



**HAL**  
open science

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

Camille Vandromme, Corentin Spriet, David Dauvillee, Adeline Courseaux, Jean-Luc Putaux, Adeline Wychowski, Frédéric Krzewinski, Maud Facon, Christophe d'Hulst, Fabrice Wattebled

### ► To cite this version:

Camille Vandromme, Corentin Spriet, David Dauvillee, Adeline Courseaux, Jean-Luc Putaux, et al.. PII1: a protein involved in starch initiation that determines granule number and size in Arabidopsis chloroplast. *New Phytologist*, 2019, 221 (1), pp.356-370. 10.1111/nph.15356 . hal-02106179

**HAL Id: hal-02106179**

**<https://hal.science/hal-02106179>**

Submitted on 17 May 2024

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22

**Title:**

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

Camille Vandromme<sup>a</sup> ; Corentin Spriet<sup>a</sup> ; David Dauvillée<sup>a</sup> ; Adeline Courseaux<sup>a</sup> ; Jean-Luc Putaux<sup>b</sup>; Adeline Wychowski<sup>a</sup> ; Maud Facon<sup>a</sup> ; Christophe D’Hulst<sup>a</sup> ; Fabrice Wattebled<sup>a\*</sup>.

<sup>a</sup> Univ. Lille, CNRS, UMR8576 – UGSF – Unité de Glycobiologie Structurale et Fonctionnelle, F-59000 Lille, France.

<sup>b</sup> Univ. Grenoble Alpes, CNRS, CERMAV, F-38000, Grenoble, France.

\* Author for correspondence: Fabrice Wattebled

Email: [fabrice.wattebled@univ-lille.fr](mailto:fabrice.wattebled@univ-lille.fr);

Phone: +33 320434881

23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43

## 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*

44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65  
66  
67  
68  
69  
70  
71  
72  
73  
74  
75

## 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  $\alpha$ -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  $\alpha$ -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; Wattedled *et al.*, 2005; Streb *et al.*, 2008; Wattedled *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  $\mu\text{m}$ . Starch granules in the *ss4* mutant are larger (3-5  $\mu\text{m}$ ) 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 Y2H<sup>TM</sup> 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<sup>®</sup>) completed with Murashige  
153 and Skoog medium and 0.8% plant agar (Duchefa Biochemie). Roots develop in a culture  
154 solution (1.1 mM MgSO<sub>4</sub>, 2mM KNO<sub>3</sub>, 805 μM Ca(NO<sub>3</sub>)<sub>2</sub>, 695 μM KH<sub>2</sub>PO<sub>4</sub>, 60 μM  
155 K<sub>2</sub>HPO<sub>4</sub>, 20 μM FeSO<sub>4</sub>, 20 μM Na<sub>2</sub>EDTA, 9.25 μM H<sub>3</sub>BO<sub>3</sub>, 3.6 μM MnCl<sub>2</sub>, 74 nM  
156 (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>, 3 μM ZnSO<sub>4</sub>, 785 nM CuSO<sub>4</sub>), 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<sup>™</sup> directional TOPO<sup>®</sup> cloning kit, Invitrogen). Using LR clonase  
163 (Gateway<sup>®</sup> LR clonase<sup>™</sup> 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 CaCl<sub>2</sub>, 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 CaCl<sub>2</sub>, 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 MgCl<sub>2</sub>, 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) PEG<sub>4000</sub>, 0.4 M mannitol, 0.1M Ca(NO<sub>3</sub>)<sub>2</sub> 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  $550\text{ nm}$  (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 H<sub>2</sub>O 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  $\mu\text{m}$  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  $\mu\text{m}$  (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  $\mu\text{m}$  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).

414

415

416

417

## 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 *piil-2*. Although these two lines were confirmed mutant by RT-  
446 PCR (Figure 1) we cannot exclude that a small amount of active PIII is still produced at a low  
447 level in the *piil-1* mutant while it is completely abolished in *piil-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 *piil-2* (*piil-1* is probably not a null allele), starch granules of PIII 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 *piil* mutants  
453 remain lenticular, a form comparable to that of wild-type starch.

454 Another difference observed between the *piil* and the *ss4* mutants is related to plant  
455 growth. Indeed, while the *ss4* mutants show significant growth retardation, the *piil* 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 PIII 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 *piil* 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 PIII.

466

#### 467 ***PIII 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, PIII was identified as  
477 an SS4-interacting partner. When PIII 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

## Acknowledgments

499 The authors thank Christine Lancelon-Pin (CERMAV, Grenoble) for the SEM  
500 observations and the NanoBio-ICMG platform (FR 2607, Grenoble) for granting access to the  
501 Electron Microscopy facility.

502 We are indebted to the Research Federation FRABio (Univ. Lille, CNRS, FR 3688,  
503 FRABio, Biochimie Structurale et Fonctionnelle des Assemblages Biomoléculaires) for  
504 providing the scientific and technical environment conducive to achieving this work

505 We thank the Agence Nationale de la Recherche for funding (Project “CaSta-DivA”  
506 Programmes non thématiques 2011 section SVSE6).

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.

514

515

516

517

518  
519  
520  
521  
522  
523  
524  
525  
526  
527  
528  
529  
530  
531  
532  
533  
534  
535  
536  
537  
538  
539  
540  
541  
542  
543  
544  
545  
546  
547  
548  
549  
550  
551  
552  
553  
554  
555  
556  
557  
558  
559  
560  
561  
562

## References

- Abel GJW, Springer F, Willmitzer L, Kossmann J. 1996.** Cloning and functional analysis of a cDNA encoding a novel 139 kDa starch synthase from potato (*Solanum tuberosum* L.). *The Plant Journal* **10**(6): 981-991.
- Adamson JG, Zhou NE, Hodges RS. 1993.** Structure, function and application of the coiled-coil protein folding motif. *Current Opinion in Biotechnology* **4**(4): 428-437.
- Ahmed Z, Tetlow IJ, Ahmed R, Morell MK, Emes MJ. 2015.** Protein-protein interactions among enzymes of starch biosynthesis in high-amylose barley genotypes reveal differential roles of heteromeric enzyme complexes in the synthesis of A and B granules. *Plant Science* **233**: 95-106.
- Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, et al. 2003.** Genome-Wide Insertional Mutagenesis of *Arabidopsis thaliana*. *Science* **301**(5633): 653-657.
- Bionda T, Tillmann B, Simm S, Beilstein K, Ruprecht M, Schleiff E. 2010.** Chloroplast Import Signals: The Length Requirement for Translocation In Vitro and In Vivo. *Journal of Molecular Biology* **402**(3): 510-523.
- Blauth SL, Yao Y, Klucinec JD, Shannon JC, Thompson DB, Guiltinan MJ. 2001.** Identification of Mutator Insertional Mutants of Starch-Branching Enzyme 2a in Corn. *Plant Physiology* **125**(3): 1396-1405.
- Boyer L, Roussel X, Courseaux A, Ndjindji OM, Lancelon-Pin C, Putaux JL, Tetlow IJ, Emes MJ, Pontoire B, D'Hulst C, et al. 2016.** Expression of *Escherichia coli* glycogen branching enzyme in an *Arabidopsis* mutant devoid of endogenous starch branching enzymes induces the synthesis of starch-like polyglucans. *Plant, Cell & Environment* **39**(7): 1432-1447.
- Coutinho PM, Deleury E, Davies GJ, Henrissat B. 2003.** An evolving hierarchical family classification for glycosyltransferases. *Journal of Molecular Biology* **328**(2): 307-317.
- Crofts N, Abe N, Oitome NF, Matsushima R, Hayashi M, Tetlow IJ, Emes MJ, Nakamura Y, Fujita N. 2015.** Amylopectin biosynthetic enzymes from developing rice seed form enzymatically active protein complexes. *Journal of Experimental Botany* **66**(15): 4469-4482.
- Crofts N, Sugimoto K, Oitome NF, Nakamura Y, Fujita N. 2017.** Differences in specificity and compensatory functions among three major starch synthases determine the structure of amylopectin in rice endosperm. *Plant Molecular Biology* **94**(4-5): 399-417.
- Crumpton-Taylor M, Grandison S, Png KMY, Bushby AJ, Smith AM. 2012.** Control of starch granule numbers in *Arabidopsis* chloroplasts. *Plant Physiology* **158**(2): 905-916.
- Crumpton-Taylor M, Pike M, Lu K-J, Hylton CM, Feil R, Eicke S, Lunn JE, Zeeman SC, Smith AM. 2013.** Starch synthase 4 is essential for coordination of starch granule formation with chloroplast division during *Arabidopsis* leaf expansion. *New Phytologist* **200**(4): 1064-1075.



- 563 **D'Hulst C, Wattedled F, Szydlowski N. 2015.** Starch biosynthesis in leaves and its  
564 regulation. in *Starch: Metabolism and Structure*, ed. Y. Nakamura. (Tokyo:  
565 Springer Japan): 211-237.
- 566 **Delatte T, Trevisan M, Parker ML, Zeeman SC. 2005.** Arabidopsis mutants *Atisa1* and  
567 *Atisa2* have identical phenotypes and lack the same multimeric isoamylase,  
568 which influences the branch point distribution of amylopectin during starch  
569 synthesis. *The Plant Journal* **41**(6): 815-830.
- 570 **Delorenzi M, Speed T. 2002.** An HMM model for coiled-coil domains and a comparison  
571 with PSSM-based predictions. *Bioinformatics* **18**(4): 617-625.
- 572 **Delvallé D, Dumez S, Wattedled F, Roldán I, Planchot V, Berbezy P, Colonna P, Vyas  
573 D, Chatterjee M, Ball S, et al. 2005.** Soluble starch synthase I: a major  
574 determinant for the synthesis of amylopectin in *Arabidopsis thaliana* leaves. *The  
575 Plant Journal* **43**(3): 398-412.
- 576 **Dumez S, Wattedled F, Dauvillee D, Delvalle D, Planchot V, Ball SG, D'Hulst C. 2006.**  
577 Mutants of Arabidopsis Lacking Starch Branching Enzyme II Substitute Plastidial  
578 Starch Synthesis by Cytoplasmic Maltose Accumulation. *The Plant Cell* **18**(10):  
579 2694-2709.
- 580 **Edwards A, Borthakur A, Bornemann S, Venail J, Denyer K, Waite D, Fulton D,  
581 Smith A, Martin C. 1999.** Specificity of starch synthase isoforms from potato.  
582 *European Journal of Biochemistry* **266**(3): 724-736.
- 583 **Emanuelsson O, Nielsen H, von Heijne G. 1999.** ChloroP, a neural network-based  
584 method for predicting chloroplast transit peptides and their cleavage sites.  
585 *Protein Science* **8**(5): 978-984.
- 586 **Feike D, Seung D, Graf A, Bischof S, Ellick T, Coiro M, Soyk S, Eicke S, Mettler-  
587 Altmann T, Lu K-J, et al. 2016.** The starch granule-associated protein EARLY  
588 STARVATION1 (ESV1) is required for the control of starch degradation in  
589 *Arabidopsis thaliana* leaves. *The Plant Cell* **28**(6): 1472-1489.
- 590 **Ferreira SJ, Senning M, Fischer-Stettler M, Streb S, Ast M, Neuhaus HE, Zeeman SC,  
591 Sonnewald S, Sonnewald U. 2017.** Simultaneous silencing of isoamylases ISA1,  
592 ISA2 and ISA3 by multi-target RNAi in potato tubers leads to decreased starch  
593 content and an early sprouting phenotype. *PLoS ONE* **12**(7): e0181444.
- 594 **Gámez-Arjona FM, Raynaud S, Ragel P, Mérida Á. 2014.** Starch synthase 4 is located  
595 in the thylakoid membrane and interacts with plastoglobule-associated proteins  
596 in Arabidopsis. *The Plant Journal* **80**(2): 305-316.
- 597 **Grefen C, Donald N, Hashimoto K, Kudla J, Schumacher K, Blatt MR. 2010.** A  
598 ubiquitin-10 promoter-based vector set for fluorescent protein tagging facilitates  
599 temporal stability and native protein distribution in transient and stable  
600 expression studies. *The Plant Journal* **64**(2): 355-365.
- 601 **Hennen-Bierwagen TA, Lin Q, Grimaud F, Planchot V, Keeling PL, James MG, Myers  
602 AM. 2009.** Proteins from Multiple Metabolic Pathways Associate with Starch  
603 Biosynthetic Enzymes in High Molecular Weight Complexes: A Model for  
604 Regulation of Carbon Allocation in Maize Amyloplasts. *Plant Physiology* **149**(3):  
605 1541-1559.
- 606 **Hennen-Bierwagen TA, Liu F, Marsh RS, Kim S, Gan Q, Tetlow IJ, Emes MJ, James  
607 MG, Myers AM. 2008.** Starch Biosynthetic Enzymes from Developing Maize  
608 Endosperm Associate in Multisubunit Complexes. *Plant Physiology* **146**(4): 1892-  
609 1908.



- 610 **Leterrier M, Holappa LD, Broglie KE, Beckles DM. 2008.** Cloning, characterisation  
611 and comparative analysis of a starch synthase IV gene in wheat: functional and  
612 evolutionary implications. *BMC Plant Biology* **8**: 98.
- 613 **Liu F, Ahmed Z, Lee EA, Donner E, Liu Q, Ahmed R, Morell MK, Emes MJ, Tetlow IJ.**  
614 **2012.** Allelic variants of the amylose extender mutation of maize demonstrate  
615 phenotypic variation in starch structure resulting from modified  
616 protein-protein interactions. *Journal of Experimental Botany* **63**(3): 1167-1183.
- 617 **Liu F, Makhmoudova A, Lee EA, Wait R, Emes MJ, Tetlow IJ. 2009.** The amylose  
618 extender mutant of maize conditions novel protein-protein interactions between  
619 starch biosynthetic enzymes in amyloplasts. *Journal of Experimental Botany*  
620 **60**(15): 4423-4440.
- 621 **Lu K-J, Pfister B, Jenny C, Eicke S, Zeeman SC. 2018.** Distinct Functions of STARCH  
622 SYNTHASE 4 Domains in Starch Granule Formation. *Plant Physiology* **176**(1):  
623 566-581.
- 624 **Makhmoudova A, Williams D, Brewer D, Massey S, Patterson J, Silva A, Vassall KA,**  
625 **Liu F, Subedi S, Harauz G, et al. 2014.** Identification of multiple  
626 phosphorylation sites on maize endosperm starch branching enzyme IIb, a key  
627 enzyme in amylopectin biosynthesis. *The Journal of Biological Chemistry* **289**(13):  
628 9233-9246.
- 629 **Malinova I, Fettke J. 2017.** Reduced starch granule number per chloroplast in the  
630 *dpe2/phs1* mutant is dependent on initiation of starch degradation. *PLoS ONE*  
631 **12**(11): e0187985.
- 632 **Mouille G, Maddelein ML, Libessart N, Talaga P, Decq A, Delrue B, Ball S. 1996.**  
633 Preamylopectin Processing: A Mandatory Step for Starch Biosynthesis in Plants.  
634 *The Plant Cell* **8**(8): 1353-1366.
- 635 **Myers AM, Morell MK, James MG, Ball SG. 2000.** Recent Progress toward  
636 Understanding Biosynthesis of the Amylopectin Crystal. *Plant Physiology* **122**(4):  
637 989-998.
- 638 **Nakamura Y, Utsumi Y, Sawada T, Aihara S, Utsumi C, Yoshida M, Kitamura S. 2010.**  
639 Characterization of the Reactions of Starch Branching Enzymes from Rice  
640 Endosperm. *Plant and Cell Physiology* **51**(5): 776 - 794.
- 641 **Pfister B, Zeeman SC. 2016.** Formation of starch in plant cells. *Cell Mol Life Sci* **73**(14):  
642 2781-2807.
- 643 **Ragel P, Streb S, Feil R, Sahrawy M, Annunziata MG, Lunn JE, Zeeman S, Mérida Á.**  
644 **2013.** Loss of Starch Granule Initiation Has a Deleterious Effect on the Growth of  
645 Arabidopsis Plants Due to an Accumulation of ADP-Glucose. *Plant Physiology*  
646 **163**(1): 75-85.
- 647 **Raynaud S, Ragel P, Rojas T, Mérida Á. 2016.** The N-terminal Part of Arabidopsis  
648 thaliana Starch Synthase 4 Determines the Localization and Activity of the  
649 Enzyme. *Journal of Biological Chemistry* **291**(20): 10759-10771.
- 650 **Regina A, Kosar-Hashemi B, Ling S, Li Z, Rahman S, Morell M. 2010.** Control of starch  
651 branching in barley defined through differential RNAi suppression of starch  
652 branching enzyme IIa and IIb. *Journal of Experimental Botany* **61**(5): 1469-1482.
- 653 **Roldán I, Wattebled F, Lucas MM, Delvallé D, Planchot V, Jiménez S, Pérez R, Ball S,**  
654 **D'Hulst C, Mérida Á. 2007.** The phenotype of soluble starch synthase IV  
655 defective mutants of *Arabidopsis thaliana* suggests a novel function of elongation  
656 enzymes in the control of starch granule formation. *The Plant Journal* **49**(3): 492-  
657 504.

- 658 **Roussel X, Lancelon-Pin C, Vikso-Nielsen A, Rolland-Sabate A, Grimaud F, Potocki-**  
659 **Veronese G, Buleon A, Putaux JL, D'Hulst C. 2013.** Characterization of  
660 substrate and product specificity of the purified recombinant glycogen branching  
661 enzyme of *Rhodothermus obamensis*. *Biochimica et Biophysica Acta* **1830**(1):  
662 2167-2177.
- 663 **Samson F, Brunaud V, Balzergue S, Dubreucq B, Lepiniec L, Pelletier G, Caboche M,**  
664 **Lecharny A. 2002.** FLAGdb/FST: a database of mapped flanking insertion sites  
665 (FSTs) of *Arabidopsis thaliana* T-DNA transformants. *Nucleic Acids Research*  
666 **30**(1): 94-97.
- 667 **Schwall GP, Safford R, Westcott RJ, Jeffcoat R, Tayal A, Shi Y-C, Gidley MJ, Jobling**  
668 **SA. 2000.** Production of very-high-amylose potato starch by inhibition of SBE A  
669 and B. *Nature Biotechnology* **18**(5): 551-554.
- 670 **Scialdone A, Mugford ST, Feike D, Skeffington A, Borrill P, Graf A, Smith AM,**  
671 **Howard M. 2013.** *Arabidopsis* plants perform arithmetic division to prevent  
672 starvation at night. *Elife* **2**: e00669.
- 673 **Seung D, Boudet J, Monroe JD, Schreier TB, David LC, Abt M, Lu K-J, Zanella M,**  
674 **Zeeman SC. 2017.** Homologs of PROTEIN TARGETING TO STARCH Control  
675 Starch Granule Initiation in *Arabidopsis* Leaves. *The Plant Cell* **29**(7): 1657-1677.
- 676 **Seung D, Soyk S, Coiro M, Maier BA, Eicke S, Zeeman SC. 2015.** PROTEIN TARGETING  
677 TO STARCH Is Required for Localising GRANULE-BOUND STARCH SYNTHASE to  
678 Starch Granules and for Normal Amylose Synthesis in *Arabidopsis*. *PLoS Biol*  
679 **13**(2): e1002080.
- 680 **Streb S, Delatte T, Umhang M, Eicke S, Schorderet M, Reinhardt D, Zeeman SC.**  
681 **2008.** Starch Granule Biosynthesis in *Arabidopsis* Is Abolished by Removal of All  
682 Debranching Enzymes but Restored by the Subsequent Removal of an  
683 Endoamylase. *The Plant Cell* **20**(12): 3448-3466.
- 684 **Subasinghe RM, Liu F, Polack UC, Lee EA, Emes MJ, Tetlow IJ. 2014.** Multimeric  
685 states of starch phosphorylase determine protein-protein interactions with  
686 starch biosynthetic enzymes in amyloplasts. *Plant Physiology and Biochemistry*  
687 **83**(0): 168-179.
- 688 **Szydowski N, Ragel P, Hennen-Bierwagen TA, Planchot V, Myers AM, Mérida A,**  
689 **d'Hulst C, Wattedled F. 2011.** Integrated functions among multiple starch  
690 synthases determine both amylopectin chain length and branch linkage location  
691 in *Arabidopsis* leaf starch. *Journal of Experimental Botany* **62**(13): 4547-4559.
- 692 **Szydowski N, Ragel P, Raynaud S, Lucas MM, Roldan I, Montero M, Munoz FJ,**  
693 **Ovecka M, Bahaji A, Planchot V, et al. 2009.** Starch Granule Initiation in  
694 *Arabidopsis* Requires the Presence of Either Class IV or Class III Starch Synthases.  
695 *The Plant Cell* **21**(8): 2443-2457.
- 696 **Tanaka N, Fujita N, Nishi A, Satoh H, Hosaka Y, Ugaki M, Kawasaki S, Nakamura Y.**  
697 **2004.** The structure of starch can be manipulated by changing the expression  
698 levels of starch branching enzyme IIb in rice endosperm. *Plant Biotechnology*  
699 *Journal* **2**(6): 507-516.
- 700 **Tetlow IJ. 2012.** Branching enzymes and their role in determining structural and  
701 functional properties of polyglucans. in *Starch: Origins, Structure and Metabolism.*,  
702 ed. IJ. Tetlow. (London, UK: Society for Experimental Biology): 141-178.
- 703 **Tetlow IJ, Beisel KG, Cameron S, Makhmoudova A, Liu F, Bresolin NS, Wait R,**  
704 **Morell MK, Emes MJ. 2008.** Analysis of Protein Complexes in Wheat  
705 Amyloplasts Reveals Functional Interactions among Starch Biosynthetic  
706 Enzymes. *Plant Physiology* **146**(4): 1878-1891.

- 707 **Tetlow IJ, Wait R, Lu Z, Akkasaeng R, Bowsher CG, Esposito S, Kosar-Hashemi B,**  
708 **Morell MK, Emes MJ. 2004.** Protein Phosphorylation in Amyloplasts Regulates  
709 Starch Branching Enzyme Activity and Protein-Protein Interactions. *Plant Cell*  
710 **16(3): 694-708.**
- 711 **Wattebled F, Dong Y, Dumez S, Delvalle D, Planchot V, Berbezy P, Vyas D, Colonna**  
712 **P, Chatterjee M, Ball S, et al. 2005.** Mutants of Arabidopsis Lacking a  
713 Chloroplastic Isoamylase Accumulate Phytoglycogen and an Abnormal Form of  
714 Amylopectin. *Plant Physiol.* **138(1): 184-195.**
- 715 **Wattebled F, Planchot V, Dong Y, Szydlowski N, Pontoire B, Devin A, Ball S, D'Hulst**  
716 **C. 2008.** Further Evidence for the Mandatory Nature of Polysaccharide  
717 Debranching for the Aggregation of Semicrystalline Starch and for Overlapping  
718 Functions of Debranching Enzymes in Arabidopsis Leaves. *Plant Physiology*  
719 **148(3): 1309-1323.**
- 720 **Yao Y, Thompson DB, Guiltinan MJ. 2004.** Maize starch-branching enzyme isoforms  
721 and amylopectin structure. In the absence of starch-branching enzyme IIb, the  
722 further absence of starch-branching enzyme Ia leads to increased branching.  
723 *Plant Physiology* **136(3): 3515-3523.**
- 724 **Zhang X, Myers AM, James MG. 2005.** Mutations affecting starch synthase III in  
725 Arabidopsis alter leaf starch structure and increase the rate of starch synthesis.  
726 *Plant Physiology* **138(2): 663-674.**
- 727 **Zhang X, Szydlowski N, Delvalle D, D'Hulst C, James M, Myers A. 2008.** Overlapping  
728 functions of the starch synthases SSII and SSIII in amylopectin biosynthesis in  
729 Arabidopsis. *BMC Plant Biology* **8(1): 96.**
- 730 **Zimmermann L, Stephens A, Nam S-Z, Rau D, Kübler J, Lozajic M, Gabler F, Söding J,**  
731 **Lupas AN, Alva V. 2017.** A Completely Reimplemented MPI Bioinformatics  
732 Toolkit with a New HHpred Server at its Core. *Journal of Molecular Biology*: in  
733 press.  
734

735

736

737

## 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  $\mu\text{m}$

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.

789 Scale bar = 10  $\mu\text{m}$

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  $\mu\text{m}$  (*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  $\mu\text{m}$ .

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  $\mu\text{m}$ , 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, *pri1-1*, *ss4-1*. Panel **(d)**: from left to right: Ws,  
815 *pri1-2*, *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)**: *pri1-2*.

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  $\mu$ 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)**: *pri1-1*, **(c)**: WS. **(d)**: *pri1-2*. Each profile is the mean of two analysis carried  
845 out with starch extracted from two independent cultures.



846

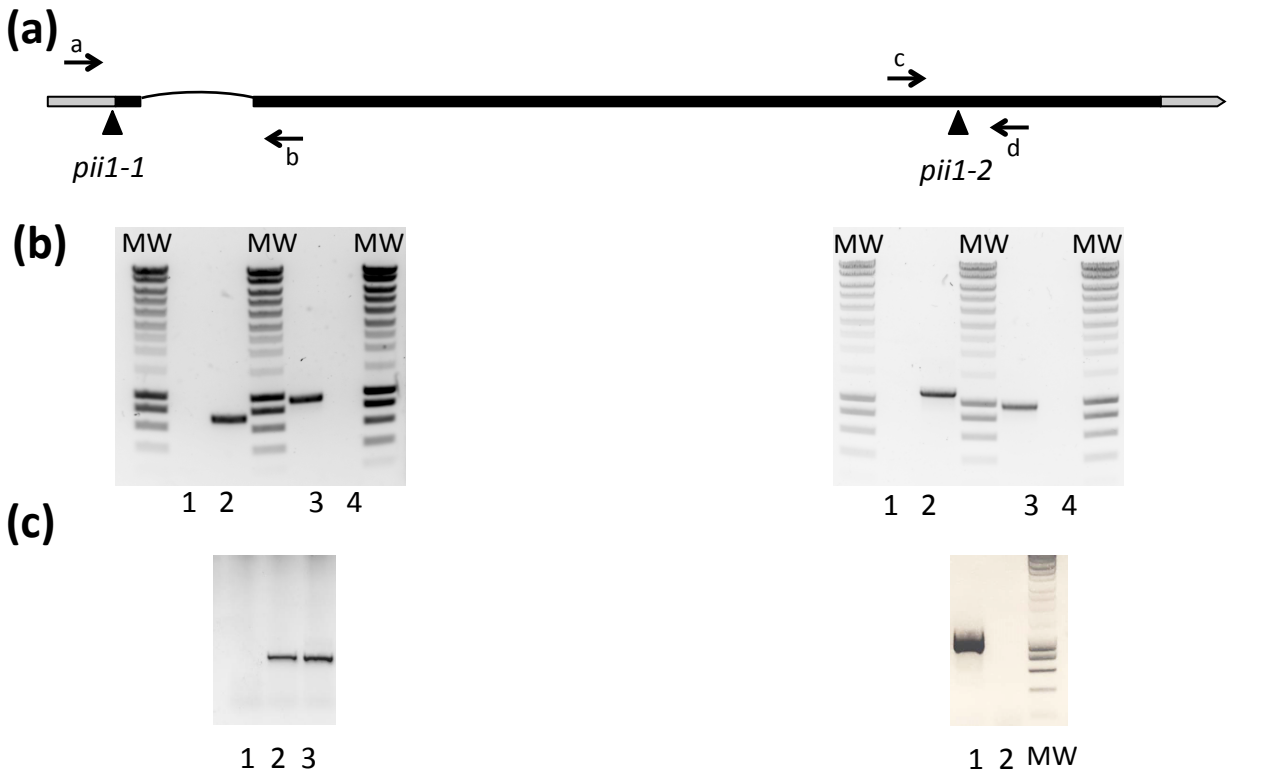
847 **Fig. 9: Localization of SS4 in Arabidopsis protoplasts.**

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  $\mu$ m

852

853



### Fig. 1: Selection of *pii1* mutants.

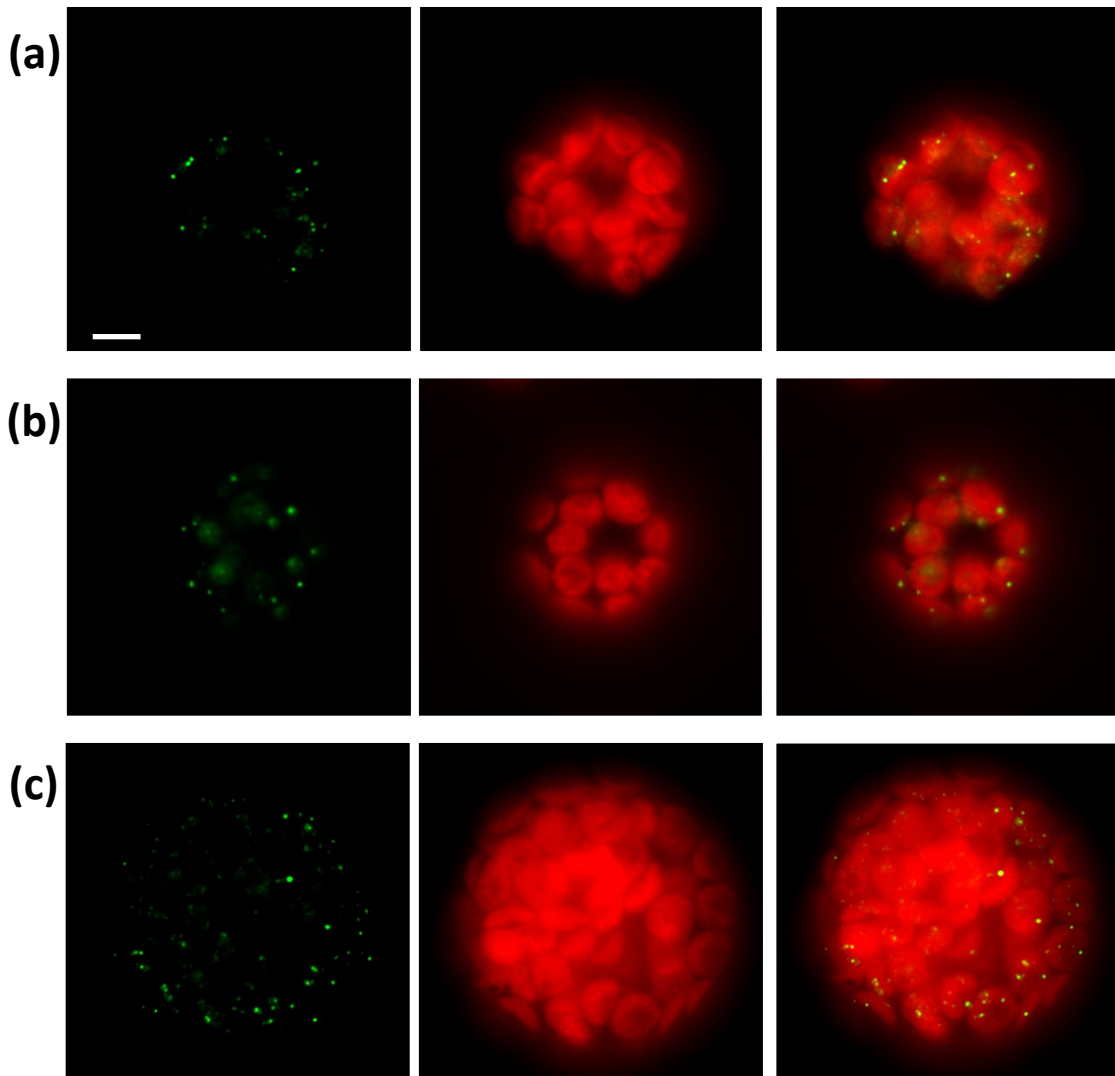
**(a)** Structure of the *At4g32190* locus encoding PII1. UTR are indicated by grey boxes while introns and exons are depicted as black lines and black boxes respectively. T-DNA insertion corresponding to *pii1-1* and *pii1-2* alleles are indicated by triangles. Primer position used for selection are indicated by arrows (not in scale)

**(b)** Left panel represents the selection of the *pii1-1* homozygote mutant. gDNA from one mutant plant (lanes 1 and 3) and one wild-type control (lanes 2 and 4) was used. Amplification products of wild type allele (using primer a and b) are in lanes 1 and 2. Amplification products of mutant allele (using T-DNA primer and primer b) are in lanes 3 and 4. Right panel represents the selection of the *pii1-2* homozygote mutant. gDNA from one mutant plant (lanes 1 and 3) and one wild-type control (lanes 2 and 4) was used. Amplification products of wild type allele (using primer c and d) are in lanes 1 and 2. Amplification products of mutant allele (using T-DNA primer and primer c) are in lanes 3 and 4.

**(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.

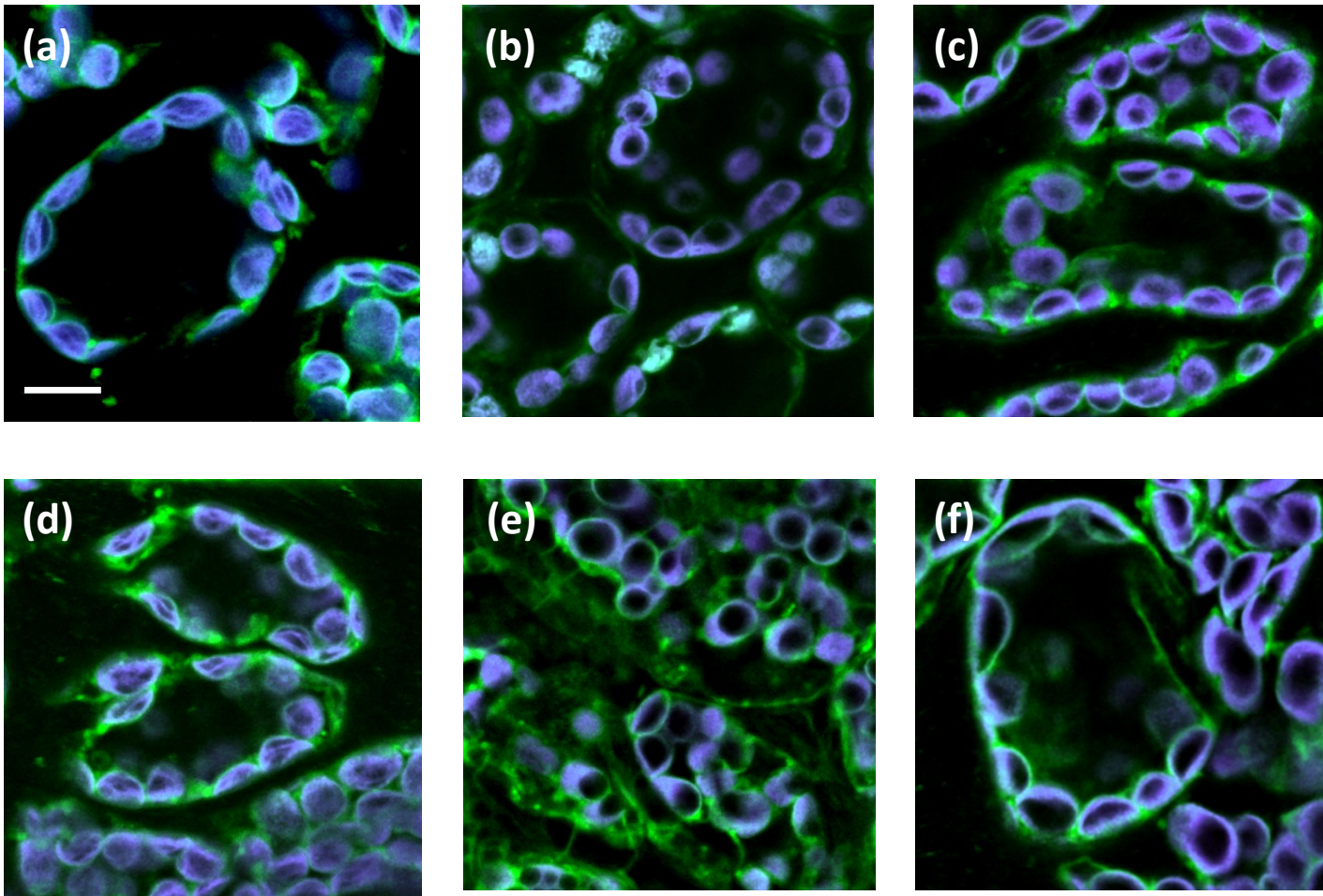




**Fig. 2: Subcellular localization of PII1 in Arabidopsis protoplasts.**

Protoplasts were prepared from Col-0 **(a)**, *pii1-1* **(b)** or *ss4-1 pii1-1* **(c)**. Image acquisition was performed using a video microscope. In each row, the first image corresponds to the GFP signal of the PII1-GFP fusion protein. Chlorophyll fluorescence is displayed in the central panels. The merged images are displayed on the right.

Scale bar = 10  $\mu$ m

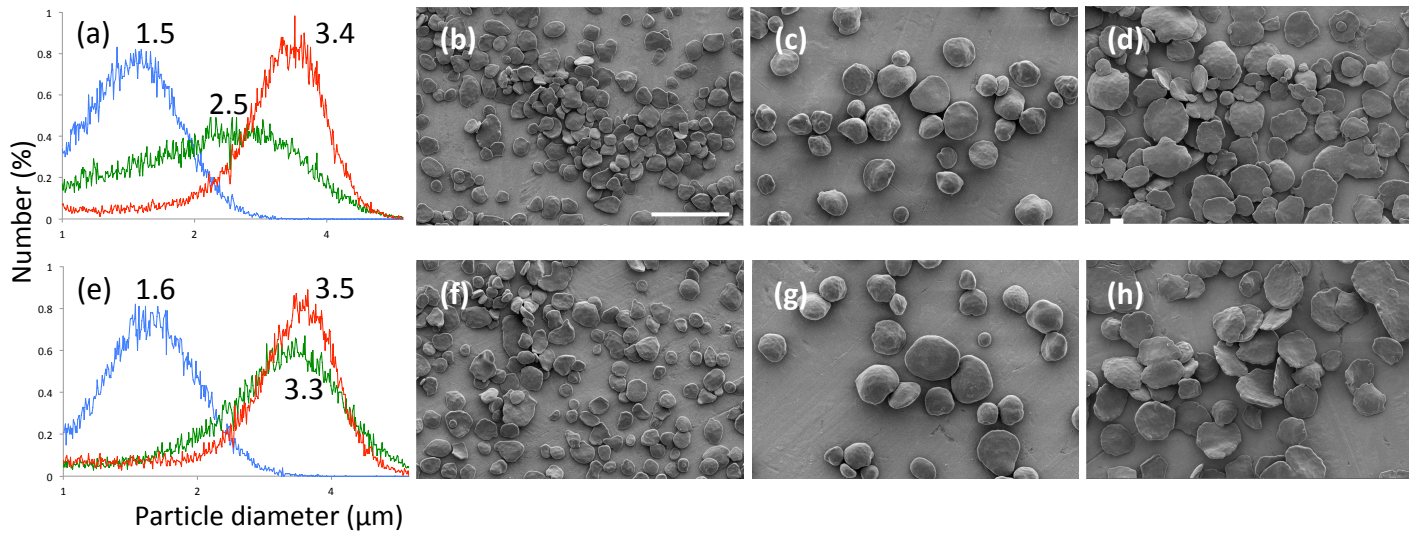


**Fig. 3: Starch granule number per chloroplast.**

Isolated leaf cells were prepared from Col-0 (a); *ss4-1* (b); *pii1-1* (c); *Ws* (d); *ss4-2* (e) and *pii1-2* (f).

Leaves samples were collected at the end of the day and tissues were fixed in paraformaldehyde before disruption in EDTA. Pictures were collected using a confocal microscope. Chlorophyll fluorescence in purple delimits the chloroplast volume, while starch granules are black.

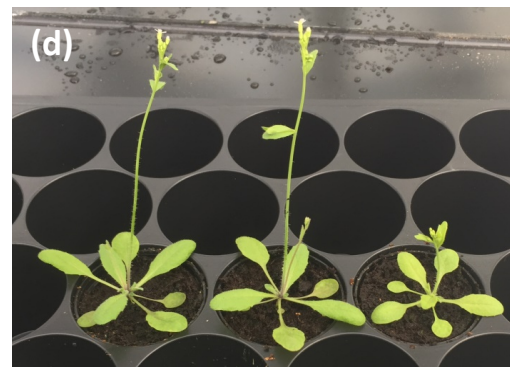
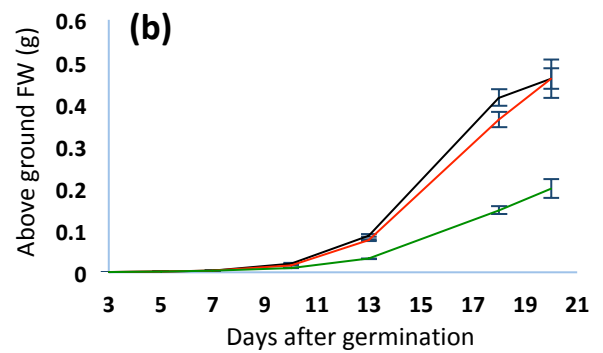
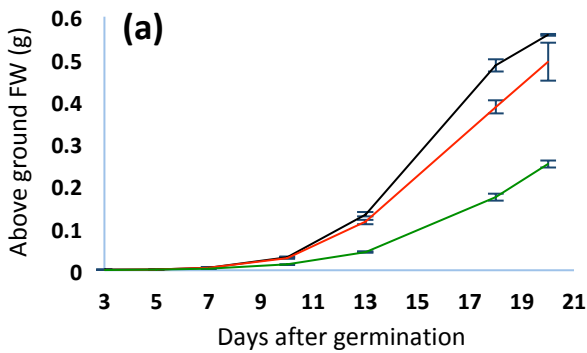
Scale bar = 10  $\mu$ m



#### Fig. 4: Starch granule size and morphology.

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

The starch granule size distribution was determined by analyzing 30,000 particles extracted for each genotype. The results are expressed in relative percentage (*y*-axis) of particles of a diameter ranging from 1 to 6  $\mu\text{m}$  (*x*-axis, logarithmic scale). In (a) and (e), starch from wild type, *ss4* and *pii1* lines are in blue, red and green, respectively. The value at the peak of the Gaussian distribution is indicated in  $\mu\text{m}$ . (b) to (d) and (f) to (h): starch granules were observed using scanning electron microscopy. (b) and (f): wild-type (Col-O and Ws, respectively); (c) and (g): *ss4-1* and *ss4-2*; (d) and (h): *pii1-1* and *pii1-2*. Scale bar = 10  $\mu\text{m}$ , all images are at the same scale.



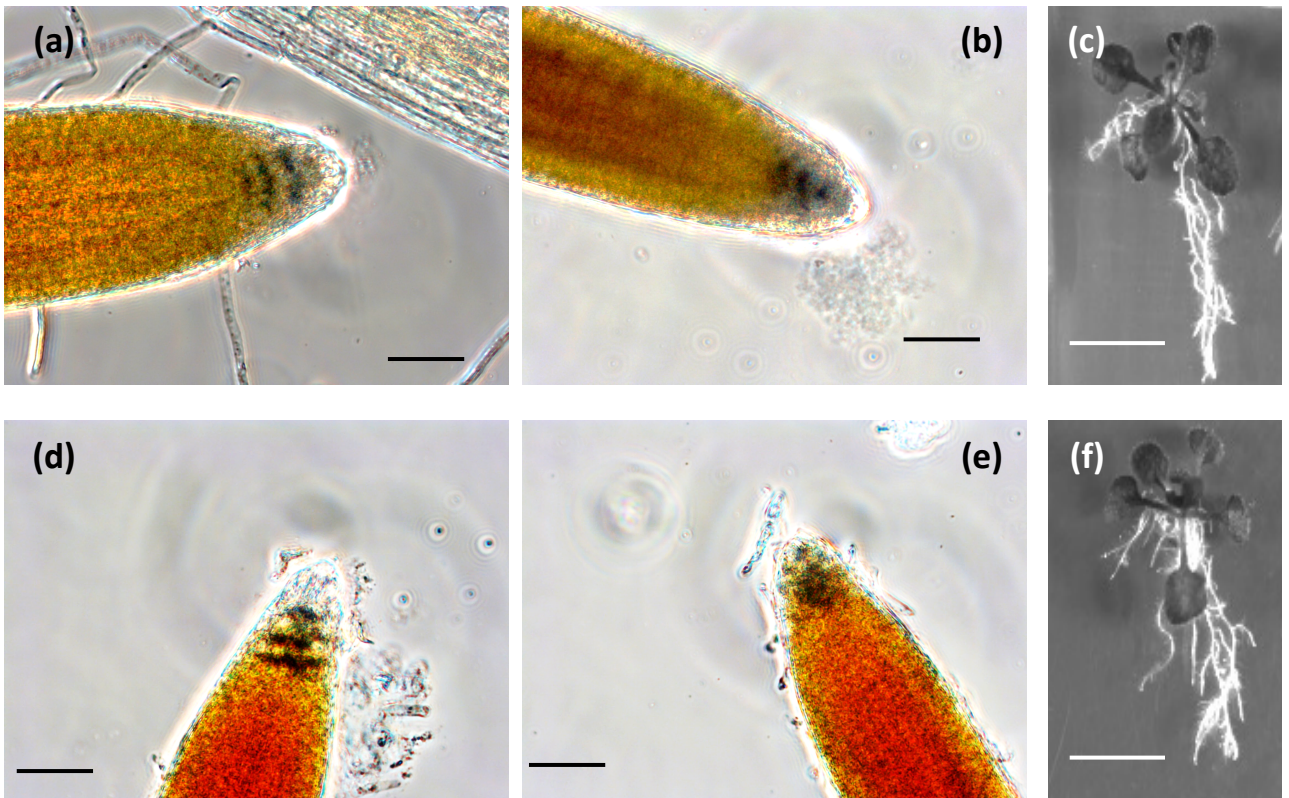
**Fig. 5: *pii1* growth phenotype compared to *ss4* and wild type plants.**

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

Panels (c) and (d) are pictures of 3-week-old plants grown in the same conditions as above.

Panel (c): from left to right: Col-0, *pii1-1*, *ss4-1*. Panel (d): from left to right: Ws, *pii1-2*, *ss4-2*.





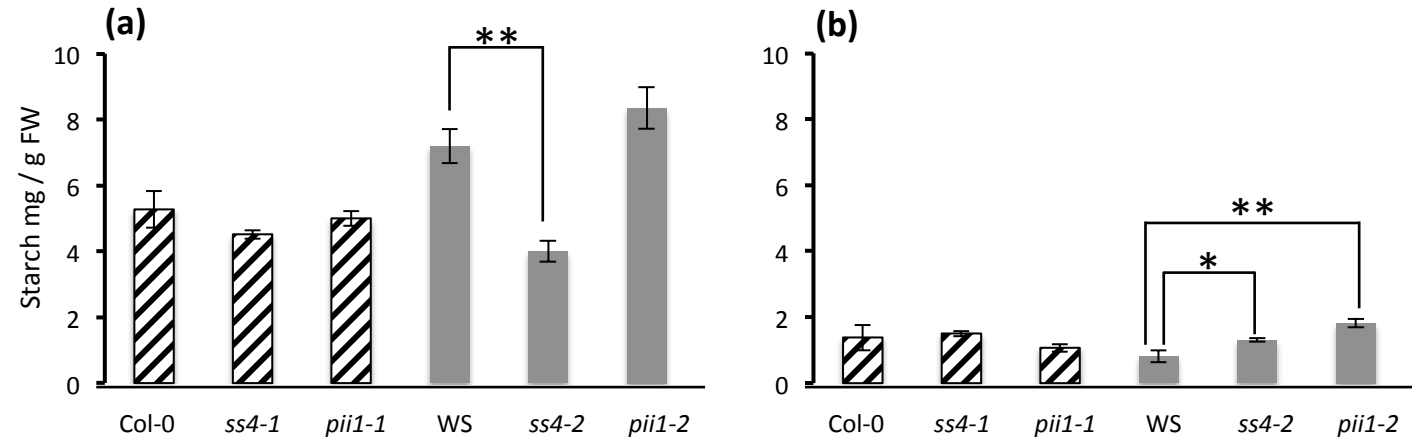
**Fig. 6: Starch accumulation in root and root development.**

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

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

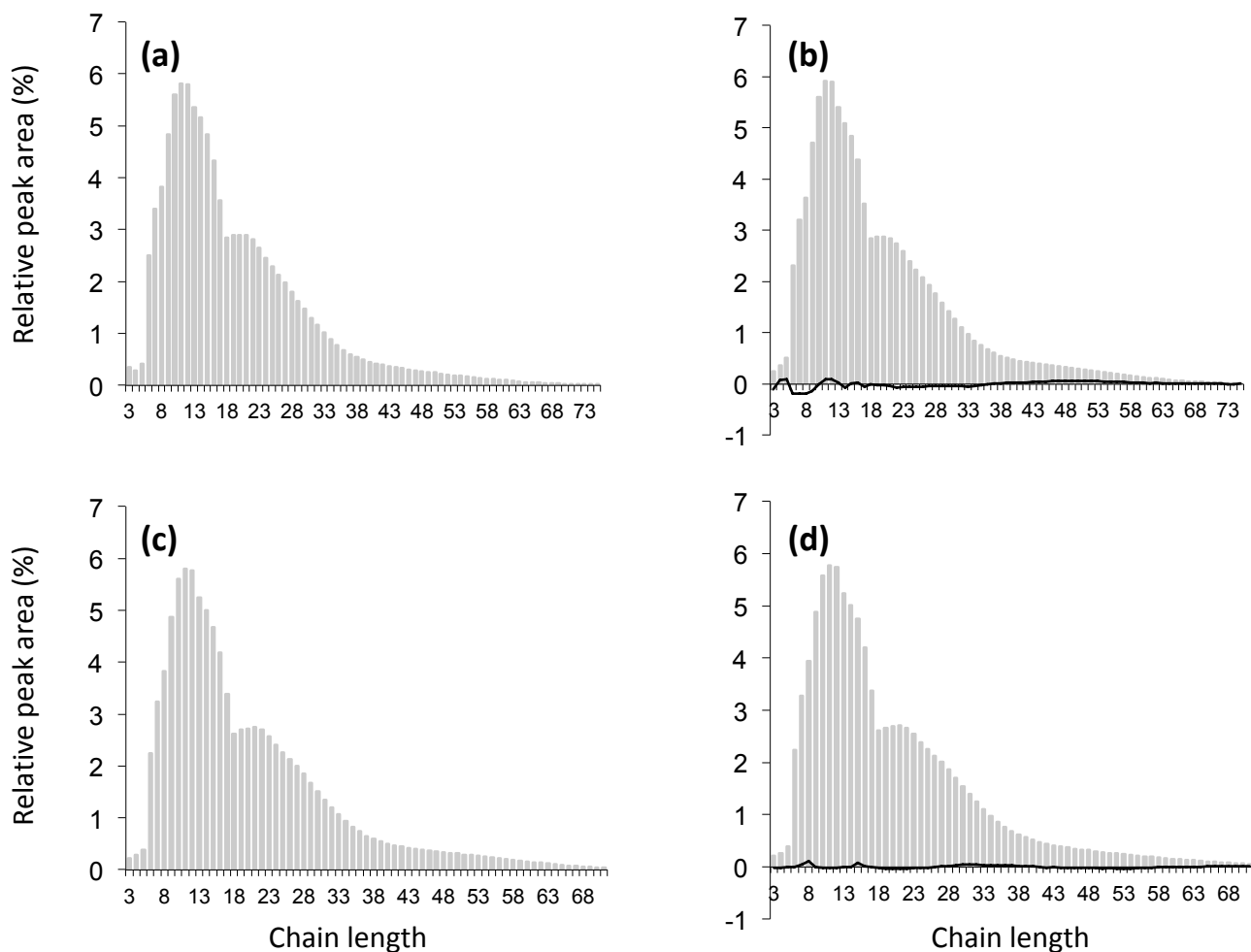
Panels (c) and (f): seeds were sown on agar plates that were maintained vertically after seed germination. Pictures were taken after 2 weeks of growth.

Black bar = 50  $\mu$ m; white bar = 1 cm



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

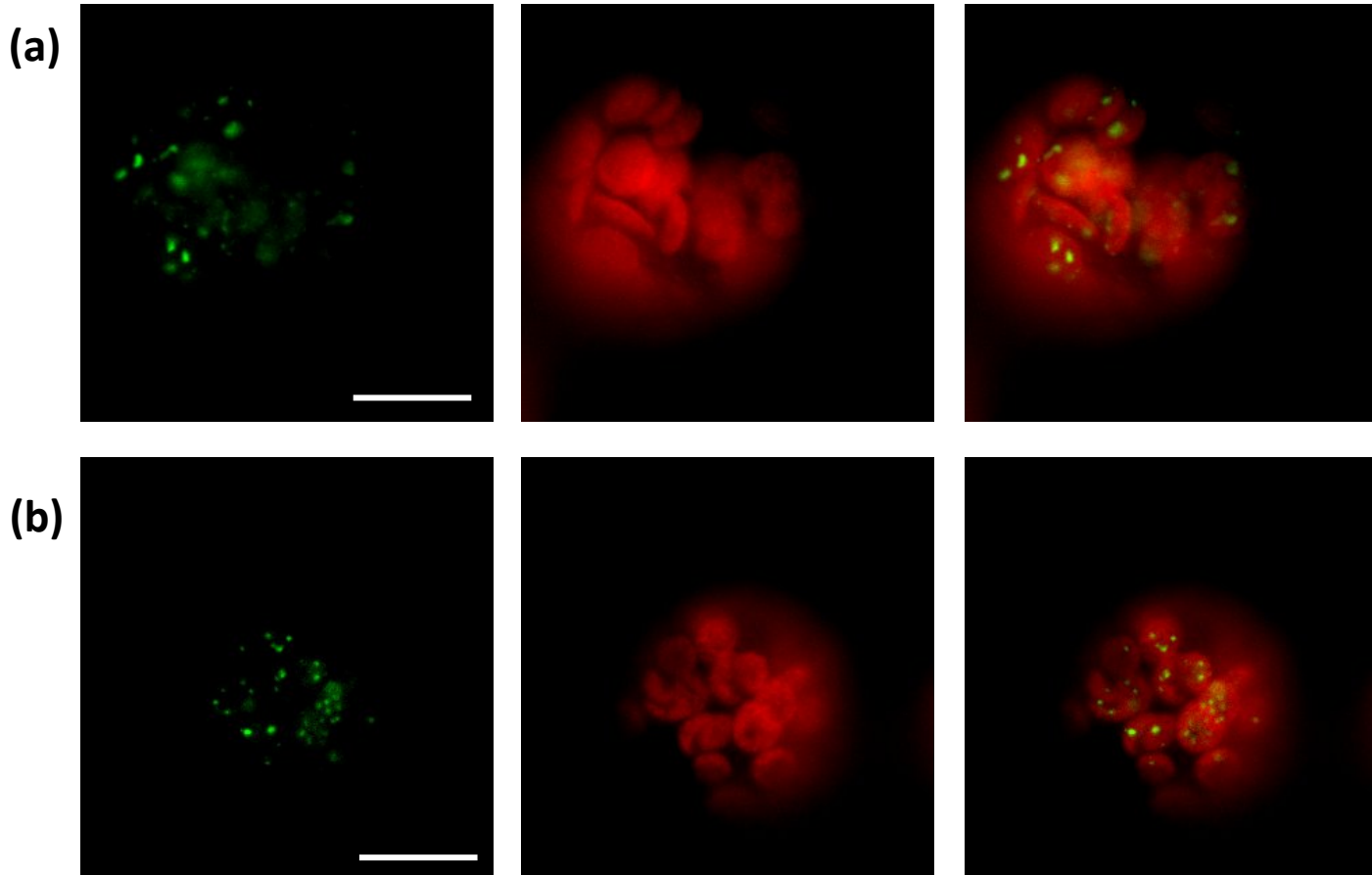
Plants were grown in a culture room under 16 h : 8 h, light : dark photoperiod. Three weeks after germination leaves were harvested either at the end of the day **(a)** or at the end of the dark phase **(b)**. Three independent cultures were performed and for each culture three independent extractions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



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

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

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



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

Protoplasts were prepared from *ss4-2* (a), *ss4-2 pii1-2* (b). Image acquisition was performed using a video microscope. In each row, from left to right: SS4-GFP fluorescence; Chlorophyll fluorescence; Merged images. All images are at the same scale. Bar = 10  $\mu\text{m}$