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Dynamic responses of PA to environmental stimuli imaged by a genetically encoded mobilizable fluorescent sensor

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ABSTRACT

Membrane fluidity, permeability, and surface charges are controlled by phospholipid metabolism and transport. Despite the importance of phosphatidic acid (PA) as a bioactive molecule, the mechanical properties of PA translocation and subcellular accumulation are unknown. Here, we used a mobilizable, highly responsive genetically encoded fluorescent indicator, green fluorescent protein (GFP)–N160RbohD, to monitor PA dynamics in living cells. The majority of GFP–N160RbohD accumulated at the plasma membrane and sensitively responded to changes in PA levels. Cellular, pharmacological, and genetic analyses illustrated that both salinity and abscisic acid rapidly enhanced GFP–N160RbohD fluorescence at the plasma membrane, which mainly depended on hydrolysis of phospholipase D. By contrast, heat stress induced nuclear translocation of PA indicated by GFP–N160RbohD through a process that required diacylglycerol kinase activity, as well as secretory and endocytic trafficking. Strikingly, we showed that gravity triggers asymmetric PA distribution at the root apex, a response that is suppressed by PLDζ2 knockout. The broad utility of the PA sensor will expand our mechanistic understanding of numerous lipid-associated physiological and cell biological processes and facilitate screening for protein candidates that affect the synthesis, transport, and metabolism of PA.

Key words: phospholipid, phosphatidic acid, RbohD, heat stress, salinity, abscisic acid


INTRODUCTION

Phospholipids are important components of biological membranes; they are involved in cellular segregation, energy storage, membrane trafficking, molecular recognition, and signal transduction (Noack and Jaillais, 2020; Munnik et al., 2021). Phospholipids are metabolized by diverse enzymes and accumulate in specialized membranes, forming distinct biochemical and biophysical identities in response to different developmental and environmental stimuli (Gronnier et al., 2018; Colin and Jaillais, 2020). Anionic phospholipids including phosphatidic acid (PA), phosphatidylserine (PS), and phosphatidylinositol (PtdIns) have been reported to regulate vascular trafficking, cell signaling, and cell division and development, primarily by modulating the physical properties of membranes and interactions with targeted proteins (Platre et al., 2019; Wang et al., 2019; Noack and Jaillais, 2020).

PA is a simple membrane phospholipid and a precursor in the biosynthesis of complex lipids (Testerink and Munnik, 2005; Wang, 2005). PA is generated by three principal routes: lysophosphatidic acid acyltransferase (LPAT)-dependent de novo synthesis, phospholipase D (PLD)-catalyzed hydrolysis,
Figure 1. A fluorescent sensor for PA in Arabidopsis.

(A) Schematic representation of a sensor for phosphatidic acid (PA). GFP–N160RbohD contains the N-terminal 1–160 amino acids (N160) of respiratory burst oxidase homolog D (RbohD) ligated with GFP under the control of the UBQ10 promoter. GFP–N160M_RbohD was generated by mutation of four arginine residues (149, 150, 156, and 157) in GFP–N160RbohD. PA is produced from phospholipids by phospholipase D (PLD), from diacylglycerol (DAG) by phospholipase A (PLA), and from 1-stearoyl-2-arachidonoyl-phosphatidylcholine (LPA) by LPA phosphatidylethanolamine (PAP). PA is then degraded by diacylglycerol kinase (DGK) to produce DAG.

(B) Detection of PA using an anti-His antibody. The graph shows the optical density (OD) at 405 nm as a function of protein concentration.

(C) Detection of PA using an anti-His antibody at different concentrations (0 µg and 10 µg).

(D) Confocal microscopy images of GFP-N160RbohD in Arabidopsis cells. The images show GFP fluorescence (green), FM4-64 (red), and DAPI (blue) staining. The merged image combines all three channels.

(E) Confocal microscopy images of GFP-N160M_RbohD in Arabidopsis cells. The images show GFP fluorescence (green), FM4-64 (red), and DAPI (blue) staining. The merged image combines all three channels.

(F) Confocal microscopy images of GFP-N160RbohD in Arabidopsis cells. The images show GFP fluorescence (green), FM4-64 (red), and DAPI (blue) staining. The merged image combines all three channels.

(G) Confocal microscopy images of GFP-N160M_RbohD in Arabidopsis cells. The images show GFP fluorescence (green), FM4-64 (red), and DAPI (blue) staining. The merged image combines all three channels.

(H) Confocal microscopy images of GFP-N160RbohD in Arabidopsis cells. The images show GFP fluorescence (green), FM4-64 (red), and DAPI (blue) staining. The merged image combines all three channels.

(I) Confocal microscopy images of GFP-N160M_RbohD in Arabidopsis cells. The images show GFP fluorescence (green), FM4-64 (red), and DAPI (blue) staining. The merged image combines all three channels.

(J) Confocal microscopy images of GFP-N160RbohD in Arabidopsis cells. The images show GFP fluorescence (green), FM4-64 (red), and DAPI (blue) staining. The merged image combines all three channels.

(K) Confocal microscopy images of GFP-N160M_RbohD in Arabidopsis cells. The images show GFP fluorescence (green), FM4-64 (red), and DAPI (blue) staining. The merged image combines all three channels.

(L) Confocal microscopy images of GFP-N160RbohD in Arabidopsis cells. The images show GFP fluorescence (green), FM4-64 (red), and DAPI (blue) staining. The merged image combines all three channels.

(M) Confocal microscopy images of GFP-N160M_RbohD in Arabidopsis cells. The images show GFP fluorescence (green), FM4-64 (red), and DAPI (blue) staining. The merged image combines all three channels.

(N) Graph showing pixel intensity for GFP, FM4-64, and DAPI stained Arabidopsis cells along the distance (µm).

(O) Graph showing pixel intensity for GFP, FM4-64, and DAPI stained Arabidopsis cells along the distance (µm).

(P) Immunoblot analysis of Arabidopsis extracts with anti-GFP and Rubisco L antibodies. The blots show the expression levels of GFP-N160 and GFP-N160M_RbohD.

(Q) Immunoblot analysis of Arabidopsis extracts with anti-GFP and Rubisco L antibodies. The blots show the expression levels of GFP-N160M_RbohD.

(R) Immunoblot analysis of Arabidopsis extracts with anti-GFP and Rubisco L antibodies. The blots show the expression levels of GFP-N160M_RbohD.

(S) Graph showing the ratio of GFP PM/cyt intensity for Arabidopsis extracts treated with DMSO, E64d, and MG132. The graph shows the effect of these treatments on the GFP signal.

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and phosphorylation of diacylglycerol (DAG) by DAG kinase (DGK) (Kim et al., 2005; Arisz et al., 2009; Selvy et al., 2011; Tan et al., 2018). In plants, the conversion of lysophosphatidic acid (LPA) into PA by LPAT occurs in plastids and the endoplasmic reticulum (ER). Disruption of plastid LPAT1 causes embryo lethality in Arabidopsis (Yu et al., 2004), suggesting that LPAT-derived PA is required for plant embryogenesis and cell development. PA as a signaling molecule mainly originates from distinct phospholipase pathways (Testerink and Munnik, 2011); PLD usually produces PA at the plasma membrane (PM) through the hydrolysis of structural phospholipids such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Wang, 2005; Xing et al., 2021). There are 12 PLD members in Arabidopsis with distinct enzymatic properties; although these members regulate diverse plant processes, no defective developmental phenotype has been found in the pld mutants (Hong et al., 2016). Instead, PLD-derived PA acts as an essential mediator in plant responses to drought, salinity, and nutrient deficiency (Yu et al., 2010; Zhang et al., 2012; Su et al., 2018). For example, Arabidopsis PLDδ-1 and PLDδ-derived PA bind to PINOID kinase; these interactions enhance PINOID-dependent phosphorylation of PIN-FORMED2 to mediate auxin efflux and redistribution upon salt stress (Wang et al., 2019); PLD’2 and PA mediate clathrin-dependent endocytosis of PIN-FORMED2 during root gravitropism and halotropism (Li and Xue, 2007; Galvan-Ampudia et al., 2013).

Many proteins have been identified as PA targets, including kinases, phosphatases, oxidases, cytokine-associated proteins, and transcription factors (Anthony et al., 2004; Testerink et al., 2004, 2007; Zhang et al., 2009, 2012; Yu et al., 2010; Gao et al., 2013; McLoughlin et al., 2013; Yao et al., 2013; Julkowska et al., 2015; Wang et al., 2019), suggesting that PA functions both at the PM and in the nucleus. Our previous study showed that PA specifically binds respiratory burst oxidase homolog D (RbohD) to regulate abscisic acid (ABA) responses in Arabidopsis; the PA binding sites are restricted to four N-terminal arginine residues (149, 150, 156, and 157) (Zhang et al., 2009). We recently developed PAleon, a PA-specific biosensor based on Förster resonance energy transfer that is anchored to the PM, using amino acids 1–250 of RbohD (Li et al., 2019). However, our conceptual understanding of where and to what extent PA is increasing and/or decreasing in subcellular compartments remains poor because of the lack of a ubiquitous and mobilizable PA probe in plants.

RESULTS AND DISCUSSION

**Construction and verification of the PA-specific sensor GFP–N160RbohD**

To overcome the technical limitation to observe PA dynamics, we used the N-terminal 1–160 amino acids of Arabidopsis RbohD tagged with green fluorescent protein (GFP) to construct a PA sensor (designated as GFP–N160RbohD); as a control, we generated a mutant GFP–N160M<sub>RbohD</sub> in which the four arginine residues of truncated RbohD were mutated to alanine (N160M; Figure 1A). A 6xHis tag was fused to the N terminus of N160 and N160M. The recombinant proteins were produced in *Escherichia coli*, affinity purified (Figure 1B, inset), and subjected to a lipid–protein binding assay. Enzyme-linked immunosorbent assay results showed that His-tagged N160, but not N160M, exhibited strong, concentration-dependent binding to PA (Figure 1B; Zhang et al., 2009; Li et al., 2019). N160 did not bind to PC, phosphatidylinositol (PI), phosphatidylglycerol (PG), LPA, or PE in vitro (Figure 1C).

To investigate the use of GFP–N160<sub>RbohD</sub> in plant cells, we generated stable transgenic *Arabidopsis* plants expressing GFP–N160<sub>RbohD</sub> and GFP–N160M<sub>RbohD</sub> under the control of the *Arabidopsis* UBO10 promoter. Four days after germination, we observed high GFP fluorescence in whole seedlings (Supplemental Figure 1A); most of the GFP–N160<sub>RbohD</sub> signal was localized at the PM in cells of the embryo, cotyledon, apical meristem, lateral root primordia, and root hair (Supplemental Figures 1B–1F), indicating that GFP–N160<sub>RbohD</sub>...
can identify cellular PA distribution at a high spatial resolution in various tissues and developmental stages. In root epidermal cells, GFP–N160RbohD showed characteristic fluorescence at the PM, where it colocalized with the endocytic dye FM4-64 (Figures 1D–1H). By contrast, the GFP–N160MRbohD probe was ubiquitously distributed in the PM, cytosol, and nucleus (Figures 1 I–1O). We noticed some PM labeling of GFP–N160MRbohD in root cells, presumably because of N160M binding to additional lipids or as-yet unknown proteins at the PM (Figure 1J). In addition, GFP–N160MRbohD reduced bulk protein levels by about 50% (Figure 1P), the degradation of which was attenuated by treatment with the protease inhibitor E64d or the proteasome inhibitor MG132 (Figure 1 Q), consistent with confocal observations (Figures 1R and 1S). Therefore, GFP–N160MRbohD becomes unstable and is prone to degradation in plant cells.

To further characterize the PA sensor in vivo, Arabidopsis lines expressing GFP–N160RbohD were treated with different types of phospholipids, including PA, PS, LPA, PC, PG, and PE. As shown in Figure 2A, a substantial increase in probe fluorescence was immediately observed upon the addition of 10 μM PA (Figure 2A). As the concentration of PA reached 50 μM, the PM versus cytoplasmic intensity ratio for GFP–N160RbohD increased by 42% compared with control cells (Figures 2A and 2B), suggesting enhanced accumulation of PA at the PM. To examine the specificity of the response, we replaced the PA sensor with a mutant N160M domain, which did not bind PA. As expected, GFP–N160M did not impact the PM abundance of GFP–N160RbohD (Figures 2A and 2B). Treatment with other phospholipids (e.g., LPA, PC, PG, or PE) did not increase the levels of PA, whereas PS exhibited less-efficient change than PA treatment (Figures 2C and 2D), suggesting a minor cross-interaction between PS and GFP–N160RbohD. Furthermore, short-term treatment with phenylarsine oxide, a PtdIns(4) kinase inhibitor, disrupted the PM localization of the PtdIns4P biosensor but had no effect on GFP–N160RbohD (Supplemental Figure 2A). In addition, GFP–N160RbohD- or GFP–N160M-expressing Arabidopsis plants showed no obvious morphological or developmental differences from wild-type plants (Supplemental Figure 2B–2D). Together, these results suggest that GFP–N160RbohD is a PA biosensor in Arabidopsis.

PA dynamics during salt and ABA exposure in Arabidopsis

The production of PA is rapidly induced upon exposure to salinity, drought, and hyperosmotic stress (Frank et al., 2000; Munnik et al., 2000; Hong et al., 2008; Zhang et al., 2009; Yu et al., 2010), thus facilitating targeted protein recruitment to cellular membranes and mediating downstream responses. To further examine the reliability and consistency of our PA reporter system, we analyzed in vivo PA dynamics in roots of 4-day-old Arabidopsis seedlings expressing GFP–N160RbohD after...
exposure to salt or ABA. Fluorescence of GFP–N160RbohD, but not GFP–N160MRbohD, at the PM was transiently promoted 5 min after 50 mM NaCl exposure; it increased to higher levels after 10 min of salt treatment (Figure 3A; Supplemental Figure 3A and 3B). Moreover, PA signals reached higher values after 5 and 10 min of treatment with 100 mM NaCl (Figure 3A; Supplemental Figure 4A), consistent with a previous analysis of PA levels using electrospray ionization tandem mass spectrometry (Wang et al., 2019). The GFP–N160RbohD signal showed similar tendencies upon ABA treatment (Figure 3B; Supplemental Figure 4B). Notably, most of the stress-enhanced PA signals were localized at the PM of root cells (Figure 3A and 3B), suggesting that some unknown process mediates the specification of PA-enriched membrane domains.

To test the importance of PA-generating enzymes in the production of PA at the PM of stressed plants, 1-butanol (1-But), CI-976, and R59949 were used; these compounds inhibit PLD-, LPAT-, and DGK-derived PA production, respectively (Munnik et al., 1995; Drecktrah et al., 2003; Platre et al., 2018). Treatment with 1-But reduced bulk PA levels by 19% and 25% compared with treatment with NaCl or ABA for 10 min, respectively, whereas the LPAT inhibitor only weakly inhibited PA production, and the DGK inhibitor had no effect (Figures 3A and 3B; Supplemental Figure 4). Phosphatidylbutanol treatment had no impact on the GFP–N160RbohD signal, confirming that the effects of 1-But were caused by inhibition of intracellular PA production (Supplemental Figure 3C and 3D; Munnik et al., 1995). As a negative control, 2-But (an isomer of 1-But) was used, but it had no effect on the GFP–N160RbohD signature (Figure 3A and 3B). In addition, neither NaCl nor ABA affected sensor expression levels in the WT or pld mutant lines (Supplemental Figure 5), suggesting that the stress-induced fluorescence signal at the PM imaged by GFP–N160RbohD is due to PA enhancement rather than increased expression levels of sensors.

PLDα1 and PLDδ are the most abundant PLDs in Arabidopsis; their activities increase upon exposure to diverse stresses (Bargmann et al., 2009a; 2009b; Yu et al., 2010; Wang et al., 2019). To gain insight into the function of PLDs in stress-induced PA production, we isolated representative knockout mutants of PLDα1 and PLDδ, respectively (Munnik et al., 2001; 2002; Shiva et al., 2016). Although PLDα1 was substantially decreased in these double mutants (Wang et al., 2019), we introduced GFP–N160RbohD into pldα1pldδ-1 and pldα1pldδ-2. Because total PLD activity was monitored at higher levels in nuclei after 10 min of heat stress. Four-day-old seedlings expressing GFP–N160RbohD were exposed to 45°C for the time periods indicated. GFP fluorescence began to detach from the PM and accumulated at higher levels in nuclei after 10 min of heat stress. At 30 min of heat stress, most of the GFP–N160RbohD signal was translocated to the nucleus, both in the cotyledon and the root (Figure 4A). By contrast, heat treatment had little impact on the localization of GFP–N160MRbohD (Supplemental Figure 6A and 6B). The GFP–N160RbohD signal was translocated to the PM from the nucleus after withdrawal of heat (Supplemental Figure 6C and 6D), suggesting that PA undergoes reversible movement in response to temperature stimuli.

Although the recently developed PA sensor mCIT-1×PASS was tagged with a nuclear export signal to exclude the fusion protein from the nucleus (Zhang et al., 2014; Platre et al., 2018), nuclear translocation of PtdIns(4,5)P2 was observed in heat-stressed root epidermal cells (Figures 4D and 4E). Intriguingly, heat stress also induced the nuclear translocation of PtdIns3P imaged by mCIT-2×FYVE-FHs but had no impact on the localization of DAG, PtdIns4P, or PtdIns(4,5)P2 imaged by EYFP-C1αPKC, mCIT-1×PH_FAPP1, and mCIT-2×PHEPLC, respectively (Figures 4B and 4C; Simon et al., 2016; Vermeer et al., 2017). Although the level of PtdIns(4,5)P2 was significantly enhanced at the PM when roots were exposed to heat stress, we did not observe heat-induced nuclear translocation of PtdIns(4,5)P2 (Figures 4B and 4C), inconsistent with a previous report (Mishkind et al., 2009). This apparent contradiction may be due to the use of different plant species (Arabidopsis versus Nicotiana tabacum) or cell types (root epidermal versus BY-2 cells). Treatment with 1-But partially inhibited heat-induced nuclear translocation of PA, consistent with confocal assays of pldα1pldδ mutants (Figures 4D and 4E). Surprisingly, the application of R59949 to inhibit DGK activity abolished nuclear PA translocation, whereas less inhibition was observed after treatment with the LPAT inhibitor CI-976 (Figures 4D and 4E). Cold or hypersmotic stress did not induce nuclear PA translocation (Supplemental Figure 7). Overall, these findings indicate that DGK is required for heat-stress-induced mobilization of PA for nuclear import.

Multiple lipid species generated at the ER are rapidly and efficiently transferred to different target membranes through vesicular and nonvesicular trafficking pathways (Lev, 2012). Because phospholipids are the major constituents of most biological membranes and transport vesicles, vesicular pathways may have an important role in the cellular transport of phospholipids. We used brefeldin A, a vesicle transport inhibitor that blocks cell exocytosis, and wortmannin (Wm), a
Figure 3. PA production upon stimulation by salt stress and ABA.

(A and B) Confocal images of GFP–N160RbohD fluorescence upon NaCl (A) and ABA (B) treatment with or without inhibitors. Four-day-old seedlings expressing GFP–N160RbohD were exposed to NaCl or ABA for the indicated time. For drug treatment, 0.1% (v/v) 1-butanol (1-But) or 2-But, the LPAT inhibitor CI-976 (50 μM), or the DGK inhibitor R59949 (12.5 μM) was added for 60 min prior to NaCl or ABA treatment. White arrowheads indicate PA at the PM. Co, cortex; Ep, epidermis. Bars: 25 μm.

(C and D) Knockout of PLDα1 and PLDδ2 prevented NaCl- and ABA-induced PA enhancement at the PM as indicated by GFP–N160RbohD. Bars: 25 μm.

(E) Quantification of GFP–N160RbohD PM/Cyt intensity ratios after treatments indicated in (C) and (D). Data are means ± SDs of three independent experiments (n > 40 cells from at least 10 roots). n.s., no significance.

(F) Manipulation of medium pH affected salt-induced PA accumulation at the PM. Plants expressing GFP–N160RbohD were grown on medium at the indicated pH values for 4 days and treated with 50 mM NaCl for 10 min. White arrowheads indicate PA at the PM. Bars: 25 μm.

(G) Quantification of GFP–N160RbohD PM/Cyt intensity ratios after the treatments indicated in (F). Different letters indicate statistically significant differences determined by one-way ANOVA; p < 0.05.
Figure 4. Heat-stress-induced translocation of PA to the nucleus.

(A) Confocal images of GFP–N160RbohD fluorescence in cotyledons (top) and roots (bottom) of wild-type Arabidopsis upon exposure to heat stress. Four-day-old seedlings expressing GFP–N160RbohD were exposed to 45°C for the indicated time before confocal observation. Magnified images of root cells expressing GFP–N160RbohD, with DAPI counterstaining, are shown on the right. The numbers (inset) indicate the nuclear versus PM-associated (Nu/PM) intensity ratios of GFP–N160RbohD in roots. Data are means ± SDs of three independent experiments (n > 40 cells from at least 10 roots). Different letters indicate statistically significant differences determined by one-way ANOVA; p < 0.05.

(B) Effect of heat stress on the cellular localization patterns of PA, DAG, PtdIns3P, PtdIns4P, and PtdIns(4,5)P2 as probed by mCIT-1×PASS, EYFP-CA1αPKC, mCIT-2×FYVEHRS, mCIT-1×PHFAPP1, and mCIT-2×PHPLC, respectively. Four-day-old seedlings expressing sensors were exposed to 45°C for 20 min prior to confocal observation. White arrowheads indicate nuclei. Bars: 25 μm.

(C) Quantification of the Nu/PM intensity ratios of probe fluorescence indicated in (B). Different letters indicate statistically significant differences determined by one-way ANOVA; p < 0.05. n.s., no significance; n.d., not detectable.

(D) Confocal images of GFP–N160RbohD fluorescence in wild-type and ptda1ptide mutants exposed to heat stress with or without inhibitor treatment. Four-day-old seedlings were pretreated with 0.1% (v/v) 1-But or 2-But, R59949 (12.5 μM), or CI-976 (50 μM) for 60 min before exposure to 45°C for 10 min. Bars: 25 μm.

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PI3K inhibitor that inhibits protein cargo trafficking to vacuoles, to elucidate the effects of vesicular transport on PA translocation. After treatment with BFA, PA accumulated in BFA bodies, and PA translocation to the nucleus was decreased (Figures 4F and 4G). PA trafficking was also sensitive to Wm treatment, which led to the aggregation of cytoplasmic PA and its relocalization to punctate structures in heat-stressed root cells (Figures 4F and 4G). Furthermore, BFA treatment inhibited the nuclear accumulation of fluorescent nitrobenzoxadiazole (NBD)–PA (Figure 4H), suggesting that heat enhancement of nuclear PA is largely caused by cellular transport from other membranes rather than local PA production in the nucleus. Together, our results suggest that heat-triggered nuclear transport of PA requires functional vesicular trafficking.

**Gravity stimulation mediates the establishment of a PA gradient in roots**

Because PLD and PA mediate gravitropic, hydrotropic, and halotropic responses via auxin signaling (Li and Xue, 2007; Galvan-Ampudia et al., 2013; Tan et al., 2018), we examined the dynamic distribution of PA after gravity stimulation. In gravity-stimulated roots expressing GFP–N160RbohD, asymmetric PA distribution can be detected by comparing the upper and lower sides of the root. Within 1.5 h of gravity stimulation, PA-specific signals were weaker at the upper, rather than the lower, side of vertically positioned roots. This difference was most pronounced in the outermost lateral root cap (Figure 5A). Most importantly, the gravity-induced lateral PA gradient was completely disrupted in the pldΔ2 mutant but inhibited to a lesser extent in the pldΔ1pldΔ6 mutant, consistent with their gravitropic phenotypes (Figures 5A and 5B; Li and Xue, 2007; Wang et al., 2019). Together, our results implicate the intracellular distribution of PA in root gravity regulation.

**Subcellular targeting of GFP–N160RbohD**

PA is found in various biological membranes; it is thought to be confined to the vicinity of lipid metabolism and to be shaped by lipid transport. To generate transformants targeting GFP–N160RbohD to distinct subcellular compartments, we fused peptide tags to the N or C terminus of the sensor to confer localization at the PM or nuclear membrane or in plastids, the ER, or peroxisomes (Figure 6). Targeting of GFP–N160RbohD to the ER (ER–GFP–N160RbohD) was achieved by fusing the HDEL motif to the C terminus of GFP–N160RbohD (Figure 6E; Geldner et al., 2009). PA has been reported to mediate the dynamics of lipid droplets, underlying their biogenesis at the ER (Fei et al., 2011). To better understand whether PA is involved in the formation of lipid droplets upon nutrient stress, Arabidopsis seedlings stably expressing ER–GFP–N160RbohD were subjected to fixed-carbon starvation induced by darkness. As shown in Supplemental Figure 8, several green bodies containing ER–GFP–N160RbohD were present in cells of hypocotyls and roots and colocalized with the lipid droplet marker Nile Red, suggesting that PA is associated with lipid droplets during lipid metabolism and energy homeostasis. Therefore, comparative analyses of PA dynamics combining distinct subcellular GFP–N160RbohD with genetic materials should allow monitoring of the spatiotemporal dynamics of PA metabolism and transport, distinguishing PA signatures emanating from membrane stores or enzyme-derived pools.

There is increasing research focus on the links between lipid-based signaling pathways and plant stress responses, including subcellular localization-dependent lipid metabolism and membrane trafficking. Several fluorescent fusion proteins and PA-binding domains that probe cellular PA in plants have been reported previously (Potocký et al., 2014; Platre et al., 2018; Li et al., 2019), but it has been difficult to determine...
Figure 6. Subcellular targeting of GFP–N160RbohD in *N. benthamiana* and *A. thaliana*.
N- or C-terminal fusion of targeting peptides direct the GFP–N160RbohD sensor to specific cellular sites including the PM (A), nuclear membrane (B), plastid (C), peroxisome (D), and endoplasmic reticulum (E). Different sensors were transiently expressed in *N. benthamiana* or stably expressed in *A. thaliana*. White arrows indicate merged signals. PM, plasma membrane; NM, nuclear membrane; Pd, plastid; Px, peroxisome; ER, endoplasmic reticulum.
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Plant material and growth conditions

All Arabidopsis (A. thaliana) plants in this study were generated using the Col-0 ecotype background. The double mutants pldΔ1;pldΔ2-1 and pldΔ1;pldΔ2-2 were described previously (Wang et al., 2019). Arabidopsis seeds were surface sterilized and plated on half-strength Murashige and Skoog (1/2 MS) medium containing 1% (w/v) sucrose and 1% (w/v) plant agar, stratified for 1 day at 4 °C, and moved to a growth chamber with a light intensity of 160 mol m⁻² s⁻¹ and a day/night regimen of 14/10 h (23°C/21°C). Root length and fresh weight were determined using 12-day-old plants that had been grown on 1/2 MS medium.

For carbon starvation, seeds were sown on 1/2 MS medium lacking sucrose and grown for 4 days. The plates were then covered with a double layer of aluminum foil and incubated in the same growth chamber for 24 h. Before microscopy analysis, the seedlings were stained with a Nile Red solution consisting of 50 mM Tris-maleate buffer (pH 7.5) with 20 mg/ml polylvinylypyrrolidone and 2.5 μg/ml Nile Red Oxazone (Sigma) for 15 min (Greenspan et al., 1985; Thines et al., 2000).

Plasmid constructs and generation of transgenic lines

To obtain transgenic plants expressing UBQ10::GFP-N160/N160M, the N-terminal amino acids 1–160 (N160) from RbohD were cloned and inserted into the pCAMBIA1301 vector. The four PA-binding arginine residues were replaced with alanine in N160M (Li et al., 2019). The GFP sequence without a stop codon was amplified and cloned into N160/N160M-pCAMBIA1301. The UBQ10 promoter was cloned to generate UBQ10::GFP-N160/N160M. For the constructs for subcompartments, peptides including SKL (Zu et al., 2017), HDEL (Dean and Pelham, 1999), P2C3 (C-terminal 33 amino acids from AIPAP2; Sun et al., 2012), and K-Ras4B (Nishioka et al., 2010) were ligated to the C terminus of UBQ10::GFP-N160 with a stop codon. For nuclear localization, the WPP domain was amplified and inserted before GFP to generate UBQ10::WPP-GFP-N160 (Rose and Meier, 2001). Peptide sequences are listed in Supplemental Table 1. The constructs were transformed into Col-0 by the floral dip method to generate stable transgenic plants using Agrobacterium strain GV3101. The primers used to generate constructs are listed in Supplemental Table 2.

Recombinant protein expression and immunoblot analysis

The N160 and N160M coding regions were amplified and inserted into the pCold I-His vector to generate His-tagged proteins. The sequences of the primers used to generate recombinant proteins are listed in Supplemental Table 2. All constructs were transformed into E. coli (BL21); recombinant protein expression was induced by incubation with 0.5 mM isopropyl β-d-1-thiogalactopyranoside at 16°C for 8 h. Cultures were collected by high-speed centrifugation (12 000 g) at 4°C. Pellets were resuspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 10 mM imidazole [pH 7.4]) and incubated for 30 min on ice. Cells were lysed by sonication on ice for 8 min and then centrifuged at 12 000 g for 30 min at 4°C. The supernatant was purified using Ni-IDA Resin (GenScript). Proteins were separated from beads using elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 250 mM imidazole [pH 8]). Proteins were separated by SDS–PAGE for immunoblotting using an anti-His antibody (1:5000, Sigma-Aldrich) and goat anti-mouse immunoglobulin G (IgG) secondary antibody (1:10,000, Sigma-Aldrich). Images were acquired using a ChemiDoc XRS1 System (Tanon).

For immunoblot analysis of sensor expression levels, protein extracts were prepared using a protein extraction buffer (50 mM Tris–HCl, 10 mM KCl, 1 mM ethylenediaminetetraacetic acid, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail), then centrifuged at 12 000 g for 20 min at 4°C. Proteins were separated by 10% SDS–PAGE and then subjected to immunoblotting using anti-GFP antibodies (1:5000, Abclonal) and the corresponding secondary goat anti-mouse IgG antibody (1:10 000, Sigma-Aldrich). Images were acquired using a ChemiDoc XRS1 System (Tanon).

Lipid–protein binding assay

For enzyme-linked immunosorbent assays to test protein–lipid interactions, 16:0–18:2 PA, PC, PI, PG, LPA, and PE (Avanti Polar Lipids, Alabaster, AL, USA) stored in chloroform were dried with nitrogen gas and dissolved in methanol. The lipids were then coated onto a 96-well microplate and dried for 2 h to remove the methanol. The microplate was blocked with 3% bovine serum albumin for 1 h, and tagged N160 and N160M were then added to each well and incubated overnight at 4°C. Subsequently, the microplate was incubated with a mouse anti-His antibody, followed by the corresponding goat anti-mouse IgG–alkaline phosphatase antibody.
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(1:5000; Sigma-Aldrich). After reaction in chromogenic solution at 37 °C for 10 min, the optical density was measured at 405 nm.

Cell staining

Four-day-old Arabidopsis seedlings were stained with 100 μM propidium iodide (Invitrogen), 5 μM FM4-64 (Invitrogen), or 5 μM DAPI (Sigma-Aldrich) for 2 min before confocal observation. Distilled water or dimethyl sulfoxide was used as a negative control.

Confocal microscopy and analysis

For live-cell GFP–N160RbohD and GFP–N160MRbohD imaging, fluorescence was visualized using a Leica TCS SP8 X confocal laser scanning microscope with the following parameters: GFP (excitation, 488 nm; emission, 505–555 nm), phosphatidylinositol and FM4-64 (excitation, 561 nm; emission, 571–639 nm), and DAPI (excitation, 405 nm; emission, 410–550 nm). All scans were conducted at 1024 × 1024-pixel resolution with repeated scanning of two lines. Data were analyzed using ImageJ software. The following ratios were calculated: PM/Cyt = fluorescence intensity of PM/fluorescence intensity of cytosol, and Nu/PM = fluorescence intensity of nucleus/fluorescence intensity of PM.

Stress and lipid treatment

For stress treatment, 4-day-old seedlings were transferred to half-strength MS medium with NaCl, ABA, or heat stress for the indicated time, prior to confocal observation. For lipid treatment, PA or other lipids obtained in chloroform from Avanti Polar Lipids were dried in a stream of N2 and emulsified by sonication in distilled water on ice and then subjected to filter sterilization. The lipids were added to 1/2 MS medium for the indicated time.

For NBD–PA, 4-day-old seedlings of WT Arabidopsis were incubated with 5 μM NBD–PA for 60 min, then exposed to 45 °C for 30 min with or without BFA prior to confocal observation.

Analysis of gravitropism

The root gravity response was measured using 5-day-old seedlings reoriented 90°. Photographs of plants were taken at predetermined time points after reorientation using a confocal microscope. Data were analyzed using ImageJ software.

Structural modeling

The three-dimensional protein structure of the RbohD molecule was obtained from the UniProt database (https://www.uniprot.org). The structure was edited and illustrated using PyMOL (v.2.5.0).

Drug treatment

For drug treatments, 4-day-old Arabidopsis plants were preincubated with 1-But (0.1%, v/v), 2-But (0.1%, v/v), R59949 (12.5 μM), BFA (50 μM), or Wm (25 μM) for 60 min before exposure to NaCl, ABA, or heat treatment for the indicated time.

SUPPLEMENTAL INFORMATION

Supplemental information is available at Plant Communications Online.

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AUTHOR CONTRIBUTIONS

Q.Z., T.L., and X.X. designed the experiments and wrote the paper. T.L., X.X., Q.L., and W.L. conducted the experiments and analyzed the data. L.L., W.Z., T.M., and X.W. provided suggestions and revised the paper. All authors discussed the results and edited the manuscript.

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REFERENCES

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separation and protein segregation in living bacteria. EMBO J. 41: e109800.


A genetically encoded mobilizable fluorescent PA sensor


