

1 **A genetically validated approach to detect inorganic polyphosphates in plants.**

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14

15 **Abstract**

16 Inorganic polyphosphates (polyPs) are linear polymers of orthophosphate units linked by
17 phosphoanhydride bonds. PolyPs represent important stores of phosphate and energy, and are
18 abundantly found in many pro- and eukaryotic organisms. In plants, the existence of polyPs has been
19 established using microscopy and biochemical extraction methods that are now known to produce
20 artifacts. Here we use a polyP-specific dye and a polyP binding domain to detect polyPs in plant and
21 algal cells. To develop the staining protocol, we induced polyP granules in *Nicotiana tabacum* and
22 *Arabidopsis* cells by heterologous expression of *E. coli* polyphosphate kinase 1 (PPK1). Over-expression
23 of PPK1 but not of a catalytically impaired version of the enzyme lead to severe growth phenotypes,
24 suggesting that ATP-dependent synthesis and accumulation of polyPs in the plant cytosol is toxic. We
25 next crossed stable PPK1 expressing *Arabidopsis* lines with plants expressing the polyP-binding
26 domain of *E. coli* exopolyphosphatase (PPX1c), which co-localized with PPK1-generated polyP
27 granules. These granules were stained by the polyP-specific dye JC-D7 and appeared as electron dense
28 structures in transmission electron microscopy (TEM) sections. Using the polyP staining protocol
29 derived from these experiments, we screened for polyP stores in different organs and tissues of both
30 mono- and dicotyledonous plants. While we could not detect polyP granules in higher plants, we could
31 visualize the polyP-rich acidocalicisomes in the green algae *Chlamydomonas reinhardtii*. Together, our
32 experiments suggest that higher plants may not contain large polyPs stores.

33

34 **Keywords**

35 inorganic polyphosphate, energy metabolism, polyphosphate kinase, polyphosphate phosphatase,
36 Arabidopsis, Chlamydomonas

37

38 **Significance Statement**

39 A chemical dye and an inorganic polyphosphate binding domain are shown to specifically label
40 inorganic polyphosphate granules in transgenic Arabidopsis lines and Chlamydomonas
41 acidocalcisomes. Using these tools, we show that in contrast to many prokaryotic and eukaryotic
42 organisms, higher plants do not seem to contain large inorganic polyphosphate stores.

43

44 **Introduction**

45 PolyPs are linear inorganic phosphate (Pi) polymers, with sizes ranging from tripolyphosphate
46 (3 Pi units) to long-chain polyPs (~1,000 Pi units) (Kornberg, 1956; Kornberg *et al.*, 1957; Kulaev and
47 Vagabov, 1983; Clark and Wood, 1987). In bacteria, polyPs accumulate in volutin granules (Babes,
48 1895; Widra, 1959; Tumlirsch and Jendrossek, 2017) or in membrane-surrounded acidocalcisomes
49 (Seufferheld *et al.*, 2003). Bacterial polyPs are synthesized by two different classes of polyphosphate
50 kinases PPK1 (Kornberg, 1957; Ahn and Kornberg, 1990) and PPK2 (Zhang *et al.*, 2002) from ATP or
51 GTP, respectively. In yeast and other fungi, large amounts of long-chain polyPs (Macfarlane, 1936)
52 generated by the vacuolar transporter chaperone (VTC) complex (Hothorn *et al.*, 2009) accumulate in
53 the vacuole (Urech *et al.*, 1978) and in the cell wall (Werner *et al.*, 2007b). The eukaryotic VTC
54 complex is conserved among other unicellular eukaryotes such as protozoa (Fang *et al.*, 2007) and
55 green algae (Aksoy *et al.*, 2014), organisms that store polyPs in specialized vacuoles termed
56 acidocalcisomes (Docampo and Huang, 2016) and also in their cell walls (Werner *et al.*, 2007a). The
57 eukaryotic amoeba *Dictyostelium discoideum* contains polyP accumulating acidocalcisomes but lacks
58 the VTC complex. Instead *Dictyostelium* features two polyphosphate kinases that are similar to
59 bacterial PPK1 (Zhang *et al.*, 2005) and to an actin-related protein (Gómez-García and Kornberg,
60 2004), respectively. Neither PPK1, PPK2 nor the VTC complex appear to be present in higher
61 eukaryotes (Hooley *et al.*, 2008), but polyPs of various chain-length have been identified in
62 mitochondria and lysosomes of different animal and human cells and tissues (Pisoni and Lindley, 1992;
63 Kumble and Kornberg, 1995). PolyPs also accumulate in acidocalcisomes in human platelet and mast
64 cells (Ruiz *et al.*, 2004; Moreno-Sanchez *et al.*, 2012).

65 Invasive and non-invasive polyP detection techniques have been developed and applied to
66 detect specific polyP stores in bacteria, fungi and algae, all of which accumulate polyP to high levels.

67 Initially, polyP bodies were detected in microorganisms as metachromatic granules in light microscopy
68 experiments, and stained with unspecific dyes such as toluidine blue (Meyer, 1904). Using this method,
69 polyP granules were reported from lower plants, including algae and different mosses (Keck and Stich,
70 1957). In TEM sections, electron dense granules have been interpreted as polyP bodies or
71 acidocalcisomes, for example in the seeds of different palm species and in rice anthers (DeMason and
72 Stillman, 1986; Mamun *et al.*, 2005).

73 PolyPs can also be biochemically purified and initially the acid-labile polymer was converted to
74 Pi using strong acids. This rather unspecific and insensitive method was used early on to characterize
75 polyP stores in spinach leaves and in parasitic plants (Miyachi, 1961; Tewari and Singh, 1964). The
76 identification of highly specific bacterial and fungal polyP metabolizing enzymes, such as *E. coli* PPK1
77 (which can also catalyze the reverse reaction, the phosphorylation of ADP to ATP using polyP as a
78 phosphate donor) or the *E. coli* (Akiyama *et al.*, 1993) and *S. cerevisiae* (Wurst and Kornberg, 1994)
79 exopolyphosphatase PPX1, has enabled the enzymatic identification and quantification of polyPs
80 extracted from cells and tissues (Ault-Riché and Kornberg, 1999; Bru *et al.*, 2016). This method has, to
81 the best of our knowledge, not been applied to study polyP stores in higher plants.

82 The isolated polyP-binding domain of *E. coli* PPX1 has been used to immunolocalize polyPs in
83 fungal, algae and human cells (Saito *et al.*, 2005; Werner *et al.*, 2007b; Werner *et al.*, 2007a; Jimenez-
84 Nuñez *et al.*, 2012). PolyPs of different chain length can also be visualized on UREA-PAGE gels,
85 either using radioactive ³²P, toluidine blue or 4',6-diamidino-2-phenylindol (DAPI) (Ogawa *et al.*, 2000;
86 Smith and Morrissey, 2007). *In vivo* ³¹P nuclear magnetic resonance spectroscopy has been used to
87 define phosphate-containing metabolites in cells and tissues, including polyP in bacteria and lower
88 eukaryotes (Moreno *et al.*, 2000). Raman spectroscopy has been used to detect Pi-rich inositol
89 polyphosphates, but not inorganic polyphosphates in wheat grains (Kolozsvari *et al.*, 2015). *In situ*
90 staining methods for polyP have been developed using DAPI (Kulakova *et al.*, 2011). Its unique
91 emission spectrum allows to distinguish polyP from nucleic acids (also interacting with DAPI), but not
92 from inositol polyphosphates also abundant in plants (Kolozsvari *et al.*, 2014). Two specific dyes for
93 polyP, JC-D7 and JC-D8 have been developed and employed to localize polyP stores in mammalian
94 cells.

95 We have recently uncovered a molecular connection between the Pi starvation response
96 controlled by inositol pyrophosphate (PP-InsPs) signaling molecules and their SPX receptors, and
97 polyP metabolism in yeast (Wild *et al.*, 2016). PP-InsPs and SPX domains also control the Pi starvation
98 response in Arabidopsis (Zhu *et al.*, 2018), and we have characterized the short-chain polyphosphatase
99 TRIPHOSPHATE TUNNEL METALLOENZYME 3 (AtTTM3) (Martinez *et al.*, 2015; Lorenzo-Orts,

100 Witthoef, *et al.*, 2019) and a specific polyP-binding domain (Lorenzo-Orts, Hohmann, *et al.*, 2019) in
101 plants. We thus speculate that polyPs may exist in plants and that they could form a relevant Pi store.
102 Here we make use of both the JC-D7 dye (Angelova *et al.*, 2014) and the PPX1 polyP-binding domain
103 to probe for the presence of polyP stores in different plant cells and tissues.

104

105 **Results**

106 To test if JC-D7 (Angelova *et al.*, 2014) can label polyP in intact plant cells we sought to
107 introduce defined polyP stores, allowing for a genetically validated detection of polyP. To this end, we
108 generated transgenic Arabidopsis lines expressing the bacterial polyphosphate kinase PPK1 from *E.*
109 *coli*, which polymerizes long-chain polyPs from ATP (Kornberg, 1957; Ahn and Kornberg, 1990),
110 under the control of the Ubi10 promoter and carrying a C-terminal mCitrine (mCit) tag (see Methods).
111 EcPPK1 has been previously characterized as a histidine kinase, and mutation of His435 to alanine
112 impaired polyP accumulation in *E. coli* (Kumble *et al.*, 1996). The crystal structure of EcPPK1 in
113 complex with a non-hydrolyzable substrate analog revealed that His435 and His592 are in direct
114 contact with the β - and γ -phosphates of the ATP substrate (Figure 1a) (Zhu *et al.*, 2005). Based on this
115 analysis, we generated a control line expressing a catalytically impaired PPK1 (mPPK1), in which
116 His435 and His592 are replaced by alanine. PPK1-mCit T3 lines displayed a strong growth phenotype
117 with small purple leaves, which correlates with PPK1-mCit protein levels (Figure 1b,c). The mPPK1-
118 mCit control lines are similar to wild-type.

119 mPPK1-mCit control plants show a uniform expression of the fusion protein in root tips (Figure
120 2). In contrast, expression of the catalytically active PPK1-mCit is patchy, with some cells
121 accumulating the fusion protein to high levels and others showing small punctate structures. These
122 punctate structures appear as dense granules in the corresponding bright field images (Figure 2).

123 We next tested if ectopic expression of PPK1 introduces polyP stores in plant cells and if those
124 stores can be detected using the JC-D7 dye. We transiently expressed the PPK1-mCit and mPPK1-mCit
125 constructs in *Nicotiana tabacum* leaves, where we again found mPPK1-mCit to be evenly distributed in
126 the cytosol, while PPK1-mCit localized in punctate structures (Figure 3a). We stained epidermal cells
127 with JC-D7 (see Methods), and found the dye to co-localize with the punctate structures in PPK1-mCit
128 expressing cells, but not in the mPPK1-mCit control (Figure 3a). We hypothesized that PPK1-mCit
129 localized to polyP granules generated by the bacterial enzyme, which are labeled by JC-D7. To test this
130 hypothesis, we used the polyP binding domain of the *E. coli* exopolyphosphatase PPX1 (Akiyama *et al.*,
131 1993), which has been previously employed to specifically stain polyP stores in fungal, algal and
132 human cells (Saito *et al.*, 2005; Werner *et al.*, 2007a; Werner *et al.*, 2007b; Jimenez-Nuñez *et al.*,

133 2012). To this end, we generated a PPXc – mCherry (mChe) fusion protein (residues 311-508; Figure
134 3b). We found PPXc-mChe to localize to the cytoplasm and nucleus when transiently expressed in
135 *Nicotiana tabacum*, and in stable transgenic Arabidopsis lines (Figure 3c). Next, we crossed PPX1c-
136 mChe with our PPK1-mCit and mPPK1-mCit lines, which revealed PPXc-mChe to co-localize with
137 PPK1-mCit in JC-D7 stained granules in hypocotyl cells (Figure 3d). This was not observed in the
138 mPPK1-mCit control lines (Figure 3d). Co-localization of PPK1-mCit, PPXc-mChe and the JC-D7 dye
139 could also be observed in fixed leaf epidermal and hypocotyl cells in Arabidopsis (Figure S1). TEM
140 analysis of hypocotyl sections revealed the presence of electron-dense granules in PPK1-mCit but not
141 in mPPK1-mCit expressing lines (Figure 4). Together, these experiments suggest that PPK1 introduces
142 polyP granules in Arabidopsis, which can be detected by both the JC-D7 dye and the PPX1c polyP-
143 binding domain.

144 While we could readily detect PPK1-generated polyP granules in different cells and tissues
145 from Arabidopsis and tobacco, we could not reproducibly detect JC-D7 stained structures in Col-0
146 wild-type (Figures S2, S3). PolyP also did not accumulate in a previously characterized loss-of-
147 function mutant of AtTTM3 (*ttm3-1*) (Moeder *et al.*, 2013; Lorenzo-Orts, Witthoef, *et al.*, 2019).
148 (Figures S2, S3). We next stained different mono- and dicots using the JC-D7 dye (examples are shown
149 in Figure S4) but again could not detect a polyP-specific signal. In contrast, JC-D7 stained the
150 previously characterized polyP containing acidocalcisomes of *Chlamydomonas reinhardtii*, which
151 appeared enriched in a sulfur depleted growth condition (Gal *et al.*, 2018; Aksoy *et al.*, 2014) (Figure
152 5).

153

154 Discussion

155 PolyPs are evolutionary ancient energy polymers and have been identified in a variety of pro-
156 and eukaryotic organisms (Rao *et al.*, 2009). Based on earlier reports (Keck and Stich, 1957; Miyachi,
157 1961; Tewari and Singh, 1964; DeMason and Stillman, 1986), polyPs have been assumed to exist in
158 higher plants (Clark and Wood, 1987; Kornberg *et al.*, 1999; Kulaev, 2005). However, just like in
159 animals the synthesis, storage and breakdown of polyP has been poorly characterized and no
160 physiological functions have been assigned to polyP in plants. In the green algae *Chlamydomonas*
161 *reinhardtii* polyP stores have been identified in acidocalcisomes (Ruiz *et al.*, 2001; Gal *et al.*, 2018) and
162 in the cell wall compartment (Werner *et al.*, 2007a), as it has been seen in other unicellular eukaryotes
163 such as yeast or trypanosomes (Docampo and Huang, 2016). PolyP in *Chlamydomonas* is synthesized
164 by the vacuolar transporter chaperone VTC complex (Aksoy *et al.*, 2014), whose catalytic subunit Vtc4
165 generates polyP from ATP (Hothorn *et al.*, 2009), and translocates the growing polymer to the

166 vacuole/acidocalcisome using a membrane pore (Gerasimaitė *et al.*, 2014). While the VTC complex is
167 apparently absent in higher plants, proteins sharing structural homology with the Vtc4 catalytic domain
168 exist in Arabidopsis (Moeder *et al.*, 2013; Martinez *et al.*, 2015; Ung *et al.*, 2017). We have previously
169 characterized AtTTM3 as a specific short-chain inorganic polyphosphatase (Martinez *et al.*, 2015),
170 which is transcribed in a bicistronic transcript also encoding the cell cycle regulator AtCDC26
171 (Lorenzo-Orts, Witthoef, *et al.*, 2019).

172 Since the polyP-synthesizing enzymes in higher eukaryotes remain to be identified, we set out
173 to develop a genetically validated method that would allow for the specific detection of polyP stores in
174 living cells. DAPI has been used to visualize polyP stores in various pro- and eukaryotic cells using a
175 unique DAPI-polyP emission spectrum (Aschar-Sobbi *et al.*, 2008; Puchkov, 2010; Kulakova *et al.*,
176 2011; Gomes *et al.*, 2013; Martin and Van Mooy, 2013). However, DAPI has recently been shown to
177 also interact with inositol phosphates (Kolozsvari *et al.*, 2014) and many of the staining procedures
178 lacked suitable genetic controls that would confirm the identity of the stained structures as *bona fide*
179 polyP stores. We thus chose to heterologously express a bacterial polyP kinase PPK1 to induce specific
180 polyP granules in *Nicotiana tabacum* and Arabidopsis cells (Figures 1,3). We found that expression of
181 PPK1 but not of a catalytically inactive version of the enzyme led to stunted growth and anthocyanin
182 accumulation in stable transgenic lines. This is possibly due to the accumulation of polyPs in the
183 cytosol, which has been previously shown to have severe toxic effects in yeast (Gerasimaitė *et al.*,
184 2014). As polyPs are very potent chelators of divalent cations, the presence of polyPs in the cytosol
185 may inhibit the activity of many enzymatic processes and disrupt cellular structures. In line with this,
186 we observe patchy expression of a PPK1-mCit but not the mPPK1-mCit fusion protein in the root tip of
187 Arabidopsis (Figure 2).

188 The PPK1-generated polyP granules in Arabidopsis co-localize with the PPK1-mCit fusion
189 protein itself and with the polyP-specific binding domain PPXc from *E. coli* (Figure 3). Importantly,
190 these structures were specifically stained with the JC-D7 dye, previously shown to detect polyP
191 granules in human cells (Angelova *et al.*, 2014). The same structures appear as electron dense granules
192 in TEM analysis (Figure 4). Together, our experiments suggest that JC-D7 is a specific dye for polyP
193 detection in plants. In line with this, JC-D7 stained the known, VTC-generated polyP stores in
194 *Chlamydomonas* (Ruiz *et al.*, 2001; Aksoy *et al.*, 2014). However, we failed to reproducibly detect
195 polyP granules in wild-type Arabidopsis, tobacco, maize, *Phaesolus vulgaris* or *Ricinus communis*
196 plants (Figures. 3, S2-4), the latter of which contains the polyP-specific binding protein RcCHAD
197 (Lorenzo-Orts, Hohmann, *et al.*, 2019). Also, we could not detect polyP accumulation in *ttn3-1* mutant,
198 which we hypothesized to have reduced polyP phosphatase activity (Figures S2,S3) (Martinez *et al.*,

2015; Lorenzo-Orts, Witthoef, *et al.*, 2019). These findings suggest that either plants do not contain polyP stores, that they are present in concentrations too low to be detected by JC-D7, or that they do not accumulate in the tissues and/or growth conditions analyzed. In line with this, inositol polyphosphates including phytic acid (inositol hexakisphosphate) but not inorganic polyPs were detected by Raman spectroscopy in wheat grains (Kolozsvari *et al.*, 2015). However, our results are in stark contrast with earlier reports relying on biochemical extraction and hydrolysis methods (Miyachi, 1961; Tewari and Singh, 1964; DeMason and Stillman, 1986), which inadvertently may have detected inositol polyphosphates and/or other organic phosphates.

Taken together, we could demonstrate that indeed JC-D7 can specifically stain dense polyP granules in living and fixed plant and algal cells, but we failed to detect significant polyP stores in wild-type mono- or dicotyledonous plants. Of course, we cannot rule out the possibility that higher plants only accumulate polyP in specific organs or cells, only in certain developmental stages, or in response to certain environmental stimuli. The lack of significant polyP stores however raises the question, why conserved, broadly expressed polyP phosphatases exist in plants (Moeder *et al.*, 2013; Martinez *et al.*, 2015; Lorenzo-Orts, Witthoef, *et al.*, 2019) and why some species adopted polyP-specific binding proteins (Lorenzo-Orts, Hohmann, *et al.*, 2019). At this point, despite our efforts the statement by Harold (1966) remains true: “The case for the occurrence of polyP in higher plants and animals would be materially strengthened by the demonstration of appropriate biosynthetic enzymes” (Harold, 1966).

218

219 **Experimental procedures**

220

221 **Plant material and growth conditions**

222 Plants were grown at 21°C with 50 % humidity and a 16 h light : 8 h dark cycle. For confocal imaging, Arabidopsis seedlings were generally grown on vertical plates with half-strength Murashige and Skoog (¹/₂MS, Duchefa) media containing 1% (w/v) sucrose, 0.5 g/L MES buffer and 0.8 % (w/v) agar (Duchefa), pH 5.7 for 7 to 8 days. Seeds were sequentially surface-sterilized using 70 % (v/v) ethanol, a 5 % hypochlorite solution (Javel, 13 - 14 %), and rinsed 4 times with sterilized water.

227 The Arabidopsis *ttm3-1* (SALK_133625) mutant was obtained from the European Arabidopsis Stock Center (<http://arabidopsis.info/>) (Lorenzo-Orts, Witthoef, *et al.*, 2019). *Chlamydomonas reinhardtii* strain WT 2C (mt⁺) cells were grown in tris-acetate phosphate medium (Kropat *et al.*, 2011), in white light originating from fluorescent tubes at 60 μM photons m⁻² s⁻¹ and at 25°C. The -sulfur medium was prepared by substituting sulfates by their corresponding chloride salts.

232 **Generation of Arabidopsis transgenic lines**

233 The coding sequences of *Escherichia coli* PPK1 (UniProt ID C3T032) and the mPPK1
234 catalytically inactive mutant (H435A; H592A) were assembled together with a Ubi10 promoter and a
235 C-terminal mCit tag and introduced into destination vector pH7m34GW2 and pB7m34GW2
236 respectively as described (Lorenzo-Orts, Hohmann, *et al.*, 2019). The coding sequence of the C-
237 terminal polyP binding domain of *Escherichia coli* exopolyphosphatase (PPXc, UniPro ID P0AFL6)
238 was cloned into a pDONR221 vector using forward primer
239 GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGCATCAGGATGTGCGTAGTCGC and
240 reversed primer GGGGACCACTTTGTACAAGAAAGCTGGGTAAGTACTTTCTTCTTCAATTTTC.
241 The PPXc domain was fused to a C-terminal mChe tag and expression was driven by a Ubi10
242 promoter, assembled in destination vector pH7m34GW2. All of the constructs were transformed into *A.*
243 *tumefaciens* strain pGV2260 by heat shock. All of transgenic lines were generated in Col-0 background
244 using the floral dip method (Clough and Bent, 1998). T3 generation transformants were selected in
245 ^{1/2}MS medium (Duchefa), supplement with 50 µg/mL BASTA or 20 µg/mL Hygromycin.

246

247 **Confocal imaging**

248 All light microscopy imaging was performed on a Zeiss LSM780 confocal microscope using a
249 40 x / 1.3 water objective. Various confocal settings were set to record the emission of mCitrine
250 (excitation 514 nm; emission 517-550 nm), mCherry (excitation 594 nm; emission 606-632nm), and
251 JC-D7 (excitation 405 nm; emission 480-510 nm) using a GaAsP detector, and chlorophyll (excitation
252 594 nm; emission 653-698 nm) was imaged with a PMT detector. The sequential scanning mode was
253 used for imaging multiple-color images. JC-D7 (Angelova *et al.*, 2014) (Glix Laboratories Inc.) was
254 applied at a concentration of 100 µM for 1.5 h at room temperature in HEPES buffered salt solution
255 (HBSS) composed of 10 mM HEPES/NaOH (pH 7.4), 156 mM NaCl, 3 mM KCl, 2 mM MgSO₄, 1.25
256 mM KH₂PO₄, 2 mM CaCl₂, 10 mM glucose, as described (Angelova *et al.*, 2014). 50 mM stock
257 solution were prepared in 100% (v/v) DMSO. Live cell imaging was performed in root, hypocotyl, and
258 leaf epidermis cells. Plant tissues or *C. reinhardtii* strain WT 2C (mt⁺) were incubated together with JC-
259 D7 in HBSS buffer. To image fixed plant tissues, samples were fixed in vacuum in PBS buffer
260 containing 4 % (v/v) para-formaldehyde, 0.1 % (v/v) Triton X-100 and 1 mM DTT (in the case of
261 roots), then sequentially washed two times in PBS and two times in HBSS buffer.

262 To co-localize PPK1-induced polyP granules with PPXc-mChe and JC-D7, Arabidopsis lines
263 harboring PPK1-mCit or PPK1m-mCit were crossed with plants expressing PPXc-mChe. F1 generation
264 seeds were grown on ^{1/2}MS medium for 8-9 d and then stained by JC-D7 as described above. To co-

265 localize transiently expressed PPK1-mCit and PPK1m-mCit with JC-D7, *A. tumefaciens* cell cultures
266 were infiltrated into *Nicotiana benthamiana* leaves as described (Lorenzo-Orts, Hohmann, *et al.*, 2019).
267 Epidermal cells were imaged by confocal microscopy after 3 d incubation at room temperature.

268

269 **Transmission electron microscope (TEM) imaging**

270 Samples for TEM were fixed overnight at 4 °C in 2.5% (v/v) glutaraldehyde, 0.01% (v/v)
271 Tween-20 in 100 mM sodium cacodylate pH 7.0, after vacuum infiltration. A first post-fixation was
272 done in 1.5 % (w/v) osmium tetroxide for 2 h at 4 °C, and a second post-fixation in 1 % (w/v) uranyl
273 acetate for 1 h at 4 °C. Seedlings were then embedded in 1.5 % (w/v) agarose, dehydrated in a graded
274 ethanol series and finally embedded in Epon 812. 85 nm ultra-thin sections were made using a UCT
275 microtome (Leica) and deposited on Formvar films on copper grids; they were stained for 25 min with
276 2 % (w/v) uranyl acetate, then for 20 min with Reynolds lead citrate, and finally observed with a Tecnai
277 G2 Sphera (FEI) at 120kV equipped with a high-resolution digital camera.

278

279 **Plant protein extraction for western blot**

280 Around 250 mg plant tissue was harvested from the shoots of 4 week old plants, frozen in liquid
281 N₂ in 2 ml eppendorf tubes with metal beads, and ground in a tissue lyzer (MM400, Retsch). Ground
282 plant material was resuspended in 500 µl extraction buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 10
283 % [v/v] glycerol, 1 % [v/v] Triton X-100, 5 mM DTT) containing a protease inhibitor cocktail (P9599,
284 Sigma, 1 tablet / 20 ml) and centrifuged for 30 min and 1700 x g at 4°C. Protein concentrations were
285 estimated in Bradford protein assays. Around 100 µg protein was mixed with 2x SDS loading buffer,
286 boiled at 95 °C for 10 min, and separated on 8 % SDS-PAGE gels. Anti-GFP antibody coupled with
287 horse radish peroxidase (HRP, Miltenyi Biotec) at 1:2000 dilution was used to detect eGFP/mCit
288 tagged protein constructs.

289

290 **Structural diagrams**

291 for the crystals structures of *E. coli* PPK1 (<http://rcsb.org> PDB-ID 1XDO) (Zhu *et al.*, 2005)
292 and PPX (PDB-ID 1U6Z) (Alvarado *et al.*, 2006) were prepared using Pymol
293 (<https://sourceforge.net/projects/pymol/>).

294

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303

304 **Figure legends**

305 **Figure1. Heterologous expression of *E. coli* PPK1 results in dose-dependent stunted growth in** 306 ***Arabidosis*.**

307 (a) Structural view of the PPK active site. PPK1 is show in blue (PDB-ID 1XDO, ribbon diagram) with
308 the catalytic His435 and His592 (in bonds representation, in yellow) contacting the γ and β phosphates
309 of the ATP substrate (in bonds representation). (b) Growth phenotype of PPK1 and mPPK1 expressing
310 plants. Shown are rosettes of 4 week old plants (upper panel) and 5 week old flowering plants (lower
311 panel). Three independent lines are shown for each PPK1-mCit or mPPK1-mCit. (c) Western blot of
312 the lines shown in (b, lower panel), using an anti-GFP antibody.

313

314 **Figure 2. PPK1-mCit but not mPPK1-mCit expression results in the formation of punctate** 315 **structures surrounded by the enzyme.**

316 Confocal imaging of root tips of 7 DAG seedlings grown on $^{1/2}$ MS plates. Col-0, PPK1-mCit and
317 mPPK1-mCit expressed seedlings were imaged with similar confocal settings. Enlarged squares
318 highlight the presence of dense punctate structures (highlighted by arrow heads) in PPK1 but not
319 mPPK1 expressing lines. Scale bar = 20 μ m.

320

321 **Figure 3. JC-D7 and PPXc mark polyP granules in PPK1-mCit but not mPPK1-mCit expressing** 322 **plants.**

323 (a) Transiently expressed PPK1-mCit (green) co-localizes with JC-D7 (magenta) stained polyP
324 granules outside chloroplasts (blue) in tobacco epidermal cells. White arrow heads highlight the
325 granule structures, and black arrow heads indicate putative nuclear structures.

326 (b) Overview of the PPX polyP binding domain PPXc (PDB-ID 1U6Z, residues 311-508, ribbon
327 diagram, in magenta) bound to sulfate ions which mimic the position of the polyP substrate (Alvarado
328 *et al.*, 2006) (in bonds representation).

329 (c) Sub-cellular localization of mChe tagged PPXc (magenta) expressed in isolation in *Nicotiana*
330 *tabacum* epidermal cells and Arabidopsis hypocotyl cells. Arrow heads indicate putative nuclear
331 structures.

332 (d) Arabidopsis lines that stably express PPXc-mChe were crossed with lines that stably express PPK1-
333 mCit and mPPK1-mCit. Active PPK1-mCit (green, upper panel) but not its catalytic impaired mutant
334 version (green, lower panel) co-localizes with PPXc-mChe (magenta) on JC-D7 (cyan) stained polyP
335 granules. Scale bars = 10 μm .

336

337 **Figure 4. Electron dense granules are present in TEM sections of PPK1-mCit but not mPPK1-**
338 **mCit expressing plants.**

339 Hypocotyl sections (upper panel, scale bar = 50 μm) together with a zoomed in view on xylem cells
340 (lower panel, scale bar = 5 μm) of PPK1-mCit and mPPK1-mCit expressing Arabidopsis lines. Arrow
341 heads highlight electron dense granules.

342

343 **Figure 5. JC-D7 can specifically stain polyP granules in *Chlamydomonas reinhardtii*.**

344 JC-D7 (green) stained polyP granules in *Chlamydomonas* cells grown in tris-acetate phosphate
345 medium. The -sulfur medium was prepared by substituting sulfates by their corresponding chloride
346 salts. Chlorophyll auto-fluorescence is shown in magenta. Scale bars = 10 μm .

347

348 **Figure S1. PolyP granules can be detected in fixed Arabidopsis cells expressing PPK1-mCit.**

349 Arabidopsis seeds from PPXc-mChe / PPK1-mCit (upper panel) or PPXc-mChe / mPPK1-mCit (lower
350 panel) double transgenic lines were germinated on ^{1/2}MS medium. 9 d old seedlings were fixed by para-
351 formaldehyde and subsequently stained with JC-D7. Arrow heads highlight structures where PPXc-
352 mChe (magenta) and active PPK1-mCit (green) co-localize with JC-D7 (cyan) stained polyP granules
353 in leaf and hypocotyl cells. No co-localization or JC-D7 staining was observed in mPPK1-mCit
354 expressing control lines. Scale bars = 10 μm .

355

356 **Figure S2. JC-D7 fails to stain specific structures in hypocotyl cells of Col-0 and *ttn3-1* plants.**

357 9 d old Col-0 and *ttn1-3* seedlings grown on ^{1/2}MS plates were stained by JC-D7 and imaged by
358 confocal scanning microscopy, the PPK1-mCit line is shown as a control alongside. White arrow heads
359 show PPK1-mCit (green) and JC-D7 (magenta) stained polyP granules in PPK1-mCit expressed
360 hypocotyl cells, no specific JC-D7 staining was observed in Col-0 or *ttn3-1* hypocotyl cells. Scale bars
361 = 20 μm .

362 **Figure S3. JC-D7 fails to stain specific structures in fixed hypocotyl cells of Col-0 and *ttm3-1***
363 **plants.**

364 Samples were grown and imaged as described in Figure S2, using a fixation protocol specified in
365 Experimental Procedures. Scale bars = 20 μm .

366

367 **Figure S4. No JC-D7 stained polyP granules could be detected in epidermal cells of mono- and**
368 **dicotyledonous plants.**

369 Epidermal cells from *Zea mais*, *Phaseolus vulgaris* and *Ricinus communis* were stained using the JC-
370 D7 dye. No specific polyP signal was detected by confocal scanning microscopy.

371

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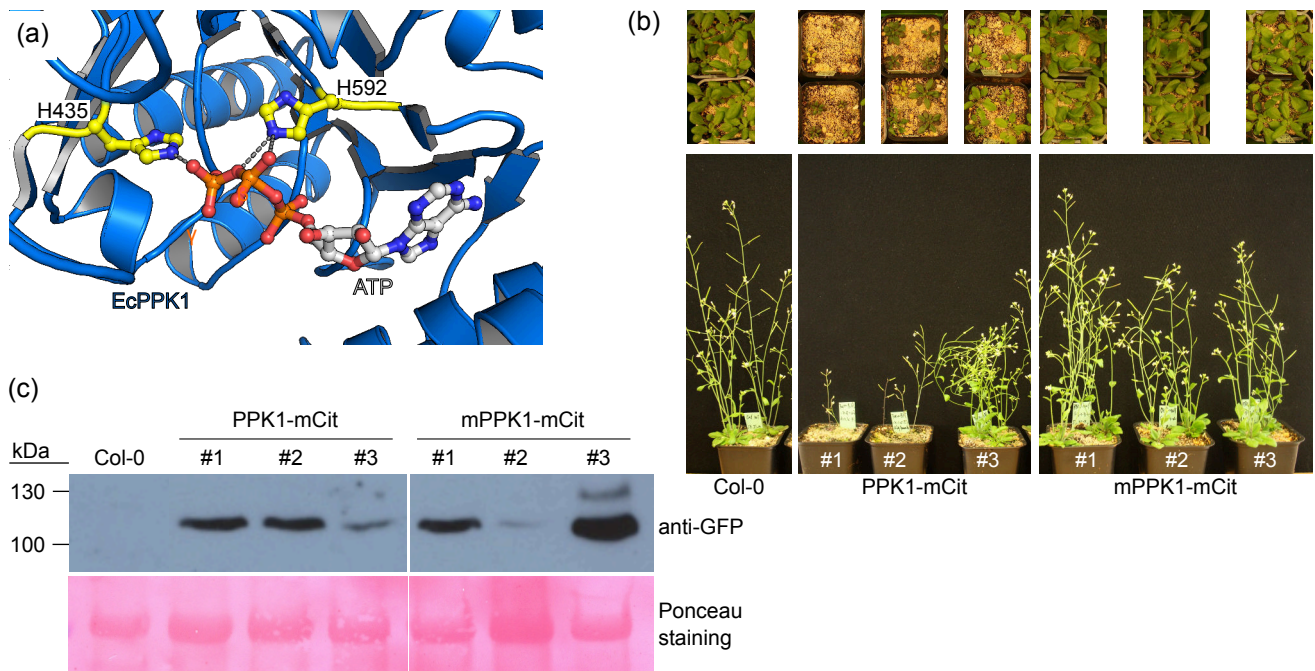


Figure1. Heterologous expression of *E. coli* PPK1 results in dose-dependent stunted growth in Arabidopsis.

(a) Structural view of the PPK active site. PPK1 is shown in blue (PDB-ID 1XDO, ribbon diagram) with the catalytic His435 and His592 (in bonds representation, in yellow) contacting the γ and β phosphates of the ATP substrate (in bonds representation). (b) Growth phenotype of PPK1 and mPPK1 expressing plants. Shown are rosettes of 4 week old plants (upper panel) and 5 week old flowering plants (lower panel). Three independent lines are shown for each PPK1-mCit or mPPK1-mCit. (c) Western blot of the lines shown in (b, lower panel), using an anti-GFP antibody.

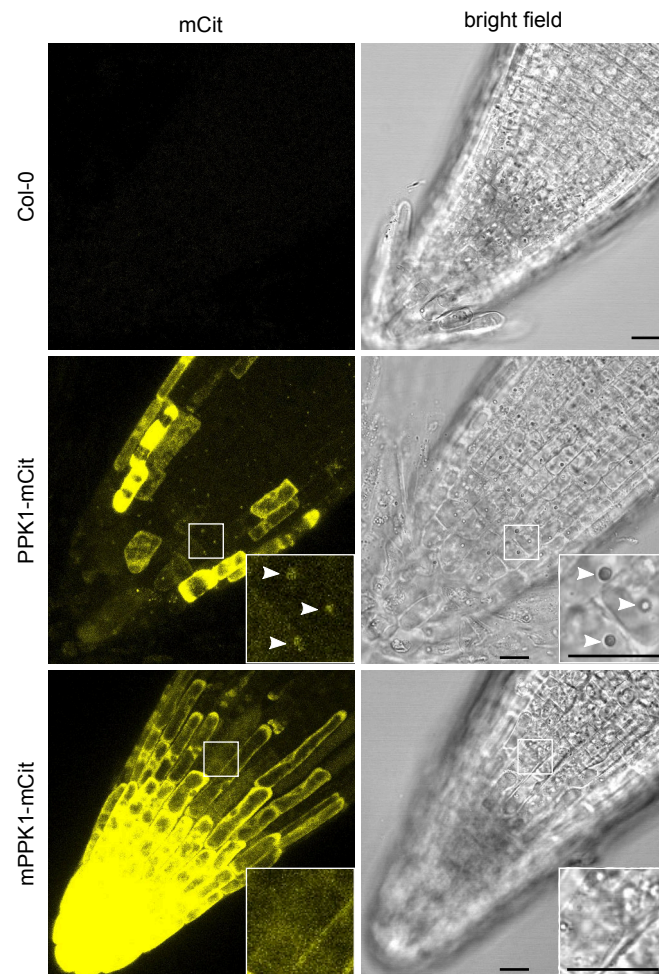


Figure 2. PPK1-mCit but not mPPK1-mCit expression results in the formation of punctate structures surrounded by the enzyme.

Confocal imaging of root tips of 7 DAG seedlings grown on ^{12}C MS plates. Col-0, PPK1-mCit and mPPK1-mCit expressed seedlings were imaged with similar confocal settings. Enlarged squares highlight the presence of dense punctate structures (highlighted by arrow heads) in PPK1 but not mPPK1 expressing lines. Scale bar = 20 μm .

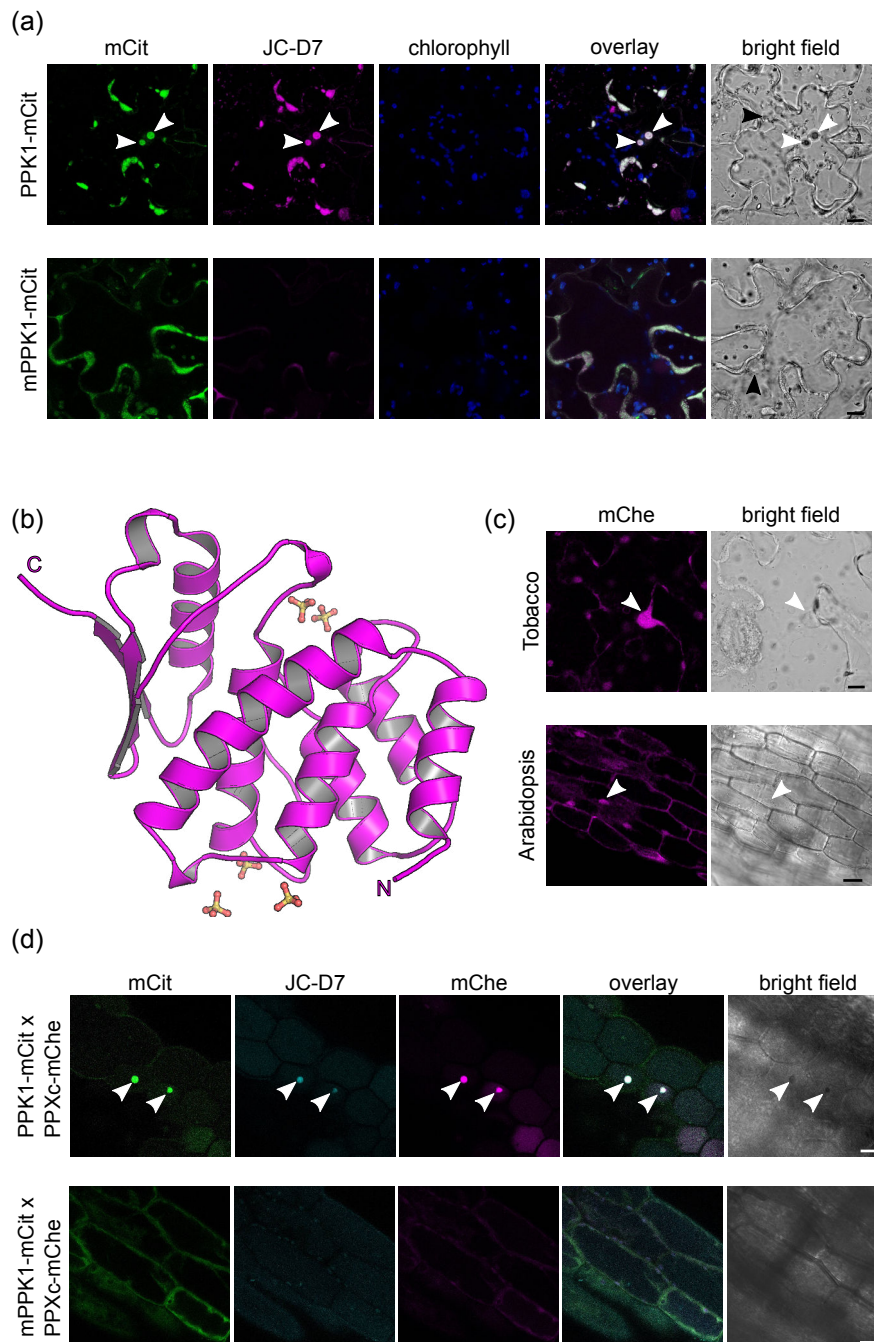


Figure 3. JC-D7 and PPXc mark polyP granules in PPK1-mCit but not mPPK1-mCit expressing plants.

(a) Transiently expressed PPK1-mCit (green) co-localizes with JC-D7 (magenta) stained polyP granules outside chloroplasts (blue) in tobacco epidermal cells. White arrow heads highlight the granule structures, and black arrow heads indicate putative nuclear structures.

(b) Overview of the PPX polyP binding domain PPXc (PDB-ID 1U6Z, residues 311-508, ribbon diagram, in magenta) bound to sulfate ions which mimic the position of the polyP substrate (Alvarado *et al.*, 2006) (in bonds representation).

(c) Sub-cellular localization of mChe tagged PPXc (magenta) expressed in isolation in *Nicotiana tabacum* epidermal cells and Arabidopsis hypocotyl cells. Arrow heads indicate putative nuclear structures.

(d) Arabidopsis lines that stably express PPXc-mChe were crossed with lines that stably express PPK1-mCit and mPPK1-mCit. Active PPK1-mCit (green, upper panel) but not its catalytic impaired mutant version (green, lower panel) co-localizes with PPXc-mChe (magenta) on JC-D7 (cyan) stained polyP granules. Scale bars = 10 μ m.

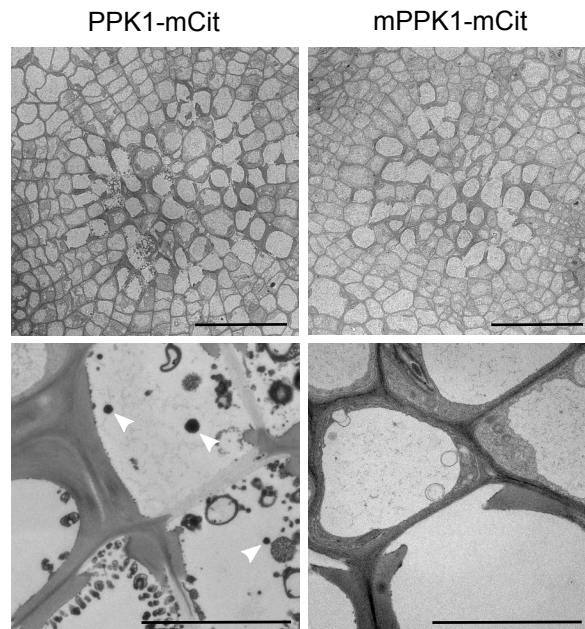


Figure 4. Electron dense granules are present in TEM sections of PPK1-mCit but not mPPK1-mCit expressing plants. Hypocotyl sections (upper panel, scale bar = 50 μm) together with a zoomed in view on xylem cells (lower panel, scale bar = 5 μm) of PPK1-mCit and mPPK1-mCit expressing Arabidopsis lines. Arrow heads highlight electron dense granules.

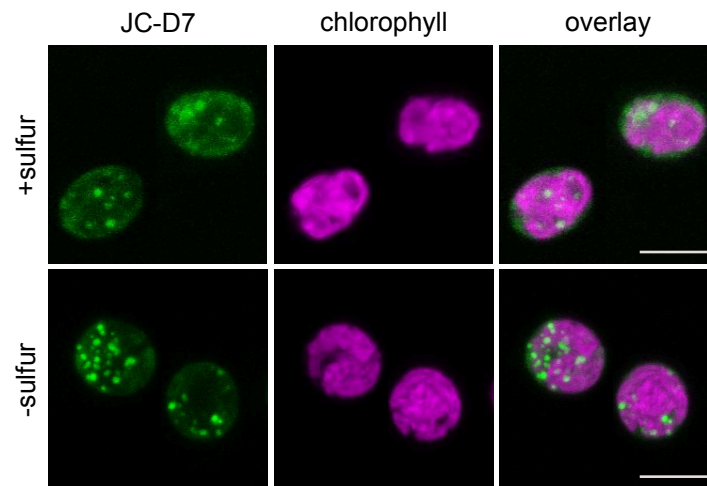


Figure 5. JC-D7 can specifically stain polyP granules in *Chlamydomonas reinhardtii*.

JC-D7 (green) stained polyP granules in *Chlamydomonas* cells grown in tris-acetate phosphate medium. The -sulfur medium was prepared by substituting sulfates by their corresponding chloride salts. Chlorophyll auto-fluorescence is shown in magenta. Scale bars = 10 μ m.

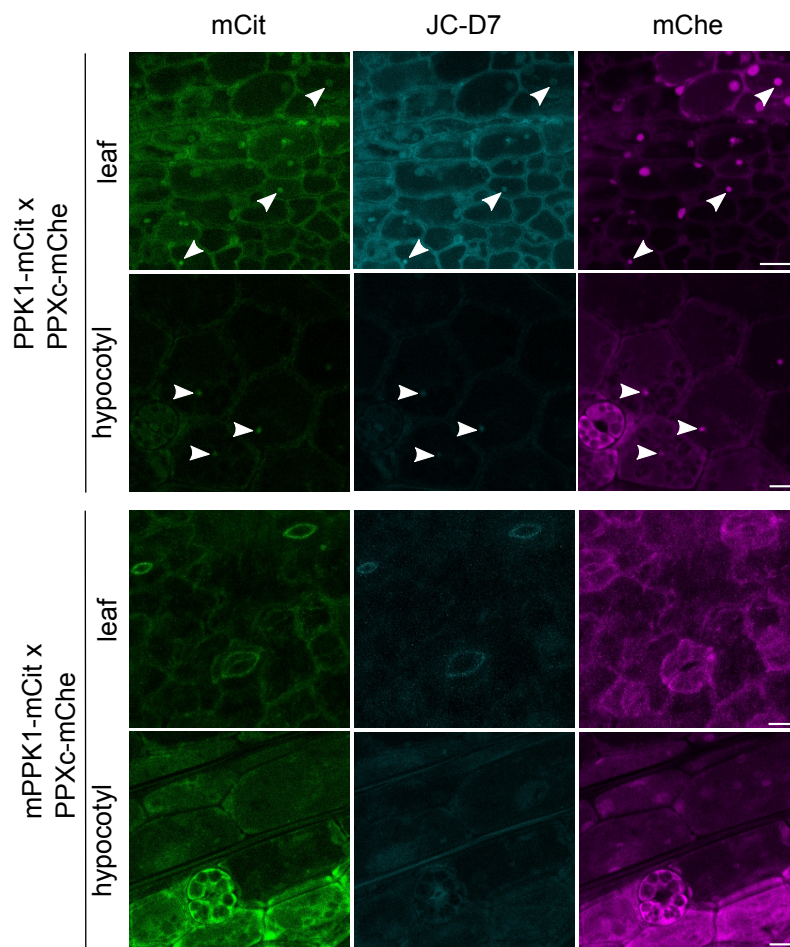


Figure S1. PolyP granules can be detected in fixed Arabidopsis cells expressing PPK1-mCit.

Arabidopsis seeds from PPXc-mChe / PPK1-mCit (upper panel) or PPXc-mChe / mPPK1-mCit (lower panel) double transgenic lines were germinated on $^{1/2}$ MS medium. 9 d old seedlings were fixed by para-formaldehyde and subsequently stained with JC-D7. Arrow heads highlight structures where PPXc-mChe (magenta) and active PPK1-mCit (green) co-localize with JC-D7 (cyan) stained polyP granules in leaf and hypocotyl cells. No co-localization or JC-D7 staining was observed in mPPK1-mCit expressing control lines. Scale bars = 10 μ m.

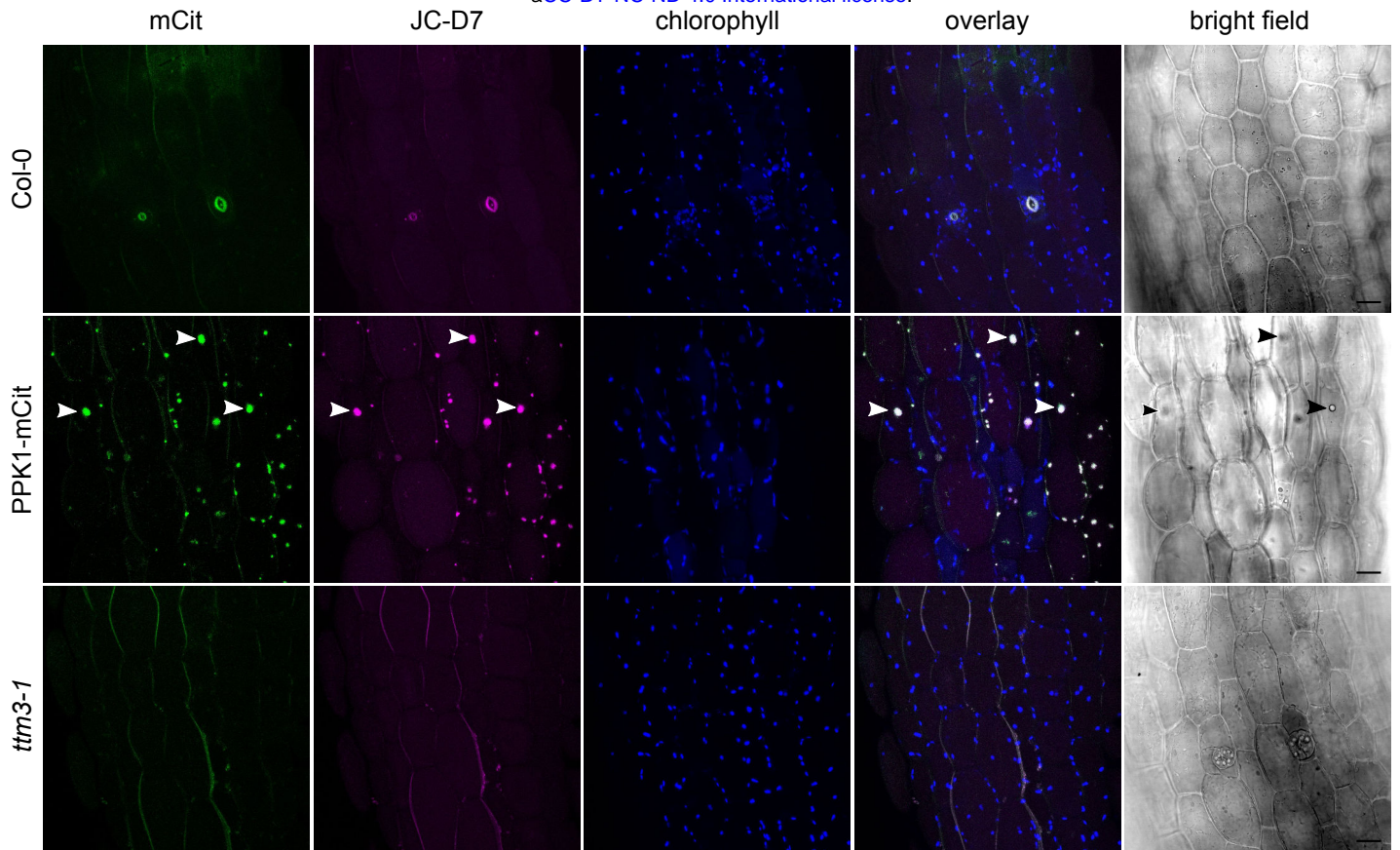


Figure S2. JC-D7 fails to stain specific structures in hypocotyl cells of Col-0 and *ttm3-1* plants.

9 d old Col-0 and *ttm3-1* seedlings grown on ^{12}C MS plates were stained by JC-D7 and imaged by confocal scanning microscopy, the PPK1-mCit line is shown as a control alongside. White arrow heads show PPK1-mCit (green) and JC-D7 (magenta) stained polyP granules in PPK1-mCit expressed hypocotyl cells, no specific JC-D7 staining was observed in Col-0 or *ttm3-1* hypocotyl cells. Scale bars = 20 μm .

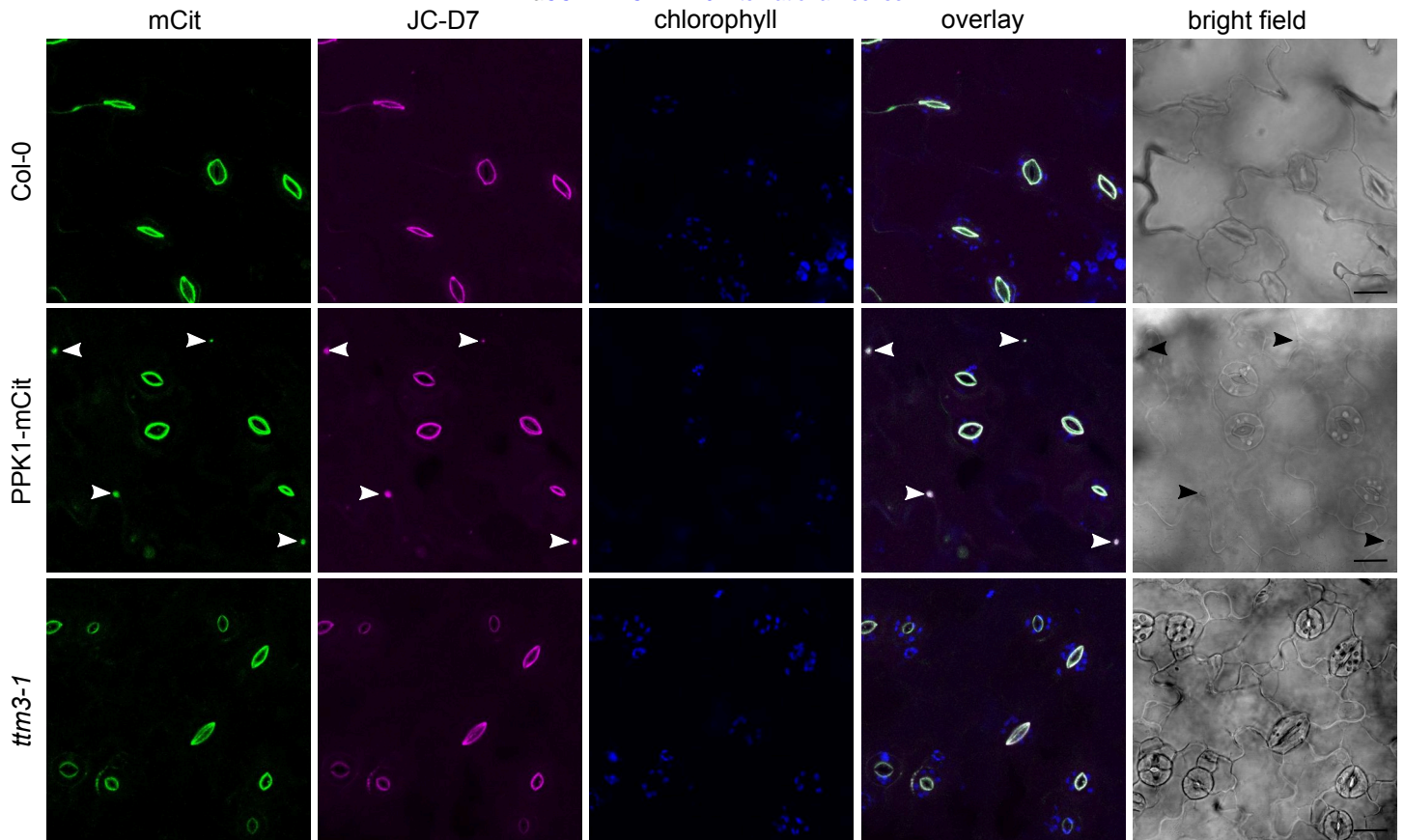


Figure S3. JC-D7 fails to stain specific structures in fixed hypocotyl cells of Col-0 and *ttm3-1* plants.

Samples were grown and imaged as described in Figure S2, using a fixation protocol specified in Experimental Procedures. Scale bars = 20 μ m.

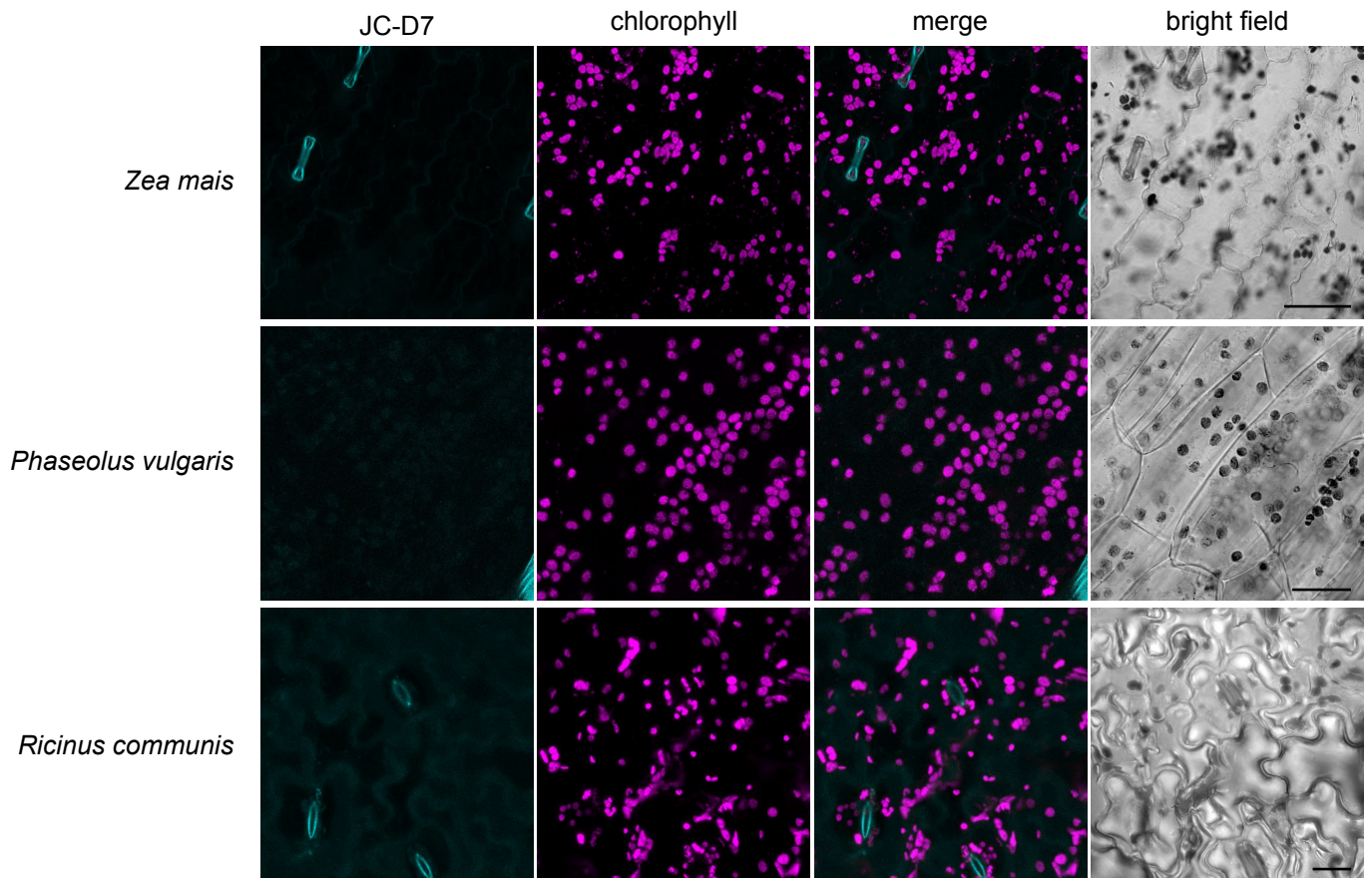


Figure S4. No JC-D7 stained polyP granules could be detected in epidermal cells of mono- and dicotyledonous plants.

Epidermal cells from *Zea mais*, *Phaseolus vulgaris* and *Ricinus communis* were stained using the JC-D7 dye. No specific polyP signal was detected by confocal scanning microscopy.