27,28,29]. The production and removal of PA are mediated by multiple enzymes (Fig. 1), some of which are associated with nuclei. In addition, PA produced by extranuclear enzymes can move to nuclei [22].

2.1. Cellular production of PA associated with nuclei

Phospholipase D (PLO) and diacylglycerol (DAG) kinase (DGK) are

Abbreviation: PA, phosphatidic acid; DAG, diacylglycerol; PLA, phospholipase A; PLD, phospholipase D; DGK, DAG kinase; PLC, phospholipase C; NPC, nonspecific phospholipase C; PI-PLC, phosphatidylinositol-specific PLC; TAG, triacylglycerol; PC, phosphatidylcholine; MGDG, monogalactosyldiacylglycerol; PG, phosphatidylglycerol; ABA, abscisic acid; GA, gibberellic acid; MAPK, mitogen-activated protein kinase; PAP, phosphatidic acid phosphatase; PKC, protein kinase C; LPP, lipid phosphate phosphatase; !AA, indole acetic acid; BL, brassinolide; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; PI(4)P, phosphatidylinositol-4-phosphate; PI, phosphatidylinositol; ABil, ABA insensitive 1; ABA2, ABA insensitive 2; GAPC, cytosolic glyceraldehyde-3-phosphate dehydrogenase; NADPH, nicotinamide adenine dinucleotide phosphate; SPHK, sphingosine ldnase; WER, werewolf..

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two major families of enzymes that produce signaling PA. PLD hydrolyzes membrane phospholipids, such as phosphatidylcholine (PC), to PA (Fig. 1). DGK produces PA by phosphorylating DAG that can be produced by phosphoinositide-specific phospholipase C (PI-PLC) or non-specific PLC (NPC) (Fig. 1). PA can be removed by PA phosphohydrolase (PAH), lipid phosphate phosphatase (LPP), phospholipase A (PLA), or PA kinase (PAK) (Fig. 1). Each of the PA-metabolizing enzyme families has multiple members and many of them within the same family have different biochemical and regulatory properties, temporal and spatial expression, and/or subcellular associations [7,8]. For example, the differences, such as Ca^2+ requirements, substrate preferences, and stimulus-induced activation of different PLDs (Table 1), can lead to specific temporal and spatial patterns of PA production, as well as molecular species of PA produced, underlying a basis for diverse cellular effects of PA.

Some of the PA-producing and removal enzymes are associated with nuclei. Early studies in animal systems indicate that PA-metabolizing enzymes are associated with the nucleus, and isolated nuclei can synthesize PA [38,39,40]. PAH is translocated between the endoplasmic reticulum (ER) and nucleus in yeast [41]. Among 12 PLDs in Arabidopsis, PLDy was associated with isolated nuclei, revealed by an antibody raised against PLDy [42]. Arabidopsis has three PLDys with high sequence similarity, and whether all PLDys are associated with nuclei remains to be determined. Arabidopsis has seven DGKs that are grouped into cluster I (DGKl and 2), II (DGK3, 4 and 7), and III (DGK5 and 6). A portion of cluster III DGK5 has been found to be associated with nuclei [43,44]. Moreover, disruption of *DGKS* decreased, whereas overexpressing it increased nuclear PA levels [44].

Since DGK in nuclei phosphorylates DAG to PA, the DAG-producing enzymes, including PI-PLC and NPC, can indirectly affect nuclear PA levels. PI-PLC3 in Arabidopsis is associated with both the nucleus and plasm membrane [45], and is involved in lateral root initiation and thermotolerance [46,47]. The total cellular PA was not changed *inplc3,* but the effect of PLC3 on nuclear PA levels remains to be determined [47]. Three of the six NPCs in Arabidopsis (NPCl, NPC2, and NPC5) are localized to ER (reviewed in [48]). Since the outer nuclear membrane is continuous with the ER membrane, the ER-associated PLCs could potentially affect the DAG levels in the outer nuclear membrane, but their effect on nuclear PA levels is not tested.

2.2. PA movement to nuclei

In addition to PA-metabolizing enzymes associated with nuclei, extranuclear PA has been reported to move into nuclei. In animal cells, PA outside the nucleus enters the nucleus via PA trafficking, while the precise mechanism remains to be elucidated [49]. In plants, PLD6 is involved in heat-induced PA accumulation associated with nuclei [22]. PLD6 is associated with the plasma membrane [50]. The heat-induced PA elevation in nuclei was diminished by knockout of ***PLDo*** and by the vesicle trafficking inhibitor brefeldin A (BFA), as shown using mass spectrometry (MS)-based analysis of nuclear PA and by live-cell imaging [22]. The heat-induced nuclear PA elevation that was blocked by BFA was also shown using a PA biosensor [51]. The results suggest the possibility that PA moves to the nucleus via vesicle trafficking under heat stress (Fig. 2). We propose a topologically possible model for the PA-GAPC co-movement through the outer and inner nuclear membranes without flipping of any lipids or proteins (Fig. 2), and such nuclear envelope trafficking has been proposed in other systems [52,53,54,55]. However, it remains unclear whether the transport is direct or via other subcellular compartments, such as Golgi complex or endoplasmic reticulum, and hence, the specific route by which PA and GAPC move into the nucleus requires further elucidation. In addition, other possibilities exist as described below.

The geometrical shape of PA renders it a high propensity to distort membranes. PA in membranes exists in a cone-like shape because it contains two bulky fatty acids and a relatively small head group [56]. This structure of PA decreases the packing stability of the lipid bilayer and induces negative (concave) curvature on the membrane, which could be involved in membrane budding and vesicle formation [57]. PA has been shown to interact with proteins in vesicle fission/fusion [58,59,60,61]. In addition, PA can transverse membrane lipid bilayer spontaneously or be assisted by proteins. PA has been found extracellularly in phloem associated with proteins as mobile signals in long-distance signaling [62]. Furthermore, the nuclear envelope is comprised of double membranes that are physically continuous with ER. ER is connected directly to the outer nuclear membrane (ONM), which is contiguous with the inner nuclear membrane (INM) via membranes surrounding nuclear pore complexes [63]. Thus, extranuclear PA can be associated with nuclei potentially via direct movement through continuous membrane connections, vesicular trafficking, and/or membrane contact sites. The complex PA behaviors may account for its diverse effects on vesicular trafficking and membrane fusion/fission

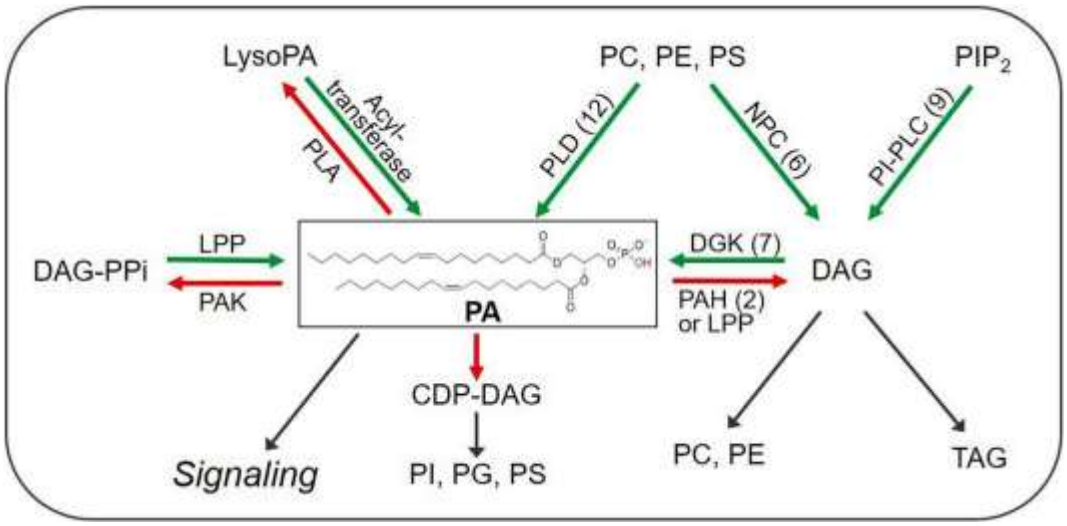

Fig. 1. Multiple enzymes that produce and remove PA. Green and red arrows indicate the production and removal of PA, respectively. Numbers in parenthesis indicate the number of genes in Arabidopsis. Please refer to the text for abbreviations of lipids and enzymes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Signature properties and functions of arabidopsis PLDs.

Group	Stimulation			Substrate preference	Subcellular pssociation	Mutant phenotypea	Referencea
	Ca^{2+}	PIP2	Oleate				
PLDal	mM	No	No	PC> PE	Cyto, Mic	Water loss	[30]
PLDa3	mM	No	No	PC< PE	Mic	Salt, drought	[31]
PLDpl	µM	Yes	No	PC PE	Mic	Pathogens	[32]
PLDyl	µM	Yes	No	PC< PE	**Mic, Nuc**	Al	[33]
PLDB	µM-mM	Yes	Yes	PC< PE	PM	Salt, temp	[34]; [22]
PLDe	µM-mM	Yes	No	PC< PE	PM,Mic	N deficit	[24]
PLDl;l	No	Yes	No	PC	**PM, Mic**	P, deficit	[35]
PLD1;2	No	Yes	No	PC	**Vacuole**	P, deficit	[36]; [37]

a Selected phenotypes and references only. **Mic, microsome;** Nuc, nucleus; PM, plasma membrane; Temp, temperature; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

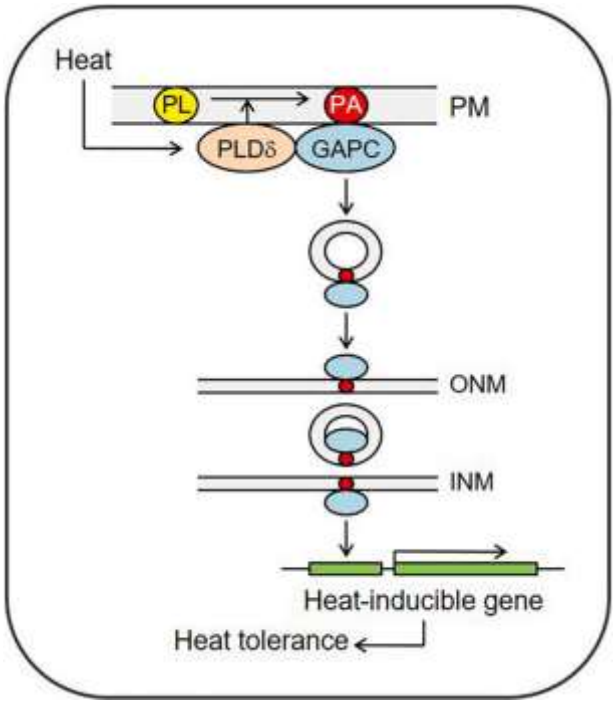

Fig. 2. A proposed model for PLD6 derived PA interacting with GAPC and PA-GAPC co-movement into nuclei under heat. In response to heat stress, PA produced by PLD5 activity promotes GAPC translocation into the nucleus via vesicle trafficking, where GAPC increases the expression of heat-inducible genes, rendering Arabidopsis tolerant to heat stress. For simplicity, membranes are depicted as monolayer and PA acyl chains in vesicle omitted, and outer nuclear membrane (ONM) undervalued. PL, phospholipid; !NM, inner nuclear membrane.

depending on cell types and conditions [64,65,66,67]. Thus, the specific route by which PA moves into nuclei could be specific to a given cue, which requires further elucidation.

2.3. Enzymes degrading PA

PA can be removed by various enzymes, such as dephosphorylation by PAHs and LPPs and deacylation by acyl-hydrolases and PLA. The PAH homologs in mammals and invertebrates have been found to be associated with ER and nuclear membranes and regulate gene expression [68]. Arabidopsis has two PAHs, and both are localized into the cytosol [69,70]. The double knockout of *PAHl* and *PAH2* caused overexpansion of the ER membrane [69]. One of nine LPPs in Arabidopsis, LPPa2, is localized to the ER membrane and regulates ER phospholipid biosynthesis [71]. PLA hydrolyzes the acyl group of phospholipids to produce lysophospholipid and free fatty acids. The secretory *PLA2-a* was found to move into the nucleus, but its activity toward PA is very low [72,73]. Several members of Arabidopsis PLAs can use PA as substrate and have also been reported to be localized to ER (reviewed in [74]). Since the outer nuclear membrane is continuous with the ER membrane, these ER-localized PA-hydrolyzing enzymes could potentially contribute to the regulation of PA levels in nuclei.

3_ Detection of PA associated with nuclei

The ability to detect and quantify PA in nuclei is crucial to determine its function in nuclei. Different approaches have been used to analyze PA, such as MS-based lipidomic profiling and PA biosensors, as well as traditional lipid separation and quantification. PA associated with nuclei has been measured using MS-based lipidomic profiling with the ability to quantify molecular species of PA and PA biosensors for live-cell monitoring of PA dynamics. In addition, fluorescently labeled lipids, including PA, have been applied to detect lipid distribution and dynamics in nuclei.

3.1. MS-based lipid profiling of nuclear fracti.on

MS-based lipid profiling has been used to quantify PA from isolated nuclei without apparent cytoplasmic contamination [17]. Lipids are extracted from the nuclear fraction and analyzed using electrospray ionization tandem MS (ESI-MS/MS, Fig. 3A). For example, a recent study measured PA levels in nuclei isolated from heat-treated WT, *pld/5,* and BFA-treated WT to determine whether heat stress induced nuclear PA accumulation [22]. Upon heat stress, total cellular PA levels increased in all the plants, with most PA molecular species being increased. However, heat-induced PA increases were only detected in WT, but not *inpld/5* or BFA-treated WT.

One precaution in nuclear fractionation is to prevent lipolytic activity during mechanical disruption of the cells. Major lipolytic activities can be inhibited by including in all buffers used for extraction and fractionation millimolar levels of Ca^{2+}-chelating EGTA that inhibit key lipolytic enzymes, such as PLD [17]. To test whether the lipolytic activity is inhibited, we compared the PA levels of nuclear fractions between WT and *pldalpld/5* treated without and with EGTA (Fig. 3B). PLDal and PLD6 are the two most active PLDs in Arabidopsis. The results indicate that including EGTA is required to minimize PA production during nuclear isolation (Fig. 3B).

3.2. PA biosensors detecti.ng PA associated with nuclei in-cell

To detect PA changes at subcellular levels, PA biosensors have been used, including membrane translocation-based single fluorescent PA biosensors and Forster resonance energy transfer (FRET)-based PA sensors. The translocation PA biosensors feature a PA-binding domain (PABD) attached to a fluorescent protein, such as GFP [75,76,77,51,64,78]. The GFP-tagged PABD from the N-terminal region of Arabidopsis respiratory burst oxidase homolog D (GFP-Nl60Rboho)

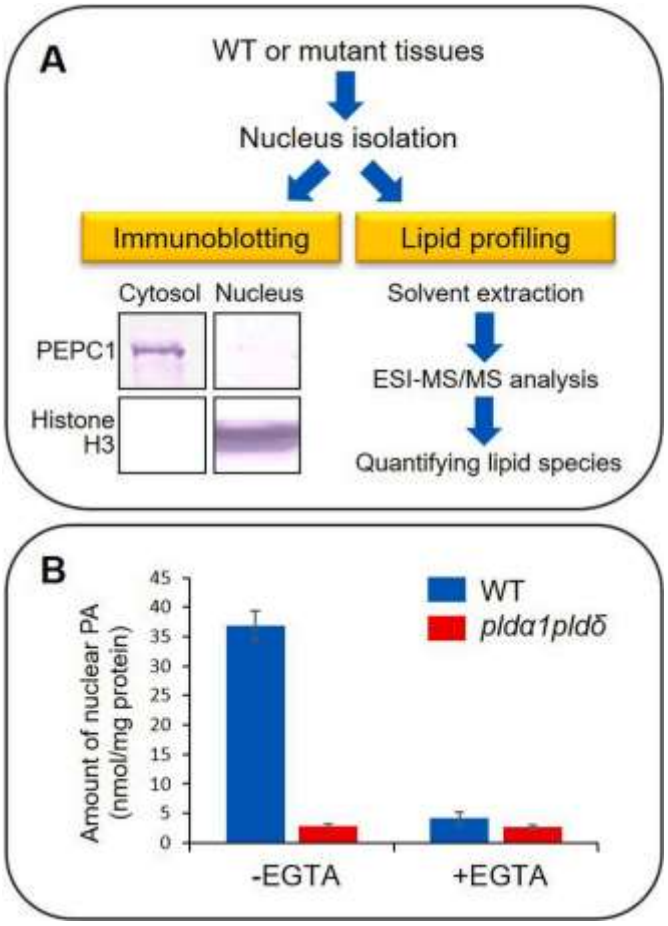

Fig. 3. Quantification of nuclear PA. A. A workflow for nuclear isolation and lipid analysis. Isolated nuclei were verified by immunoblotting Histone H3 as a nuclear marker and phosphoenolpyruvate carboxylase (PEPC) as a cytosolic marker. Lipids from nuclei were analyzed using ESI-MS/MS. **B.** Suppression of PA formation by EGTA during nuclear isolation. Nuclei were isolated from 10-day-old Arabidopsis seedlings by Percoll gradient centrifugation with or without 50 mM EGTA. PA was quantified by ESI-MS/MS and shown as per total nuclear proteins.

successfully detected salt-induced PA increase at the plasma membrane (PM) of root cells, while the PA binding-abolished mutant GFP-N160MRbohD failed to detect the PA increase at PM [51]. Moreover, heat stress induced the translocation of GFP-Nl60MRbohD from PM to nuclei, which was impaired by suppressing PLO- and DGK-mediated PA production [51].

In FRET-based PA probes, the emission spectrum of a donor fluorophore overlaps with the excitation spectrum of an acceptor fluorophore [79,80). When the donor gets close to the acceptor, the emission of the acceptor can be detected under the excitation wavelength of the donor. FRET detection is based on ratio imaging, thus having advantages over single-intensity probes because the effects of probe concentration or photobleaching cancel out when two images are divided to yield a ratio. FRET-based PA biosensors have been used to monitor PA changes in plant cells. The construct features the PABD from NADPH oxidase that was fused between cyan (CFP) and yellow (YFP) fluorescent proteins through rigid a-helical linkers consisting of repeated EAAAR sequences [81]. Also, FRET sensors can be targeted to specific subcellular membranes to measure local changes in PA, such as using the non-raft plasma membrane-targeted domain of K-Ras4B [80]. The FRET PA biosensor has detected in real-time PA increases in the PM induced by salt and abscisic acid (ABA) [81].

3.3. Detection of nuclear PA using fluorescent lipids and FRET

The application of fluorescent lipids has greatly facilitated the study of lipid distribution and dynamics, including PA in nuclei [49,22,82]. When nitrobenzoxadiazole (NBD)-labeled lipids were infiltrated into Arabidopsis seedlings, NBD-PA bound to proteins, which were pulled down by immunoprecipitation, and this helped to verify PA interactions with transcription factors [21). To provide spatial information about the interactions, FRET between a CFP-tagged protein and an NBD-labeled lipid has been used to detect lipid-protein interaction *in vivo* at a subcellular level, including nuclei, as the excitation spectrum of NBD (488 nm) overlaps with the emission spectrum of CFP ([22]; Fig. 4A).

In addition, the fluorophore BODIPY (TopFluor)-labeled lipids offer some advantages over the NBD-lipids because TopFluor ()1.Max *exl"-Max* em = 505 nm/513 nm) is more photostable and hydrophobic [84,85]. TopFluor has low excitation at 405 nm, so it could be a better FRET acceptor than NBD to be paired with CFP (Fig. 4A). The emission spectrum of TopFluor tetramethylrhodamine (TMR)-PA excited at 561 nm shows less overlap with the emission of CFP excited at 406 nm compared with that of NBD excited at 488 nm [83]. TopFluor TMR-PA and CFP-tagged cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPC) have been used to document PA-GAPC interaction *in planta* (Fig. 4B; [83]). Without stress, GAPC mainly interacts with PA in the cytosol (Fig. 4B), whereas some GAPC-PA co-move into nuclei under heat stress [22). In addition, recent developments in using click-chemistry to control and detect PA production in mammalian cells [86] are promising to be adapted to visualize nuclear PA and investigate nuclear PA functions. It is worth noting some limitations of the above methods. The fluorophores on PA may hinder the binding of PA to its target protein. The FRET-based PA probes may not detect PA that would be partially embedded in a protein. Hence, a lack of FRET signals does not necessarily mean an absence of PA-protein interaction.

4. PA interactions with proteins that function in nuclei

PA binding to proteins is one of the modes of PA's cellular actions and the identification of PA-interacting proteins and subsequent ana lyses have shed light on how PA acts as cellular mediators (Fig. 5; [9]). Physiochemical properties of PA, such as pH-dependent dual deprotonation and the ability to act as an electrostatic/hydrogen-bond switch, make PA a unique class of lipids in interacting with proteins [87). The study of PA-interacting proteins has led to the identification of several PA-binding proteins in nuclei [17,21].

4.1. PA binding to transcription regulators

An earlier study identified WEREWOLF (WER) as PA binding protein that functions in nuclei. WER is a MYB transcription factor that regulates cell differentiation, such as root hair patterning, and the PA-WER interaction affects the WER's nuclear localization and root hair development [88]. To explore lipid interactions with transcription factors, an Arabidopsis transcription factor library was screened for lipid binding [21]. This led to the finding that PA binds to two closely related MYB transcription factors, CIRCADIAN CLOCK ASSOCIATED (CCAl) and LATE ELONGATED HYPOCOTYL (LHY), which are core regulators of the circadian clock in Arabidopsis [21]. In addition, PA was found to bind the AT-hook motif-containing nuclear localized (AHL) protein, AHL4. Further analysis revealed that the PA-AHL4 interaction modulates transcriptional regulation of triacylglycerol (TAG) degradation and seed germination [17].

The PA interactions with LHY/CCAl and AHL4 were shown with filter binding, liposomal binding, surface plasmon resonance (SPR), and co-immunoprecipitation [17,21]. The binding specificity has been tested against other phospholipids such as PC, phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylserine (PS), and moreover, different PA molecular species. For example, LHY binds to PA-

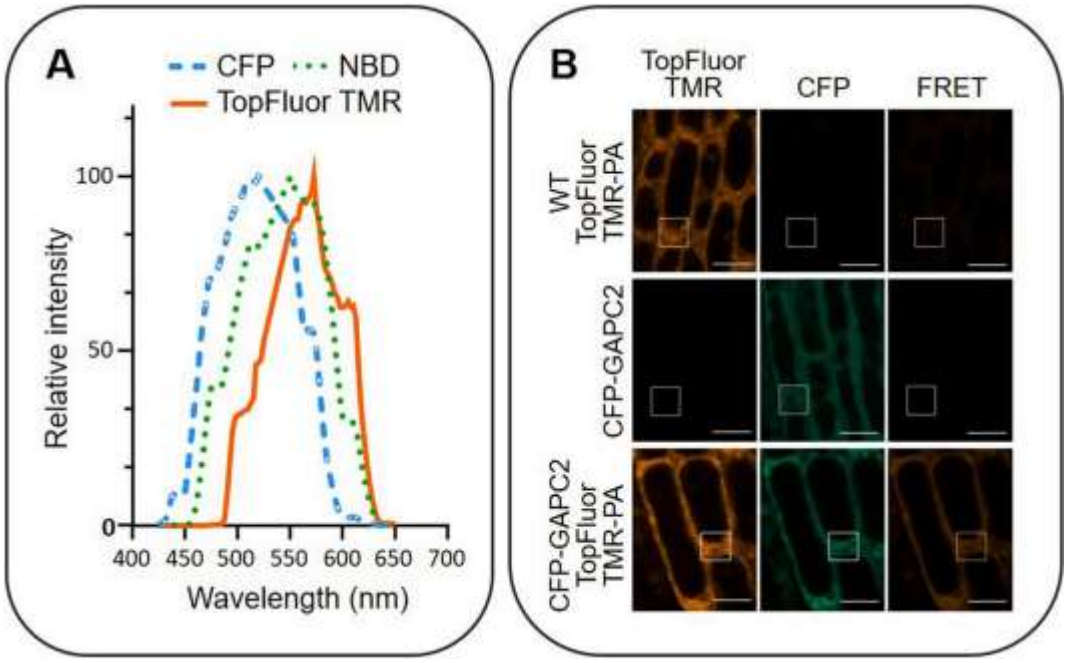

Fig. 4. Spectra of CFP, NBD, and TopFluor TMR fluorophores and FRET between CFP-GAPC and TopFluor TMR-PA in Arabidopsis roots. A. Spectra in the regions of interest for CFP (dashed line), NBD (dotted line), and TopFluor TMR (solid line). **B.** Images of WT root cells with 10 μM TopFluor TMR-PA and CFP-GAPC2-expressing root cells stained without or with 10 μM TopFluor TMR-PA. Scale bar= 10 μm. The regions of interest, such as the boxed or elsewhere, could be used to calculate FRET efficiency as described in Yao and Wang [83].

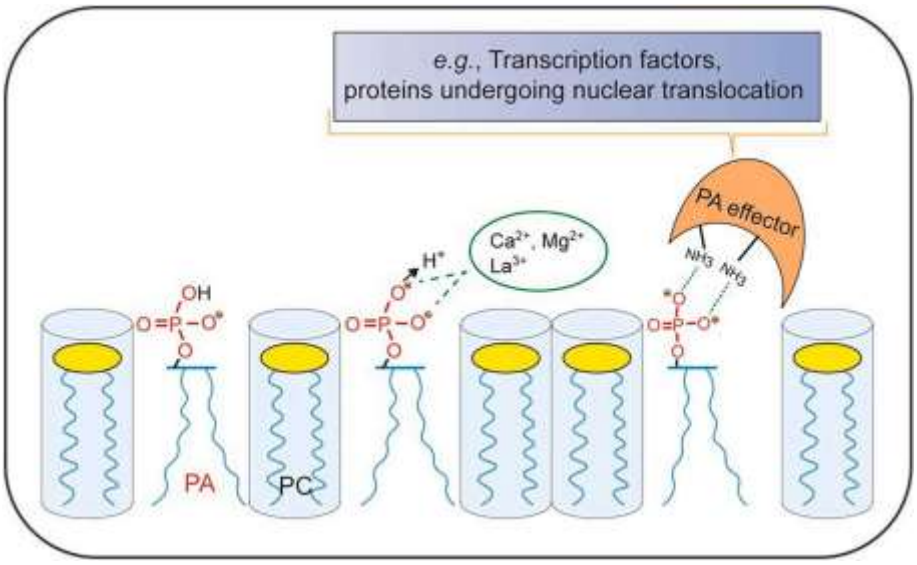

Fig. 5. Interaction of PA **with** proteins that act in nuclei. *Lower,* PA has pH-dependent dual protonation and hydrogen bonding to effector proteins that distinguish it from other lipids. *Upper,* Examples of PA-binding proteins with nuclear functions.

containing, but not PC-only, liposomes, and it binds to specific PA species (e.g., di16:0-PA) but not di18:l-PA (Fig. 6). In addition, LHY and PA were co-immunoprecipitated from plant extracts. Furthermore, fluorescence-labeled PA in plants was co-immunoprecipitated with PA-binding LHY/CCAl, but not with a non-PA binding transcription factor [21].

4.2. PA binding to proteins that translocate into nuclei in response to stimuli

PA was shown to interact with GAPCs in Arabidopsis, a cytosolic glycolytic enzyme [89,90]. A portion of GAPC molecules undergoes nuclear translocation from the cytoplasm in response to various stressors, including heat [91,92,93]. A recent study showed that PA is involved in the heat-induced nuclear localization of GAPC [22]. The loss of PLD5 activity in Arabidopsis abolished nuclear PA accumulation and nuclear translocation of GAPC, both induced by heat, suggesting that PLD5 mediates the heat-induced PA accumulation in nuclei and the nuclear localization of GAPC [22].

Several other proteins that interact with PA also undergo nuclear translocation. GIBBERELLIN (GA)-INSENSITIVE DWARFl (G!Dl) is a soluble GA receptor that moves into nuclei in response to GA. PA binds to G!Dl in rice, and the mutations of arginines 79 and 82 of G!Dl that are required for PA binding abolish its nuclear localization [18]. A

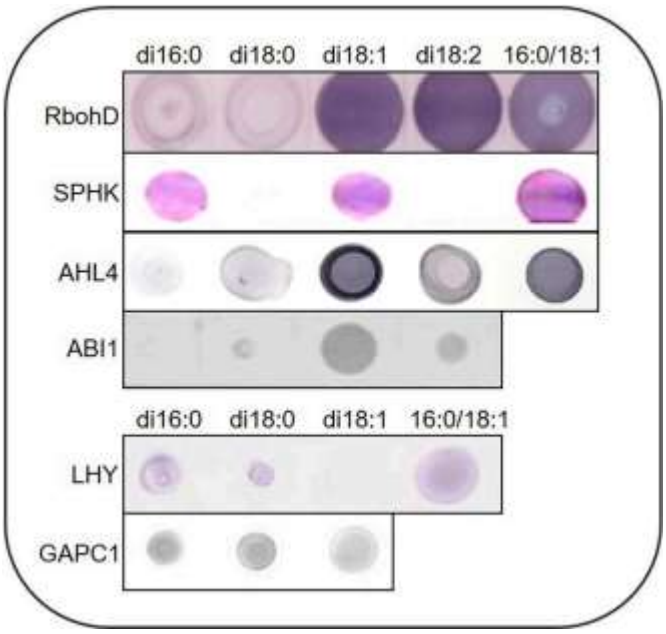

Fig. 6. Protein binding with different PA species. Lipid immunoblotting of different PA species with various PA-binding proteins.

recent study shows that PA binds ABA DEFICIENT 2 (ABA2) and suppresses its enzymatic activity. ABA2 was detected in and outside nuclei, and the loss of the nuclear *DGKS* decreased the nuclear association of ABA2. Those results indicate that DGK5 and PA interact with ABA2 and suppress ABA production [44]. In addition, the scaffolding Al subunit of protein phosphatase 2A (PP2AA1) mediates the dephosphorylation of auxin transporter PIN-FORMED 1 (PINI) and regulates the distribution of auxin. PA binds to PP2AA1 in Arabidopsis, and exogenous PA induces the accumulation of PP2AA1 on the membrane. The inhibition of PLO-mediated PA production by 1-butanol causes the perinuclear aggregation of PP2AA1 [94].

A recent study indicates that PA promotes the nuclear translocation of the calcium-dependent protein kinase (CDPKs /CPK) CPK12 in Arabidopsis upon low oxygen (hypoxia) stress [19]. CPKs play important roles in plant development and stress response by sensing the calcium signals induced by developmental and environmental stimuli and phosphorylating different substrate proteins [95]. CPK12 is activated during hypoxia stress and translocated from the cytoplasm to the nucleus, where it interacts and stabilizes the core regulator of hypoxia sensing group VII ethylene-responsive transcription factors (ERF-VII). PA binds to CPK12, and the application of PA promotes the nuclear translocation of CPK12, whereas the inhibition of PLO-mediated PA production abolishes it upon hypoxia stress [19].

4.3. PA molecular species display binding selectivity for protein interactions

PA can exist as various molecular species due to the number of carbons and double bonds of two fatty acyl chains. PA-binding proteins exhibit varied binding preferences to different PA molecular species [96,89,88,28]. For example, ABSCISIC ACID INSENSITIVE 1 (ABil), a protein phosphatase 2C that negatively regulates plant response to the stress hormone ABA, displayed binding to dil8:l-PA but not to dil6:0-PA (Fig. 6; [28]). This binding property is in stark contrast with LHY that binds 16:0-containing PA species (e.g., dil6:0 and 16:0/18:1-PA) but not dil8:l-PA ([21]; Fig. 6). Another PA-binding transcription factor AHL4 binds PA containing unsaturated fatty acids (e.g. 16:0/18:1 and di18:l-PA), but not PA containing two saturated fatty acids (e.g., di16:0 and di18:0-PA; [17]). By comparison, sphingosine kinases bind to 16:0/18:1-PA and di18:l-PA equally well, but not di18:0-PA or dil8:2-PA ([96]; Fig. 6), whereas GAPCs displayed no specific preference for PA species tested [89]. The binding specificity has been verified using liposomal binding and, in some cases, with SPR [96,89]. In addition, LHY immunoprecipitated from plants has 16C-containing PA associated [21].

Lipidomic analyses revealed selective changes of PA species abundance as affected by genetic alterations of lipid metabolic enzymes and stress conditions. For example, among 23 PA species measured, the level of 34:3-PA was decreased by disruption of *pPLAIIIβ*, whereas the levels of 34:3-PA and 7 major PA species, such as 34:1, 34:2, 36:2, 36:3, 36:4, 36:5 and 36:6-PA, were increased by overexpression *ofpPLAIII/J* [97]. A recent study reported that the nuclear levels of 34:2-, 34:3-, and 36:6-PA were decreased in *DGKS-KO,* while 34:1, 34:2, 36:2, 36:3, and 36:4-PA were increased in *DGKS-OE,* compared to WT, with the levels of other PA species comparable to those of WT [44]. NaCl stress decreased the nuclear levels of 34:3, 36:2, 36:4, and 36:5-PA in *DGKS-KO* and increased those of 34:2-, 34:3-, and 36:4-PA in *DGKS-OE,* with the nuclear levels of other PA species in the DGKS-altered plants being comparable to those in WT [44]. Heat stress increased the levels of 34:2, 34:3, 36:3, and 36:4-PA in nuclei while increasing levels of most species of total cellular PA [22]. The contents of PA species vary depending on photoperiod and circadian conditions as well. 34:2, 34:3, 34:6, 36:4, 36:5 and 36:6-PA highly accumulated at dawn when compared to dusk, and 34:4 and 36:6-PA levels oscillated diurnally [21,98].

Such specific interactions and their biological significances are supported by structural analysis. For example, the hydrophobic residues at the C-terminus of the Arabidopsis actin capping protein (AtCP) are inserted into the lipid bilayer containing PA, and this interaction is likely to be regulated by the length of the PA acyl chains [99,100]. Changes in PA levels in specific membranes can directly affect the PA-targeted proteins, their localization, activity, and biological functions. These results suggest that the acyl chain composition of PA is an important determinant of PA interactions with specific target proteins.

5. PA effects on nuclear and physiological processes

The involvement of PA in nuclear functions is currently supported by two categories of evidence. One is the interaction of PA with proteins that function in nuclei, such as transcription factors. The other is the impact of the genes and enzymes that affect nuclear PA production and cellular processes. Manipulations of the nuclear PA-producing reactions shed light on the role of nuclear PA in specific cellular and physiological processes.

5.1. Lipid modulation of circadian clock function

The finding that PA binding to the core regulators of the circadian clock LHY/CCAl provided valuable insights into the interconnection between lipid metabolism and molecular clock functioning (Fig. 7). LHY and CCAl, together with *TIMING OF CAB EXPRESSION] (TOCl),* constitute a central loop in the molecular clock of Arabidopsis. LHY and CCAl accumulate in the morning and suppress *TOCl* expression by binding to its promoter, while TOCl, in turn, represses *LHY/CCAl* expression in the evening [101,102,103]. Increasing levels of 16C-PA species that bind to LHY impeded the LHY binding to *TOClpro,* interfering with LHY's association with target gene promoters [21]. The effect of the PA-LHY/CCAl interaction on the circadian clock is supported by the finding that perturbations of PA metabolism alter clock function (Fig. 7A, B). The *pahlpah2* that had elevated levels of PA exhibited longer periods in *TOCl* expression and leaf movements than WT (Fig. 7B). Conversely, when the PA production by PLO or DGK was suppressed, the oscillation of leaf movement was approximately one hour shorter than solvent controls [21]. The opposing changes in circadian period length from the increased and decreased PA levels suggest that altered PA metabolism affects the PA-LHY/CCAl

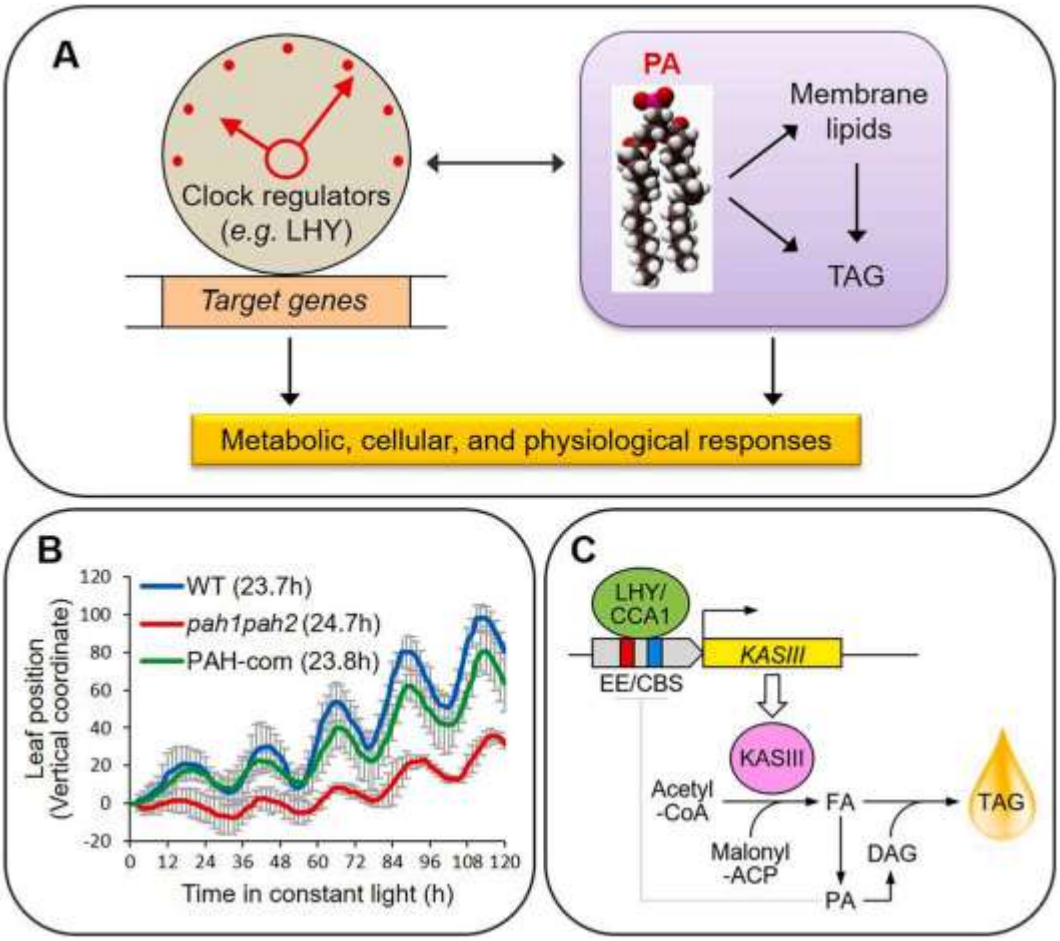

Fig. 7. Interconnection between the circadian clock and lipid metabolism, A. Reciprocal regulation between LHY/CCAl and PA, B. Altered PA levels affect clock outputs. Increased PA by loss of *PAHl andPAH2 (pahlpah2)* lengthens the oscillation of vertical leaf movement. Plants were entrained to 12-h light/12-h dark cycle for 5 days, and leaf movement was monitored under constant light. Values are means± S.D. *(n* = 12) normalized to the initial leaf position. The period length is in parenthesis. PAH-com, *pahlpah2* complemented with *PAHl.* C. LHY/CCAl binds the evening element (EE) and/or CBS of *KASIII* and promotes its expression that mediates the first condensation reaction of fatty acid biosynthesis. PA inhibits LHY/CCAl binding to *KASIII* promoter, suppressing LHY/CCAl's promotion.

interaction and impacts clock function [21].

Studies in animal systems also indicate an interplay between lipids and the circadian clock. Clock misalignments, such as shift work, chronic jet lag, sleep deprivation, and clock gene mutations, are associated with various lipid metabolic diseases, such as obesity and nonalcoholic fatty liver disease [104,105,106]. The core clock regulators BMALl and CLOCK play an important role in the modulation of fat storage, utilization, and adipocyte differentiation [107]. C16:0, a common fatty acid in the human diet, alters the expression of clock-regulated genes and disrupts circadian rhythms [108], and high-fat diets impaire BMALl recruitment to target chromatin sites and rhythmic expression of clock genes [109,110,111,112,113]. The effect of a high-fat diet on circadian gene expression is mediated in part through PPARy effect on BMAL/CLOCK [110]. PPARs bind to CRYl/2 [114], and PPARa binds to PER2 and modulates the circadian expression of *BMALl* [115,116], which is also regulated by PPARy [117]. PPARs' role in circadian regulation is through interaction with lipids because fatty acids, eicosanoids, and PA are ligands for PPAR activity. PA binds to PPARa and reduces its ability to bind the promoter of epidermal growth factor receptor (EGFR), repressing the *EGFR* expression in cancer cells [118]. In addition, the level of an 18:0-containing PA species oscillates in the nucleus in mouse liver cells [119]. Suppression of PA production inhibited the rhythmic expression of *BMALl* in human cells, and the effect was associated with its attenuation of the mTOR pathway [120]. These results indicate that PA may directly and/or indirectly regulate clock functions in mammals.

5.2. *Transcriptional regulation of lipid synthesis and seed oil production*

Genetic alterations of the clock transcriptional regulators LHY/CCAl affect lipid metabolism and accumulation [121,21]. The levels of specific species, including 16C-containing PA, display cycling with a period of -24-h in WT, but not in *lhy ccal* [21]. Similar lipid oscillation was observed previously, but whether it was due to die! cycles in the environment or under circadian regulation was unclear [98]. The analysis with *lhy ccal* mutants suggests that the lipid level changes observed in WT are influenced by the circadian clock, rather than just as a response to a light-dark cycle. Furthermore, the storage TAG content in seeds was decreased in *lhy ccal* but increased in *LHY-OE* plants, compared to WT [20,21]. Similarly, *CCAl-OE* plants also displayed an increase in seed oil content [121]. The opposite effects on oil content in *lhy ccal* and their OE plants are consistent with their effect on circadian periods because *lhy ccal* plants are short-period (i.e. -19 h) whereas LHY-/CCAl-OE plants are long-period to arrhythmic, as the level of overexpression increases [122,123].

As evidence for how the perturbation of the clock and changed circadian periods lead to the opposite effects on lipid accumulation, our recent study shows that LHY/CCAl regulates the initial condensation step of fatty acid biosynthesis in Arabidopsis [20]. Increased *LHY* expression enhanced FA synthesis, and the expression of *KASIII* that

encoded -ketoacyl-ACP synthase III was oppositely changed in developing seeds of *LHY!CCAl-OEs* and *lhyccal.* Chromatin immunoprecipitation, electrophoretic mobility shift, and transactivation assays indicated that LHY directly bound and activated the promoter of *KASIII* (Fig. 7C). PA, a metabolic precursor of TAG, inhibited LHY binding to *KASIII* promoter elements, suggesting that PA acts as a negative effector modulating the LHY/CCAl promotion of storage lipid production (Fig. 7C; [20]).

5.3. Transcriptional regulation of lipid degradation and seed gennination

Transcriptional control plays important roles in metabolic regulation because it can affect the expression of a network of multiple genes. In lipid biosynthesis, transcription factors, such as WRINKLED 1 (WRil), LEAFY COTYLEDONs (LECs), and FUSCA 3 (FUS3), regulate the expression of multiple genes contributing to lipid synthesis and TAG accumulation [124,125,126,127,128,129]. However, little was known about the transcriptional regulation of lipid degradation in plants.

The characterization of the PA-AHL4 interaction led to the finding that AHL4 suppresses the expression of genes for TAG lipases and for enzymes in fatty acid -oxidation during seedling establishment and growth [17]. AHL4 bound to the promoter regions of the genes encoding the TAG lipases SDPl and DALLS and acyl-thioesterase KATS involved in fatty acid -oxidation [17]. Those genes contained AHL4-binding *cis*-elements, and the AHL4 interaction with the promoter region was suppressed by PA species that bound to AHL4. The expression levels of AHL4-targeted genes, *SDPl, DALLS,* and *KATS,* were decreased in *pldalpldi5* that had a lower PA content but increased in *pahlpah2* mutants with a higher PA level [17]. The rate of seed TAG degradation during and after germination was lower in the seeds and seedlings of *AHL4* OEs but higher in those of *AHL4* KOs. These results indicate that an increase in PA releases the suppression of AHL4 on its target genes, and, in tum, increases TAG hydrolysis and fatty acid oxidation to provide the energy and substrates for seedlings establishment and development.

5.4. PA in honnone signaling and production

PLD, NPC, and PA have been shown to mediate ABA signaling and auxin distribution [130,30,131,132,28]. The protein phosphatase 2C ABil negatively regulates ABA response, which requires its catalytic activity and nuclear localization [133,134]. PA binds ABil and inhibits phosphatase activity of ABil, which in tum promotes ABA response. The *plda* displayed decreased ABA-induced PA production and impaired ABA-mediated stress response but had increased nuclear accumulation of ABil [30,28]. PA application increased the membrane association of the PA-binding protein PP2AA1, which enhances protein phosphatase 2 A (PP2A) activity [94]. In addition, PA binds PINOID kinase (PID), increases the plasma membrane association of PID, and promotes PID-dependent phosphorylation of PIN, which regulates the efflux and redistribution of auxin [132]. These results indicate that PA is involved in ABA response and auxin distribution by modulating the protein activity and/or subcellular distribution in and outside nuclei.

Recent results indicate that PA action in nuclei is involved in hormone signaling and metabolism (Fig. 8). PA interacts with the GA receptor GIDl in rice and affects the translocation of GIDl into nuclei [18]. The GA-induced nuclear localization of GIDl and degradation of the DELLA protein SLENDER RICEl (SLRl) are impaired *inplda6* [18]. Another study suggested that the inhibition of PA production by 1-butanol abolished the salicylic acid (SA)-induced nuclear localization of the SA receptors non-expresser of pathogenesis-related protein (NPRl) [135]. SA plays a critical role in plant defense response, and the nuclear localization of NPRl is required to activate the expression of pathogenesis-related (PR) genes [136,137].

In addition, DGKS and its product PA bind the ABA synthesizing enzyme ABA2 and reduce the enzymatic activity of ABA2 (Fig. 8; [44]). ABA content was increased in *DGKS-KO* plants but decreased in *DGKS*-OE plants. *DGKS-KO* plants were more resistant to water and salt stress, but *DGKS-OE* plants were more sensitive to those stressors. In addition, both DGKS and ABA2 were localized in and outside nuclei, and the *in vivo* interaction between DGKS and ABA2 mainly occurred in nuclei. Moreover, ABA2 was accumulated less in nuclei in DGKS-KOs plants [44]. These results indicate that DGKS and PA regulate ABA production by regulating the enzymatic activity and/or subcellular distribution of ABA2.

5.5. PA in hypoxia responses

PA levels have been reported to increase in response to hypoxia in Arabidopsis and wheat [138,139]. Both *plda* and *pld/5* displayed increased sensitivity to hypoxia [23]. Hypoxia stress, which is usually caused by root waterlogging and submergence in plants, has a negative effect on plant growth and production [140,141]. The submergence of plants in water causes the gaseous hormone ethylene to entrap in the submerged tissues, and ethylene has been found to play an important role in hypoxia acclimation and metabolic adjustment in response to flooding-induced hypoxia stress [142]. In the absence of ethylene, the Raf-like protein kinase CONSTITUTIVE TRIPLE RESPONSEl (CTRl)

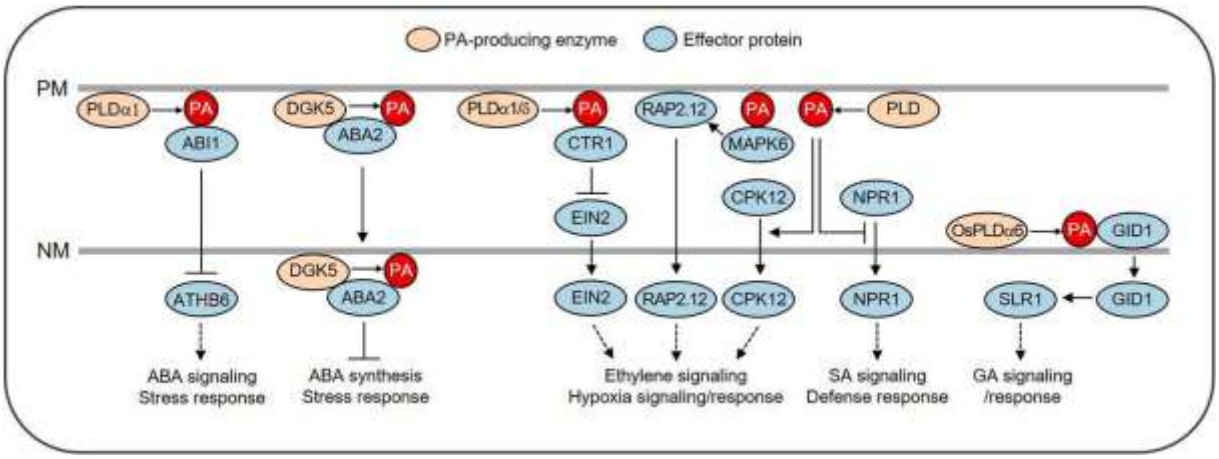

Fig. 8. Effects of PA on nuclear processes in hormone signaling and production. Refer the text for the effect on specific processes. For brevity, effect on specific pathways is shown, but crosstalk among pathways exists. In addition, PA from PLDal and PLDii both are involved, but in different steps of ABA signaling. Please refer to text for abbreviations and details.

phosphorylates the C-terminal domain of ETHYLENE-INSENSITIVE2 (EIN2), preventing the nuclear localization of EIN2, whereas, in presence of ethylene, CTRl perceives the signal from the ethylene receptor complex and becomes inactive (Fig. 8). The unphosphorylated C-terminal domain of EIN2 is then cleaved and translocated from the ER membrane into nuclei, where it regulates its downstream transcription factors [143]. A previous study showed that PA bound CTRl, inhibited its kinase activity, and impaired the interaction between CTRl and the ethylene receptor ETRl [144]. Additionally, exogenous PA promotes the nuclear translocation of EIN2 [138]. A recent study shows that PA binds CPK12 and promotes its nuclear translocation ([19]; Fig. 8). The cytoplasm to nuclear translocation of CPK12 under hypoxia stabilizes the core regulator of hypoxia signaling and response [19].

In addition, PA also promotes the translocation of an ethylene-responsive transcription factor RAP2.12 from the PM into the nuclei [23]. RAP2.12 is involved in the regulation of plant metabolism under hypoxia stress, and low oxygen induces the translocation of RAP2.12 into the nucleus, where it induces the expression of hypoxia-responsive genes [145,146,147]. The binding of PA to mitogen-activated protein kinase 6 (MPK6) stimulates its kinase activity and increases the MPK6-mediated phosphorylation of RAP2.12, which activates the transcriptional activity of RAP2.12 [148,23]. These results indicate that PA is involved in plant hypoxia response via directly or indirectly modulating the nuclear accumulation of proteins that regulate hypoxia sensing and response (Fig. 8).

5.6. *GAPC nuclear moonlighting in plant stress responses*

GAPC is a cytosolic metabolic enzyme involved in glycolysis and also has moonlighting functions in plant responses to different stress conditions. One mode of GAPC's non-metabolic actions is via its intracellular translocation from the cytoplasm to the nucleus under stress, including cadmium, hydrogen sulfide, bacterial flagellin, and heat [149,150,91,93]. Nuclear GAPC has been reported to mediate stress responses as a transcriptional regulator [91,151]. In response to heat, for example, some of GAPC molecules were translocated into nuclei, where it bound and activated a transcription factor known to regulate the expression of heat-inducible genes, enhancing the thermotolerance of Arabidopsis (Fig. 2; [91]).

As a mechanism of how heat induced GAPC nuclear translocation, a recent study shows that PLD5 and its lipid product PA mediates the nuclear translocation of GAPC in Arabidopsis under heat (Fig. 2; [20]). Previously, heat stress was shown to induce a rapid PA increase, which resulted mainly from PLO activation even though specific PLD(s) for the process remained unknown [152]. In addition, both PLD5 and PA were found to interact with GAPC in Arabidopsis [153,89]. PLD5 is associated with the plasma membrane, and its intracellular distribution is not affected by heat stress [50,154]. Thus, PLD5 may not directly co-move with GAPC for the nuclear translocation of GAPC under heat stress. Instead, the PLD5-mediated PA production is required for the nuclear translocation of GAPC, and colocalization and interaction of PA-GAPC in the nucleus during heat stress are inhibited by the membrane trafficking inhibitor BFA [22]. These results indicate that during heat stress, PA produced by PLD5 mediates the translocation of GAPC into the nucleus, where GAPC promotes the expression of heat-inducible genes and regulates the thermotolerance of Arabidopsis (Fig. 2).

5.7. *PA effects on root architecture*

The development of proper root architecture is crucial to the water and nutrient uptake of plants and communication with other plants and the environment [155,156]. The analysis of DGKs in rice reveals that DGKl, which is predicted to be associated with the nucleus, and its associated lipid mediators, DAG and PA, play an important role in root architecture in rice [157]. KO plants of *OsDGKl* had more lateral roots and smaller seminal root radius than those of WT, whereas overexpression of *OsDGKl* resulted in fewer lateral roots with larger radius. Exogenous DAG and PA had the opposite effect on lateral root number and seminal root radius, and the addition of PA, which is the product of DGK-mediated phosphorylation of DAG, restored the root phenotype of *OsDGKl* KO to that of WT [157]. DAG in animal cells is a potent cellular messenger [158,159], but the signaling functions of DAG remain elusive in plants. The loss of NPC5, which hydrolyzes phospholipids to produce DAG, decreased the DAG content in roots and caused fewer lateral roots under mild salt stress [160]. The application of exogenous DAG restored the lateral root number of *NPCS-KO* to that of WT, but the addition of PA failed to rescue the later root phenotype of *NPCS-KO.* In contrast to the inhibitory effect of PA on lateral root development, PA promotes the elongation of primary roots. Inhibition of DAG conversion to PA by a DGK inhibitor caused shorter primary roots [161]. These results indicate that PA and DAG have opposite effects on lateral root development.

Several PLDs, which hydrolyze phospholipids to produce PA, are involved in primary root elongation. The *pldalpldo* mutants, which had lower PA contents, had shorter primary roots under salt stress, and exogenous PA restored the primary root length of *pldalpldo* to that of WT under salt stress [132]. The loss of *PLDl;* in Arabidopsis decreased the primary root length under phosphate deficiency [35,37]. Moreover, the overexpression of *PLDc,* which increased PA contents, increased primary root length in Arabidopsis, canola, and soybean [24,162,163]. These results together show that PA and DAG play important roles in the development of root architecture; PA enhances primary root elongation but inhibits lateral root development, whereas DAG promotes lateral root development. However, further studies are needed to establish the direct connection between nuclear PA and its effects on root architecture.

6. PA in nuclear membrane remodeling and homeostasis

In addition to their signaling roles, PA and DAG are metabolic precursors for membrane synthesis, and changes in their cellular levels affect nuclear membrane remodeling, homeostasis, and functions. Defects in PAH that convert PA to DAG lead to an abnormal nuclear envelope (NE) in yeast and metazoan. The loss of PAH homolog Smp2 in yeast caused enlarged nuclei with long nuclear membranes [164]. Down-regulation of PAH homolog Lipin-1 by RNAi in *Caenorhabditis elegans* impaired the breakdown of NE during mitosis and resulted in binucleated cells [165]. The catalytic activity of animal PAH homolog lipin was also found to be involved in increased nuclear eccentricity [166]. One possibility is that the PA/DAG ratio serves as a signal for feedback regulatory pathways that control overall lipid homeostasis. In budding yeast, PA accumulates in nuclear envelope herniations that form from the hyperactivation of the ESCRT-III (endosomal sorting complex required for transport III) nuclear envelope remodeling machinery [167]. The ESCRT machinery plays key roles in membrane remodeling and protecting the nuclear envelope integrity [168]. PA binds to the NE-specific ESCRT, Chm?, and an increase in cellular PA levels leads to the translocation of Chm? from the cytosol to the NE/ER membrane [167]. The data suggest that the local accumulation of PA on NE recruits Chm? to the fusion sites of the inner and outer nuclear membranes and contributes to the NE sealing during nuclear pore complex mis-assembly [167].

In Arabidopsis, *pahlpah2* that lost both PA phosphohydrolases exhibited overexpansion of the ER membrane, although the volume of the nucleus was not greatly enlarged in the cells of *pahlpah2* leaves [69]. Compared to WT, *pahlpah2* had a higher level of nuclear PA, and the major nuclear PA species (34:2, 34:3, 34:4, 36:2, 36:3, 36:4, 36:5, and 36:6) were all increased [17]. Conversely, the PLO mutants *pldalpldo* had a lower level of nuclear PA, with the levels of major nuclear PA species being decreased compared to those in WT. The nuclear PA changes in response to stress, such as high temperature and salinity. Under heat stress, nuclear PA levels increased in WT but not in *pldo,*

suggesting that PA produced by PLD5 in the plasma membrane possibly moves to the nucleus [22]. Under salt stress, *DGKS-KOs* had a 40% lower nuclear PA level but a 17% higher nuclear DAG level than WT, whereas *DGKS-OEs* displayed a 45% higher nuclear PA level but a 16% lower nuclear DAG level than WT. Thus, the nuclear PA/DAG ratio was lower in *DGKS-KOs* but higher in *DGKS-OEs* [44]. These results indicate that DGKS regulates the nuclear PA/DAG homeostasis in Arabidopsis. Mechanisms that control nuclear membrane remodeling are essential to maintain the integrity and function of the nucleus, but they remain to be fully elucidated.

7. Future perspectives

PA has emerged as an important class of cellular mediators, and perturbations of its metabolism and signaling function affect various cellular and physiological processes. The mode of PA's action has been studied primarily outside the nuclei, but the recent findings of PA signaling and function in nuclei, as described here, open a new direction to investigate and understand the regulatory function of PA. The nuclear function of PA may underlie a basis for its role in regulating gene expression, cell proliferation, and stress responses. However, the precise mechanisms of PA actions in nuclei require further elucidation. One open question is whether nuclear PA changes are associated with the nuclear envelope, nucleoplasm reticulum, and/or nucleoplasm. Developing effective nuclear PA probes enabling nuclear PA detection and quantification *in vivo* will help address this question. Another question is how the PA binding to a nuclear protein affects the protein functions, such as its structure, membrane association, catalytic activity, and/or interaction with other proteins or other nuclear components, such as nucleic acids and chromatin. In addition, how does PA move into and out of nuclei, such as via membrane contact site, lipid movements in the continuous membrane between ER and nuclear envelope, or vesicular trafficking? How does PA affect protein trafficking into nuclei, such as via vesicular trafficking, nuclear pore complex, and/or interaction with other proteins? Increasing results indicate the importance of the acyl chain composition of PA in PA-protein interactions, which could underlie a basis for the specificity and diverse functions of PA. However, except for binding specificity for proteins, little is known about the effect of the acyl groups on the biological functions of PA and biochemical reactions and specific conditions by which the specific PA acyl species are produced. Furthermore, besides being the substrate and product of PA metabolism (Fig. 1), DAG is a mediator in plants, and the role of PA/DAG homeostasis in nuclei and plant growth, development, and stress responses requires more attention.

Moreover, how PA, despite having the simplest structure, has such diverse functions has been a long-standing question in the field. The characterization of PA-metabolizing enzymes, such as PLO, DGK, and NPC families, has begun to shed light on the issue. Many of the individual members of a given family, such as PLO, display distinguishable regulatory properties, subcellular associations, stimulus-induced temporal and spatial expression, and/or substrate preferences (Table 1). It is conceivable that those characteristics collectively lead to cellular regulation of PA changes in terms of specific temporal and spatial patterns and acyl composition, as well as in response to specific stimuli. The PA signature is decoded in the cell, at least in part, by its interaction with different effector proteins, such as transcription factors and those involved in hormone signaling, transport, and production (Fig. 8). The production and distribution of the effector proteins themselves are tightly regulated. Those properties, in combination, could underly a basis for the diverse cellular effects of PA. The discovery of PA signaling in nuclei and further elucidation of the process will advance the understanding of the mechanism by which PA mediates cellular functions, which may unveil new regulatory mechanisms for gene expression, lipid metabolism, and stress responses.

CRediT authorship contribution statement

Shuaibing Yao: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft, Writing - review & editing. **Sang-Chui Kim:** Data curation, Formal analysis, Methodology, Validation, Visualization, Writing - original draft, Writing - review & editing. **Jianwu Li:** Data curation, Formal analysis, Investigation, Validation, Visualization, Writing - review & editing. **Shan Tang:** Data curation, Formal analysis, Validation. **Xuemin Wang:** Conceptualization, Funding acquisition, Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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