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Title:

PII1: a protein involved in starch initiation that determines granule number and size in Arabidopsis chloroplast.

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Abstract

The initiation of starch granule formation is still poorly understood. However, soluble starch synthase 4 (SS4) appears to be a major component of this process since it is required to synthesize the correct number of starch granules in the chloroplasts of *Arabidopsis thaliana* plants. A yeast-2-hybrid screen allowed the identification of several putative SS4 interacting partners. We identified the product of *At4g32190* locus as a chloroplast-targeted PROTEIN INVOLVED IN STARCH INITIATION (named PII1). *Arabidopsis* mutants devoid of PII1 display an alteration of starch initiation process and accumulate, on average, one starch granule per plastid instead of the 5 to 7 granules found in plastids of wild-type plants. These granules are larger than in wild type and they remain flat and lenticular. *pil* mutants display wild-type growth rates and accumulate standard starch amounts. Moreover, starch characteristics, such as amylopectin chain length distribution, remain unchanged. Our results reveal the involvement of PII1 in starch priming process in *Arabidopsis* leaves through interaction with SS4.

Keywords: starch, starch priming, starch granule size, starch initiation, SS4, PII1, *Arabidopsis*

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Introduction

Starch is the main storage polysaccharide produced by plants. It accumulates as water-insoluble semi-crystalline granules in the chloroplast of photosynthetic organ cells or in the amyloplasts of storage organ cells (potato tubers, endosperm of cereal seeds). Starch is a mix of two structurally distinct α -glucan polymers, amylose and amylopectin in which glucose residues are linked in $\alpha(1\rightarrow4)$ and branched in $\alpha(1\rightarrow6)$. Amylopectin, the major fraction of starch, is moderately branched containing up to 6% of $\alpha(1\rightarrow6)$ linkages while the frequency of branching of amylose is much below 1%.

Starch synthesis is a complex process that implies tens of proteins, enzymatically active or not, and each step is catalyzed by several genetically independent isoforms (D'Hulst *et al.*, 2015). For instance, up to five starch-synthases catalyze the elongation of the α -glucan polymers by transferring the glucose residue from ADP-glucose to the non-reducing end of the molecules (Abel *et al.*, 1996; Edwards *et al.*, 1999; Delvallé *et al.*, 2005; Zhang *et al.*, 2005; Zhang *et al.*, 2008; Crofts *et al.*, 2017). The formation of the branch points and the control of their distribution in amylopectin is monitored by up to three branching enzymes that create $\alpha(1\rightarrow6)$ bonds (Schwall *et al.*, 2000; Blauth *et al.*, 2001; Tanaka *et al.*, 2004; Yao *et al.*, 2004; Dumez *et al.*, 2006; Nakamura *et al.*, 2010; Regina *et al.*, 2010; Tetlow, 2012) and by debranching enzymes (isoamylases and pullulanase) that hydrolyze some of them (Mouille *et al.*, 1996; Myers *et al.*, 2000; Delatte *et al.*, 2005; Wattebled *et al.*, 2005; Streb *et al.*, 2008; Wattebled *et al.*, 2008; Ferreira *et al.*, 2017). This process induces the formation of a cluster-like structure of amylopectin responsible for its specific physicochemical properties (Pfister & Zeeman, 2016).

A major current challenge is to understand how the activity of these enzymes is controlled to generate new starch granules. It is now well established that enzymes such as branching enzymes and starch synthases are engaged in hetero-multimeric complexes (Tetlow *et al.*, 2004; Hennen-Bierwagen *et al.*, 2008; Tetlow *et al.*, 2008; Hennen-Bierwagen *et al.*, 2009; Ahmed *et al.*, 2015; Crofts *et al.*, 2015). However, the regulation of the formation of these complexes remains to be elucidated even if it is strongly suspected that the protein phosphorylation state is a key factor controlling protein-protein interaction (Liu *et al.*, 2009; Liu *et al.*, 2012; Makhmoudova *et al.*, 2014; Subasinghe *et al.*, 2014). Moreover, an increasing number of non-catalytic proteins have been described to be involved in starch

76 metabolism with functions that are essential for correct starch synthesis or degradation (Seung
77 *et al.*, 2015; Feike *et al.*, 2016; Seung *et al.*, 2017).

78 One step of starch synthesis that remains poorly understood is the initiation of granule
79 formation. This process is of prime importance since it defines, *in fine*, the number, the size,
80 and the morphology of the starch granules. Arabidopsis accumulates on average 5 to 7 starch
81 granules per plastid in mature leaves. This number is rather constant, implying a finely tuned
82 regulation *in planta*, and depends on the chloroplast volume (Crumpton-Taylor *et al.*, 2012;
83 Crumpton-Taylor *et al.*, 2013). It has been shown that starch synthase 4 (SS4) is a major
84 factor affecting the priming of starch synthesis. Arabidopsis *ss4* mutants accumulate one
85 (sometimes none, rarely two) starch granule(s) per chloroplast (Roldán *et al.*, 2007).
86 Interestingly, this reduction in the number of starch granules per plastid is accompanied by a
87 modification of their shape. Wild-type (WT) granules are generally flat and lenticular with a
88 diameter of 1-2 μm . Starch granules in the *ss4* mutant are larger (3-5 μm) and spheroidal
89 (Roldán *et al.*, 2007). The synthesis of the unique granule in the *ss4* mutant depends on the
90 presence of another starch synthase: SS3. Indeed, starch synthesis collapses in the *ss3 ss4*
91 double mutant (Szydlowski *et al.*, 2009) and the synthesis of one starch granule observed in
92 few chloroplasts are probably due to stochastic initiation events (Crumpton-Taylor *et al.*,
93 2013).

94 SS4 is a protein composed of two distinct domains. The C-terminal moiety of the
95 protein corresponds to the glycosyl-transferase 5 (GT5) domain of the CAZy classification that
96 is shared by all starch synthases (Coutinho *et al.*, 2003). The N-terminal half of the protein is
97 specific to SS4 and is essentially composed of coiled-coil motifs (Leterrier *et al.*, 2008;
98 Gámez-Arjona *et al.*, 2014). These two domains have specific functions in granule formation.
99 While the C-terminal part of the protein determines the number of initiation events, the N-
100 terminal moiety is involved in protein localization and controls granule shape (Lu *et al.*,
101 2018). Indeed, SS4 is not evenly distributed within the chloroplast but is associated with
102 plastoglobules where it has been described to interact with fibrillins 1 (Gámez-Arjona *et al.*,
103 2014; Raynaud *et al.*, 2016). It was also reported that SS4 interacts with itself and with
104 PTST2, a non-catalytic protein that, together with PTST3, was proposed to deliver a substrate
105 allowing SS4 to initiate starch granule formation (Seung *et al.*, 2017).

106 In this article, we report the identification of a new protein that physically interacts
107 with SS4 and is involved in starch priming. This protein was named PII1 for “Protein
108 Involved in starch Initiation” (*At4g32190*). Mutants lacking PII1 have a reduced number of
109 larger starch granules compared to the wild type. The phenotype observed is not a strict

110 phenocopy of that of the *ss4* mutant, because plant growth and starch granule morphology are
111 unaltered in *pil1* mutant compared to wild type. We propose that PII1 is required for starch
112 granule initiation by controlling the catalytic activity of SS4.

113

114 **Material and methods**

115

116 **Plant material and growth conditions**

117 The ULTimate Y2HTM was carried out by Hybrigenics-services (Paris, France) using
118 SS4 as bait (amino acids 43 to 1040) against a library prepared from one-week-old seedlings.
119 Among 125 millions interaction tested, 369, corresponding to 80 different proteins, were
120 positives. Using the ChloroP algorithm prediction (Emanuelsson *et al.*, 1999), we were able to
121 select proteins with predicted chloroplast targeting peptides. We ended-up with six candidates
122 among which PII1 (*At4g32190*) was selected (Table S1). Hybrigenics-services provides
123 interaction results associated to a predicted biological score (PBS). This score indicates the
124 interaction reliability and is divided in 6 different classes (A to F): A: very high confidence in
125 the interaction. B: high confidence in the interaction. C: good confidence of interaction. D:
126 moderate confidence of interaction. E: interaction involving highly connected prey domains.
127 This class is subjected to non-specific interactions. F: experimentally proven artifacts.

128 *Arabidopsis thaliana* lines were obtained from NASC (Nottingham Arabidopsis Stock
129 Centre; <http://Arabidopsis.info>; (Alonso *et al.*, 2003)) or from the collection generated at
130 URGV (INRA of Versailles; (Samson *et al.*, 2002)). Wassilewskija (Ws) and Columbia (Col-
131 0) lines were used as wild type references. T-DNA insertion lines used are: *pil1-1*
132 (SALK_122445); *pil1-2* (FLAG_137A02); *ss4-1* (GABI_290D11); *ss4-2* (FLAG_559H08).
133 Both *pil1-1* and *ss4-1* are in Columbia genetic background while *pil1-2* and *ss4-2* were
134 generated in Ws genetic background. *ss4* alleles were already described in (Roldán *et al.*,
135 2007). Oligonucleotides used for selection and RT-PCR experiments are described in Table
136 S2.

137 Depending on experiments, plants were grown either in a greenhouse (16 h : 8 h,
138 light : dark photoperiod at 23 °C during the day and 20 °C during the night, 150 $\mu\text{mol photon}$
139 $\text{m}^{-2} \text{s}^{-1}$) or in a growth chamber (16 h : 8 h, light : dark photoperiod at 23 °C during the day
140 and 20 °C during the night, 120 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$). Seeds are incubated at 4 °C in 0.1 %
141 Phytigel solution (w/v) during 3 days before being sown on peat-based compost.

142 The selection of homozygous mutant lines was performed by PCR amplification on
143 genomic DNA according to standard procedures described in Wattedled *et al.*, 2008. RNA
144 were extracted from leaves harvested at the middle of the light phase using Nucleospin RNA
145 plant kit from Macherey-Nagel following manufacturer instructions. 500 ng of RNA were
146 used to complete RT-PCR amplification using the One-Step RT-PCR kit from Qiagen. To
147 ensure that RNA extraction was correctly performed, we have systematically amplified, as a
148 negative control, template without the step of retro transcription. Detailed primer sequences
149 are listed in table S2.

150 The analysis of starch accumulation in roots was performed on plants grown under
151 hydroponic conditions. The seeds were sterilized in ethanol 75% and stratified at 4 °C during
152 3 days. Seeds were then deposited on the Seedholder (araponics[®]) completed with Murashige
153 and Skoog medium and 0.8% plant agar (Duchefa Biochemie). Roots develop in a culture
154 solution (1.1 mM MgSO₄, 2mM KNO₃, 805 μM Ca(NO₃)₂, 695 μM KH₂PO₄, 60 μM
155 K₂HPO₄, 20 μM FeSO₄, 20 μM Na₂EDTA, 9.25 μM H₃BO₃, 3.6 μM MnCl₂, 74 nM
156 (NH₄)₆Mo₇O₂₄, 3 μM ZnSO₄, 785 nM CuSO₄), pH is adjusted to 5.8. After 2 weeks of
157 growth, plant roots were collected and stained with iodine. Roots were observed under phase
158 contrast microscope (20X plan fluor, NA = 0.45, objective) and subsequently photographed.

159

160 **Protoplasts preparation and transformation**

161 SS4 and PIII1 cDNAs were cloned in a Gateway entry vector following manufacturer
162 instructions (pENTR[™] directional TOPO[®] cloning kit, Invitrogen). Using LR clonase
163 (Gateway[®] LR clonase[™] II enzyme mix, Invitrogen), cDNA were transferred in the
164 destination vector pUBC-GFP-Dest allowing expression of the protein fused to GFP (Grefen
165 *et al.*, 2010).

166 Arabidopsis protoplasts were isolated from 2-week-old plants grown on Murashige
167 and Skoog agar (1.2 %) medium. Leaves were cut in 15 ml of 500 mM mannitol. After
168 mannitol removal, preparations were incubated without shaking overnight in darkness at room
169 temperature in enzyme solution (400 mM mannitol, 5 mM MES, 1 M CaCl₂, 1% (w/v)
170 cellulase Onozuka R10, 0.25 % (w/v) Macerozyme R10 at pH 5.6). Protoplasts were filtered
171 through two layers of Miracloth (Calbiochem, EMD Biosciences, La Jolla, CA), centrifuged
172 during 5 min at 50 g in swing out rotor at room temperature. Protoplasts were resuspended in
173 5 ml of W5 solution (154 mM NaCl, 125 mM CaCl₂, 5mM KCl, 5 mM glucose, 1.5 mM
174 MES, pH 5.6). 2.5 ml protoplasts were deposited on 6 ml 21 % sucrose solution and
175 centrifuged 10 min at 50 g. Intact protoplasts, that accumulate on the sucrose surface were

176 resuspended in 0.3 ml of MaMg solution (0.4 M mannitol, 15 mM MgCl₂, 5 mM MES
177 pH 5.6). Transformation was performed using 50 µg of plasmid DNA and 50 µg salmon
178 sperm DNA used as sheared carrier DNA. 325 µl of transfection buffer was immediately
179 added (40 % (w/v) PEG₄₀₀₀, 0.4 M mannitol, 0.1M Ca(NO₃)₂ pH7-8). Protoplasts were
180 incubated for 30 min in darkness, washed in 10 ml of W5 solution, centrifuged 5 min at 50 g
181 at room temperature and resuspended in 2 ml of W5 solution. Protoplasts were observed, 48-
182 72 h after transformation, under a video microscope (Leica AF6000LX) with a Plan Apo 100x
183 Oil (NA = 1.4) objective. We have observed protein expression with $\lambda_{\text{ex}} = 484\text{-}500\text{nm}$ and
184 $\lambda_{\text{em}} = 514\text{-}554\text{ nm}$ (green channel), and protoplasts autofluorescence with $\lambda_{\text{ex}} = 564\text{-}586\text{nm}$
185 and $\lambda_{\text{em}} = 602\text{-}662\text{ nm}$ (red channel).

186

187 **Determination of starch granule number per chloroplast**

188 One leaf of 2-weeks-old plants grown under 16 h : 8 h, light : dark photoperiod in a
189 growth chamber was harvested at the end of the light phase and placed under vacuum in 1 ml
190 fixating solution (4 % (w/v) paraformaldehyde, 4 % (w/v) sucrose, 1x PBS at pH 7.3). The
191 leaf was then deposited between microscope slide and coverslip. Samples were observed
192 under A1 Nikon confocal microscope (Nikon Instruments Europe B.V.) with a Plan Apo 60x
193 Oil (NA = 1.4) objective. Auto fluorescence was acquired with $\lambda_{\text{ex}} = 488\text{ nm}$ and $\lambda_{\text{em}} = 500\text{-}$
194 550 nm (green channel), and with $\lambda_{\text{ex}} = 561\text{ nm}$ and $\lambda_{\text{em}} = 570\text{-}620\text{ nm}$ (purple channel).

195

196 **Polysaccharide extraction and purification**

197 After 3 weeks of culture in a growth chamber, Arabidopsis leaves were harvested at
198 the end of the day or at the end of the night. Samples were immediately frozen in liquid
199 nitrogen, and stored at -80 °C until use. Depending on the subsequent analysis, two different
200 extraction methods were performed.

201 For polysaccharide quantification we used a perchloric acid method (adapted from
202 (Delatte *et al.*, 2005)): About 0.3 g of leaves was homogenized with a Polytron blender in 5
203 mL of 1 M perchloric acid. The crude lysate was centrifuged at 4,500 g for 10 min at 4 °C to
204 separate the pellet, which contains starch, and the supernatant containing the water soluble
205 polysaccharides (WSP). The pellet was rinsed three times with sterile deionized water and
206 resuspended in 1 ml H₂O before quantification.

207 To determine starch granule size, polysaccharides chain length distribution profile and
208 to perform scanning electron microscopy, starch was extracted as follow: Approximately 5 g
209 of fresh material were homogenized using a polytron blender in 30 mL of the following

210 buffer: 100 mM MOPS, pH 7.2; 5 mM EDTA; 10% (v/v) ethylene glycol. The homogenate
211 was filtered through two layers of Miracloth (Calbiochem, EMD Biosciences, La Jolla, CA)
212 and centrifuged for 15 min at 4 °C and 4,000 g. The pellet was resuspended in 2 ml Percoll
213 90% (v/v) and centrifuged for 40 min at 4 °C and 10,000 g. The starch pellet was washed with
214 sterile distilled water (10 min at 4 °C and 10,000 g) and one time with 80% ethanol. Starch
215 was finally stored at 4 °C in 20% ethanol.

216

217 **Granule size distribution and scanning electron microscopy**

218 Purified starch granules were dispersed in 20 ml of IsoFlow Sheath (Beckman
219 Coulter) and analyzed in a multisizer 4 Coulter-counter (Beckman) with a 20 µm aperture
220 tube. The Multisizer software was set to determine the size of 30 000 particles ranging from 1
221 to 6 µm. 300 bins are logarithmically spaced between 1 and 6 µm (X-axis) and the size
222 frequency distribution was expressed as relative percentage of total amount (Y-axis).

223 For scanning electron microscopy observation, drops of dilute aqueous suspensions of
224 purified starch granules were deposited on a piece of glow-discharged copper tape and
225 allowed to dry. The specimens were coated with Au/Pd and secondary electron images were
226 recorded with an FEI Quanta 250 scanning electron microscope equipped with a field
227 emission gun and operating at 2 kV.

228

229 **Starch content and ultrastructure**

230 Leaves of 3-weeks-old plants were collected at the end of the day or at the end of the
231 night and immediately frozen. Starch was extracted from 0.3 to 0.5g of leaves by the
232 perchloric acid method described above. Starch content was determined by a
233 spectrophotometric method following manufacturer's instructions (Enzytec™ R-Biopharm).
234 For each genotype, three independent cultures were performed. For each culture, three
235 different samples were collected (each sample contains leaves from 3 plants).

236 Therefore, for each genotype, a mean and a standard error was calculated from nine
237 different values (eight values for Col-O). A two-tailed *t*-test was applied to compare mutant
238 lines to their respective wild-type.

239 The polysaccharide chain length distribution profile was determined on purified starch
240 granules as described in (Boyer *et al.*, 2016). 1 mg of purified starch was debranched with a
241 mix of 4 U isoamylase (*Pseudomonas sp*, megazyme) and 2 U pullulanase (*Klebsiella*
242 *planticola*, megazyme) in sodium acetate buffer (55 mM, pH 3.5) during 12 h at 42 °C. After
243 desalting (Grace™ Alltech™ Extract-Clean™ Carbograph columns) and lyophilization,

244 samples were suspended in 300 µl of deionized water. CLD was determined by high-
245 performance anion exchange chromatography with pulsed amperometric detection analysis
246 (Dionex® ICS-300 – PA200 CarboPac column 250x3 mm, Thermo Fisher, Sunnyvale, CA,
247 USA) as fully described in (Roussel *et al.*, 2013).

248

249

250

Results

251

252 ***Selection of pii1 lines***

253 SS4 is a major protein involved in starch initiation. To identify other members of the
254 starch-priming complex, proteins that potentially interact with SS4 were identified by a yeast-
255 2-hybrid screen. An ULTImate Y2H™ was carried out by Hybrigenics-services (Paris,
256 France) using SS4 as bait (amino acids 43 to 1040) against a library prepared from one-week-
257 old seedlings. Among 125 millions interactions tested, 369, corresponding to 80 different
258 proteins, were positive. During this screen, SS4 was identified in the prey proteins,
259 confirming that it is able to interact with itself and validating the approach. Using ChloroP
260 algorithm prediction (Emanuelsson *et al.*, 1999) on SS4-interacting candidates, we have
261 selected proteins with predicted chloroplast targeting peptides. Among six SS4-interacting
262 selected candidates having a predicted chloroplast targeting peptide (Table S1), we have
263 identified the PII1 protein (*At4g32190*). For each interaction identified during the screen a
264 predicted biological score (PBS) was given. This score indicates the reliability of the
265 identified interaction and ranges from A (very high confidence of the interaction) to F
266 (experimentally proven artifacts). The protein encoded by the gene *At4g32190* displayed a
267 PBS of “C” corresponding to a “good confidence of interaction”. Indeed, SS4 used as bait
268 was found to interact with 5 yeast clones expressing different fragments of the prey protein.
269 An *in vivo* interaction between SS4 and PII1 was also observed by bimolecular fluorescence
270 complementation (BiFC) experiments. Tobacco plants were transformed to allow the transient
271 co-expression of SS4 and PII1. Each protein was fused to a moiety of YFP. The fluorescence
272 corresponding to YFP reconstitution reveals the proximity of SS4 and PII1 in tobacco
273 chloroplasts (Supporting information Fig. S1).

274 To determine the biological significance of the potential interaction between this
275 protein and SS4, we have engaged a phenotypic analysis of lines impaired in the
276 corresponding gene. Two Arabidopsis lines with T-DNA insertion within *At4g32190* gene

277 were obtained from NASC resource center. Lines N679037 and 137A02 were in Col-0 and
278 Ws genetic background respectively. After selection of homozygous lines, we have evaluated
279 *At4g32190* mRNA integrity by RT-PCR (Fig. 1). In both cases the RNA integrity is
280 compromised by the T-DNA insertion. In line N679037, the insertion is located within 5'
281 UTR (three nucleotides before the ATG codon), while in line 137A02, T-DNA is inserted in
282 the second exon. The two mutant alleles were named *pil1-1* (Col-0 background) and *pil1-2*
283 (Ws background), respectively.

284 Even if PIII is annotated as a « myosin heavy chain-related protein » in databases, no
285 function has been reported for this protein. Analysis performed using MARCOIL server
286 (Delorenzi & Speed, 2002; Zimmermann *et al.*, 2017) indicates that PIII is composed of
287 several coiled-coil domains. Considering only the amino acids that have a probability above
288 50% to be involved in a coiled-coil motif, four distinct domains are identified (regions 106-
289 331; 340-426; 448-529 and 629-723). Altogether these motifs represent more than 60% of the
290 protein sequence. The coiled-coil motifs are known to favor protein-protein interaction
291 (Adamson *et al.*, 1993) and interestingly, it has already been reported that SS4 also contains
292 such motifs (Letierrier *et al.*, 2008).

293 Phytozome server allowed the identification of Arabidopsis PIII homologs in other
294 dicots, such as *Solanum tuberosum* (XP_006344374) and in monocots (ex: *Brachypodium*
295 *distachyon* XP_010233922; *Oryza sativa*: XP_015627751; *Zea mays*: XP_008679905).
296 Homologs can also be found in lycopodiophytes (*Selaginella moellendorffii*: XP_002983651),
297 but no protein similar to PIII was identified in bryophytes (*Physcomitrella patens*) or green
298 algae such as *Chlamydomonas reinhardtii* or *Ostreococcus lucimarinus*. Interestingly no clear
299 homologs of SS4 can be found in these green algae indicating that a co-evolution of PIII and
300 SS4 may have occurred.

301

302

303 ***Intracellular localization of the PIII protein.***

304 *At4g32190* encodes a protein of 783 amino acids. Use of ChloroP software (Emanuelsson *et*
305 *al.*, 1999) predicts a chloroplast targeting peptide of 27 amino acids in length. Even if the size
306 of this predicted targeting peptide is relatively small, it is not incompatible with the predicted
307 subcellular localization (Bionda *et al.*, 2010). Nevertheless PIII localization was
308 experimentally determined by protoplast transient transformation. Protoplasts were prepared
309 from Arabidopsis wild-type or *pil1* plants and transformed with a construct allowing the
310 transient expression of PIII fused in its C-terminus to the green fluorescent protein (GFP).

311 Images obtained using a fluorescence microscope confirm the chloroplastic localization of the
312 protein. Moreover fluorescence appears as dots (Fig. 2), a pattern that is similar to that already
313 reported for SS4 (Raynaud *et al.*, 2016). Protoplasts of the *ss4* mutant were also transformed
314 with the PIII-GFP fused chimeric construct. Again, the PIII-GFP protein was located within
315 chloroplasts and the same dot-like distribution pattern of the protein was maintained
316 indicating that SS4 is not required for proper PIII localization inside the chloroplast.

317

318

319 ***Starch granule number and morphology are altered in *pii1* lines***

320 The starch granule number per chloroplast was determined from light microscopy
321 observations of Arabidopsis leaf cells. This technique allows the visualization of starch
322 granules without the need to produce sections of the leaf tissue. While a typical number of 5
323 to 7 starch granules were observed in the chloroplasts of wild-type cells, most plastids of *pii1*
324 leaf cells contained only one large starch granule (Fig. 3). This observation was made
325 whatever the genetic background of *pii1* lines (i.e., Ws or Col-0) indicating a reduction of
326 starch-granule priming efficiency in these mutants.

327 The granule size was determined from starch extracted at the end of the day from the
328 rosette leaves of 3-week-old plants. The use of a Coulter counter allows the determination of
329 30,000 particles size within few seconds. In our growth conditions (16 h : 8 h, light : dark
330 photoperiod), wild-type (WT) starch displays a unimodal distribution of granules size with a
331 maximum at 1.5 and 1.6 μm for Col-0 and Ws ecotypes, respectively. As already reported,
332 starch granules of *ss4* lines are larger with a maximum of the Gaussian distribution between
333 3.4 and 3.5 μm (Fig. 4). Starch granules extracted from *pii1* lines are also larger compared to
334 the WT counterpart. Distributions of granule size remain unimodal in both mutant lines with a
335 peak of the Gaussian distribution at 2.5 and 3.3 μm in Col-0 and Ws background, respectively
336 (Fig. 4).

337 The morphology of purified starch granules was also examined using scanning
338 electron microscopy. The granules extracted from wild-type Arabidopsis leaves were
339 lenticular in shape with a smooth surface. Several mutations affecting Arabidopsis starch-
340 metabolizing enzymes were reported to affect starch granule morphology (Szydlowski *et al.*,
341 2011; Malinova & Fettke, 2017). This is also the case of the *ss4* mutants that accumulate
342 large and spherical granules with a smooth surface. While similar in size to granules extracted
343 from *ss4* mutants, those purified from *pii1* remain lenticular but display an indented surface
344 (Fig. 4).

345

346 ***Impact of PIII deficiency on plant growth and root development***

347 *ss4* mutants display growth retardation and pale green leaves phenotype. These
348 characteristics were proposed to be caused by the reduction of initiation events leading to
349 accumulation of unused ADP-glucose especially in starch free chloroplasts (Ragel *et al.*,
350 2013). Since *pil1* mutants lines also display a reduction of initiation events, growth kinetics of
351 these lines were analyzed and compared to wild-type and *ss4* lines. Plants were grown in a
352 green house under 16 h light and 8 h dark cycling conditions during 3–weeks. Growth rate of
353 *pil1* lines was similar to wild type while growth retardation of *ss4* lines was confirmed
354 whatever the genetic background (Fig. 5). Pale green leaves phenotype was recorded only in
355 SS4 deficient lines.

356 Starch accumulation in roots and roots development were also evaluated in *pil1*
357 mutants (Fig. 6). Plants were cultivated in a growth chamber using hydroponic systems and
358 starch accumulation in the columella of primary and lateral roots was visualized under
359 microscope after iodine staining. The alteration of starch synthesis at the apex of *ss4* roots
360 was previously described and this alteration was associated with a modification of root
361 architecture and response to gravity (Crumpton-Taylor *et al.*, 2013). After iodine staining of
362 2-weeks-old *pil1* seedlings, the apex of both primary and lateral roots appear dark blue
363 indicating that *pil1* mutants accumulate starch granules in the columella. The intensity of the
364 coloration and number of stained granules in *pil1* appear to be similar to wild type. Moreover,
365 the architecture of root development was visualized on agar plates that were placed vertically
366 after seed germination. Again, the *pil1* line behaves like wild-type plants and the roots of 2-
367 week-old seedlings respond correctly to gravity and no aberrant development was observed
368 (Fig. 6).

369

370 ***Impact of PIII deficiency on starch amount and ultrastructure***

371 The mutation at the *PIII* locus led to the increase of starch granule size associated to a
372 reduction of granule number. To determine if these phenotypes are associated to a
373 modification of total starch amount, we also assayed the starch content in these lines and
374 compared data to wild-type and *ss4* lines. Leaves of 3-week-old plants, grown in a growth
375 chamber under 16 h : 8 h, light : dark photoperiod, were harvested at the end of the
376 illuminated or dark periods and the starch content was enzymatically assayed. It was similar
377 to the amount already reported for wild-type and SS4-deficient lines (Roldán *et al.*, 2007).
378 The loss of SS4 led to a slight reduction of starch content at the end of the day but a higher

379 amount at the end of the night (Fig. 7). This effect is exacerbated in *Ws* background. In *pii1*
380 mutants, no reduction of the starch content was recorded at the end of the day. Moreover, in
381 *Ws* genetic background, the residual starch accumulated at the end of the night was
382 significantly higher in *pii1* or *ss4* mutants compared to the corresponding wild-type line
383 (Fig. 7). No significant accumulation of water-soluble polysaccharides could be detected in
384 the different analyses.

385 Starch fractionation was carried out by size exclusion chromatography on Sepharose CL-2b
386 matrix. No modification of the amylose / amylopectin ratio was recorded in *pii1-1* or *pii1-2*
387 lines when compared to their respective wild types (Supporting information Fig. S2). The
388 starch ultrastructure was also analyzed by establishing the chain length distribution (CLD)
389 profile of the glucans. The purified starch was enzymatically debranched and linear glucans
390 were then separated using high-performance anion exchange chromatography and detected by
391 pulsed amperometric detection (HPAEC-PAD). The CLD profile determined for starch
392 extracted from *PII1* deficient lines was identical to that of the corresponding wild types
393 (Fig. 8).

394

395 ***PII1* deficiency does not alter other starch metabolizing enzymes.**

396 The activity level of several enzymes was estimated by zymogram analysis. All tested
397 activities (starch synthases SS1 and SS3, branching enzymes, debranching enzymes,
398 phosphorylases) remained unaffected in the *pii1* mutant (Supporting information Fig. S3).

399 Since the reduction of granules number without alteration of starch structure observed
400 in *pii1* line was similar to that observed in *ss4*, *ptst2* or *ptst3* lines, we verified that these
401 genes were still correctly expressed in the absence of *PII1*. Total RNAs of *pii1* lines were
402 extracted and purified from leaves harvested in the middle of the day. RT-PCR performed to
403 amplify RNA signal of SS4, PTST2 and PTST3 gave signals in *pii1* lines indicating that these
404 genes are expressed (Supporting information Fig. S4). In the same manner, the RNAs were
405 extracted and purified from *ss4* lines and RT-PCR revealed that *PII1* is expressed in this
406 mutant (Fig. 1).

407 Moreover, SS4 was reported to be located in specific regions of the chloroplast. SS4 is
408 preferentially distributed near the thylakoid membranes (Gómez-Arjona *et al.*, 2014). The N-
409 terminal moiety of SS4, containing several coiled-coil domains known to be involved in
410 protein-protein interactions, is responsible of the proper location of SS4 (Lu *et al.*, 2018). To
411 determine if *PII1* has an impact on SS4 subchloroplastic location, we expressed the SS4 -GFP

412 fused protein in the *piil* mutant. SS4 distribution was not modified in the presence or absence
413 of PII1 (Fig. 9).

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Discussion

418

PII1 and SS4 interact in planta

420 In this study we describe a new protein involved in starch priming. PII1 was identified
421 by yeast-2-hybrid screen as physically interacting with SS4. The number of positive clones
422 with different PII1 fragments gave a good confidence of the interaction between the two
423 proteins. This result was reinforced by other observations such as subcellular protein
424 localization or BiFC experiment.

425 *In silico* analysis of PII1 coding sequence predicts the presence of a small transit
426 peptide allowing the targeting of the protein to the chloroplast (Table S1). The chloroplastic
427 localization of PII1 was confirmed by expression of the recombinant PII1-GFP fluorescent
428 chimeric protein. Moreover this approach also revealed a dotted distribution of the protein in
429 the plastid (Fig.2) similarly to what was previously described for SS4 (Raynaud *et al.*, 2016).
430 Although the formation of aggregates of PII1 could not be totally excluded, it is unlikely that
431 such dotted distribution of PII1 arises from the over-expression of the protein, since we have
432 made use of the moderate expression “ubiquitin promoter-10” for the expression of the
433 transgene (Grefen *et al.*, 2010). In addition, the transformations were carried out with
434 protoplasts prepared from cells of the *piil* mutant lacking the endogenous protein limiting the
435 over accumulation of the protein. Finally, the spatial proximity of PII1 and SS4 has also been
436 confirmed *in planta* by a BiFC approach (Supporting information Fig. S1).

437 As a whole, these results (yeast-2-hybrid, distribution within the chloroplast and BiFC)
438 provide good evidence for the direct physical interaction between SS4 and PII1 in the
439 chloroplast.

440

PII1 is required in determination of starch granule number in Arabidopsis chloroplast.

442 In this work, we have phenotypically characterized two independent mutant alleles of
443 the PII1 gene: the *piil-1* in the Col-0 background and *piil-2* in the Ws background. T-DNA
444 insertion lies in the 5'UTR, only 4 nucleotides upstream of the START codon in *piil-1*, while

445 it is localized in exon 2 in *pil-2*. Although these two lines were confirmed mutant by RT-
446 PCR (Figure 1) we cannot exclude that a small amount of active PII1 is still produced at a low
447 level in the *pil-1* mutant while it is completely abolished in *pil-2*.

448 Nevertheless, the phenotypes of the two mutants are very similar. Both lines have less
449 (typically one) but bigger starch granules per chloroplast. Although the effect is more
450 pronounced in *pil-2* (*pil-1* is probably not a null allele), starch granules of PII1 deficient
451 lines are 2 to 3 times larger than wild type granules, and of a size similar to those of the *ss4*
452 mutant. However, while *ss4* starch is spherical in shape, those extracted from *pil* mutants
453 remain lenticular, a form comparable to that of wild-type starch.

454 Another difference observed between the *pil* and the *ss4* mutants is related to plant
455 growth. Indeed, while the *ss4* mutants show significant growth retardation, the *pil* behave
456 like their corresponding wild types. The stunting growth of *ss4* seems, at least partly, related
457 to the accumulation of higher ADP-glucose content in the leaves (Ragel *et al.*, 2013) probably
458 because a significant fraction of chloroplasts are starch-free in these mutants (Roldán *et al.*,
459 2007; Lu *et al.*, 2018). Although the absence of PII1 leads to a reduction in the number of
460 starch granules per chloroplast, none or very few organelles are completely free of starch (a
461 much smaller fraction than *ss4*). In addition, the *ss4* mutants have a root growth defect related
462 to the perturbation of starch accumulation in the columella (Crumpton-Taylor *et al.*, 2013).
463 Starch accumulation in roots and root development of *pil* lines are similar to wild type
464 plants. The combination of these two factors, absence of starch-free chloroplast and normal
465 root development, may explain the correct growth of plants lacking PII1.

466

467 ***PII1 is proposed to be required for SS4 catalytic activity***

468 SS4 is a major component involved in starch priming. This protein is composed of two
469 distinct parts. The C-terminal part of the protein corresponds to the catalytic domain that is
470 shared by all starch-synthases of the GT5 family. The N-terminal part of SS4, essentially
471 composed of coiled-coil domains, is unique (Raynaud *et al.*, 2016). Both parts have specific
472 function in determination of starch granule size and morphology. Expression in *ss4* mutants
473 of a truncated protein lacking the N-terminal part or a chimeric protein composed of N-
474 terminal part of SS4 fused to the glycogen-synthase demonstrates that the catalytic domain
475 (C-terminal) of SS4 determines the number of starch granule, while the N-terminal moiety
476 control the shape of the granule (Lu *et al.*, 2018). In the present work, PII1 was identified as
477 an SS4-interacting partner. When PII1 was knocked down, the major phenotype was the
478 reduction of starch granule number per chloroplast without alteration of granule shape. This

479 phenotype can be interpreted as an inactivation of SS4 catalytic part (C-terminal) without
480 alteration of the function of SS4 N-terminal moiety. We therefore propose that PII1 is a
481 protein involved in starch priming that interacts with SS4 and is required for proper catalytic
482 activity of the starch synthase controlling the number of initiation events generating new
483 starch granules. PII1 could be needed to provide an adequate substrate to SS4 and / or to
484 prevent the degradation of the substrate presented to SS4 to prime granule formation. A
485 similar function was already proposed for PTST2 another SS4-interacting protein (Seung *et*
486 *al.*, 2017). Alternatively, PII1 could be requested to the correct folding of SS4 or its
487 association with any other factor requested in the starch-priming machinery. Leaves are
488 submitted to diurnal fluctuations of the starch content. The precise control of the starch
489 content is of prime importance in leaves to prevent starvation at night (Scialdone *et al.*, 2013).
490 Such precise control could only be achieved if the priming of starch synthesis (number, size,
491 and morphology of the granules) is itself highly controlled. Consequently, starch granule
492 initiation is a complex mechanism involving enzymes and non-catalytic proteins. To date,
493 several components of this machinery have been identified including SS4, SS3, PTST2,
494 PTST3 and the new PII1 protein described in this work. Further investigations are needed to
495 determine the precise function of each protein within the starch-priming complex and
496 understanding how plants control the number and the size of the starch granules.

497

498

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507

508

Author Contribution

509

510 CV performed most experiments. AC, DD, MF participated to experiments. AW performed
511 chain length distribution analysis. JLP has supervised the electron microscopy observations,

512 CS performed optical microscopy observations. FW and CDH designed experiments and
513 wrote the manuscript.

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733 press.
734

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738 **Supporting information**

739 **Table S1:** Proteins identified during the yeast-2-hybrid screen, that are predicted to be
740 targeted to the chloroplast.

741

742 **Table S2:** Information on T-DNA lines and primers used for selection and RT-PCR
743 experiments.

744

745 **Fig. S1:** SS4 and PII1 interaction (BiFC).

746

747 **Fig. S2:** Starch fractionation.

748

749 **Fig. S3:** Zymograms of starch metabolizing enzymes.

750

751 **Fig. S4:** expression of *SS4*, *PTST2* and *PTST3*.

752

753

754 **Figure legends:**

755

756 **Fig. 1: Selection of *pil1* mutants.**

757 **(a)** Structure of the *At4g32190* locus encoding PII1. UTR are indicated by grey boxes
758 while introns and exons are depicted as black lines and black boxes respectively. T-DNA
759 insertion corresponding to *pil1-1* and *pil1-2* alleles are indicated by triangles. Primer
760 position used for selection are indicated by arrows (not in scale)

761 **(b)** Left panel represents the selection of the *pil1-1* homozygote mutant. gDNA from one
762 mutant plant (lanes 1 and 3) and one wild-type control (lanes 2 and 4) was used.
763 Amplification products of wild type allele (using primer a and b) are in lanes 1 and 2.
764 Amplification products of mutant allele (using T-DNA primer and primer b) are in lanes
765 3 and 4. Right panel represents the selection of the *pil1-2* homozygote mutant. gDNA
766 from one mutant plant (lanes 1 and 3) and one wild-type control (lanes 2 and 4) was
767 used. Amplification products of wild type allele (using primer c and d) are in lanes 1 and
768 2. Amplification products of mutant allele (using T-DNA primer and primer c) are in
769 lanes 3 and 4.

770 **(c)** Left panel: RT-PCR amplification products obtained using primers a and b on total
771 RNA extracted from *pil1-1* (lane 1), *ss4-1* (lane 2) and Col-0 (lane 3). Right panel: RT-PCR
772 amplification products obtained using primers c and d on total RNA extracted from Ws
773 (lane 1), *pil1-2* (lane 2).

774 Molecular weight (MW): SmartLadder, Eurogentec.

775

776 **Fig. 2: Subcellular localization of PII1 in Arabidopsis protoplasts.**

777 Protoplasts were prepared from Col-0 **(a)**, *pil1-1* **(b)** or *ss4-1 pil1-1* **(c)**. Image
778 acquisition was performed using a video microscope. In each row, the first image
779 corresponds to the GFP signal of the PII1-GFP fusion protein. Chlorophyll fluorescence is
780 displayed in the central panels. The merged images are displayed on the right.

781 Scale bar = 10 μm

782

783 **Fig. 3: Starch granule number per chloroplast.**

784 Isolated leaf cells were prepared from Col-0 **(a)**; *ss4-1* **(b)**; *pii1-1* **(c)**; *Ws* **(d)**; *ss4-2* **(e)**
785 and *pii1-2* **(f)**. Leaves samples were collected at the end of the day and tissues were fixed
786 in paraformaldehyde before disruption in EDTA. Pictures were collected using a
787 confocal microscope. Chlorophyll fluorescence in purple delimits the chloroplast
788 volume, while starch granules are black.

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790

791 **Fig. 4: Starch granule size and morphology.**

792 Starch granules were extracted and purified from leaves of 3-week-old plants harvested
793 at the end of the day. The plants were grown in 16 h : 8 h, light : dark photoperiod.
794 Panels **(a)** - **(d)**: plants of the Col-0 genetic background. Panels **(e)** - **(h)** plants of the *Ws*
795 genetic background.

796 The starch granule size distribution was determined by analyzing 30,000 particles
797 extracted for each genotype. The results are expressed in relative percentage (*y*-axis) of
798 particles of a diameter ranging from 1 to 6 μm (*x*-axis, logarithmic scale). In **(a)** and **(e)**,
799 starch from wild type, *ss4* and *pii1* lines are in blue, red and green, respectively. The
800 value at the peak of the Gaussian distribution is indicated in μm .

801 **(b)** to **(d)** and **(f)** to **(h)**: starch granules were observed using scanning electron
802 microscopy. **(b)** and **(f)**: wild-type (Col-0 and *Ws*, respectively); **(c)** and **(g)**: *ss4-1* and
803 *ss4-2*; **(d)** and **(h)**: *pii1-1* and *pii1-2*. Scale bar = 10 μm , all images are at the same scale.

804

805 **Fig. 5: *pii1* growth phenotype compared to *ss4* and wild type plants.**

806 Plants were grown in a greenhouse under 16 h : 8 h, light : dark photoperiod. Panels **(a)**
807 and **(b)**: the weight of above ground organs (g/plant) is plotted against the number of
808 days after germination. Black lines correspond to wild type plants (Col-0 and *Ws* in
809 panels **(a)** and **(b)** respectively), red lines correspond to *pii1-1* and *pii1-2* in panels **(a)**
810 and **(b)** respectively and green lines correspond to *ss4-1* and *ss4-2* in panel **(a)** and **(b)**
811 respectively. Each value corresponds to the mean of three samplings (each sample being
812 composed of several plants). Thin vertical bars represent the standard error.

813 Panels **(c)** and **(d)** are pictures of 3-week-old plants grown in the same conditions as
814 above. Panel **(c)**: from left to right: Col-0, *pii1-1*, *ss4-1*. Panel **(d)**: from left to right: Ws,
815 *pii1-2*, *ss4-2*.

816

817 **Fig. 6: Starch accumulation in root and root development.**

818 Plants were grown during 2 weeks under 16 h : 8 h, light : dark photoperiod. Panels **(a)**,
819 **(b)** and **(c)**: Ws. Panels **(d)**, **(e)** and **(f)**: *pii1-2*.

820 Panels **(a)**, **(b)**, **(d)** and **(e)**: roots of 2-week-old plants cultured with hydroponic
821 systems were soaked in lugol, rinsed with water and observed under microscope. **(a)**
822 and **(d)**: primary root. **(b)** and **(e)**: lateral root.

823 Panels **(c)** and **(f)**: seeds were sown on agar plates that were maintained vertically after
824 seed germination. Pictures were taken after 2 weeks of growth.

825 Black bar = 50 μ m; white bar = 1 cm

826

827 **Fig. 7: Starch content in leaves.**

828 Plants were grown in a culture room under 16 h : 8 h, light : dark photoperiod. Three
829 weeks after germination leaves were harvested either at the end of the day **(a)** or at the
830 end of the dark phase **(b)**. Three independent cultures were performed and for each
831 culture three independent extractions were realized. Values correspond to the mean of
832 nine assays (except for Col-0 end of dark phase corresponding to 8 assays). Thin vertical
833 bars represent the standard error. Values obtained for mutant lines were compared to
834 their respective wild-type by a two-tailed *t*-test. Asterisk represents statistically
835 significant difference at $p < 0.05$ (*) or $p < 0.001$ (**)

836

837 **Fig. 8: Chain length distribution of purified starches.**

838 Purified starches were enzymatically debranched. Linear glucans were then separated
839 and detected using high performance anion exchange chromatography with pulsed
840 amperometric detection (HPAEC-PAD). Grey bars represent the proportion of each DP
841 expressed as a percentage of the total amount presented in the figure. The black line
842 (panels **(b)** and **(d)**) corresponds to the differential plot between the mutant and wild
843 type profiles.

844 **(a)**: Col-0. **(b)**: *pii1-1*, **(c)**: WS. **(d)**: *pii1-2*. Each profile is the mean of two analysis carried
845 out with starch extracted from two independent cultures.

846

847 **Fig. 9: Localization of SS4 in Arabidopsis protoplasts.**

848 Protoplasts were prepared from *ss4-2* **(a)**, *ss4-2 pii1-2* **(b)**. Image acquisition was
849 performed using a video microscope. In each row, from left to right: SS4-GFP
850 fluorescence; Chlorophyll fluorescence; Merged images. All images are at the same scale.

851 Bar = 10 μ m

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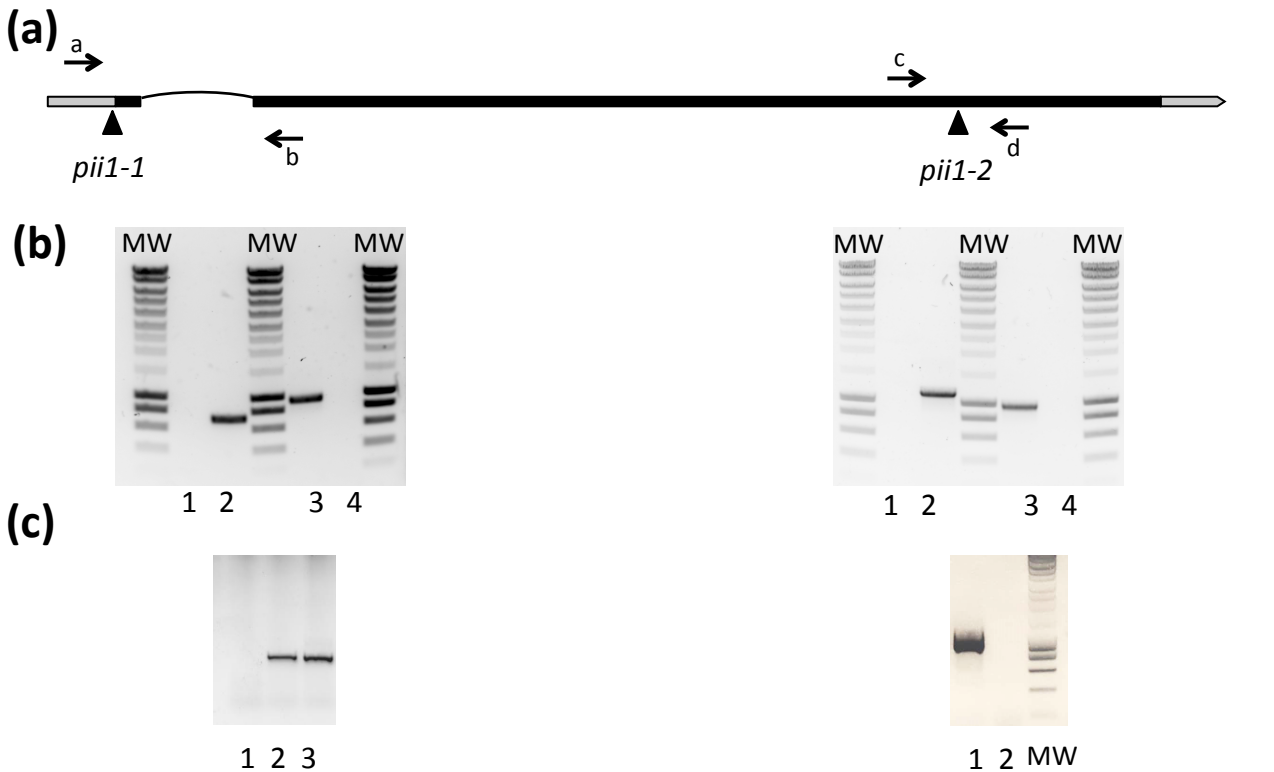


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(c) Left panel: RT-PCR amplification products obtained using primers a and b on total RNA extracted from *pii1-1* (lane 1), *ss4-1* (lane 2) and Col-0 (lane 3). Right panel: RT-PCR amplification products obtained using primers c and d on total RNA extracted from Ws (lane 1), *pii1-2* (lane 2).

Molecular weight (MW): SmartLadder, Eurogentec.

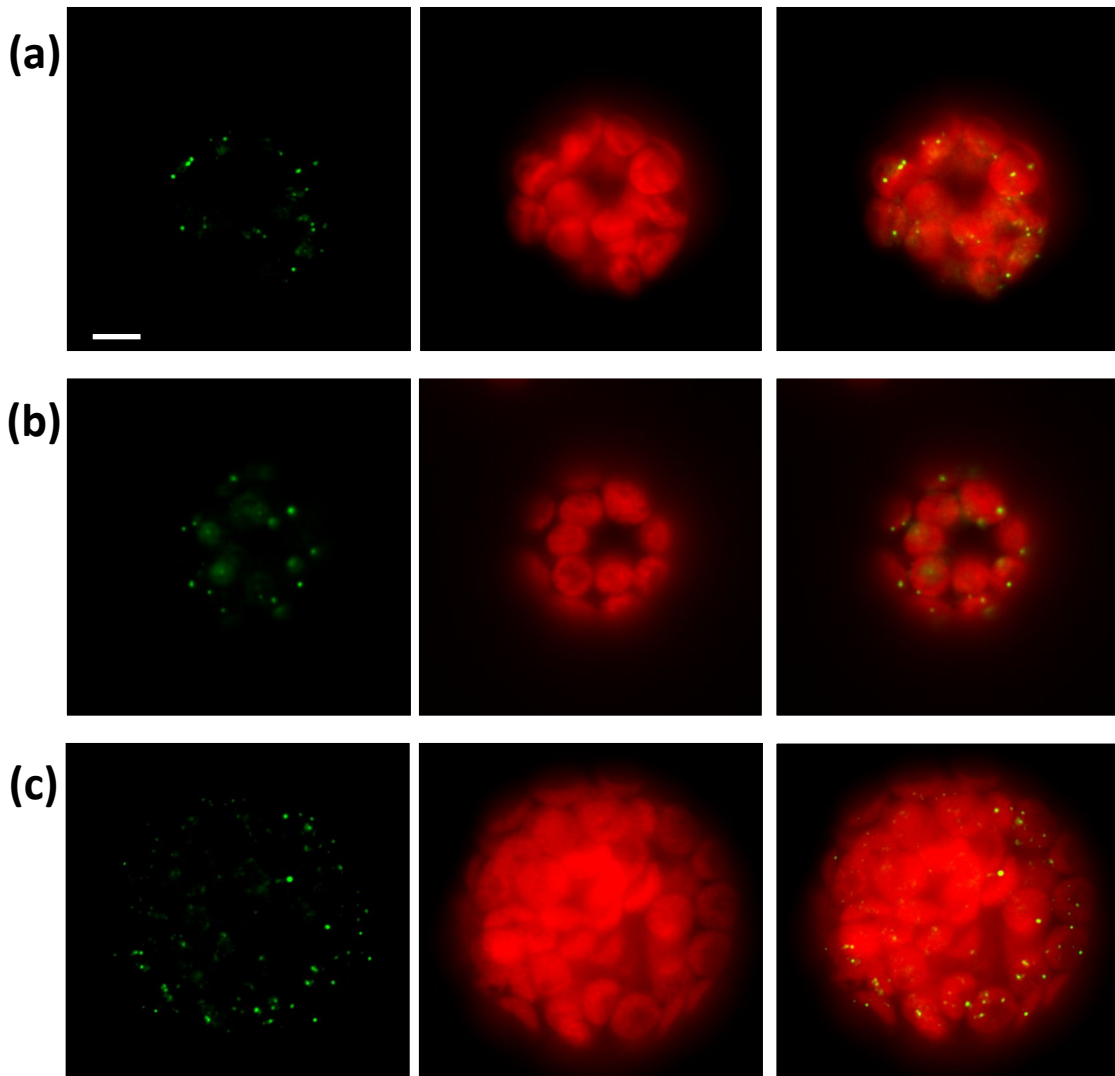


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Scale bar = 10 μ m

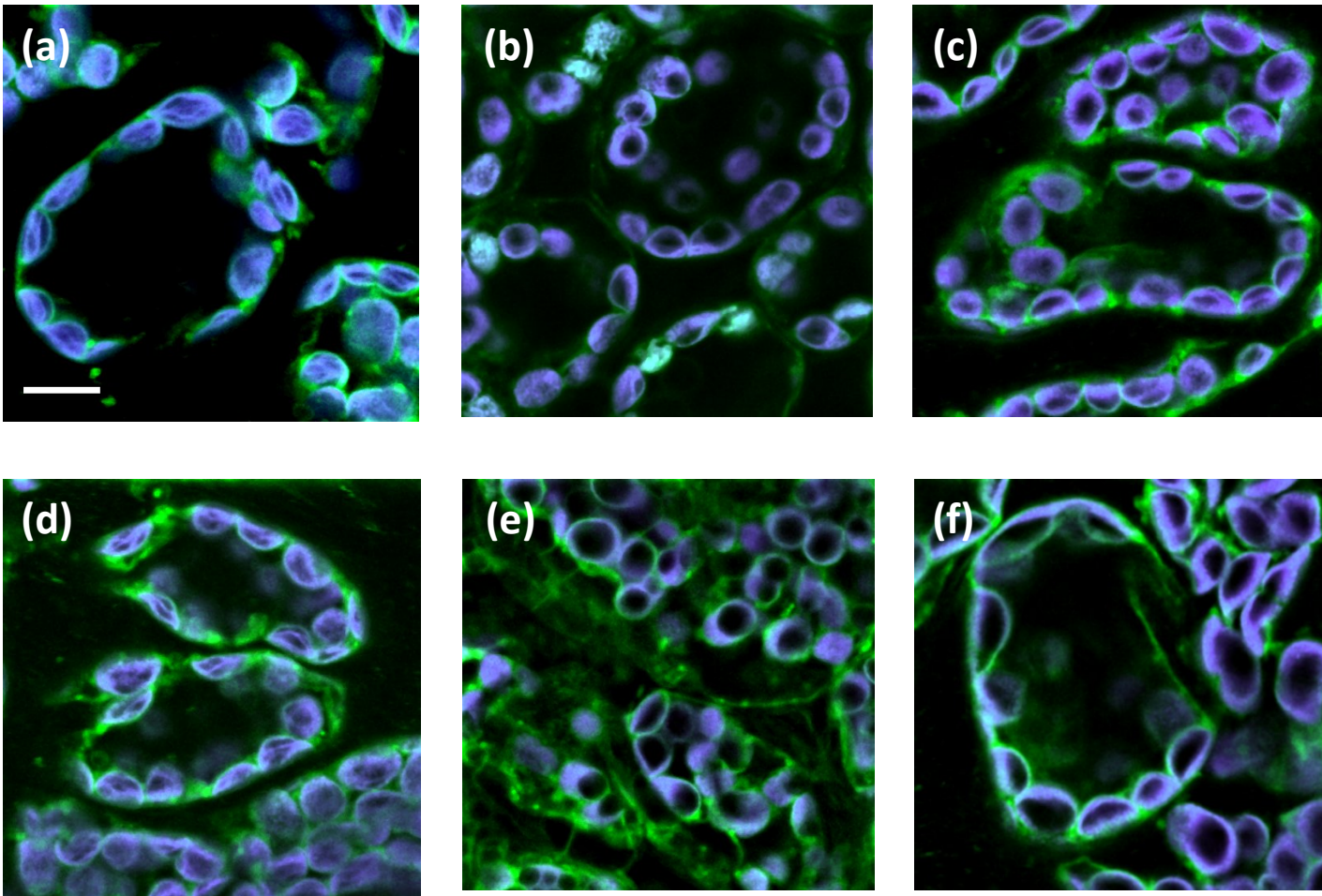


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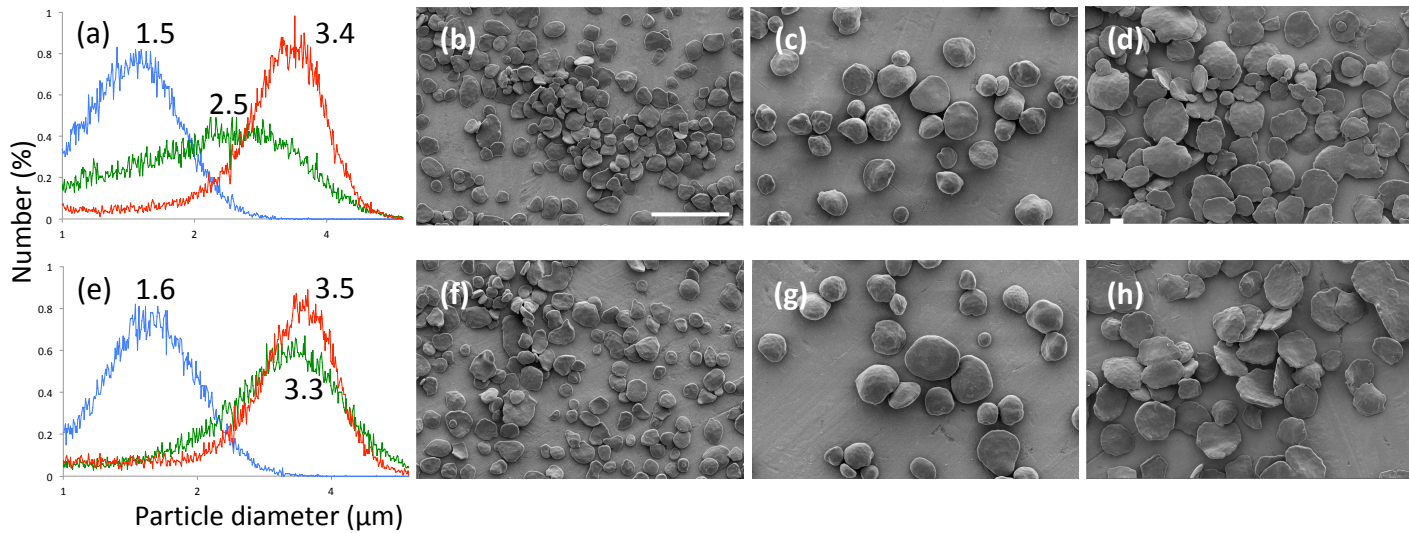


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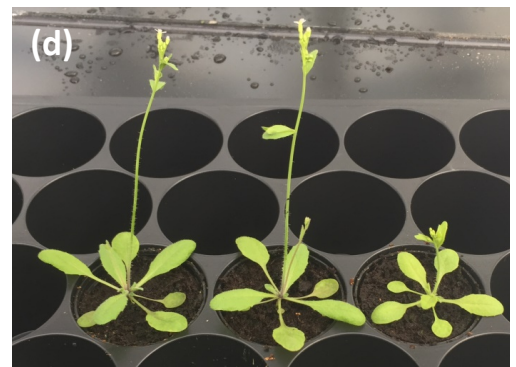
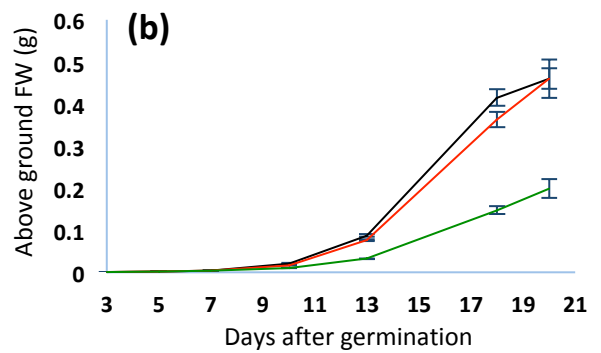
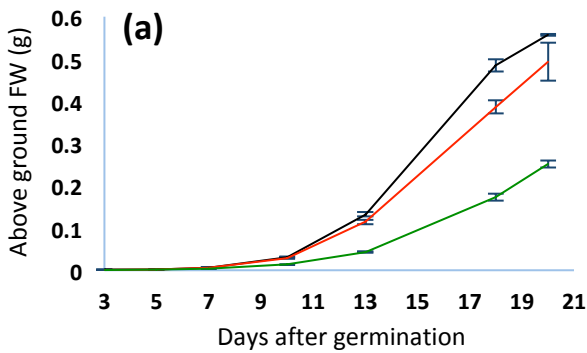


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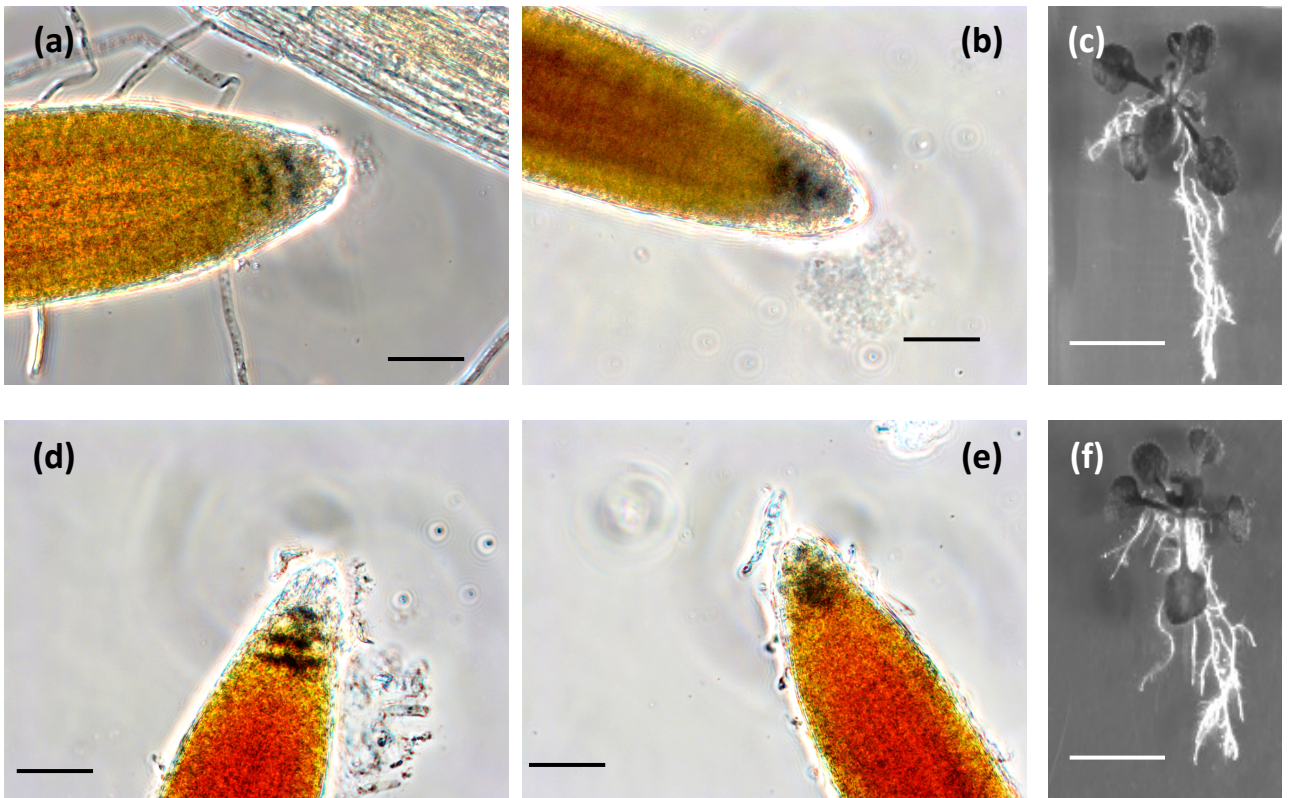


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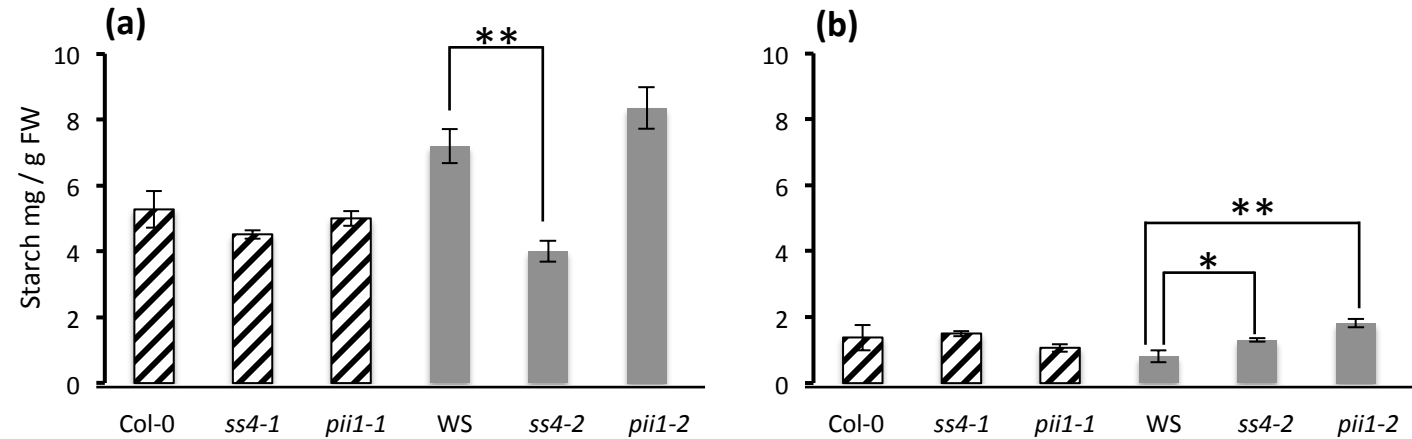


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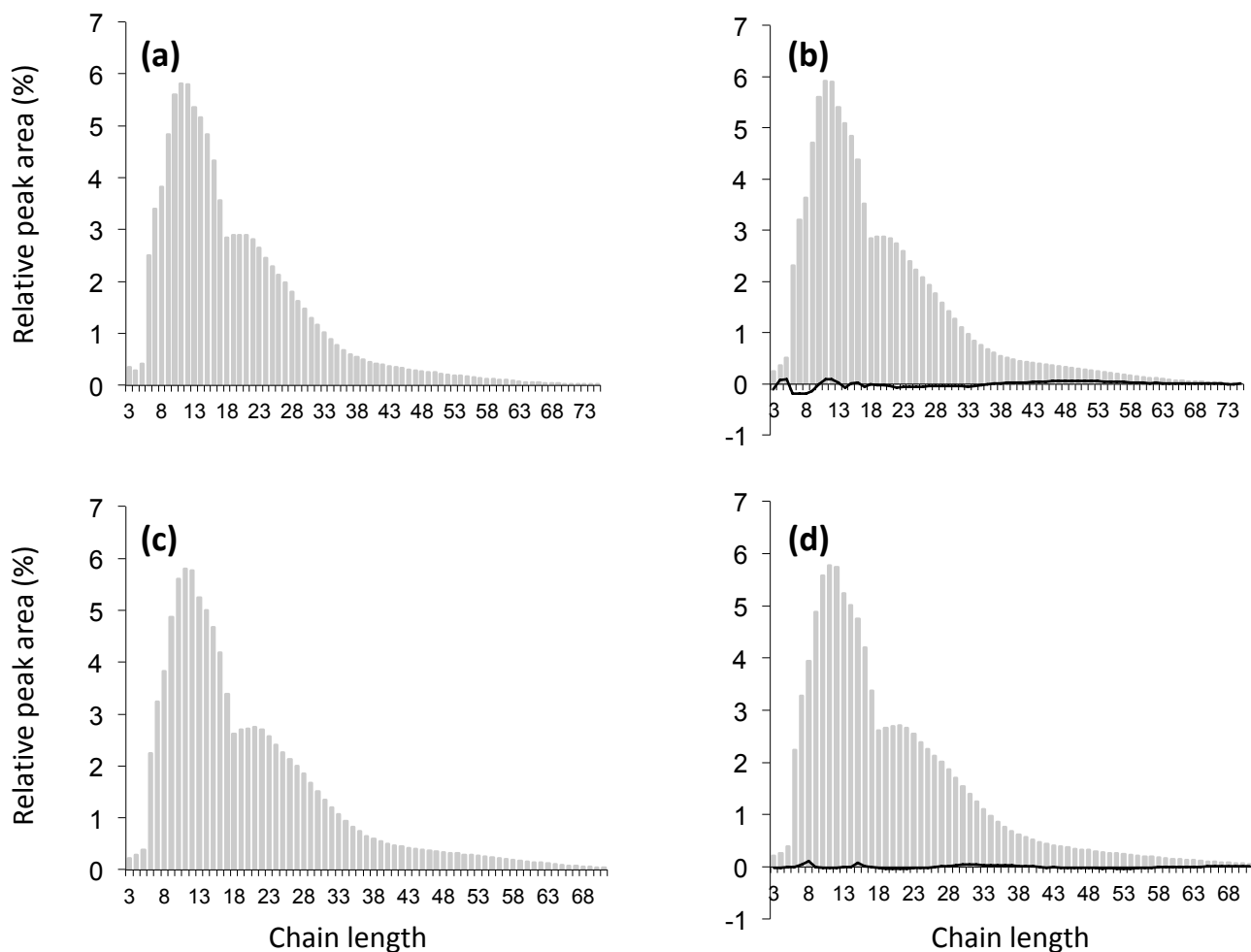


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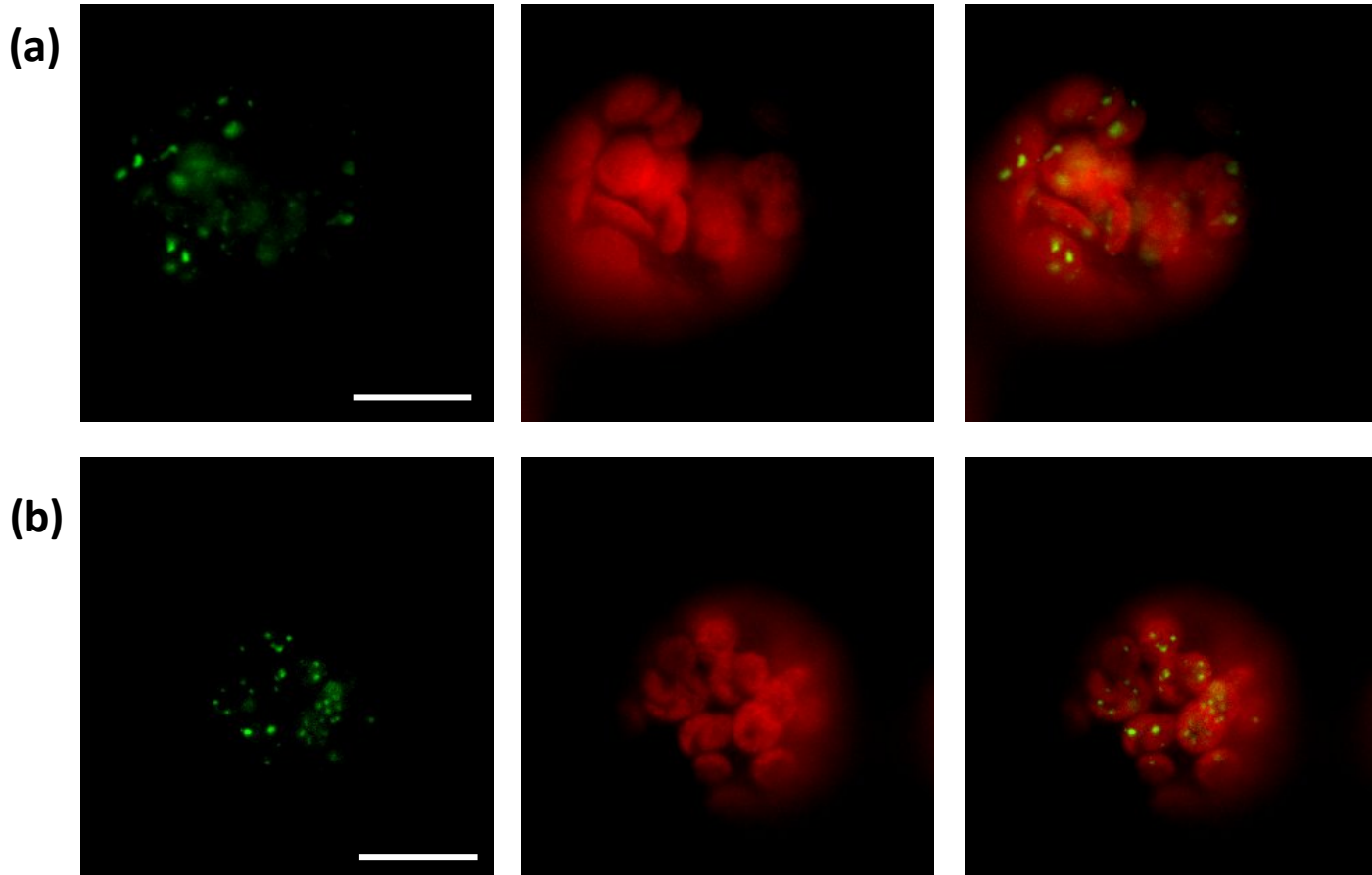


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