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# **iDePP: a genetically encoded system for the inducible depletion of PI(4,5)P<sub>2</sub> in *Arabidopsis thaliana*.**

Mehdi Doumane<sup>1</sup>, Léia Colin<sup>1</sup>, Alexis Lebecq<sup>1</sup>, Aurélie Fangain<sup>1</sup>, Joseph Bareille<sup>1</sup>, Olivier Hamant<sup>1</sup>, Youssef Belkhadir<sup>2</sup>, Yvon Jaillais<sup>1\*</sup>, Marie-Cécile Caillaud<sup>1\*</sup>.

1. Laboratoire Reproduction et Développement des Plantes (RDP), Université de Lyon, ENS de Lyon, UCB Lyon 1, CNRS, INRAE, F-69342 Lyon, France.

2. Gregor Mendel Institute (GMI), Austrian Academy of Sciences, Vienna Biocenter (VBC), Dr. Bohr-Gasse 3, 1030 Vienna Austria.

\* Corresponding authors: [yvon.jaillais@ens-lyon.fr](mailto:yvon.jaillais@ens-lyon.fr), [marie-cécile.caillaud@ens-lyon.fr](mailto:marie-cécile.caillaud@ens-lyon.fr).

## ***ABSTRACT***

Phosphatidylinositol 4,5-bisphosphate [PI(4,5)P<sub>2</sub>] is a low abundant lipid present at the plasma membrane of eukaryotic cells. Extensive studies in animal cells revealed the pleiotropic functions of PI(4,5)P<sub>2</sub>. In plant cells, PI(4,5)P<sub>2</sub> is involved in various cellular processes including the regulation of cell polarity and tip growth, clathrin-mediated endocytosis, polar auxin transport, actin dynamics or membrane-contact sites. To date, most studies investigating the role of PI(4,5)P<sub>2</sub> in plants have relied on mutants lacking enzymes responsible for PI(4,5)P<sub>2</sub> synthesis and degradation. However, such genetic perturbations only allow steady-state analysis of plants undergoing their life cycle in PI(4,5)P<sub>2</sub> deficient conditions and the corresponding mutants are likely to induce a range of non-causal (untargeted) effects driven by compensatory mechanisms. In addition, there are no small molecule inhibitors that are available in plants to specifically block the production of this lipid. Thus, there is currently no system to fine tune PI(4,5)P<sub>2</sub> content in plant cells. Here we report a genetically encoded and inducible synthetic system, iDePP (Inducible Depletion of PI(4,5)P<sub>2</sub> in Plants), that efficiently removes PI(4,5)P<sub>2</sub> from the plasma membrane in different organs of *Arabidopsis thaliana*, including root meristem, root hair and shoot apical meristem. We show that iDePP allows the inducible depletion of PI(4,5)P<sub>2</sub> in less than three hours. Using this strategy, we reveal that PI(4,5)P<sub>2</sub> is critical for cortical microtubule organization. Together, we propose that iDePP is a simple and efficient genetic tool to test the importance of PI(4,5)P<sub>2</sub> in given cellular or developmental responses but also to evaluate the importance of this lipid in protein localization.

**Research Organism:** *A. thaliana*

## INTRODUCTION

1  
2 Phosphoinositides are “tiny lipids with giant impacts” on cell biology (Balla, 2013). These low  
3 abundant membrane lipids are derived from phosphatidylinositol. Phosphatidylinositol’s head can be  
4 phosphorylated on positions 3 and/or 4 and/or 5, leading to the synthesis of up to seven different  
5 phosphoinositide species. Detection of phosphoinositides revealed that each species is differentially  
6 enriched in the various membranes of eukaryotic cells (Platre and Jaillais, 2016), constituting a landmark  
7 code that allows specific targeting of proteins to given membranes (Bigay and Antonny, 2012; Simon  
8 et al., 2016). In particular, phosphatidylinositol 4,5-bisphosphate [PI(4,5)P<sub>2</sub>] is mainly present in the  
9 cytosolic leaflet of the plasma membrane in wild-type cells of *Saccharomyces cerevisiae* (Eumycota  
10 Ascomycota; Patton and Lester, 1992), human (Metazoa Mammal; Hammond et al., 2009, 2012),  
11 *Drosophila melanogaster* (Metazoa Hexapoda; El Kadhi et al., 2011), and *Arabidopsis thaliana*  
12 (Archaeplastida Angiosperm; Simon et al., 2014; Van Leeuwen et al., 2007). Pharmacological and  
13 genetically encoded systems allowing to exogenously modulate PI(4,5)P<sub>2</sub> levels in animal cells led to  
14 significant advances toward the comprehensive understanding of PI(4,5)P<sub>2</sub> functions (Idevall-Hagren  
15 and De Camilli, 2015). While phosphoinositide manipulation via inhibitors and/or activators of enzymes  
16 involved in phosphoinositide metabolism is easily achievable in vitro, the use of these drugs in cell  
17 cultures is often constrained by the permeability of the plasma membrane which in turn limits their  
18 activities to narrow ranges of concentrations. Thus, the use of such molecules is even less tractable on  
19 pluricellular organisms. Second, drugs often have unknown off-target effects that affect cellular  
20 processes distinct from phosphoinositide metabolism. With these limitations in mind, the  
21 pharmacological targeting of phosphoinositide kinases, phosphatases and phospholipases is nevertheless  
22 a valuable tool to study, relatively fast, the effects of phosphoinositide levels modulation. Genetically-  
23 encoded fast and reversible systems are privileged for the study of the role of the phosphoinositide in  
24 animal cell culture, however there is still a striking gap between state-of-the-art acute phosphoinositide  
25 manipulation carried out in cultured cells and those carried out in pluricellular organisms.

26 At the plasma membrane of animal cells, PI(4,5)P<sub>2</sub> has a wide spectra of actions. It is for example a  
27 precursor for second messengers such as inositol 1,4,5-trisphosphate (IP<sub>3</sub>), diacylglycerol (DAG) and  
28 PI(3,4,5)P<sub>3</sub> secondary messengers (Antal and Newton, 2013). PI(4,5)P<sub>2</sub> can be seen as a second  
29 messenger itself, since it recruits a host of proteins to the plasma membrane in a highly controlled and  
30 regulated manner (Rajala and Anderson, 2010). It can also activate ion channels, and it regulates many  
31 cellular processes including clathrin-mediated endocytosis, exocytosis, cell polarity, membrane contact  
32 sites and actin cytoskeleton remodeling (Hammond et al., 2012; Schmid and Mettlen, 2013; Yin and  
33 Janmey, 2003). Typically, PI(4,5)P<sub>2</sub> is known to promote the formation of actin filament structures—  
34 beneath the plasma membrane by inhibiting or activating distinct sets of proteins (Senju et al., 2017).  
35 Together with phosphatidylinositol 4-phosphate (PI4P), PI(4,5)P<sub>2</sub> also contributes to plasma membrane

36 surface charge (Hammond et al., 2012), a basic property of the plasma membrane, which recruits many  
37 proteins through electrostatic interaction (Platre and Jaillais, 2017).

38

39 In plant cells, less is known about PI(4,5)P<sub>2</sub>. Intriguingly, it seems that some functions of PI(4,5)P<sub>2</sub>  
40 in animals are not conserved in plants. For example, plasma membrane surface charges relies on PI4P,  
41 phosphatidylserine (PS) and phosphatidic acid (PA), but not PI(4,5)P<sub>2</sub> (Simon et al., 2016a; Platre and  
42 Jaillais, 2017). Yet, mutants in PI4P 5-kinases (PIP5K) suggest that PI(4,5)P<sub>2</sub> production is essential  
43 and has critical roles in development, immunity and reproduction (Heilmann, 2016; Noack and Jaillais,  
44 2017a). However, i) the synthesis of each phosphoinositide species is tied to each other and to other  
45 lipids, ii) PI(4,5)P<sub>2</sub> has pleiotropic cellular functions and iii) PI(4,5)P<sub>2</sub> interacts with many different  
46 proteins. Thus, it is likely that steady-state depletion of PI(4,5)P<sub>2</sub> triggers a range of indirect effects  
47 involving several types cellular and developmental compensatory mechanisms. It is therefore essential  
48 to manipulate the levels of this lipid in a rapid manner if we want to untangle direct and indirect functions  
49 of PI(4,5)P<sub>2</sub>. However, there are limited tools available for researcher to modulate PI(4,5)P<sub>2</sub> levels in  
50 plant cells and organs in an inducible way. To date, most of the available tools allow to increase the  
51 amount of PI(4,5)P<sub>2</sub>. This includes U-73122, a pan-phospholipases C (PLC) inhibitor (Barbosa et al.,  
52 2016; Lee et al., 2019; Van Leeuwen et al., 2007). However, because phosphoinositide-specific PLCs  
53 (PI-PLC) use both PI(4,5)P<sub>2</sub> and PI4P as substrates in plants, such treatment is likely to also impact the  
54 levels of both lipids (Delage et al., 2012). Another method is the exogenous treatment to membrane  
55 permeable PI(4,5)P<sub>2</sub> analogues (Ozaki et al., 2000). However, the stability of these lipids is questionable  
56 given that phospholipids tend to be degraded into DAG upon exogenous treatment (Grabski et al., 1993).  
57 Finally, inducible expression of PIP5Ks efficiently increase the level of PI(4,5)P<sub>2</sub> in plants (Gujas et al.,  
58 2017a; Ischebeck et al., 2013). Yet, to date, a strategy that allows to specifically and inducibly deplete  
59 PI(4,5)P<sub>2</sub> in plants remains to be implemented. Indeed, long treatment with the PI4K inhibitor  
60 phenylarsine oxide (PAO) reduces both PI(4,5)P<sub>2</sub> and PI4P levels (Platre et al., 2018; Simon et al., 2016)  
61 and there is no PIP5K inhibitor available (Kusano et al., 2008; Mei et al., 2012; Menzel et al., 2019;  
62 Stanislas et al., 2018).

63 Here, we built a system for the inducible depletion PI(4,5)P<sub>2</sub> in plants, that we coined iDePP  
64 (Inducible Depletion of PI(4,5)P<sub>2</sub> in Plants). iDePP is a dexamethasone inducible genetically encoded  
65 system that allows fast and specific PI(4,5)P<sub>2</sub> depletion in an entire organism, the model plant  
66 *Arabidopsis*. This system triggers the expression of a plasma membrane anchored PI(4,5)P<sub>2</sub> 5-  
67 phosphatase domain from the *Drosophila melanogaster* OCRL upon dexamethasone treatment. Using  
68 iDePP, we detected a strong and specific PI(4,5)P<sub>2</sub> depletion starting as soon as 90 min after induction.  
69 We show that the iDePP system is suitable to study the function of PI(4,5)P<sub>2</sub> in both above and below-  
70 ground plant in *Arabidopsis*. Finally, we use iDePP to provide evidence that the appropriate cortical-  
71 microtubule orientation requires PI(4,5)P<sub>2</sub> in the *Arabidopsis* root epidermis.

72

## 73 **RESULTS**

### 74 **dOCRL phosphatase domain acts as a PI(4,5)P<sub>2</sub> phosphatase *in vitro***

75 In cultured mammalian cells, an inducible system for the depletion PI(4,5)P<sub>2</sub> was previously design  
76 (Idevall-Hagren et al., 2012). They used a truncation (amino acids 234 to 539) of human OCRL  
77 (OCRL1) corresponding to its PI(4,5)P<sub>2</sub> 5-phosphatase domain. We were concerned that this domain,  
78 that underwent natural selection in an endotherm organism with set-point temperature of 37°C, would  
79 be less active in *Arabidopsis thaliana*, a plant of temperate climate which we usually grow at 21°C.  
80 Therefore, we decided to use the phosphatase domain of dOCRL, *Drosophila melanogaster* homolog,  
81 as *Drosophila* are ectotherm and live at ~ 20°C. Alignment of OCRL1<sub>234-539</sub> and dOCRL allowed us to  
82 identify dOCRL<sub>168-509</sub> as the homolog region of OCRL1 generally used in optogenetic systems (Figure  
83 1A). We first assessed dOCRL activity, which has not been reported so far, even though *in vivo* data  
84 suggest a conserved PI(4,5)P<sub>2</sub> 5-phosphatase activity (Carim et al., 2019; Ben El Kadhi et al., 2011; Del  
85 Signore et al., 2017). We ordered a synthetic gene corresponding to dOCRL<sub>168-509</sub> and codon optimized  
86 for expression in *Arabidopsis*. We cloned a recombinant dOCRL protein (His-SUMO-dOCRL<sub>168-509</sub>-  
87 His) tagged with two 6-histidine tags and a SUMO tag, making it suitable for expression in *Escherichia*  
88 *coli* and purification. We purified His-SUMO-dOCRL<sub>168-509</sub>-His using a cobalt resin-based affinity  
89 chromatography and protein concentration (Figure 1B). We subjected the purified protein fractions C1  
90 and C2 to Malachite Green Phosphatase assays, that detects phosphate released by dephosphorylations,  
91 in presence of short chain (diC:8) water-soluble phosphoinositides (Maehama et al., 2000). Note that  
92 we only assessed dOCRL<sub>168-509</sub> activity toward phosphoinositides reported in plant cells (Platre and  
93 Jaillais, 2016; Simon et al., 2014). We observed that dOCRL<sub>168-509</sub> displayed a strong and specific  
94 phosphatase activity toward PI(4,5)P<sub>2</sub> (Figure 1C), demonstrating that dOCRL activity is conserved  
95 between Mammals and *Drosophila*. Therefore, dOCRL<sub>168-509</sub> is a domain suitable for specific PI(4,5)P<sub>2</sub>  
96 depletion at room temperature (~ 20°C).

97

### 98 **Inducible expression and subcellular targeting of dOCRL**

99 After validating *in vitro* the enzymatic activity of the catalytic domain of dOCRL<sub>168-509</sub>, we next  
100 addressed whether this enzyme could efficiently hydrolyze PI(4,5)P<sub>2</sub> in plant cell when targeted to the  
101 plasma membrane. We fused the codon optimized phosphatase domain (here after named dOCRL) to a  
102 Myristoylation And Palmitoylation (MAP) sequence, which is responsible for membrane targeting  
103 (Martinière et al., 2012) and to mCHERRY (mCH) fluorescent protein to monitor protein expression  
104 and subcellular localization of MAP-mCHERRY-dOCRL (Figure 2A). In order to observe short term  
105 effects of PI(4,5)P<sub>2</sub> depletion, we took advantage of dexamethasone (dex) - GVG system that allows  
106 efficient inducible expression of proteins in *Arabidopsis* (Aoyama and Chua, 1997; Marquès-Bueno et  
107 al., 2011; Moreno-Romero et al., 2008). We next performed site-directed D468G mutation in dOCRL

108 catalytic domain, similarly to D523G mutation already described in human OCRL1 that abolishes its  
109 phosphatase activity (Idevall-Hagren et al., 2012). We could therefore use MAP-mCH-dOCRL<sub>168-</sub>  
110 <sub>159</sub><sup>D468G</sup> (hereafter named MAP-mCH-dOCRL<sup>dead</sup>) as negative control, together with a MAP-3xmCH  
111 recombinant protein (Figure 2A).

112 We next addressed the timing of expression and the localization of each construct in *Arabidopsis*  
113 lines stably transformed with *UBQ10pro:GVG:MAP-mCH-dOCRL*, or *UBQ10pro:GVG:MAP-mCH-*  
114 *dOCRL<sup>dead</sup>*, or *UBQ10pro:GVG:MAP-3xmCH*. In root meristematic epidermal cells, without dex  
115 treatment, none of the recombinant-proteins were detected using confocal microscopy (Figure 2B). A  
116 16 h treatment with 0.5  $\mu$ M dex led to the detection of mCH fluorescence, indicating that induction of  
117 the genetic construct had occurred (Figure 2B). However, we observed a mosaic induction and a  
118 significant number of roots had no cells expressing the fluorescent reporter to detectable levels. To  
119 overcome these issues, we optimized the treatment to a 16 h induction with 5  $\mu$ M dex. Using this set up,  
120 we robustly observed red fluorescence using confocal microscopy where MAP-mCH-dOCRL, MAP-  
121 mCH-dOCRL<sup>dead</sup> and MAP-3xmCH (Figure 2B). Each of the synthetic protein was efficiently targeted  
122 to membranes, including the plasma membrane, where PI(4,5)P<sub>2</sub> accumulates, and intracellular  
123 compartments (Figure 2B). Therefore, a 16h 5 $\mu$ M dex treatment is sufficient for an effective expression  
124 of dOCRL in *Arabidopsis* stable transgenic lines.

125

## 126 **Inducible expression of MAP-mCH-dOCRL specifically and efficiently** 127 **depletes PI(4,5)P<sub>2</sub> in stable *Arabidopsis* lines**

128 Next, we tested if the targeting of dOCRL at the plasma membrane was actively depleting PI(4,5)P<sub>2</sub>.  
129 For this, we monitored the localization of genetically encoded fluorescent biosensors, made of a lipid-  
130 binding domain (LBD) fused to a fluorescent protein such as mCITRINE (mCIT), which allow  
131 monitoring lipid subcellular localization and levels, using confocal microscopy (Colin and Jaillais,  
132 2020). We previously reported that upon phosphatidylinositol 4-phosphate (PI4P) and  
133 phosphatidylserine (PS) depletion from the plasma membrane, PI4P and PS biosensors are partially or  
134 totally released from the plasma membrane, and are found in the cytosol, their default localization in the  
135 absence of a cognate lipid (Platre et al., 2018; Simon et al., 2016). Consistent with this idea, we observed  
136 a partial release of the mCIT-2xPH<sup>PLC</sup> PI(4,5)P<sub>2</sub> biosensor from the plasma membrane into the cytosol  
137 upon long-term treatment with PAO, a known inhibitor of PI4P and PI(4,5)P<sub>2</sub> synthesis (Simon et al.,  
138 2016). We thus predicted that the PI(4,5)P<sub>2</sub> biosensors would behave similarly upon MAP-mCH-  
139 dOCRL expression.

140 To test this, we generated transgenic lines stably expressing various mCITRINE-tagged anionic lipid  
141 sensors with the *MAP-mCH-dOCRL* line. First, we studied the impact of MAP-mCH-dOCRL induction  
142 on the localization of the high affinity mCIT-2xPH<sup>PLC</sup> PI(4,5)P<sub>2</sub> biosensors (Simon et al., 2014), in  
143 *Arabidopsis* meristematic cells. In the absence of dex, MAP-mCH-dOCRL was not expressed, and only

144 background signal was observed (Figure 3A, left panel), while mCIT-2xPH<sup>PLC</sup> mainly localized to the  
145 plasma membrane and was only weakly found in the cytosol (Figure 3A, right panel). 16h of dex  
146 treatment resulted in the expression of MAP-mCH-dOCRL and the full solubilization of mCIT-2xPH<sup>PLC</sup>  
147 (Figure 3B and G, dissociation indexes of ~ 2, Supplementary file 1-3), indicating an efficient depletion  
148 of PI(4,5)P<sub>2</sub> from the plasma membrane. Next, we crossed the mCIT-2xPH<sup>PLC</sup> reporter line with the  
149 MAP-mCH-dOCRL<sup>dead</sup> and MAP-3xmCH control lines and tested the impact of the induction of these  
150 controls on the plasma membrane targeting of the PI(4,5)P<sub>2</sub> reporter. In each case, the localization of  
151 the mCIT-2xPH<sup>PLC</sup> biosensor was not affected and remained at the plasma membrane either in the  
152 absence or presence of dex induction (dissociation indexes of ~ 1 and Figure 3 C, 3G Supplementary  
153 file 1-3).

154 To confirm the results obtained with mCIT-2xPH<sup>PLC</sup>, we crossed the *MAP-mCH-dOCRL* expressing  
155 line with the *mCIT-TUBBY-C* line, which expresses an independent PI(4,5)P<sub>2</sub> biosensor (Simon et al.,  
156 2014). As previously described, mCIT-TUBBY-C localized to the plasma membrane, the cytosol and  
157 the nucleoplasm in the absence of dex induction (Figure 3H). However, similar to the mCIT-2xPH<sup>PLC</sup>  
158 biosensor, mCIT-TUBBY-C was removed from the plasma membrane upon 16 hours induction of *MAP-*  
159 *mCH-dOCRL* expression (Figure 3G and I, Supplementary file 1-3).

160

161 We next tested whether the inducible expression of *MAP-mCH-dOCRL* would affect the subcellular  
162 accumulation of other anionic lipids. To this end, we first crossed the *MAP-mCH-dOCRL* expressing  
163 line with mCIT-P4M<sup>SidM</sup>, which reports on plasma membrane PI4P (Simon et al., 2016). We observed  
164 that mCIT-P4M<sup>SidM</sup> had the same localization in the absence or presence of *MAP-mCH-dOCRL*  
165 expression (dissociation index ~ 1 and Figure 3G, J and K, Supplementary file 1-3). Next, we crossed  
166 *MAP-mCH-dOCRL* with the mCIT-2xFYVE<sup>HRS</sup> reporter line, which express a PI3P biosensors targeted  
167 to late endosomes and the tonoplast (Simon et al., 2014). We confirmed that in the absence of dex,  
168 mCIT-2xFYVE<sup>HRS</sup> was localized in intracellular compartments and weakly at the tonoplast (Figure 3L)  
169 and that we obtained a similar localization pattern upon dex treatment and induction of MAP-mCH-  
170 dOCRL (Figure 3M).

171 Finally, we crossed *MAP-mCH-dOCRL* with a reporter line expressing the phosphatidylserine  
172 biosensor mCIT-C2<sup>Lact</sup> (Platre et al., 2018). mCIT-C2<sup>Lact</sup> accumulates at the plasma membrane and all  
173 along the endocytic pathways (Platre et al., 2018), and this pattern appeared to be conserved in both the  
174 absence or presence of dex (Figure 3 N and O, Supplementary file 1-3). However, the dissociation index  
175 of mCIT-C2<sup>Lact</sup> upon expression of *MAP-mCH-dOCRL* was slightly but significantly higher than upon  
176 expression of the 3xmCH control (dissociation index= of 0.957 versus 0.815, p-value = 0.043 and Figure  
177 3G; Supplementary file 1-3), suggesting a possible impact of the depletion of PI(4,5)P<sub>2</sub> on the  
178 homeostasis of phosphatidylserine.

179 Overall, our results show that MAP-mCH-dOCRL efficiently, specifically, and directly hydrolyzes  
180 the PI(4,5)P<sub>2</sub> pool at the plasma membrane of plant cells. We named this synthetic genetic system iDePP  
181 for Inducible Depletion of PI(4,5)P<sub>2</sub> in Plants.

182

### 183 **iDePP quickly depletes PI(4,5)P<sub>2</sub> *in vivo***

184 To challenge the ability of MAP-mCH-dOCRL to rapidly deplete PI(4,5)P<sub>2</sub>, we performed a dual  
185 color time-course analysis during dex induction, monitoring mCIT-2xPH<sup>PLC</sup> PI(4,5)P<sub>2</sub> biosensor  
186 subcellular localization and MAP-mCH-dOCRL appearance at the root tip in epidermal cells. We used  
187 an automated root tracking set-up we previously developed (Doumane et al., 2017) and imaged root  
188 epidermal cells every five minutes for 6 h 30 min. 15min after dex treatment (first time-point), mCIT-  
189 2xPH<sup>PLC</sup> labelled the plasma membrane while no signal relative to MAP-mCH-dOCRL fluorescence  
190 was observed (Figure 4A). After 90 min, a fraction of mCIT-PH<sup>PLC</sup> started to be partially solubilized in  
191 the cytosol of some cells, indicating that PI(4,5)P<sub>2</sub> had started to be depleted from the targeted  
192 membrane. (Figure 4A, arrows). After 180 min, mCIT-2xPH<sup>PLC</sup> was cytosolic in most cells, indicating  
193 near complete PI(4,5)P<sub>2</sub> depletion. Over the next few hours, mCIT-2xPH<sup>PLC</sup> retained a cytosolic  
194 localization while MAP-mCH-dOCRL fluorescence increased. We measured dissociation indexes of  
195 mCIT-2xPH<sup>PLC</sup> over-time during this time-lapse. We observed that dissociation index increased strongly  
196 between 90 and 180 min of dex exposure, before plateauing at ~ 1.8 (Figure 4B). At 180 min,  
197 dissociation indexes had increased in all cells, and at 330 min it had reached its maximum in all cells.  
198 Altogether, these results showed that iDePP is rapid and efficient to deplete in an inducible manner  
199 PI(4,5)P<sub>2</sub> at the plasma membrane of *Arabidopsis* root cells.

200

### 201 **iDePP depletes PI(4,5)P<sub>2</sub> in various organs**

202 We next determined if our system was suitable for PI(4,5)P<sub>2</sub> depletion in other cell types than  
203 meristematic root epidermal cells. We therefore tested iDePP in root hairs, cotyledons and shoot apical  
204 meristems for which the PI(4,5)P<sub>2</sub> subcellular and tissue patterning is well described (Noack and Jaillais,  
205 2017; Simon et al., 2014; Stanislas et al., 2018, Colin and Jaillais 2019). Upon dex treatment, all three  
206 MAP-mCH-dOCRL, MAP-mCH-dOCRL<sup>dead</sup> and MAP-3xmCH were expressed in root hairs, and  
207 localized to the plasma membrane and intracellular compartments (Figure 5A, B). Root hairs accumulate  
208 PI(4,5)P<sub>2</sub> on the plasma membrane at their tip (Denninger et al., 2019; Kusano et al., 2008; Stenzel et  
209 al., 2008; Van Leeuwen et al., 2007; Vincent et al., 2005) and expression of MAP-3xmCH did not  
210 affect plasma membrane localization of mCIT-2xPH<sup>PLC</sup>, which remained enriched at the tip of the cell  
211 (Figure 5A, B). Conversely, expression of MAP-mCH-dOCRL solubilized mCIT-2xPH<sup>PLC</sup> from the  
212 plasma membrane into the cytoplasm of root hair cells, indicating efficient PI(4,5)P<sub>2</sub> depletion (Figure  
213 5A, B).

214



215 We next investigated if the iDePP system was suitable to deplete PI(4,5)P<sub>2</sub> in the aerial part of the plant.  
216 For this, we first analyzed iDePP's effects in the shoot apical meristem. Upon dex treatment, MAP-  
217 mCH-dOCRL was mainly induced in the boundary region of the shoot apical meristem and had a weak  
218 or no expression in the central zone (Supplemental file 4). This result was consistent with our previous  
219 observations, since *MAP-mCH-dOCRL* is induced in the *UBQ10* promoter expression domain, which is  
220 low at the center of the shoot apical meristem in our conditions (Stanislas et al., 2018). We observed a  
221 strong correlation between *MAP-mCH-dOCRL* expression and its impact on mCIT-2xPH<sup>PLC</sup> localization  
222 (Supplemental file 4). Indeed, in the center of the shoot meristem, *MAP-mCH-dOCRL* was not expressed  
223 and mCIT-2xPH<sup>PLC</sup> labelling clearly accumulated at the cell periphery, likely representing the plasma  
224 membrane (Supplemental file 4). By contrast, in the boundary zone, where *MAP-mCH-dOCRL* was  
225 expressed, mCIT-2xPH<sup>PLC</sup> was not localized sharply at the cell edge and instead filled the entire volume  
226 of the cell except the nucleus (Supplemental file 4). This suggested that, similar to root epidermis,  
227 expression of *MAP-mCH-dOCRL* in the shoot epidermis displaced mCIT-2xPH<sup>PLC</sup> from the plasma  
228 membrane into the cytosol, and thus efficiently erased PI(4,5)P<sub>2</sub> from the plasma membrane.

229 Because *UBQ10*-driven *MAP-mCH-dOCRL* induction was not uniform at the shoot apical meristem,  
230 we next decided to test the iDePP system on N-1-Naphthylphthalamic Acid (NPA) grown shoot  
231 meristems. NPA is a polar auxin transport inhibitor, which induces naked shoot apical meristem without  
232 any organ (Sassi et al., 2012, Okada et al., 1991). In this condition, the *UBQ10* promoter is more  
233 uniformly expressed throughout the meristem, including the central and peripheral zones, as exemplified  
234 by the homogenous expression of *mCIT-2xPH<sup>PLC</sup>* (Figure 5C). In the absence of dex induction, mCIT-  
235 2xPH<sup>PLC</sup> was localized at the plasma membrane throughout the meristem, including both the central and  
236 peripheral zones (Figure 5C). By contrast, upon induction of *MAP-mCH-dOCRL* expression, mCIT-  
237 2xPH<sup>PLC</sup> was no longer sharply localizing at the cell edge (Figure 5D). Instead, mCIT-2xPH<sup>PLC</sup>  
238 accumulated in the cytosol, while still being excluded from the round central nuclei (Figure 5E). Note  
239 that after dex induction, *MAP-mCH-dOCRL* was strongly expressed in the peripheral zone, and was only  
240 weakly expressed in the central zone (Figure 5E). However, although its expression was weak in the  
241 central zone it appeared to be sufficient to impact mCIT-2xPH<sup>PLC</sup> localization (Figure 5E). Taken  
242 together, our results indicate that iDePP was efficient for PI(4,5)P<sub>2</sub> depletion in both NPA-treated and  
243 dissected shoot apical meristems (Figure 5C, D; Supplemental file 4). However, because iDePP is not  
244 uniformly induced in this tissue, the expression of *MAP-mCH-dOCRL* or its impact on mCIT-2xPH<sup>PLC</sup>  
245 localization should always be double checked before drawing any conclusions.

246  
247 Next, we analyzed the impact of iDePP in the epidermis of cotyledons. Most notably, we focused on  
248 the pavement cells, an epidermal cell with a jigsaw-like shape. Pavement cells are differentiated cells  
249 with a big central vacuole that pushes the cytoplasm against the plasma membrane and the cell wall.  
250 Thus, the fluorescent protein fusions that are localized in the cytosol in this cell type are enriched at  
251 the cell periphery. However, unlike plasma membrane targeted fluorescent fusion proteins, which have

252 a sharp localization at the cell periphery, the localization of cytosolic proteins is more diffuse and they  
253 are also present in cytoplasmic strands. In the absence of dex, MAP-mCH-dOCRL was not expressed  
254 and mCIT-2xPH<sup>PLC</sup> was sharply localized at the cell periphery without the presence of cytoplasmic  
255 strands (Figure 5E), likely being at the plasma membrane. Dex induction of MAP-mCH-dOCRL was  
256 efficient in this cell type and impacted the localization of mCIT-2xPH<sup>PLC</sup>, which became wider at the  
257 cell edge and also localized to cytoplasmic strands (Figure 5E). This localization indicated that mCIT-  
258 2xPH<sup>PLC</sup> was solubilized upon *MAP-mCH-dOCRL* induction in pavement cells and suggested that  
259 PI(4,5)P<sub>2</sub> is hydrolyzed in this condition. Similar to the situation in roots, the *MAP-mCH-dOCRL<sup>dead</sup>* and  
260 *MAP-3xmCH* controls were efficiently expressed in this cell type upon dex induction, but they had no  
261 effect on mCIT-2xPH<sup>PLC</sup> localization, which remained at the plasma membrane and was not found in  
262 the cytoplasm (Figure 5F and G).

263 Taken together, these results suggest that our iDePP system efficiently depletes PI(4,5)P<sub>2</sub> in various  
264 cell types and organs, both in roots and aerial parts.

265

## 266 **Biological relevance: iDePP-mediated PI(4,5)P<sub>2</sub> depletion affects** 267 **microtubule organization in elongating root epidermal cells**

268 Once the inducible PI(4,5)P<sub>2</sub> depletion established, we next set-out to test the cellular effects of  
269 PI(4,5)P<sub>2</sub> depletion on actin organization in plants, since this lipid is well known as a master regulator  
270 of actin dynamics in other eukaryotes (Logan and Mandato, 2006; Moss, 2012; Zhao et al., 2010). For  
271 example, depletion of PI(4,5)P<sub>2</sub> at the plasma membrane by expressing phosphatases disrupts the actin  
272 cytoskeleton leading to a cell-wide reduction in polymerized F-actin in animal cells (Raucher et al.,  
273 2000; Shibasaki et al., 1997). Since the activity of actin-remodeling protein are known to be regulated  
274 by PI(4,5)P<sub>2</sub> in plant cell (Gungabissoon et al., 1998; Guo et al., 2019; Huang et al., 2003; Staiger et al.,  
275 1997), we investigated qualitatively whether PI(4,5)P<sub>2</sub> depletion could affect the organization of F-actin  
276 cytoskeleton in plant cells in a manner similar to that of animal cells. Using live cell imaging, we  
277 observed actin filament in elongated root cell using z-projection of the F-actin marker LifeAct-YFPv  
278 before and upon PI(4,5)P<sub>2</sub> depletion using iDePP. Surprisingly, expression of MAP-mCH-dOCRL, did  
279 not led to a reduction in polymerized F-actin in epidermal root cells or to any major rearrangement of  
280 F-actin cables, at least qualitatively, compared with of the MAP-3xmCH control (Figure 6A).

281 We next investigated a possible role of PI(4,5)P<sub>2</sub> on the organization of cortical microtubules in  
282 *Arabidopsis* elongated root cells, using TUBULIN $\alpha$ 6-GFP (TUA6-GFP) protein fusion. In the root  
283 elongation zone, the cortical microtubules of interphasic epidermal cells are transversely aligned  
284 (Vaughn et al., 2011), thereby forming a network that gates unidirectional anisotropic cell expansion. In  
285 absence of dex induction in *MAP-mCH-dOCRL* line or upon expression of *MAP-3xmCH*, cortical  
286 microtubules of elongating epidermal cells were observed to form a network of transversely aligned  
287 microtubules, orthogonal to the elongation axis of the cells as expected (Figure 6B, Polko and Kieber,

288 2019). This result was quantitatively reflected by a relatively high anisotropy of the microtubule array  
289 per cell (mean  $\pm$  SEM is  $0.095 \pm 0.005$  for the MAP-mCH-dOCRL no dex,  $n = 90$  cells and mean  $\pm$   
290 SEM is  $0.099 \pm 0.003$  for the MAP-3xmCH with dex,  $n = 175$  cells; Type II Wald  $\chi^2$  test:  $\chi^2 = 12.155$ ,  
291  $p = 0.9387$ ; Figure 6D, Supplemental file 5). Depletion of PI(4,5)P<sub>2</sub> after induction of MAP-mCH-  
292 dOCRL led to the increased randomization of cortical microtubule orientations, which correlates with  
293 an increase in isotropy of microtubule arrays (mean  $\pm$  SEM is  $0.067 \pm 0.003$  for the MAP-mCH-dOCRL  
294 with dex,  $n = 238$  cells, Type II Wald  $\chi^2$  test:  $\chi^2 = 12.155$ ,  $p = 0.0047$ , Figure 6C, D, Supplemental file  
295 5). This result suggests that cortical microtubule reorganization requires PI(4,5)P<sub>2</sub> at the plasma  
296 membrane at least during the elongation process of epidermal root cells, and validate our approach to  
297 study the role of PI(4,5)P<sub>2</sub> in a time-scale compatible with plant cell morphodynamics.

298  
299

## 300 **CONCLUSION AND DISCUSSION**

301 Here, we describe iDePP, a new genetically encoded system that allows to rapidly deplete PI(4,5)P<sub>2</sub>  
302 from the plasma membrane *in planta*. The stable genetically encoded system we describe here is  
303 unprecedented and allow to specifically induce PI(4,5)P<sub>2</sub> depletion *in planta*. It is also one of the first in  
304 any multicellular organism (Guglielmi et al., 2015). We provide *in vitro* evidence that *Drosophila*  
305 *melanogaster* dOCRL phosphatase domain can specifically dephosphorylates PI(4,5)P<sub>2</sub>. The use of  
306 adequate negative controls demonstrates that the observed effect on PI(4,5)P<sub>2</sub> sensor only depends on  
307 dOCRL enzymatic activity and is not caused by side effects of the dex inducible system or the  
308 recruitment of an endogenous PI(4,5)P<sub>2</sub> phosphatase. Also, the absence of strong effect observed on  
309 various other membrane lipids, including PI4P, PS, and PI3P, indicated that the system specifically  
310 targets PI(4,5)P<sub>2</sub> without massively affecting the plasma membrane and internal membranes. Yet, our  
311 results suggest link between PI(4,5)P<sub>2</sub> and PS accumulation in plants, as it was recently suggested in  
312 animal systems (Ghai et al., 2017; Sohn et al., 2018) .

313 MAP-mCH-dOCRL fluorescence was detected as soon as three hours—sometimes even earlier—  
314 after gene induction was triggered. This roughly corresponds to the time required for readily detection  
315 of GFP-HsPIP5KI $\alpha$  after oestrogen treatment in (Gujas et al., 2017) system that increases PI(4,5)P<sub>2</sub>  
316 levels. Interestingly, we detected solubilization of the mCIT-2xPH<sup>PLC</sup> PI(4,5)P<sub>2</sub> sensor before we could  
317 detect MAP-mCH-dOCRL. This correlation strongly suggests that MAP-mCH-dOCRL is highly  
318 efficient and strongly depletes PI(4,5)P<sub>2</sub> even when its concentrations are below fluorescence detection  
319 levels or prior to the full maturation of the fluorescent reporter mCH. The system allows to rapidly —  
320 couple of hours— deplete PI(4,5)P<sub>2</sub>, thereby circumventing the long term effects that a chronic PI(4,5)P<sub>2</sub>  
321 depletion would have, for example through a constitutive overexpression of a plasma membrane-  
322 targeted 5-phosphatase or genetic elimination of key enzyme responsible for PI(4,5)P<sub>2</sub> homeostasis (e.g.  
323 PIP5Ks).

324 Importantly, solubilization of mCIT-2xPH<sup>PLC</sup> PI(4,5)P<sub>2</sub> sensor was effective in both root and shoot  
325 tissues. We can even speculate that in other cell types such as pollen tubes, this system will be relevant  
326 to study the role of the patterning of PI(4,5)P<sub>2</sub> in polarized growth. Here, we focused on epidermal  
327 tissues, because they are relatively easier to image at the same time the induction of MAP-mCH-dOCRL  
328 and the anionic lipid biosensors. However, MAP-mCH-dOCRL is induced in the broad expression  
329 domain of *UBQ10* promoter, and thus iDePP may be used in additional tissues and organs. Alternatively,  
330 MAP-mCH-dOCRL can easily be expressed in a tissue specific manner (Marquès-Bueno et al., 2016).  
331 The spatial control of PI(4,5)P<sub>2</sub> depletion, even if not reversible, would likely be of great help to  
332 elucidate PI(4,5)P<sub>2</sub> functions in cell differentiation and plant development.

333 We observed that expression of *MAP-mCH-dOCRL* led to defects in cortical microtubules  
334 orientation, which often failed to adopt a proper anisotropic organization orthogonal to cell growth  
335 direction in elongating root epidermal cells. This observation suggests that the maintenance of  
336 anisotropic-microtubules arrays in elongated cells requires PI(4,5)P<sub>2</sub>. This may affect proper cellulose  
337 microfibrils deposition by microtubule-guided cellulose synthase complexes. Phospholipids have been  
338 involved in the control of cytoskeleton cortical anchoring and dynamics both in plants and animals  
339 (Raucher et al., 2000; Zhang et al., 2012). The increased isotropy of microtubule arrays and the slower  
340 response to mechanical perturbations in mutants impaired in microtubule self-organization correlate  
341 with an altered phosphoinositide patterning within the shoot apical meristem (Colin and Jaillais, 2020;  
342 Stanislas et al., 2018). Indeed, the katanin mutant allele *botero1-7* exhibits more disorganized  
343 microtubules, reduced growth anisotropy and slower microtubule response to mechanical perturbations  
344 (Bichet et al., 2001; Lindeboom et al., 2013; Uyttewaal et al., 2012) and the PI(4,5)P<sub>2</sub> pattern is noisier  
345 in *bot1-7* than in the wild type (Stanislas et al., 2018). This results, together with our finding that the  
346 perturbation of the PI(4,5)P<sub>2</sub> patterning affects microtubules anisotropy suggest that PI(4,5)P<sub>2</sub> at the  
347 plasma membrane promotes microtubule array order. It was proposed that KTN1 function through  
348 RHO-LIKE GTPASE FROM PLANTS 6 (ROP6) and its effector ROP-INTERACTIVE CRIB MOTIF-  
349 CONTAINING PROTEIN 1 (RIC1), which binds to and activates KTN1 (Fu et al., 2009; Lin et al.,  
350 2013; Xu et al., 2010). ROP6 requires phosphatidylserine for signaling at the plasma membrane but it  
351 also interact with PI(4,5)P<sub>2</sub> in vitro (Platre et al., 2019). Future directions should thus include the  
352 characterization of the molecular player responsible for the PI(4,5)P<sub>2</sub> accumulation and signaling at the  
353 plasma membrane and their role in the remodeling of the microtubule cytoskeleton leading to directional  
354 growth.

355 The iDePP system should prove useful for the community of plant biologists and may be amended,  
356 by using tissue specific promoters for instance. Also, instead of dOCRL 5-phosphatase domain, others  
357 domains with other enzymatic activity could be similarly targeted at the plasma membrane. Such  
358 domains could allow depleting other species of plasma membrane lipids, but also constitute systems to  
359 assess the enzymatic activities of putative PI4P and/or PI(4,5)P<sub>2</sub> phosphatases and phospholipases *in*

360 *vivo*. iDePP can also be used to assess a protein requirement to PI(4,5)P<sub>2</sub> for plasma membrane targeting.  
361 Furthermore, the timing of iDePP activity is fully compatible with in vivo live imaging. Together, we  
362 believe that the iDePP system will have the potential to be useful for numerous research groups working  
363 in the fields of plant cell biology, plant development but also immunity and reproduction.

364  
365

## 366 ***MATERIAL AND METHODS***

### 367 **Sequence alignment**

368 HsOCRL<sub>234-539</sub> and dOCRL amino acid sequences were aligned using T-Coffee software  
369 (<http://tcoffee.crg.cat/apps/tcoffee/do:regular>) and the fasta\_aln files obtained were then treated with  
370 Boxshade ([http://www.ch.embnet.org/software/BOX\\_form.html](http://www.ch.embnet.org/software/BOX_form.html)) and inkscape programs.

371

### 372 **Cloning**

373 A synthetic gene (<https://eu.idtdna.com/pages>) corresponding to dOCRL<sub>168-159</sub> codon optimized for  
374 expression in *Arabidopsis thaliana* (see sequence below) was amplified and flanked with NcoI and XhoI  
375 restriction sites by PCR and cloned into pMH-HS-SUMO vectors (Christie et al., 2012) by restriction  
376 and ligation (see corresponding primers in Supplemental Figure 6).

377

378 dOCRL<sub>168-159</sub>:

379 gtaaagcaagagtaaagaagcgagagagtgagtatactgtctataaggacattatcattactgcgctacgtggaacgtgaacaataagacatgca  
380 gtgatagtaacaatccgctaagagcgtggctcgcgatgctccgaaaagccgacacatatatgcataggtcttcaggagctagacaccccgacca  
381 aggctatgctcaatagtacccaagtccaagcaattgaaaacagtgatagataagatgatggacagtgatcctgacgtagagtacgagattttg  
382 atgtctcaccgattagttgcgacgatgcttactgtcatcgttagaaaacagcttaggcagcacattatacgttgctgacccaaatccgtggcccggtga  
383 atattcaacacgctcggaaataaggaggggtggcaatatccctgcagttgaatgagggaacatatgtttgttaactcccctctgctgcacatatg  
384 ggtacgctgaagagaggaatcaagattacaacgctattgtcgaaggcatcaggtttgacgacggtagaactatctccgaccatgacatatatttg  
385 gtaggagactgaattatcgaatacaggaacctccggacagcagcgtctgggcccgttgagtacgcacagacatacagactcttattacaatac  
386 gatcaactccgtaagagatgcaaggggaaaatgctcgaaggttacaggggggagattaagttcagaccacatacaaatatgatcctgga  
387 acagacaattatgactcttcagagaaacagcagcacctgcatactgcgatagagtctatggaaaggtacacgtatcgaacagctagcatacaac  
388 agcataatggaattaggcaagcgatcataagccagttatgctggtttccaggtgaaggtaaagacacgagatgaggtgaagtataagcgtgttc  
389 aagaggaggtgctaaaggcgttgacaaaagggagaacgataatcagccacagtaa

390

391 *UBQ10pro:GVG (UBQ10pro:GVG-tE9::6xUAS-35Smini:: rigorously)* construct was cloned using  
392 Gibson technique and cloned into a *pDONR-P4PIR* gateway vector (thermofisher cat# 12537023).  
393 *MAP-mCH* was amplified from a *mCH/pDONR207* matrix using 5' phosphorylated primers and ligated  
394 into *MAP-mCH/pDONR207*. The synthetic dOCRL<sub>168-509</sub> sequence (see above) with an N-terminal  
395 GAGARS linker (ggggcaggagccagatcc) was flanked with attB2R and attB3 sequences and recombined

396 by BP gateway reaction into *pDONR-P2RP3*. Site directed mutagenesis of dOCRL was performed by  
397 PCR on *dOCRL<sub>168-509</sub>/pDONR-P2RP3* using 5' phosphorylated primers, and subsequent ligation of the  
398 linear plasmid circularized *dOCRL<sub>168-509</sub><sup>D468G</sup>/pDONR-P2RP3*. *2xmCHERRY/pDONR-P2RP3* was  
399 already available.

400 *UBQ10pro:GVG:MAP-mCH-dOCRL<sub>168-509</sub>/pH7m34GW*, *UBQ10pro:GVG:MAP-mCH- dOCRL<sub>168-</sub>*  
401 *509<sup>D468G</sup>/pH7m34GW*, and *UBQ10pro:GVG:MAP-3xmCH//pH7m34GW* were obtained by triple LR  
402 gateway reaction between *UBQ10pro:pDONR-P4P1R*, *MAP-mCH-pDONR207*, the corresponding  
403 *pDONR-P2RP3* vectors, and *pH7m34GW* destination vector (Karimi et al., 2007). See resource table  
404 for details.

405

## 406 Protein purification

407 BL21(DE3) pLys competent *E. coli* (Novagen and Supplementary file 5) were transformed with  
408 *pMH-HS-Sumo-dOCRL<sub>168-509</sub>* vector (Kan<sup>R</sup>) and cultured on Luria Broth (LB) liquid or 1 % agar medium  
409 supplemented with 50 μM Kanamycin. Two 800 ml 2xTY (10 g/L yeast extract; 16 g/L tryptone; 5 g/L  
410 NaCl) liquid cultures supplemented with kanamycin were inoculated with 20 ml from an overnight pre-  
411 culture of 150 ml, and left at 37°C with 200 rpm shaking. When OD<sub>600nm</sub> reached 0.6, (i) protein  
412 expression was induced by adding IPTG 0.625 mM final concentration, (ii) 45 ml of glycerol were  
413 added, and (iii) temperature was lowered by transferring the liquid culture on ice for 15 min. The induced  
414 culture was then grown for 18 h at 18°C with 200 rpm shaking. Bacteria were then centrifuged 30 min  
415 at 6000 g and 4 °C, and bacterial pellets scooped, resuspended in lysis buffer (200 mM NaCl; 20 mM  
416 Tris-HCl pH 7.5; 1 mM PMSF) with the aid of vortex, flash frozen and stored at -80°C. The day of  
417 protein purification, Bacterial pellets corresponding to 33 % (270 ml of the culture) were thawed at room  
418 temperature. For then on, all steps were carried on ice. Bacteria were then sonicated in four pulses of 30  
419 sec and 30 W. Sonicated lysates were centrifuge 30 min at 13000 g and 4°C in new falcon tubes.

420 Supernatants, corresponding to the soluble fraction of the lysate, were then diluted to 50 ml final  
421 volume and subjected to affinity chromatography. 2 ml of 50 % HIS-Select® Cobalt affinity resin  
422 (Sigma) was washed with milli-Q water and then with equilibration buffer (20 mM imidazole; 200 mM  
423 NaCl; 20 mM Tris-HCl pH 7.5; 1 mM). Bacterial lysate was run through the resin twice. Then, resin  
424 was washed with wash buffer (40 mM imidazole; 200 mM NaCl; 20 mM Tris-HCl pH 7.5; 1 mM).

425 Elution was performed with three fractions of 2 ml of elution buffer (250 mM imidazole; 200 mM  
426 NaCl; 20 mM Tris-HCl pH 7.5; 1 mM). Elution fractions were then either directly concentrated (C1  
427 fraction) or kept at 4 °C for a maximum of 16 h before concentration (C2 fraction). Concentration was  
428 achieved using 0.5 ml 30 kD molecular-weight cut-off concentration columns (Millipore UFC 501024)  
429 centrifuged at 4000 g and 4 °C until 2 ml of elution reached a final volume of 400 μL. Aliquots of 20  
430 μL were sampled from induced bacterial culture (IN), flow through (FT), wash (W), elution fraction (E1, E2 and E3) and concentrated fractions (C1 and C2). They were mixed with SDS loading buffer (0.0625

432 M Tris HCl pH 6.8; 2.5 % SDS; 2 % DTT (0.13 M); 10 % glycerol; bromophenol blue), vortexed and  
433 submitted for 5 min to 95°C treatment, and then stored at -20°C. Protein concentration in concentrated  
434 fraction was calculated from absorbance at 280 and 260 nm measured with a nanodrop and calculated  
435 extinction coefficient (<https://web.expasy.org/protparam/>). His-Sumo-dOCRL<sub>168-509</sub>-His has an expected  
436 molecular weight of 54.82 kD.

437

### 438 **Malachite green phosphatase assay**

439 Phosphate released was monitored using a Malachite green phosphatase kit (Echelon) and water  
440 soluble short chain (di:C8) phosphoinositides (Echelon) stored in glass vials at -20°C as stock solutions  
441 of 500 μM in CHCl<sub>3</sub>/MetOH (9:1; PI, PI3P, PI4P, PI5P), or in CHCl<sub>3</sub>/MetOH/H<sub>2</sub>O (5:5:1; PI(3,5)P<sub>2</sub>,  
442 PI(4,5)P<sub>2</sub>). di:C16 Phosphatidylserine (PS; Echelon) was stored in glass vials at -20°C as a stock solution  
443 of 10 mM in CHCl<sub>3</sub>/MetOH (9:1). Before malachite green assay, inorganic solvent was evaporated by  
444 centrifugation in a speed vac at 45 °C for 15 min. Dried lipids (PS and one phosphoinositide) were then  
445 resuspended in reaction buffer (200 mM NaCl 50 mM Tris-HCl pH 7.5; 10 mM KCl; 5 mM MgCl<sub>2</sub>)  
446 with a 30 sec of sonication at full power. Reaction were carried out in a 20 μL volume consisting in  
447 reaction buffer containing 500 μM of PS, 100 μM of a given short chain phosphoinositide species  
448 (except for the “mock” condition), and 5 μL of concentrated protein fractions (C1 or C2; except of “no  
449 enzyme” condition) corresponding to the final concentration displayed on Figure 1C. In the “iP”  
450 condition corresponds to addition of inorganic phosphate. Malachite green assay was carried out at 29  
451 °C for 75 min, with gentle shaking every 15 min, and reaction was eventually quenched by the addition  
452 of 90 μL of malachite green reagent. Pictures were taken 25 min after the addition of Malachite green  
453 reagent, and green coloration qualitatively indicated a significant phosphate release. This Experiment  
454 was carried out independently three times, with either one or two replicates each time, and gave  
455 consistent results.

456

### 457 **Growth condition and plant materials**

458 *Arabidopsis thaliana* Columbia-0 (Col-0) accession was used as wild type (WT) reference genomic  
459 background throughout this study. *Arabidopsis thaliana* seedlings *in vitro* on half Murashige and Skoog  
460 (½ MS) Basal Medium supplemented with 0.8% plant agar (pH 5.7) in continuous light conditions at 21  
461 °C. Plants were grown in soil under long-day conditions at 21 °C and 70% humidity 16 h daylight.

462

### 463 **Plant transformation and Selection**

464 Plant were transformed and selected as previously described (Platre et al., 2018). Each construct was  
465 transformed into *Agrobacterium tumefaciens* C58 GV3101 strain and selected on YEB media (5g/L beef  
466 extract; 1g/L yeast extract; 5g/L peptone; 5g/L sucrose; 15g/L bactoagar; pH 7.2) supplemented with  
467 antibiotics (Spectinomycin, Gentamycin, Rifampicin). After two days of growth at 28 °C, bacteria were

468 scooped and re-suspended in roughly 200 mL of transformation buffer (10mM MgCl<sub>2</sub>; 5% sucrose;  
469 0.25% silweet) and Col-0 *Arabidopsis* were transformed by dipping. Primary transformants (T1) were  
470 selected *in vitro* on the appropriate antibiotic/herbicide (glufosinate for mCIT, hygromycin for mCH-  
471 tagged proteins). Approximately 20 independent T1s were selected for each line. In the T2 generation  
472 at least two independent transgenic lines were selected using the following criteria when possible: (i)  
473 good expression level in the root for detection by confocal microscopy upon dexametazone induction,  
474 (ii) relatively uniform expression pattern, (iii) line with no obvious abnormal developmental phenotypes.

475 Lines were rescreened in T3 using similar criteria as in T2 with the exception that we selected  
476 homozygous lines (100% resistant). At this step, we selected one transgenic line for each marker that  
477 was used for further analyses and crosses. NPA treatments were carried out as previously described  
478 (Grandjean et al., 2004). Plants were further kept on NPA-containing medium for all the duration of the  
479 experiments.

480

## 481 **Induction of gene expression with dexamethasone**

482 In order to study the effect of iDePP system in root, seedlings of each genotype were grown vertically  
483 for five to seven-day *in vitro* on half Murashige and Skoog (½ MS) Basal Medium supplemented with  
484 0.8% plant agar (pH 5.7) in continuous light conditions at 21 °C. Part of the seedlings were next carefully  
485 transferred to ½ MS medium plates, 0.8 % agar (pH 5.7), containing 5 µM dexamethasone. Both non-  
486 treated seedlings and dex-treated seedlings were, all together, grown back in continuous daylight (see  
487 before). 16 h after transfer on dexamethasone plates, (more or less 90 min) seedlings were mounted in  
488 ½ MS medium and imaged by confocal microscopy.

489 For cotyledon analysis, seeds were sterilized and sown on Arabidopsis medium (MS medium without  
490 sugars and vitamins) for 7days. Seedling were then carefully transferred on MS medium (without sugars  
491 and vitamins) supplemented with 5µM of Dexamethasone. Cotyledons were placed at the surface of the  
492 medium, as flat as possible, while the rest of the plant was immersed into the medium.

493 Shoot apex were dissected as described by (Stanislas et al., 2017) and then placed in a shoot apex  
494 medium as described by (Grandjean et al., 2004) supplemented with 5µM of Dexamethasone. To  
495 generate naked meristems *in vitro*, seeds were first directly sown on Arabidopsis medium, supplemented  
496 with 10 µM NPA. Seedlings with naked meristems were then selected and transferred to a shoot apex  
497 medium without NPA and supplemented with 5µM of Dexamethasone. Images were taken at 18 h after  
498 induction.

499

## 500 **Time-lapse imaging**

501 Time lapse imaging of cell division and root hair growth were performed mostly as described  
502 previously (Doumane et al., 2017), with few amendments. In brief, five days old Arabidopsis seedlings  
503 were transferred in a chambered cover glass (Lab-Tek II, [www.thermoscientific.com](http://www.thermoscientific.com)), which contained



504 (i) 3 ml of ½ MS medium (pH 5.7) containing 0.8 % plant agar (Duchefa, [http://www.duchefa-](http://www.duchefa-biochemie.nl/)  
505 [biochemie.nl/](http://www.duchefa-biochemie.nl/)) and 5 µM dexamethasone and (ii) 800 µL of ½ MS medium (pH 5.7) containing 5 µM  
506 dexamethasone. Epidermal cells in the meristematic region of the root tip were subjected to time-lapse  
507 imaging with spinning disk confocal microscope. Up to three roots were observed simultaneously and  
508 images were collected at different Z-positions every 5 min for 8 h. Tracking of growing roots was  
509 automatically performed (Doumane et al., 2017).

510

## 511 **Microscopy setup**

512 Images of cotyledons, shoot apical meristem and NPA meristems (Figure 4B-D) were acquired with  
513 a Leica SP8 upright scanning confocal microscope equipped with a water immersion objective (HCX  
514 IRAPO L 25x/ 0.95 W). Fluorophores were excited using Led laser (Leica Microsystems, Wetzlar,  
515 Germany) emitting at wavelengths of 514 nm for mCIT fluorochrome and 552 nm for mCH  
516 fluorochrome. Emission fluorescence was recovered between 519nm - 548nm for mCIT and between  
517 604 nm - 662 nm for mCH. The following scanning settings were used: pinhole size 1 AE, scanning  
518 speed of 8000 Hz (resonant scanner), frame averaging between 8 and 10, Z intervals of 0.45 µm.

519 All additional imaging was performed with the following spinning disk confocal microscope set up:  
520 inverted Zeiss microscope (AxioObserver Z1, Carl Zeiss Group, <http://www.zeiss.com/>) equipped with  
521 a spinning disk module (CSU-W1-T3, Yokogawa, [www.yokogawa.com](http://www.yokogawa.com)) and a ProEM+ 1024B camera  
522 (Princeton Instrument, <http://www.princetoninstruments.com/>) or Camera Prime 95B  
523 ([www.photometrics.com](http://www.photometrics.com)) using a 63x Plan-Apochromat objective (numerical aperture 1.4, oil  
524 immersion). GFP and mCIT were excited with a 488 nm laser (150 mW) and fluorescence emission was  
525 filtered by a 525/50 nm BrightLine! single-band bandpass filter (Semrock, <http://www.semrock.com/>),  
526 mCH was excited with a 561 nm laser (80 mW) and fluorescence emission was filtered by a 609/54 nm  
527 BrightLine! single-band bandpass filter (Semrock, <http://www.semrock.com/>). For quantitative  
528 imaging, pictures of epidermal root meristem cells were taken with detector settings optimized for low  
529 background and no pixel saturation. Care was taken to use similar confocal settings when comparing  
530 fluorescence intensity or for quantification. Signal intensity was color-coded (green fire blue scale,  
531 <https://fiji.sc/>).

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## 533 **3D projections, dissociation indexes and anisotropy**

534 The fluorescence intensity in root hair was obtained using Fiji. A line of 66 pixels was drawn at the  
535 proximity of the root hair tip and the intensity of grey was plot using the Plot Profil tool. The values  
536 were transferred in excel file to obtain the graph. Shoot apical meristem and NPA-induced meristem  
537 projected images were obtained by using the MorphoGraphX software  
538 (<https://www.mpipz.mpg.de/MorphoGraphX>). The signal projections were generated by extracting the  
539 fluorescent signal at the surface of the meristem (between 2 µm and 5 µm from the meristem surface)

540 and by projecting it on the cellular mesh. Projection of root epidermal cells were obtained using Fiji  
541 “Max intensity projection” tool on Z-stacks of 21 slices distant of 0.5  $\mu\text{m}$  from each other.

542 Dissociation indexes of membrane lipid fluorescent biosensors were measured and calculated as  
543 previously described (Platre et al., 2018). Briefly, we calculated “indexNoDex” in the mock condition,  
544 defined as the ratio between the fluorescence intensity (Mean Grey Value function of Fiji software)  
545 measured in two elliptical regions of interest (ROIs) from the plasma membrane region (one at the  
546 apical/basal plasma membrane region and one in the lateral plasma membrane region) and two elliptical  
547 ROIs in the cytosol. Next, we measured a similar ratio after dexamethasone treatments (“indexDex”).  
548 The dissociation index is the ratio of (indexNoDex)/(indexDex). This dissociation index reveals the  
549 degree of relocalization of the fluorescent reporters from the plasma membrane to the cytosol, between  
550 the non-treated and perturbed conditions (pharmacological treatment or mutant). Dissociation indexes  
551 of mCIT-2xPH<sup>PLC</sup> during time-lapse induction of MAP-mCH-dOCRL (Figure 4) were measured in 30  
552 cells of the same root at each time point.

553 For quantification of the anisotropy of microtubule arrays in the different transgenic lines, Maximal  
554 z projection of z-stack of epidermal root cell in the elongation zone were obtained, using Fiji. In  
555 fibriltool, the value for ‘Multiply line length by’ was set up at 1 and ROI using the built-in Polygon tool  
556 were generated for individual cells (>100 cells per conditions). The data from the log file was used to  
557 extract the average anisotropy of microtubule arrays (a score between 0 and 1) and to run statistical  
558 analyses.

559

## 560 **Statistical analysis**

561 For dissociation index and anisotropy, we performed all our statistical analyses in R (v. 3.6.1, (R  
562 Core Team, 2019), using R studio interface and the packages ggplot2 (Wickham, 2016), lme4 (Bates et  
563 al., 2015), car (Fox and Weisberg, 2011), multcomp (Hothorn et al., 2008) and lsmeans (Lenth, 2016).  
564 For each model assuming normality, we plotted residuals values against normal quantiles to confirm  
565 their normal distribution. Graphs were obtained with R and R-studio software, and customized with  
566 Inkscape (<https://inkscape.org>).

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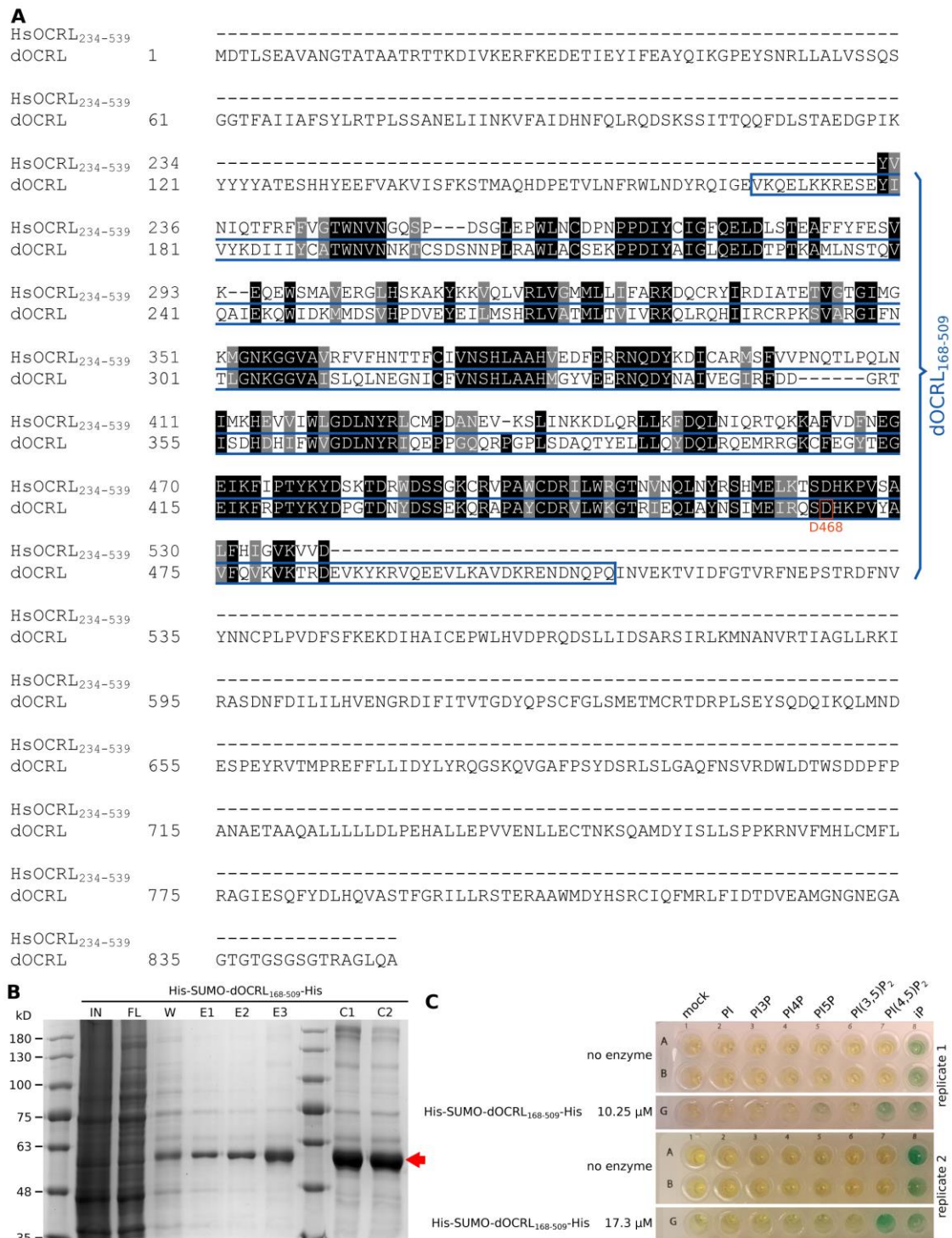
## 569 **ACKNOWLEDGMENTS**

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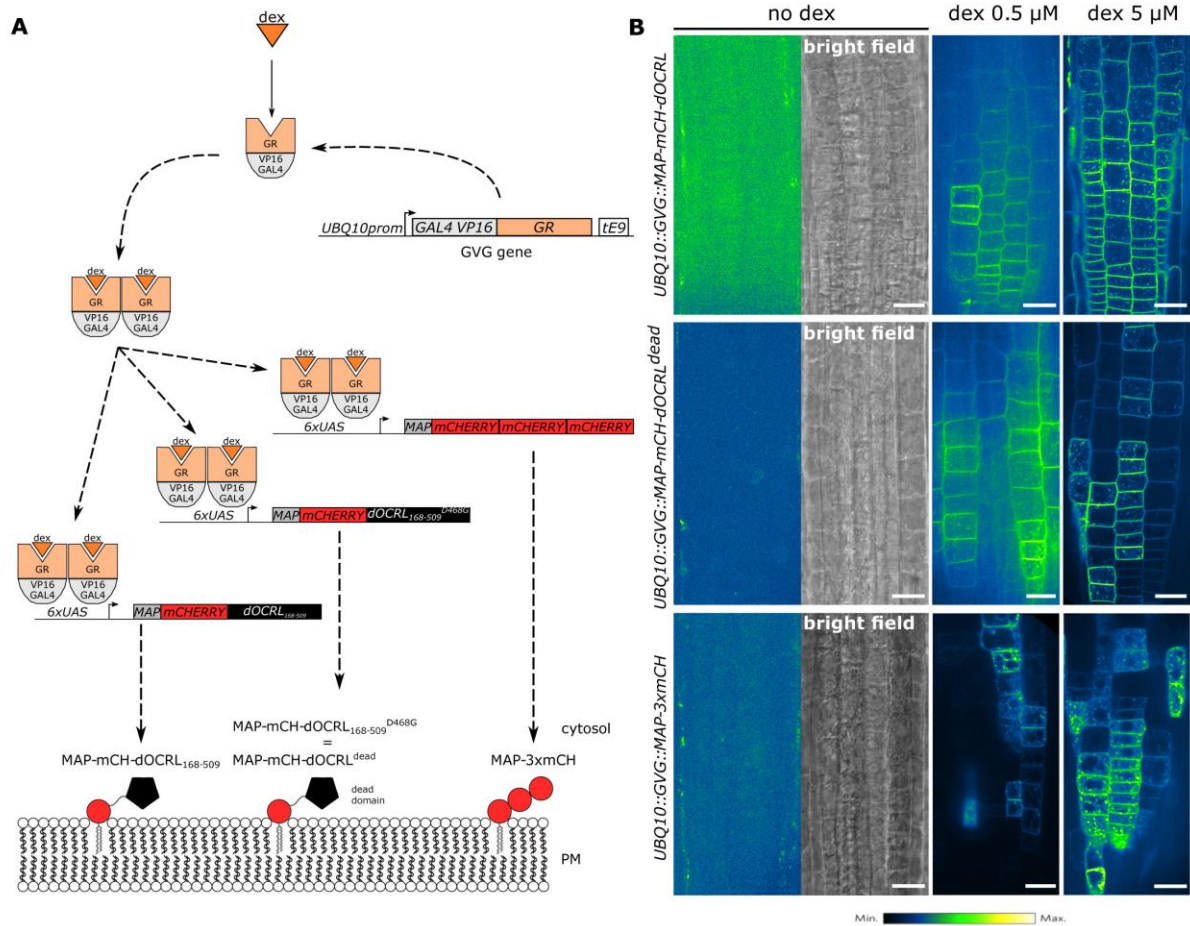
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**Figure 1. *Drosophila* dOCRL displays a PI(4,5)P<sub>2</sub> phosphatase activity in vitro.**

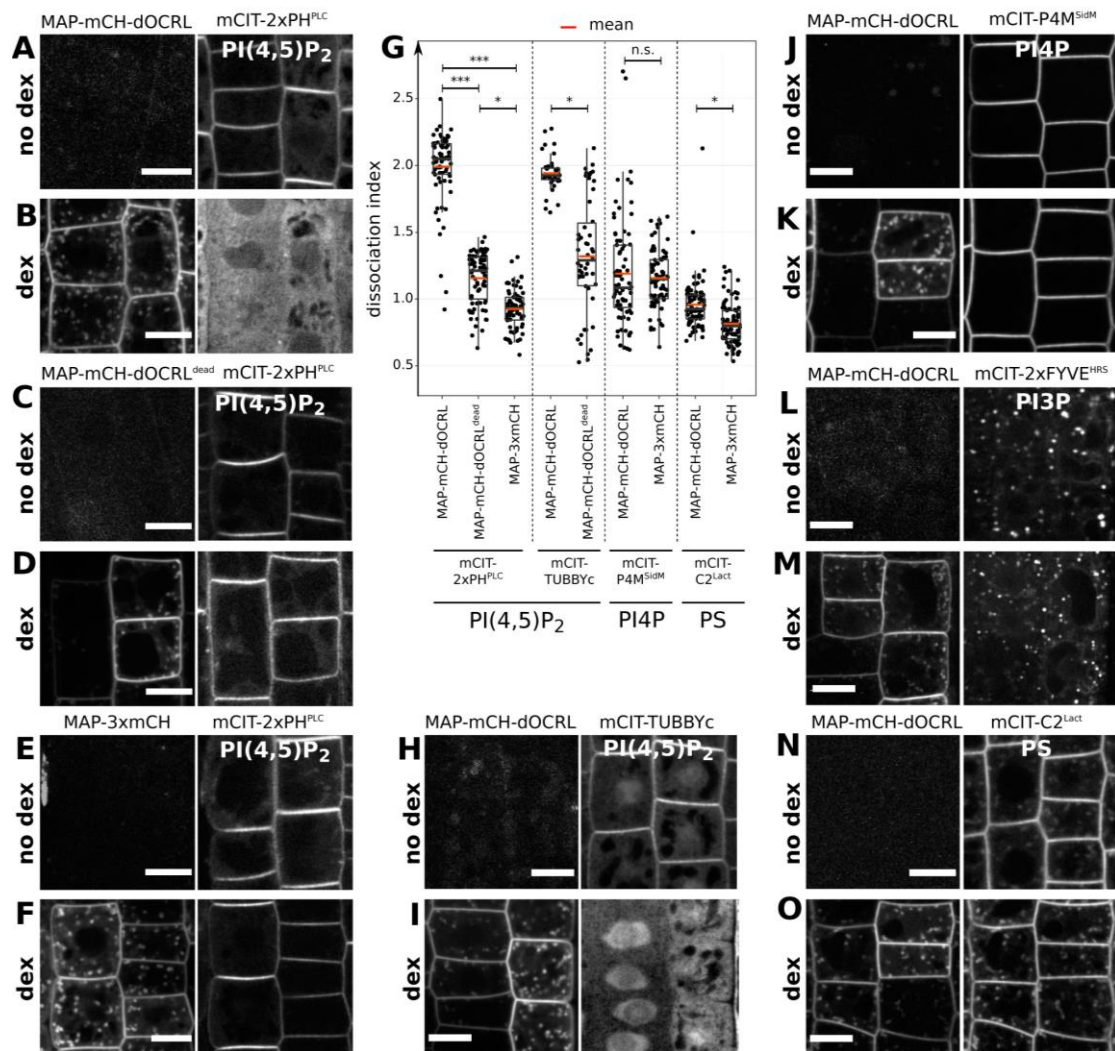
(A) Alignment of (i) *Homo sapiens* OCRL (HsOCRL or OCRL1) truncation (HsOCRL<sub>234-539</sub>) successfully used in optogenetic systems in mammalian cells (Idevall-Hagren et al., 2012), and (ii) of *Drosophila melanogaster* dOCRL. We cloned dOCRL<sub>168-509</sub> truncation, framed in blue, that is homolog to HsOCRL<sub>234-539</sub>. Aspartate 468 (D468), framed in red, is the amino acid we mutated to obtain inactive dOCRL<sub>168-509</sub> (see Figure 2 and 3). (B) Coomassie blue staining monitoring purification of recombinant dOCRL phosphatase domain (His-SUMO-dOCRL<sub>168-509</sub>-His) from *Escherichia coli*. IN: input, lysate of bacteria induced for the expression of His-SUMO-dOCRL<sub>168-509</sub>-His; FL: flow through; W: wash; E1 to E3: elution fraction 1 to 3; C1 and C2: concentrated fractions from pulled E1, E2 and E3. C1 and C2 were obtained separately on different days. His-SUMO-dOCRL<sub>168-509</sub>-His expected molecular weight is 54.8 kD. (C) Malachite green phosphatase assay on His-SUMO-dOCRL<sub>168-509</sub>-His using short chain water-soluble phosphoinositides. Replicate 1 correspond to C1 fraction. Replicate 2 to C2 fractions. Mock: no phosphoinositide added.

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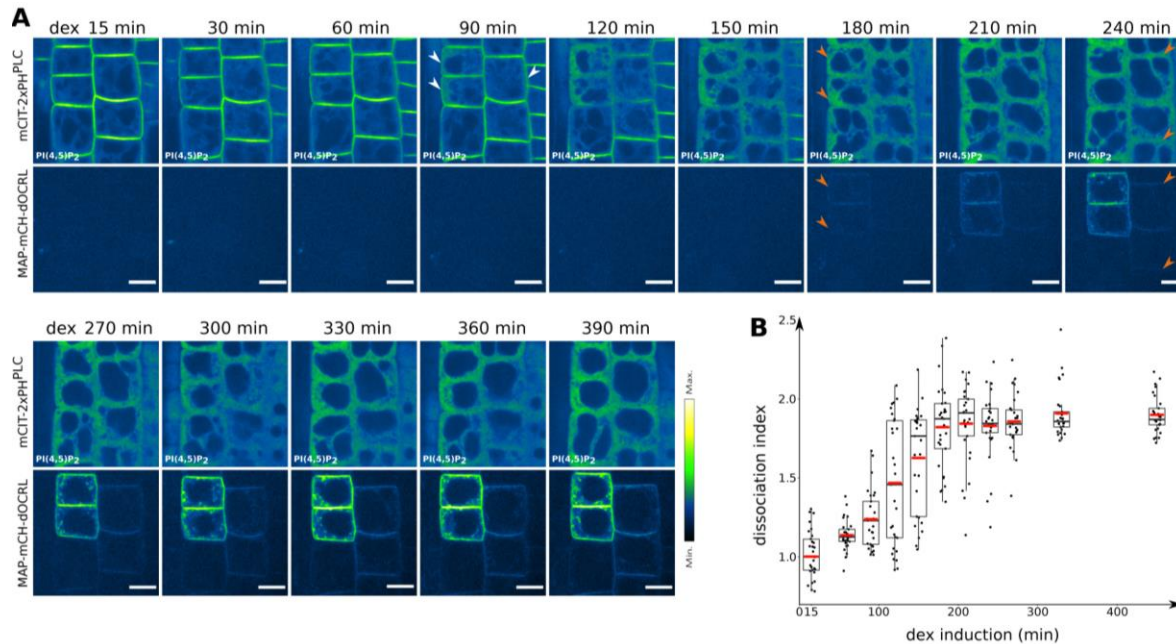
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599 **Figure 2. Successful inducible expression and subcellular targeting of MAP-mCH-dOCRL and negative**  
600 **controls.**

601 (A) Inducible system used in this study. *UBQ10* promoter is responsible for mild/strong and ubiquitous expression of  
602 *GVG* (*GAL4 VP16 GR*) synthetic gene. GR domain binds dexamethasone (dex) and subsequently induces *GVG*  
603 homodimerization and nuclear import. There, *GAL4* domain binds *UAS* DNA elements and *VP16* strongly activates  
604 the expression of downstream gene: *MAP-mCH-dOCRL<sub>168-509</sub>*, or *MAP-mCH-dOCRL<sub>168-509</sub><sup>D468G</sup>* (*MAP-mCH-*  
605 *dOCRL<sup>dead</sup>*), or *MAP-3xmCH*. *MAP* is myristoylation and palmytoylation sequence, responsible for plasma membrane  
606 targeting. *mCH* corresponds to monomeric *CHERRY* fluorescent protein. *dOCRL<sup>D468G</sup><sub>168-509</sub>* is an inactive  
607 phosphatase domain (later on called *dOCRL<sup>dead</sup>*). *3xmCH* correspond to three fused *mCHERRY*. (B) For all three  
608 inducible constructs we monitored *mCH* fluorescence without dex treatment or after a 16h-treatment with either 0.5  
609  $\mu\text{M}$  or 5  $\mu\text{M}$  dex. Signal intensity is color-coded (green fire blue scale). Scale bars: 20  $\mu\text{m}$ .  
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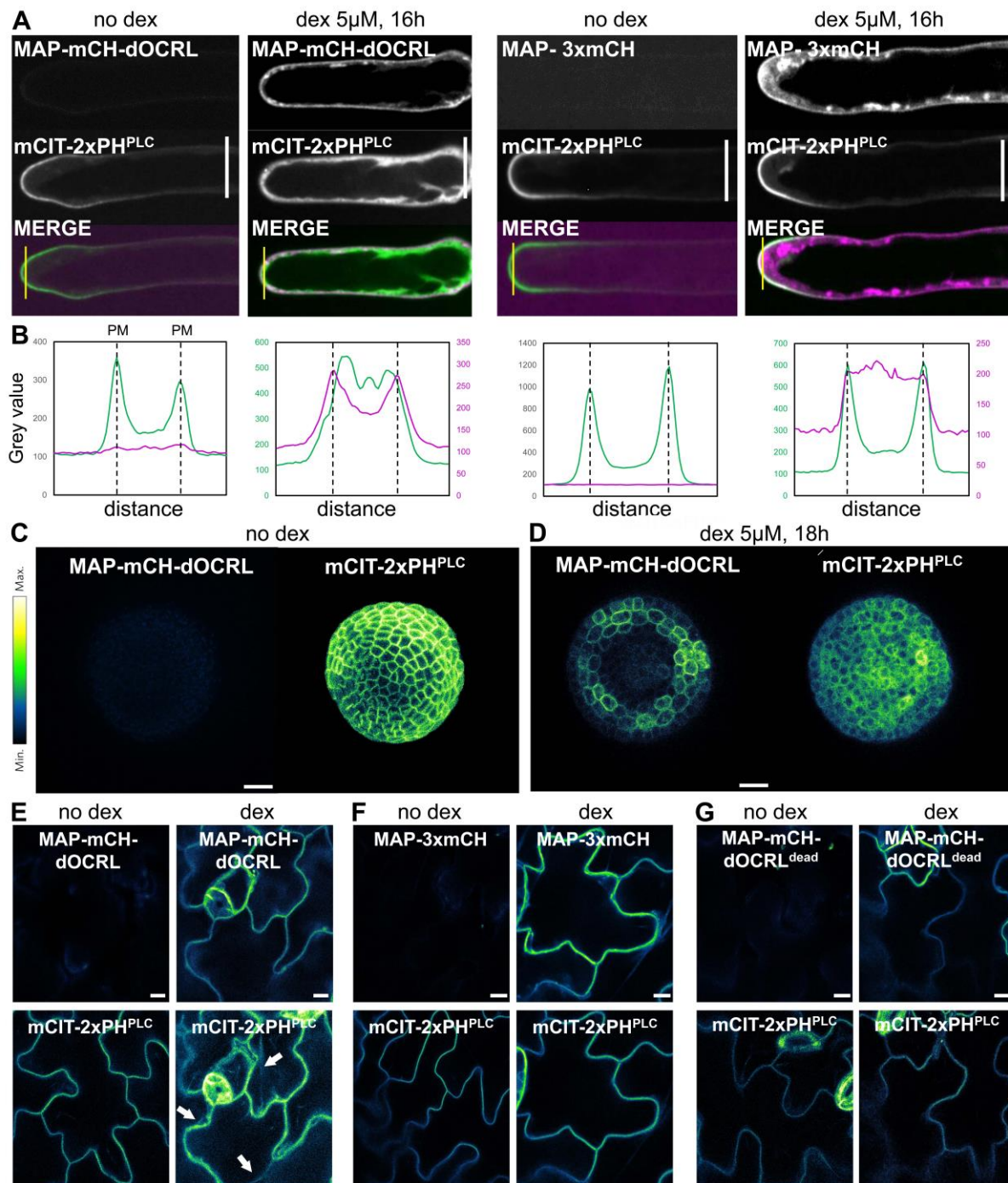


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639 corresponding to MAP-mCH-dOCRL (left panel) and the phosphatidyserine (PS) biosensor mCIT-C2<sup>Lact</sup> (right panel)  
640 in the same root cells, without dex treatment. (O) Representative images of the fluorescent signal corresponding to  
641 MAP-mCH-dOCRL (left panel) and the phosphatidyserine (PS) biosensor mCIT-C2<sup>Lact</sup> (right panel) in the same root  
642 cells, after 16h of dex treatment. Scale bars: 10  $\mu$ m.



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645 **Figure 4. Time-lapse analysis of the depletion of PI(4,5)P<sub>2</sub> in plant cells using iDePP.**  
646 Dual color time-course analysis during dex induction, monitoring mCIT-2xPH<sup>PLC</sup> PI(4,5)P<sub>2</sub> biosensor subcellular  
647 localization and MAP-mCH-dOCRL appearance at the root tip in epidermal cells every five minutes for 6h30. 15min  
648 after the beginning of the induction, mCIT-2xPH<sup>PLC</sup> labelled the plasma membrane while no signal relative to MAP-  
649 mCH-dOCRL fluorescence was observed. After 90 min, a fraction of mCIT-PH<sup>PLC</sup> started to be partially solubilized  
650 in the cytosol of some cells, indicating that PI(4,5)P<sub>2</sub> had started to be depleted from the targeted membrane (arrows).  
651 After 180 min, mCIT-2xPH<sup>PLC</sup> was cytosolic in most cells, indicating near complete PI(4,5)P<sub>2</sub> depletion (arrows). (B)  
652 Dissociation indexes of mCIT-2xPH<sup>PLC</sup> over-time. Dissociation index is the ratio of (i) plasma membrane to cytosol  
653 fluorescence ratio without dex treatment, to (ii) plasma membrane to cytosol fluorescence ratio after dex treatment.

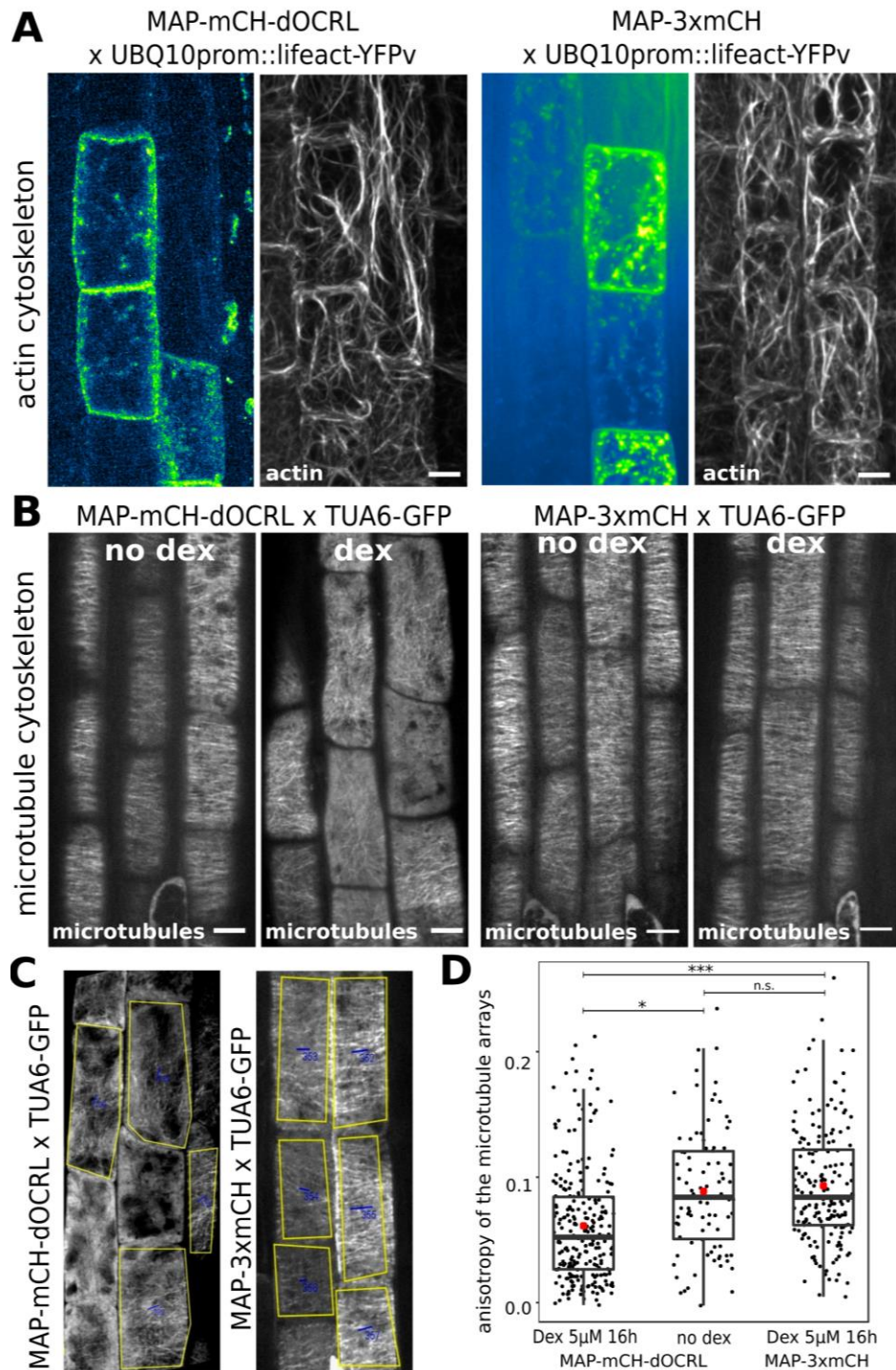


**Figure 5. iDePP enables efficient depletion of PI(4,5)P<sub>2</sub> in various tissues and organs.**

(A) PI(4,5)P<sub>2</sub> depletion in root hairs. Subcellular localization of mCIT-2xPH<sup>PLC</sup>, a PI(4,5)P<sub>2</sub> biosensor, and either MAP-mCH-dOCRL or MAP-3xmCH without and with 5  $\mu$ M dexamethasone treatment for 16h. (B) Signal intensity for mCIT-2xPH<sup>PLC</sup> (green) and MAP-mCH-dOCRL or MAP-3xmCH (magenta) with and without 5  $\mu$ M dexamethasone treatment for 16h, along the yellow line drawn on panel (A). The peaks correspond to plasma membrane (PM) positions, as indicated. Note that for the condition with dex, the signal for mCH channel was weaker than with mCIT and therefore signal intensity was plotted with a different scale, on a secondary axis on the right. (C) and (D) Subcellular localization of MAP-mCH-dOCRL (left panel) and mCIT-2xPH<sup>PLC</sup> (right panel) in a naked shoot meristem from an NPA-treated seedling, as viewed from the top. Absence of treatment (no dex, C) or 16 h with 5  $\mu$ M dexamethasone treatment (dex, D). Scale bar, 20  $\mu$ m (E to G) Subcellular localization of mCIT-2xPH<sup>PLC</sup> and either MAP-mCH-dOCRL (E), or MAP-3xmCH (F), MAP-mCH-dOCRL<sup>dead</sup>(G), in cotyledon epidermal cells. Fluorescence intensity is color-coded (green fire blue scale), cytoplasmic strands are indicated by the arrows. Scale bars: 10  $\mu$ m.

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669 **Figure 6. Effect of PI(4,5)P<sub>2</sub> depletion on cytoskeleton dynamics in epidermal root cells.**

670 (A) F-actin cytoskeleton labelled by LifeAct-YFPv in cells expressing MAP-mCH-dOCRL or MAP-3xmCH. (B)

671 Cortical microtubules labelled by TUA6-GFP, in non-treated cells (no dex) or treated (dex for 16 hours) to induce

672 MAP-mCH-dOCRL or MAP-3xmCH expression. All pictures are z-projections. no dex: no treatment. dex:

673 dexamethasone treatment. Scale bars: 10 μm. (C) Example of the images used for the quantification of the anisotropy

674 using FibrilTool macro for imageJ. (D) Average anisotropy of the microtubule arrays in MAP-mCH-dOCRL or MAP-

675 3xmCH lines. For statistical analysis, we used a linear mixed effect model and then computed post hoc tests (*lsmeans*

676 package). Details for the statistical analysis can be found in Supplemental Table 5. \*\*\*: p-value < 0.0005; \*\*: p-value

677 < 0.05; n. s.: p-value ≥ 0.05.

678 **ADDITIONAL FILES**

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LMER (Type II Wald $\chi^2$ test)					Post hoc tests ( <i>lsmeans</i> )		
Model	Biosensor	$\chi^2$	df	P-value	Pair	Estimate	P-value
Dissociation index ~ construction + (1 image ID)	mCIT-2xPH <sup>PLC</sup>	149.9	2	< 2.2*10 <sup>-16</sup>	MAP-mCH-dOCRL - MAP-mCH-dOCRL <sup>dead</sup>	0.853	< <b>0.0001</b>
					MAP-mCH-dOCRL - MAP-3xmCH	1.083	< <b>0.0001</b>
					MAP-mCH-dOCRL <sup>dead</sup> - MAP-3xmCH	0.231	<b>0.0497</b>
	mCIT-TUBBY <sub>c</sub>	6.7286	1	0.009488	MAP-mCH-dOCRL - MAP-mCH-dOCRL <sup>dead</sup>	0.622	<b>0.0357</b>
	mCIT-P4M <sup>SidM</sup>	0.1258	1	0.7229	MAP-mCH-dOCRL - MAP-3xmCH	0.0523	0.7281
	mCIT-C2 <sup>Lact</sup>	4.9880 4	1	0.02564	MAP-mCH-dOCRL - MAP-3xmCH	0.142	<b>0.0425</b>

**Supplementary file 1. Statistical analysis of lipid biosensor dissociation indexes.**

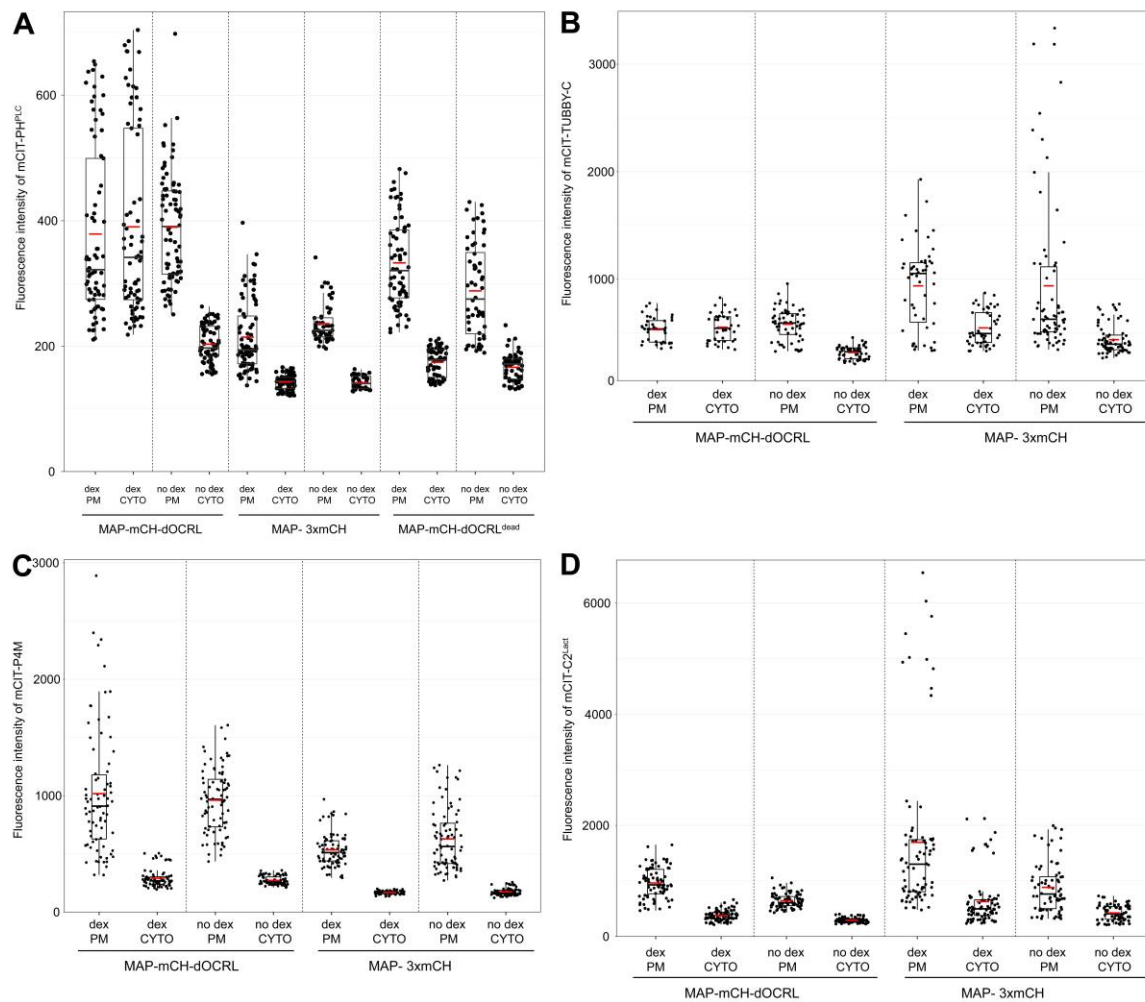
For various plasma membrane localized lipid biosensors, we compared dissociation indexes across inductions of constructions encoding MAP-mCH-dOCRL or negative controls (MAP-mCH-dOCRL<sup>dead</sup> and MAP-3xmCH). We used a linear mixed effect model accounting for image ID (*id* est root) as random factor (as one root per image were imaged, but ten cells per root used). We then computed post hoc tests (*lsmeans* package)

		dex treatment		no treatment	
		N	n	N	n
		<i>UBQ10pro:mCIT-2xPH<sup>PLC</sup></i>	<i>UBQ10pro:MAP-mCH-dOCRL</i>	7	74
	<i>UBQ10pro:MAP-mCH-dOCRL<sup>dead</sup></i>	8	80	8	80
	<i>UBQ10pro:MAP-3xmCH</i>	7	70	6	60
<i>UBQ10pro:mCIT-TUBBY<sub>c</sub></i>	<i>UBQ10pro:MAP-mCH-dOCRL</i>	4	40	6	60
	<i>UBQ10pro:MAP-mCH-dOCRL<sup>dead</sup></i>	5	50	8	80
<i>UBQ10pro:mCIT-P4M<sup>SidM</sup></i>	<i>UBQ10pro:MAP-mCH-dOCRL</i>	8	80	8	80
	<i>UBQ10pro:MAP-3xmCH</i>	8	80	8	80
<i>UBQ10pro:mCIT-C2<sup>Lact</sup></i>	<i>UBQ10pro:MAP-mCH-dOCRL</i>	8	80	8	80
	<i>UBQ10pro:MAP-3xmCH</i>	8	80	7	70

**Supplementary file 2. Size of workforces corresponding to dissociation indexes calculations.**

N: number of roots; n: number of cells (10 cells per roots except for 14 cells taken in one root (first line)).

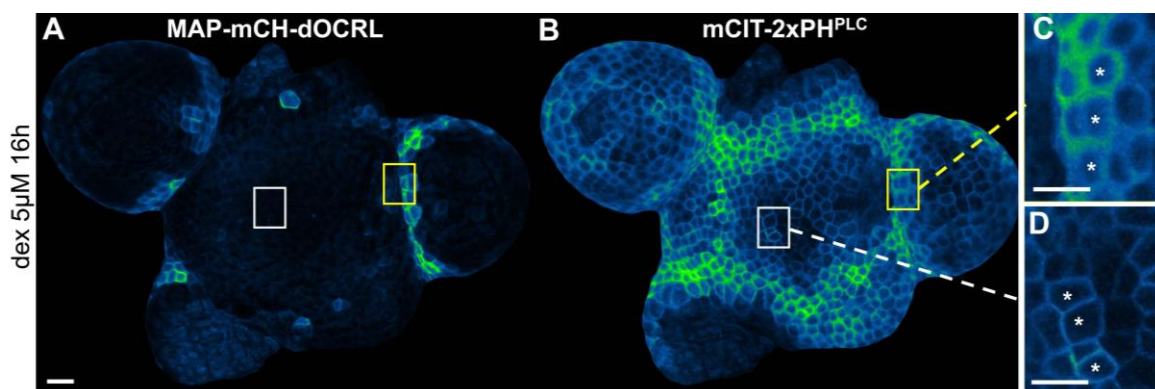
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### Supplemental file 3

Graphic representation of the fluorescent intensity observed for the different biosensors, used for the quantification of the dissociation index presented in Figure 3G.



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### Supplemental file 4

Induction of the iDePP system in the shoot apical meristem of Arabidopsis. (A) Subcellular localization of MAP-mCH-dOCRL after dex induction. (B) Subcellular localization of mCIT-2xPH<sup>PLC</sup> in the same shoot apical meristem after dex induction. (C-D) Close-ups from (B). The yellow ROI in (A), (B) and (C) represents the fluorescence observed in a zone where MAP-mCH-dOCRL was not induced. The white ROI in (A), (B) and (D) represents the fluorescence

691 observed in a zone where MAP-mCH-dOCRL was induced. Scale bar, 20  $\mu\text{m}$  in (A-B), 10  $\mu\text{m}$  in (C-D). Asterisks  
 692 represent the position of the nucleus in the cell.

LMER (Type II Wald $\chi^2$ test)					Post hoc tests (lsmeans)		
Model	Microtubule marker line	$\chi^2$	df	P-value	Pair	Estimate	P-value
Anisotropie ~ contruction + (1 image ID)	<i>TUA6-GFP</i>	12.155	2	0.002294	MAP-mCH-dOCRL DEX - MAP-mCH-dOCRL noDEX	-0.02353	0.0426
					MAP-mCH-dOCRL DEX - MAP-3xmCH DEX	-0.02710	0.0047
					MAP-mCH-dOCRL noDEX - MAP-3xmCH DEX	-0.00357	0.9387

**Supplementary file 5. Statistical analysis of microtubules anisotropy per cells.**

We used a linear mixed effect model accounting for image ID (*id* = root) as random factor (as one root per image were imaged, but five cells per root used). We then computed post hoc tests (*lsmeans* package

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Reagent and resources	Source	Identifier
<b>Bacterial strains</b>		
DH5α <i>E. coli</i> competent cells	ThermoFisher Scientific	Cat#18265
BL21(DE3) pLys <i>E. coli</i> competent cells	Novagen	H69
C58 GV3101 <i>A. tumefaciens</i> competent cells	N/A	N/A
<b>Chemicals</b>		
Dexamethasone	SIGMA	D1756
<b>Other</b>		
HIS-Select® Cobalt affinity resin	Sigma	H8162
<b>Experimental models: organisms/strains</b>		
BL21(DE3) pLys ( <i>Ec</i> )	Novagen	H69
BL21(DE3) pLys pMH-HS-dOCRL <sub>168-509</sub> ( <i>Ec</i> )	This study	
Col-0 accession ( <i>At</i> )		
<i>UBQ10pro:GVG-tE9::6xUAS-35Smini:MAP-mCH-dOCRL<sub>168-509</sub>/pH7</i> ( <i>At</i> )	This study	
<i>UBQ10pro:GVG-tE9::6xUAS-35Smini:MAP-mCH-dOCRL<sub>168-509</sub><sup>D468G</sup>/pH7</i> ( <i>At</i> )	This study	
<i>UBQ10pro:GVG-tE9::6xUAS-35Smini:MAP-3xmCH/pH7</i> ( <i>At</i> )	This study	
<i>UBQ10pro:mCIT-2xPH<sup>PLC</sup>/pB7</i> ( <i>At</i> )	(Simon et al., 2014c), NASC	N2105613
<i>UBQ10pro:mCIT-TUBBYc/pB7</i> ( <i>At</i> )	(Simon et al., 2014c), NASC	N2105610
<i>UBQ10pro:mCIT-P4M<sup>SidM</sup>/pB7</i> ( <i>At</i> )	(Simon et al., 2016c), NASC	N2107346
<i>UBQ10pro:mCIT-C2<sup>Lact</sup>/pB7</i> ( <i>At</i> )	(Platre et al., 2018a), NAC	N2107347
<i>UBQ10pro:mCIT-2xFYVE<sup>HRS</sup>/pB7</i> ( <i>At</i> )	(Simon et al., 2014c), NASC	
<i>UBQ10pro:LIFEACT-VENUS/pB7</i>	This study	
<i>35Spro:TUA6-GFP</i>		
<b>Recombinant DNA</b>		
pMH-HS cloning vector		
pMH-HS-dOCRL <sub>168-509</sub> vector for bacterial expression	This study	
Empty gateway entry vector <i>pDONR-P4PIR</i>	ThermoFisher	Cat#12537023
Empty gateway entry vector <i>pDONR221</i>	ThermoFisher	Cat#12536017
Empty gateway entry vector <i>pDONR207</i>	ThermoFisher	
Empty gateway entry vector <i>pDONR-P2RP3</i>	ThermoFisher	Cat#12537023
Empty gateway destination vector <i>pH7m34GW</i>	(Karimi et al., 2007)	
Promoter gateway entry vector <i>UBQ10pro:GVG-tE9::6xUAS-35Smini/pDONR-P4PIR</i>	This study	
Gateway entry vector <i>MAP-mCHnoSTOP/pDONR207</i>	This study, (Platre et al., 2018a)	
Gateway entry vector <i>dOCRL<sub>168-509</sub>/pDONR-P2RP3</i>	This study	
Gateway entry vector <i>dOCRL<sub>168-509</sub><sup>D468G</sup>/pDONR-P2RP3</i>	This study	
Gateway entry vector <i>2xmCH-4xMyc/pDONR-P2RP3</i>	(Marquès-Bueno et al., 2016), NASC	N2106292
Gateway destination vector <i>UBQ10pro:GVG-tE9:6xUAS-35Smini:MAP-mCH-dOCRL<sub>168-509</sub>/pH7m34GW</i>	This study	
Gateway destination vector <i>UBQ10pro:GVG-tE9:6xUAS-35Smini:MAP-mCH-dOCRL<sub>168-509</sub><sup>D468G</sup>/pH7m34GW</i>	This study	

Gateway destination vector <i>UBQ10pro:GVG-tE9:6xUAS-35Smini:MAP-3xmCH/pH7m34GW</i>	This study	
<b>Oligonucleotides</b>		
p5'_open_Rev AGCAACTTTTCTATACAAAGTTGGC	This study	
GVG-tE9_fwd caaaaaagcaggcttaaccATGAAGCTACTGTCTTC TATC	This study	
GVG-tE9_rev gggctagaGATCAGATTGTCGTTTCCC	This study	
6xUASmini35S_fwd aatctgacTCTAGCCCATCGGGTGAC	This study	
6xUASmini35S_rev gtacaagaaagctgggtTAGAGTCGAAGCTTGGGC	This study	
p5'_open_Fwd gcaagttgtacaaaaagtgaacg	This study	
NcoI_dOCRL_F TTTTACCATGgtaaagcaagagttaag	This study	
XhoI_dOCRL_R TTTTCTCGAGCTGTGGCTGATTATCGTTC	This study	
dOCRL_D469G_Fbis /5Phos/gcGGAcataagccagttatgcgg	This study	
dOCRL_D469G_Rbis /5Phos/GtccGCTTTGCCTAATTTCCATTATGC	This study	
MAPCt-mCh-pDONR207-F /5phos/catcactactctttgctgctgccgctgccgctatggtgagc aagggcgaggagg	This study	
MAPNt-pDONR2077-R /5phos/TCGACTTCTACTGCAGA GTAAGCCCATGGTAGCCTGCTTTTTTTGTAC AAACTTGGC	This study	
<b>Synthetic gene</b>		
<i>attB1-dOCRL<sub>168-509</sub>-attB2</i>	This study	

694 Supplementary file 6. Reagent and resources

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