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Visualization of Phosphatidylinositol 3,5-Bisphosphate Dynamics by a Tandem ML1N-Based Fluorescent Protein Probe in Arabidopsis

Tomoko Hirano¹, Kelly Stecker², Teun Munnik³, Haoxing Xu⁴ and Masa H. Sato¹,*

¹Laboratory of Cellular Dynamics, Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Shimogamo-nakaragi-cho, Sakyo-ku, Kyoto, 606-8522 Japan
²Biotechnology Center, University of Wisconsin, Madison, WI 53706, USA
³Section of Plant Physiology, Swammerdam Institute for Life Sciences, University of Amsterdam, Science park 904, 1098 XH Amsterdam 94216, The Netherlands
⁴Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, MI 48109, USA
*Corresponding author: E-mail, mhsato@kpu.ac.jp; Fax, +81-75-703-5448.

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Phosphatidylinositol 3,5-bisphosphate [PI(3,5)P₂] is a low-abundance phospholipid known to be associated with a wide variety of physiological functions in plants. However, the localization and dynamics of PI(3,5)P₂ in plant cells remain largely unknown, partially due to the lack of an effective fluorescent probe. Using Arabidopsis transgenic plant expressing the PI(3,5)P₂-labeling fluorescent probe (tagRFP–ML1N*2) developed based on a tandem repeat of the cytosolic phosphoinositide-interacting domain (ML1N) of the mammalian lysosomal transient receptor potential cation channel, Mucolipin 1 (TRPML1), here we show that PI(3,5)P₂ is predominantly localized on the limited membranes of the FAB1- and SNX1-positive late endosomes, but rarely localized on the membranes of plant vacuoles or trans-Golgi network/early endosomes of cortical cells of the root differentiation zone. The late endosomal localization of tagRFP–ML1N*2 is reduced or abolished by pharmacological inhibition or genetic knockdown of expression of genes encoding PI(3,5)P₂-synthesizing enzymes, FAB1A/B, but markedly increased with FAB1A overexpression. Notably, reactive oxygen species (ROS) significantly increase late endosomal levels of PI(3,5)P₂. Thus, tandem ML1N-based PI(3,5)P₂ probes can reliably monitor intracellular dynamics of PI(3,5)P₂ in Arabidopsis cells with less binding activity to other endomembrane organelles.

Keywords: Arabidopsis • Fluorescent protein probe • Late endosomes • ML1N*2 • PI(3,5)P₂ • YM201636.

Abbreviations: amiRNA, artificial microRNA; BFA, brefeldin A; CaMV, Cauliflower mosaic virus; FAB1, formation of haploid and binucleate cells 1; GFP, green fluorescent protein; LEs/MVBs, late endosomes/multivesicular bodies; ML1, Mucolipin 1; MS, Murashige and Skoog; PIP, phosphatidylinositol phosphate; PI(3,5)P₂, phosphatidylinositol 3,5-bisphosphate; RFP, red fluorescent protein; ROS, reactive oxygen species; SNX1, Sorting Nexin 1; TLC, thin-layer chromatography; TRPML1, transient receptor potential cation channel, Mucolipin 1.

Introduction

Although phosphoinositides are minor phospholipids in eukaryotic cells, they play important roles in a wide spectrum of cellular processes, which include determination of organelle identity and mediating signal transduction by recruiting various effector molecules to intracellular organelles and the plasma membrane (Balla 2013). Phosphoinositides consist of a total of seven species, which include three phosphatidylinositol monophosphates (PI3P, PI4P and PI5P), three phosphatidylinositol bisphosphates [PI(3,4)P₂, PI(3,5)P₂ and PI(4,5)P₂] and one phosphatidylinositol triphosphate [PI(3,4,5)P₃] (Di Paolo and De Camilli 2006). These phosphoinositides are interconvertible, through various PIP kinases and phosphatases that modify the phosphorylation state of the inositol head group, as well as phospholipases that hydrolyze PIPs to release the soluble inositol group into the cytosol. The combined actions of these enzymes produce various compartment-specific PIPs that determine the organelle and membrane identities (Leimmon 2008, Balla 2013).

Among those, phosphatidylinositol 3,5-bisphosphate [PI(3,5)P₂], which is produced by the phosphatidylinositol 3-phosphate 5-kinase, formation of haploid and binucleate cells1 (FAB1)/PIKfyve, localizes to the endolysosomes and vacuoles, and plays essential roles in endosomal membrane trafficking including vacuolar sorting, endocytosis of membrane proteins, ion transport, cytoskeleton dynamics and retrograde transport in animals and yeasts (Efe et al. 2005, Shisheva 2008, Ikonomov et al. 2011) despite constituting a small proportion of the total phosphoinositide pool (~0.1%) (Boss et al. 2012). In Arabidopsis, FAB1/PIKfyve or its product, PI(3,5)P₂, is essential for mediating the maturation process of the late endosomes.
Fluorescent imaging of phosphoinositides using genetically encoded probes is crucial to reveal the cellular functions of phosphoinositides in the cell. Fluorescent protein-tagged specific probes for labeling PI3P, PI4P, PI(4,5)P2, PI(3,4,5)P3 and the downstream products, diacylglycerol and phosphatidic acid, have been developed in various eukaryotic cells including animals, yeast (Oancea et al. 1998, Rizzo et al. 2000, Halet, 2005, Balla 2007, Lemmon 2008) and plants (Vermeer et al. 2006, Simon et al. 2014). In contrast, much less is known about the function and localization of PI(3,5)P2 due to its low levels and the lack of an efficient fluorescent probe in plant studies. Recently, a novel PI(3,5)P2-labeling probe, green fluorescent protein (GFP)–ML1N*2 or mCherry–ML1N*2, was developed based on a tandem repeat of the cytosolic PI(3,5)P2-interacting domain (ML1N*2) of the late endosome-localized transient receptor potential Mucolipin 1 (TRPML1) (Li et al. 2013) in mammalian cells. In mammalian cells, the probe is mostly localized in the late endosomes and lysosomes (Li et al. 2013). In yeast cells, the probe is localized in the vacuole, but also at the plasma membrane, which is most probably due to non-specific binding (Li et al. 2013). Although several follow-up studies have utilized this probe in mammalian cells, including neurons and fibroblasts (McCarty et al. 2014b, Hammond et al. 2015, Hong et al. 2015, Takatori et al. 2015), it remains controversial whether the probe is specific in vivo in a specific type of mammalian cells (Hammond et al. 2015). Currently, there is still no reported application for this probe in plant cells.

In this study, by generating transgenic Arabidopsis lines expressing tag red fluorescent protein (RFP)-fused tandem ML1N (tagRFP–ML1N*2), we investigated whether the fluorescent probes could effectively label PI(3,5)P2 in Arabidopsis cells. We found that the probe is highly co-localized with late endosomal markers, FAB1 and Sorting Nexin 1 (SNX1), in cortical cells of the root differentiation zone. Manipulating the activity and expression of FAB1 produces changes in the intensity and size of the probe in the late endosome. Using this probe, we report that reactive oxygen species (ROS) are a physiological stimulator of PI(3,5)P2 synthesis. Hence the use of the ML1N-based PI(3,5)P2 fluorescent marker may pave the way for understanding the molecular functions of PI(3,5)P2 in plants.

**Results**

**TagRFP–ML1N*2 predominantly labels FAB1A-positive vesicular compartments in Arabidopsis cortical cells of the root differential zone**

To study PI(3,5)P2 dynamics in plants, we generated the N-terminally tagRFP-fused tandem ML1N (tagRFP–ML1N*2) expression construct under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter (Supplementary Fig. S1). After Agrobacterium-mediated transformation (Clough and Bent 1998) of the probe construct, we obtained transgenic Arabidopsis plants with various levels of expression. We selected the transgenic lines with moderate levels of overexpression, and crossed them with a FAB1A–GFP-expressing line for further characterization.

FAB1/PIKfyve is the sole enzyme that mediates the synthesis of PI(3,5)P2 from PI3P in eukaryotic cells (Shisheva 2008, McCartney et al. 2014a). Although the Arabidopsis genome contains four FAB1 proteins: FAB1A, FAB1B, FAB1C and FAB1D, only FAB1A and FAB1B possess a conserved FYVE domain, and the fab1afab1b double mutant reveals a male gametophyte lethality phenotype, suggesting the functional redundancy between FAB1A and FAB1B in Arabidopsis (Whitley et al. 2009). The functions of FYVE domain-lacking unconventional FAB1C and FAB1D are still unknown, although knock-outs of FAB1B and FAB1C decreased the rate of ABA-induced stomatal closure (Bak et al. 2013), and FAB1B and FAB1D have a complementary role in the regulation of membrane recycling, vacuolar pH and homeostatic control of ROS in pollen tube growth (Serrazina et al. 2014).

Consistent with FAB1 being the PI(3,5)P2-synthesizing enzyme, we found that tagRFP–ML1N*2 was predominantly co-localized with FAB1A–GFP residing in endosomal structures (Fig. 1a–c) in the cortical cells in the root differentiation zone. In contrast, the punctate signals of tagRFP–ML1N*2 were rarely observed in the same cells in root elongation (Fig. 1d–f) and division (Fig. 1g–i) zones despite the presence of the FAB1A–GFP signal in these zones. Since the tagRFP–ML1N*2 signal was mainly detected in cortical cells of the root differentiation zone, we observed this region for further analyses.

**The TagRFP–ML1N*2 probe is predominantly localized on the FAB1- and SNX1-positive late endosomes in cortical cells in the root differentiation zone**

We next investigated the intracellular compartments in which ML1N*2 probes are localized. FAB1 is known to reside mostly in the late endosomes/multivesicular bodies (LEs/MVBs) in Arabidopsis (Hirano et al. 2015), and SNX1 is a sorting nexin protein that is also predominantly localized on LEs/MVBs, but not on the vacuoles or the early endosomes/trans-Golgi network in Arabidopsis (Jaillais et al. 2006). Consistent with this, in the tagRFP–ML1N*2- and GFP-tagged SNX1-co-expressing lines, the ML1N*2 probe is highly co-localized (83.8%) with SNX1–GFP in the cortical cells in the root differentiation zone of 5-day-old-seedlings (Fig. 2a–c). Notably, the Citrine-fused 2 × FYVEHRS marker (P18Y), which labels PI3P that is enriched in the late endosomes (Simon et al. 2014a), was partially merged (about 40.2%) with the punctate structures of tagRFP– ML1N*2 in the root differentiation zone (Fig. 2d–f). A brief (<1 h) treatment with a phosphatidylinositol 3-kinase inhibitor, wortmannin, is known to enlarge the late endosomes that are transiently transformed into donut-like structures, which disappeared upon prolonged incubation with wortmannin (Tse et al. 2004). Both tagRFP–ML1N*2 and SNX1–GFP are localized on these donut-like structures shortly after 33 μM wortmannin treatment (Fig. 2g–i). Most of the signals of a trans-Golgi
network marker, GFP–SYP43, were not merged with tagRFP–ML1N*2 punctate signals, but some parts of the two signals partially overlapped (Fig. 2j–l). Namely, the small punctate GFP–SYP43 signals were observed around the tagRFP–ML1N*2 fluorescent structures (Supplementary Fig. S2). Furthermore, another PI(3,5)P$_2$-labeling fluorescent protein probe, GFP–Raptor WD40 domain (GFP–WD40 Raptor) (Bridges et al. 2012) was transiently expressed in 5-day-old seedlings of tagRFP–ML1N*2-expressing lines (Marion et al. 2008), and was observed by confocal laser microscopy. We found that fluorescence of tagRFP–ML1N*2 was well co-localized with GFP–WD40Raptor (Supplementary Fig. S3).

Taken together, we concluded that the tagRFP–ML1N*2 probe efficiently labels PI(3,5)P$_2$ localized on the late endosomes, although we cannot rule out the possibility that tagRFP–ML1N*2 weakly binds other phosphoinositides or unknown molecules (see the Discussion).

**TagRFP–ML1N*2 can monitor rapid changes in PI(3,5)P$_2$ content in root cells**

To test the specificity of the tagRFP–ML1N*2 probe further, we acutely inhibited the activity of FAB1/PIKfyve using YM201636, a widely used specific inhibitor of FAB1/PIKfyve that can attenuate PI(3,5)P$_2$ production in both animals and plants (Jefferies et al. 2008, Hirano et al. 2015). We used this drug to examine whether the tagRFP–ML1N*2 probe could allow us to ‘monitor’ the decrease in the concentration of PI(3,5)P$_2$ in Arabidopsis cells. Using thin-layer chromatography (TLC) to measure the levels of phosphoinositides directly, we found that YM201636 treatment significantly reduced PI(3,5)P$_2$ (Fig. 7a) but not the levels of other phosphinositide (Supplementary Fig. S4). Remarkably, the number of tagRFP–ML1N*2-positive punctate structures decreased dramatically in cortical cells upon YM201636 treatment for 2.5 h (Fig. 3a, b, m; Supplementary Fig. S5). In contrast, the number of FAB1A-positive endosomes remains relatively constant during YM201636 treatment (Fig. 3d, e). Prior to YM201636 treatment, >80% of FAB1A-positive endosomes also contained tagRFP–ML1N*2 signals (Fig. 3g). However, after YM201636 treatment, the tagRFP–ML1N*2 signals were drastically reduced, and the co-localization index was reduced to only 10% (Fig. 3h). Upon washout of YM201636 for 5 h, the tagRFP–ML1N*2 signals were completely recovered to the normal level (Fig. 3c, m; Supplementary Fig. S5c, d).

PI(3,5)P$_2$ can also be depleted using wortmannin to inhibit the synthesis of PI3P, which is in turn required for PI(3,5)P$_2$ production (Whiteford et al. 1997). Indeed, in the 5-day-old seedlings that were treated with wortmannin (33 µM) for 2 h, the fluorescence...
Fig. 2 Co-localization of tagRFP–ML1N*2 with endosomal markers in the root cortical cells. Co-localization of tagRFP–ML1N*2 (a1, a2) with SNX1–GFP (b1, b2) in the cortical root cells of Arabidopsis lines expressing both tagRFP–ML1N*2 and SNX1–GFP. The numbers of merged puncta were counted from 10 images per sample for calculation of the percentage of merged puncta (c1, c2). Boxed regions in (a1–c1) are enlarged in (a2–c2). Localization of tagRFP–ML1N*2 with a late endosome marker, Citrine–2*FYVE (d–f). The numbers of merged puncta were counted from eight images per sample for calculation of the percentage of merged puncta (f). The tagRFP–ML1N*2 probe was co-localized with SNX1–GFP in the limited membranes of the enlarged vesicles shortly after (5 min) wortmannin treatment (g–i). Co-localization of tagRFP–ML1N*2 with GFP–SYP43 (j–l). Scale bars = 10 μm.
of the probe disappeared almost completely (Fig. 3j–l). YM201636, which did not affect the fluorescence of Citrine–2/C2FYVEHRS, only increased the fluorescence of tagRFP–ML1N*2. In contrast, wortmannin (33 μM) readily decreased the fluorescence of both tagRFP–ML1N*2 and Citrine–2/C2FYVEHRS signals (Fig. 4), suggesting that YM201636 specifically affects PI(3,5)P2 but not PI3P production. Collectively, these results have provided genetic evidence to support the specificity of the tagRFP–ML1N*2 probe binding to PI(3,5)P2.

We also investigated whether tagRFP–ML1N*2 is co-localized with a PI4P fluorescent protein marker, Citrine–1*PHFAPP1, because PI4P localizes to the Golgi apparatus, the early endosomes/trans-Golgi network, recycling endosomes and the plasma membrane (Simon et al. 2014). As shown in Fig. 5a–c, Citrine–1*PHFAPP1 and tagRFP–ML1N*2 were rarely co-localized on the endosomal structures, and treatment with the ARF-GEF inhibitor brefeldin A (BFA) produced large aggregated structures (so-called BFA compartments) of Citrine–1*PHFAPP1 but not tagRFP–ML1N*2, indicating that tagRFP–ML1N*2 is not localized to the Golgi apparatus, the early endosomes/trans-Golgi network or recycling endosomes.

Next, we examined whether the fluorescence of the probe could monitor the changes in the PI(3,5)P2 content when the expression of FAB1A/B is genetically manipulated. For this experiment, we have introduced tagRFP–ML1N*2 into FAB1A conditional overexpression lines, in which FAB1A overexpression was induced by the addition of estradiol (Supplementary Fig. S6a). Both the number and area of puncta of tagRFP–ML1N*2 were increased upon estradiol-induced overexpression of FAB1A (Fig. 6a–c).

Conversely, when the expression of FAB1A and FAB1B was reduced by estradiol-induced expression of artificial microRNA (amiRNA) specifically for FAB1A/B (Supplementary Fig. S6b), the punctate fluorescence of the probe was drastically decreased (Fig. 6d–f). Taken together, we concluded that the tagRFP–ML1N*2 probe can monitor rapid changes of PI(3,5)P2 content in Arabidopsis root cells.
ROS increase PI(3,5)P₂ levels in Arabidopsis root cells.

ROS, which include hydrogen peroxide (H₂O₂), superoxide anion (O₂⁻), singlet oxygen (¹O₂) and hydroxyl radical (OH⁻), are not only toxic to the cells, but are also involved in diverse signal transduction processes to regulate gene expression, enzyme activation, morphogenesis and programmed cell death (Suzuki and Mittler 2006, Suzuki et al. 2011). In Arabidopsis, salt stress and auxin robustly induce ROS production (Joo et al. 2001, Mittler et al. 2011). Given the established link between ROS production and phosphoinositide metabolism (Hee et al. 2005, Lee and Yang 2008), we investigated the

**Fig. 4** The fluorescence of Citrine–2*FYVE was decreased by wortmannin but not YM202636 treatments. Five-day-old seedlings of the Citrine–2*FYVE- (a, b, e, f) and tagRFP–ML1N*2- (c, d, e, f) co-expressing line were treated with 1 μM YM201636, and the images were taken after 0 h (a, c, e) and 2.5 h (b, d, f). The number of puncta was counted from 16 images (g, h) per sample. Five-day-old seedlings of the Citrine–2*FYVE- (i, j, m, n) and tagRFP–ML1N*2- (k, l, m, n) co-expressing line were treated with 33 μM wortmannin, and the images were taken after 0 h (i, k, m) or 2.5 h (j, l, n). The number of puncta was counted from 16 (o, p) per sample. Scale bars = 10 μm. The data were analyzed using a Mann–Whitney U-test (*P < 0.05) and are represented as box-and-whisker plots.
roles of ROS in the regulation of cellular PI(3,5)P₂ levels. Using TLC to measure the levels of phosphoinositides directly, we found that H₂O₂ induced a robust elevation of PI(3,5)P₂ (Fig. 6a) and PIPs (PI3P and PI4P) (Supplementary Fig. S4a), but not PI(4,5)P₂ (Supplementary Fig. S4b). These results suggest that ROS are a physiological regulator of PI(3,5)P₂ levels.

Under non-oxidative stress conditions, few punctate tagRFP–ML1N*2 signals were detected in cortical cells of the root division zone (Fig. 7b, d). Consistent with the TLC measurement, in the cortical cells of the root tip region, H₂O₂ treatment (10 mM for 30 min) dramatically increased the number of tagRFP–ML1N*2-positive punctate structures in the root division zone (Fig. 7c, d). Likewise, in the root differentiation zone, the fluorescent signals of tagRFP–ML1N*2 were also increased by H₂O₂ treatment (Fig. 7e–g), although in the negative controls, the wild type and tagRFP-expressing lines, H₂O₂ treatment failed to affect the fluorescence intensities (Supplementary Fig. S7). The tagRFP–ML1N*2 signals were co-localized with FAB1A–GFP after the H₂O₂ treatment (Fig. 8a–f).

In the FAB1A conditional overexpression line, the effects of H₂O₂ on the enhancement of the probes was further increased not only in the control and but also in H₂O₂-stimulated conditions (Supplementary Fig. S7). No increase in autofluorescence in the wild type (Supplementary Fig. S8a, b) or transgenic Arabidopsis plants expressing tagRFP (Supplementary Fig. S8c, d) was observed under the oxidative stress condition. These results suggest that ROS are a physiological stimulator of PIKfyve/FAB1, and that tagRFP–ML1N*2 probes can be employed to detect ROS-dependent PI(3,5)P₂ changes in Arabidopsis root cells.

**Discussion**

We have generated transgenic Arabidopsis lines that express tagRFP–ML1N*2, in which the fluorescent probe is localized to the FAB1/SNX1-positive LEs/MVBs in cortical cells in the root differentiation zone. Consistent with the TLC-mediated direct measurement of PI(3,5)P₂ levels, the punctate signals of the probe were almost completely abolished by the YM201636 treatment or FAB1A/B conditional knockdown, but increased by FAB1A conditional overexpression. Hence the fluorescent protein-tagged ML1N*2 probes could be utilized to monitor the PI(3,5)P₂ dynamics in Arabidopsis root cells.

The results that we obtained from the transgenic Arabidopsis suggest that the probe specifically binds PI(3,5)P₂, at least in Arabidopsis root cells. In the original study that reported the development of the ML1N*2 probe, as well as in several follow-up studies, the ML1N*2 probe appeared to label PI(3,5)P₂ predominantly on the late endosomes as well as lysosomes in a number of mammalian cells: CHO, COS1, NIH3T3 and HEK293 cells (Li et al. 2013), rat hippocampal neurons (McCartney et al. 2014b) and HeLa cells (Takatori et al. 2015, Guscott et al. 2016). However, upon YM201636 treatment, the probe disappeared from the Lamp1-positive compartments rather slowly (>3 h and up to 24 h) (Li et al. 2013). It is possible that in ML1N*2-expressing cells, PI(3,5)P₂ might be slowly sequestered from Lamp1-positive compartments by YM201636 inhibition. Alternatively, the probe might detect something else, especially when the PI(3,5)P₂ level is much reduced, so that the probe might migrate to the low-affinity sites. Furthermore, in yeast cells, the probe detects unknown molecules at the plasma membranes, suggesting that the specificity of the probe could be compromised in certain cell types (Li et al. 2013). Recently, Hammond et al. (2015) reported that the ML1N*2 probe showed low selectivity for PI(3,5)P₂, and the subcellular localization of the probe is largely independent of PI(3,5)P₂ in COS-7 cells. Although the reasons for the discrepancy among these reports is unknown, the probe might bind non-specifically to certain molecules (like most probes for

![Fig. 5](https://example.com/fig5.png)

**Fig. 5** The fluorescence of tagRFP–ML1N*2 was not sensitive to brefeldin A treatment. Five-day-old seedlings of the Citrine–1*PH FAPP- and tagRFP–ML1N*2-co-expressing line were treated with 50 μM BFA, and the images were taken after 0 h (a–c) and 1 h (e–f). Scale bars = 10 μm.
phosphoinositides) in specific mammalian cell types, especially when the PI(3,5)P2 level is reduced, hence exposing low-affinity sites.

In the experimental conditions of the current study in Arabidopsis cells, the ML1N*2 probe seems to bind PI(3,5)P2 specifically on the FAB1/SNX1-positive late endosomes without significant non-specific binding to other organelle membranes. It is possible that such low-affinity sites might be lacking or exist at a low expression level in Arabidopsis cells because any binding proteins of the cytosolic phosphoinositide-interacting domain (ML1N) of TRPML1 channels do not exist in Arabidopsis cells. It is also likely that when the probe is stably expressed in the transgenic lines, as opposed to transient expression studies, the probes behave much better.

In this study, we found that the punctate fluorescence of tagRFP–ML1N*2 was never observed in epidermal cells of the root elongation and division zone, despite the presence of GFP–FAB1A fluorescence in these regions in normal growth conditions. The fluorescence of tagRFP–ML1N*2 was first detected in cortical cells of the root differentiation zone but not in the root division zone in normal growth conditions. However, the punctate fluorescence of tagRFP–ML1N*2 appeared in cortical cells of root division and elongation zones after H2O2 treatment. Taken together, these results suggest that PI(3,5)P2 is synthesized by FAB1 after root cells are differentiated, or PI(3,5)P2 synthesis is enhanced under oxidative stress conditions.

Unexpectedly, we also found that no tagRFP–ML1N*2 signal was observed on the vacuolar membrane, suggesting that PI(3,5)P2 is not present on the vacuolar membrane in Arabidopsis root cells. This result is in a sharp contrast to the studies on mammalian cells and yeast, because PI(3,5)P2 is known to be localized to the lysosomal and the vacuolar membranes (Li et al. 2013, Takatori et al. 2015). In Arabidopsis, it was recently reported that phosphoinositide-phosphatases, the suppressor of actin proteins (SAC2, 3, 4 and 5), are localized on the vacuolar membrane. Indeed, the PI(3,5)P2 content is increased in the sac3sac4sac5 triple mutant, but decreased in SAC2 or SAC5 overexpression lines (Nováková et al. 2014). These data suggest that PI(3,5)P2 on the vacuolar membrane is rapidly converted to PI3P or PI5P by vacuolar-localized phosphoinositide-specific phosphatases in Arabidopsis. In fact, a PI3P marker, yellow fluorescent protein (YFP)–2*FYVE strongly labels the vacuolar membrane in leaf epidermal and guard cells in Arabidopsis (Vermeer et al. 2006). Of course, we cannot completely rule out the possibility that PI(3,5)P2 exists on the vacuolar membrane below the level of detection of the probe in Arabidopsis.

Based on the reasons mentioned above, the tandem ML1N*2-based PI(3,5)P2 probe might provide a unique advantage and sensitivity to study PI(3,5)P2 dynamics in plant cells. Using this probe, we discovered a new regulator of PI(3,5)P2 dynamics: ROS. In this study, we demonstrated that the fluorescent signals of tagRFP–ML1N*2 on the FAB1A-positive LEs/
Fig. 7 PI(3,5)P₂ levels were elevated in oxidative stress conditions. TLC measurement of PI(3,5)P₂ content in the wild type after H₂O₂ treatment in the presence or absence of YM201636. Five-day-old seedlings were labeled for 3 h with ³²P, after which they were further incubated for 30 min in the presence or absence of 1 µM YM201636 and following 10 min incubation with or without 10 mM H₂O₂. After extraction, lipids were separated by TLC and quantified by PhosphoImaging. Data are expressed as percentages of total [³²P]phospholipids. Significant differences between seedlings treated or not with YM201636 are indicated by an asterisk (Student t-test, P < 0.001). Data represent the mean ± SD. Experiments were repeated three times, and gave similar results (a). Five-day-old seedlings of the tagRFP–ML1N*2-expressing lines were treated with water (b, e) or 10 mM H₂O₂ (c, f) for 0.5 h, and then were observed by confocal microscopy. Relative fluorescence intensities or the size of fluorescent images of tagRFP–ML1N*2 were measured using six images per sample. Scale bars = 10 µm. The data were represented as box-and-whisker plots. An asterisk represents a significant difference evaluated by a Mann–Whitney U-test (P < 0.05) (d, g).

Materials and Methods

Generation of the transgenic Arabidopsis lines expressing tagRFP–ML1N*2

The codon-optimized DNA sequence encoding the tandem ML1N fragment (Li et al. 2013) was chemically synthesized (Eurofins Genomics). The synthesized DNA was introduced into the pENTR Gateway entry vector (Thermo Fisher Scientific), then the construct was cloned into pGWBS61 (Nakagawa et al. 2007) by LR cloning to generate C-terminal translational fusions with tagRFP (Evrogen).

After confirmation of the sequences, transgenic Arabidopsis expressing tagRFP–ML1N*2 were generated by Agrobacterium-mediated floral dip transformation (Clough and Bent 1998). Positive transformants were selected based on their hygromycin resistance. Homozygous T₃ plants were used for all analyses in the current study. The transgenic Arabidopsis plants co-expressing tagRFP–ML1N*2 and Citrine–2*FYVE or tagRFP–ML1N*2 and GFP–SYP43 were generated by cross-pollination. Homozygous mutant plants were then selected from the F₃ segregating population based on genotyping and marker fluorescence. All seeds were germinated on half-strength Murashige and Skoog (1/2 MS) medium. All experiments were performed with at least three independent biological replicates.

Generation of the GFP–WD40Raptor construct and Agrobacterium-mediated transient expression in Arabidopsis seedlings

The DNA fragment encoding the human Raptor WD40 domain was amplified by PCR using following primers from human Raptor cDNA: Raptor WD40 F, 5'-ttggacgaccaaatatttctgaacaggaacc-3’; and Raptor WD40 R, 5'-ttctga-cagcttcatcctccagggacag-3’. The amplified DNA fragments were cloned into the pENTR entry vector (Thermo Fisher Scientific), and then transferred to pGWBS61 (Nakagawa et al. 2007) by LR cloning to generate the C-terminal translational fusion with xGFP according to the manufacturer’s instruction (Thermo Fisher Scientific). GFP–WD40Raptor was transiently expressed in the transgenic Arabidopsis seedlings expressing tagRFP–ML1N*2 according to Marion et al. (2008).

Plant growth conditions and inhibitor treatments

Arabidopsis thaliana ecotype Columbia was used as the wild type in all experiments. Plants were grown under white light with a 16 h/8 h (light/dark) photoperiod at 22 °C. Generation of the estradiol-inducible FAB1A/B amiRNA line was described previously (Hirano et al. 2011). Transgenic marker lines in the FAB1A/B amiRNA, FAB1A–GFP (Hirano et al. 2011), SNX1–GFP (Jaillais et al. 2006), Citrine–2*FYVE (Jaillais et al. 2014) and Citrine–1*PH FAPP backgrounds (Jaillais et al. 2014) were generated by cross-pollination. Arabidopsis seeds were surface sterilized and germinated on 1/2 MS agar plates. For observation of root cells, plants were grown under long-day conditions for 5 d. For the oxidative stress treatment, Arabidopsis seedlings were subjected to 10 mM H₂O₂ for 30 min prior to harvesting of the whole seedlings before confocal microscopic observation. Five-day-old seedlings were treated for 2 h in water containing 1 µM YM201636 (Cayman) or dimethylsulfoxide as a mock control.

Phosphoinositide analysis

Polyphosphoinositide levels were measured using the TLC assay according to Munnik and Zarza (2013). Briefly, radioactive PIP₃ levels were measured by labeling 5-day-old seedlings (three per tube in triplicate) of the wild type with 370 kBq of carrier-free [³²P]orthophosphate for 3 h and then seedlings were treated with or without 1 µM YM201636 for 3 min. Five-day-old
seedlings were labeled for 3 h with $^{32}$Pi, after which they were further incubated for 30 min in the presence or absence of 1 μM 1M201636 and following 10 min incubation with or without 10 mM H$_2$O$_2$. Afterwards, the lipids were extracted, separated by TLC (Monks et al. 2001) and quantified by PhosphoImaging.

**Confocal microscopy and image analysis**

TagRFP, GFP and Citrine fluorescence and differential interference contrast (DIC) images were obtained using a Nikon ECLIPSE E600 laser scanning microscope equipped with a ×60 oil immersion lens (Nikon) or a Zeiss LSM780 confocal microscope equipped with a ×63 water immersion lens (Zeiss). The captured images were processed using Nikon EZ-C1 software or Zeiss ZEN 2012 blue software, and were analyzed by Image J software. The analyzed image data are represented as box-and-whisker plots. Boxes and solid lines in the boxes show the upper (75th) and lower (25th) quartiles and median values, respectively. Whiskers indicate the 95% confidence intervals.

**Quantitative RT-PCR**

Total RNA was extracted from transgenic lines by using the RNeasy kit (QIAGEN) following the manufacturer’s instructions. For cDNA synthesis, 1 μg of total RNA was reverse transcribed using ReverTra Ace qPCR RT Master Mix (TOYOBO). Primer pairs used in the quantitative RT-PCR were as follows: FAB1A-F (5′-AGCCGACATACAATTAAAGTGGAG-3′) and FAB1A-R (5′-AAACAACTCCTTTCAC-3′) for FAB1A; and FAB1B-F (5′-TGAGACTAATAGGATGAAGC-3′) and FAB1B-R (5′-ATCATCATTGACCTAAAC-3′) for FAB1B; and UBQ10-F (5′-GGTGTGAGTGTAATGCAGTAA-3′) and UBQ10-R (5′-AGAGATAACAGGAACG-3′) for UBQ10.

**Author contributions**

T.H., K.S. and T.M. performed the experiments and analyzed the data; M.H.S., H.X. and T.M. designed the experiments and supervised the work; M.H.S., H.X., T.M. and T.H. wrote the paper.

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**Disclosures**

The authors have no conflicts of interest to declare.

**References**


