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# A sensitive method for confocal fluorescence microscopic visualization of starch granules in iodine stained samples

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**Abbreviations:** AGP, ADP-glucose pyrophosphorylase; GBSS, granule-bound starch synthase; GFP, green fluorescent protein; SEM, scanning electron microscopy; SS, starch synthase; SuSy, sucrose synthase; WT: wild type

Synthesized by glycogen synthase and starch synthases (SS) using ADP-glucose as the sugar donor molecule, glycogen and starch accumulate as predominant storage carbohydrates in most bacteria and plants, respectively. We have recently shown that the so-called "starch-less" *Arabidopsis thaliana adg1–1* and *aps1* mutants impaired in ADP-glucose pyrophosphorylase do indeed accumulate low starch content in normal growth conditions, and relatively high starch content when plants were cultured in the presence of microbial volatiles. Our results were strongly supported by data obtained using a highly sensitive method for confocal fluorescence microscopic visualization of iodine stained starch granules. Using Arabidopsis leaves from WT plants, *aps1* plants, *ss3/ss4* plants lacking both class III and class IV SS, *gbss* plants lacking the granule-bound SS, and *sus1/sus2/sus3/sus4* plants lacking four genes that code for proteins with sucrose synthase activity, in this work we precisely describe the method for preparation of plant samples for starch microscopic examination. Furthermore, we show that this method can be used to visualize glycogen in bacteria, and pure starch granules, amylose and amylopectin.

Starch and glycogen are the most widespread glucose-based reserve polymers in plants and bacteria, respectively. Both are homopolysaccharides of α-1,4-linked glucose subunits with α-1,6-linked glucose at the branched points. Starch accumulates in the form of a quaternary structure composed of two structurally distinct polysaccharides: the highly branched amylopectin (which comprises up to ca. 80% of the starch dry weight) and the infrequently branched amylose. The synthesis of starch requires the participation of starch synthase (SS), which transfers the glucosyl moiety of the activated donor, ADP-glucose, to an elongating glucan chain. *Arabidospsis* possesses five distinct SS classes: granule-bound SS (GBSS), which is required for the synthesis of amylose, and SS classes I, II, III, and IV, the latter two being suggested to be absolutely required for starch granule initiation.[1] Since the initial demonstration that ADP-glucose serves as the precursor molecule for both plant starch and bacterial glycogen biosynthesis,[2-4] it became widely considered that ADP-glucose pyrophosphorylase (AGP) is the sole source of ADP-glucose linked to bacterial glycogen and plant starch biosynthesis. In bacteria, genetic evidence that glycogen biosynthesis occurs solely by the AGP pathway has been obtained from the characterization of *glgC*$^-$ mutants impaired in AGP such as the *Escherichia coli* AC70R1–504 strain.[5] These mutants display an apparent glycogen-less phenotype when macroscopically analyzed upon staining with iodine vapors.[6] However, recent studies have shown that these mutants can accumulate high levels of glycogen.[6] Furthermore, evidence has been provided showing the occurrence of various important sources, other than GlgC, of ADP-glucose linked to glycogen biosynthesis in different bacterial species.[7] In *Arabidopsis,* genetic evidence showing that transitory starch biosynthesis occurs solely by the AGP pathway has been obtained from the characterization of the *adg1–1* and *aps1* AGP mutants.[8-10] Leaves of these mutants display an apparent starch-less phenotype when macroscopically analyzed upon staining with iodine solutions, and when subjected to quantitative-type enzymatic tests for starch measurement. However, during the course of our studies we found that, independently of culture conditions, both *adg1–1* and *aps1* mutants accumulated ca. 2% of the wild type (WT) starch content despite the total lack of AGP activity and

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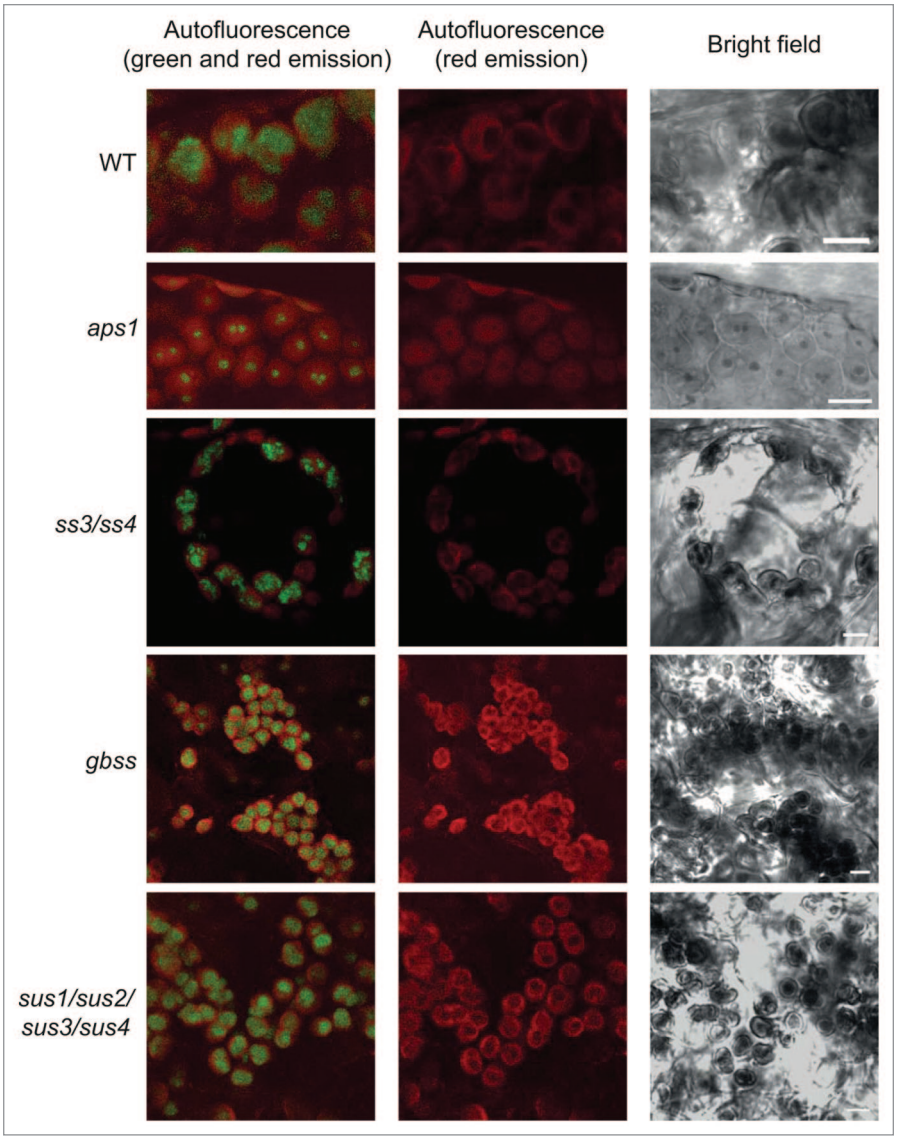


**Figure 1.** Confocal fluorescence microscopic analysis of iodine stained starch in leaves. Green fluorescence emission of iodine-stained starch granules occurring in chloroplasts of WT, *aps1*, *ss3/ss4*, *gbss* and *sus1/sus2/sus3/sus4*. Plants were grown in pots at ambient $CO_2$ (350 ppm) at 20°C under a 16 h light (90 μmol photons $sec^{-1}$ $m^{-2}$) / 8 h dark regime. Leaves were harvested at the end of the light period, fixed, stained as described in the main text, and examined using a D-Eclipse C1 confocal microscope with Ar 488 nm excitation using BA515/30 filter (detector gain setting 7.2). Note that green fluorescence associated only with oval/round structures that were identified as starch granules. Bar = 5 μm.

protein in the *aps1* mutants.[11] Furthermore, leaves of *aps1* plants exposed to microbial volatiles emitted by *Alternaria alternata* accumulated as much as 40% of the starch normally accumulated by illuminated WT leaves.[11] Moreover, *adg1–1/sex1* and *aps1/sex1* double mutants impaired in the machinery required for normal β-amylase-mediated leaf starch mobilization accumulated ca. 3 fold more starch than leaves of *adg1–1* and *aps1* mutants at the end of the light period.[11] Microscopic analyses also provided strong evidence showing the occurrence of transitory starch in leaves of both *adg1–1* and *aps1* mutants.[11] Scanning electron microscopy (SEM) of starch granules isolated from WT and *aps1* leaves confirmed not only the presence of small starch granules in *aps1* leaves, but also revealed that their shape was comparable to that of starch granules occurring in WT leaves.[11] Confocal microscopy analysis of the starch granule marker GBSS–GFP in *GBSS–GFP*-expressing *aps1* and *adg1–1* leaves further confirmed the presence of one or two small starch granules in the chloroplasts of *adg1–1* and *aps1* plants.[11]

In addition to SEM and GFP confocal fluorescence microscopic localization methods, we developed a sensitive method for confocal fluorescence microscopy detection of starch that allowed to visualize starch granules within the chloroplasts of *aps1* and *adg1–1* iodine stained leaves.[11] In principle, this method is based on the classical iodine staining protocol for macroscopic detection of glycogen and starch.[6,12] Leaves from 4-weeks old plants cultured in pots at ambient $CO_2$ (350 ppm) at 20°C under a 16 h light (90 μmol photons $sec^{-1}$ $m^{-2}$) / 8 h dark regime were fixed by immersion for 24 h at 37°C into 3.7% formaldehyde and 0.1 M phosphate buffer (pH 6.5). The fixative solution was then washed out with 0.1 M phosphate buffer (pH 6.5) for 24 h at 37°C. Dehydration and decoloration of samples was performed by transferring the samples to 50% (v/v) ethanol for 24 h, and 96% (v/v) ethanol for 2 x 24 h, all steps being conducted at 37°C (at this stage samples can be stored for months at 4°C). Samples were then rehydrated in 50% (v/v) ethanol for 30–60 min, transferred to distilled water for 20–30 min, and stained in iodine solution (2% KI (w/v) and 1% $I_2$ (w/v)) for 60 min in the darkness and at room temperature. Samples were then rinsed gently in distilled water for about 1 min, mounted on microscopic slides, and examined using a D-Eclipse C1 confocal microscope (NIKON, Japan) with Ar 488 nm excitation. Starch granule-specific green fluorescence emission was detected using BA515/30 filter (detector gain setting 7.2), whereas red autofluorescence emission was detected using BA650LP filter (detector gain setting 7.2) (as a reference, GFP fluorescence in *GBSS-GFP* expressing plants[11] was detected with gain settings ranging between 5 and 6.5). No green fluorescence emission could be detected in samples that were not stained with iodine solution (not shown). Pictures were processed by EZ-C1 software (Gold Ver. 3.40). Cropping and final arrangement of images were made in Adobe Photoshop CS3, Ver. 10.0.1.

Using this method, we could observe large, green fluorescence emitting oval/round structures that were negative for red

autofluorescence, and identified as starch granules in bright-field images of WT plants (**Fig. 1**). Consistent with the presence of reduced starch content in *aps1* leaves, the green fluorescence emitted by iodine stained starch granules in chloroplasts of mesophyll cells of *aps1* leaves was much smaller than that of WT leaves (**Fig. 1**). We also analyzed mesophyll cells of the *ss3/ss4* mutant impaired in SSIII and SSIV isoforms that are suggested to be absolutely required for starch granule initiation.[1] Although Szydlowski et al.[1] reported that this double mutant displays a starch-less phenotype, a more recent work has provided evidence that *ss3/ss4* leaves accumulate as much as 10% of the WT starch content.[13] Consistently, analyses performed using the method for confocal fluorescence microscopic observation of starch granules confirmed the occurrence of starch granules in mesophyll cells of the *ss3/ss4* mutant (**Fig. 1**).

GBSS is involved in the synthesis of amylose, and impairments in this activity result in changes of amylopectin structure.[14] Whether the method for confocal fluorescence microscopic observation of starch granules is suitable to visualize starch granules with reduced amylose content was investigated by using a T-DNA *gbss Arabidopsis* mutant impaired in GBSS activity (GABI_914G01). As shown in **Figure 1** these analyses revealed that starch granules from *gbss* leaves emitted green fluorescence.

In many heterotrophic organs, sucrose synthase (SuSy) activity acts as a major determinant of sink strength that highly controls the conversion of incoming sucrose into starch. SuSy has also been suggested to be involved, at least in part, in the sucrose-starch conversion process in autotrophic organs.[15,16] Earlier studies[17,18] have shown that different organs of the *sus1/sus2/sus3/sus4* quadruple Arabidopsis mutant accumulate WT starch content. We employed the method for confocal fluorescence microscopic observation described in this work to visualize the starch granules in the chloroplasts of *sus1/sus2/sus3/sus4* mutant. As shown in **Figure 1**, these analyses revealed the occurrence of large starch granules in the *sus1/sus2/sus3/sus4* mesophyll cells, which is consistent with the view that *sus1/sus2/sus3/sus4* leaves accumulate nearly WT starch content.

The method for confocal fluorescence microscopic observation of iodine stained branched homopolysaccharides of α-1,4- and α-1,6-linked glucose molecules was valid not only for starch granules occurring in plant cells, but also for glycogen granules occurring in bacteria. *E. coli* cells cultured in M9 minimal medium (4 mM NaCl, 9 mM $NH_4Cl$, 0.1 mM $CaCl_2$, 2 mM $MgSO_4$, 48 mM $Na_2HPO_4$ and 22 mM $KH_2PO_4$) supplemented with glucose were harvested at the end of the exponential growth,[19] fixed by immersion for 1 h in 3.7% formaldehyde and 0.1 M phosphate buffer (pH 6.5), and centrifuged at 6000 rpm for 5 min. The pellet thus obtained was washed with 0.1 M phosphate buffer and stained in iodine solution (2% KI (w/v) and 1% $I_2$ (w/v)) for 10 min. Stained cells were then transferred to microscopic slides and examined as described above using a D-Eclipse C1 confocal microscope (NIKON, Japan) with Ar 488 nm excitation and green emission detected using BA515/30 filter. As shown in **Figure 2**, these analyses revealed the presence in the poles of the cells of fluorescence emitting iodine-stained glycogen, which is consistent with previous electron microscopy studies on topographic distribution of glycogen granules in *E. coli*.[19] In clear contrast, glycogen-less Δ*glgBXCAP* mutants lacking the whole glycogen biosynthetic machinery[20] did not exhibit any green fluorescence.

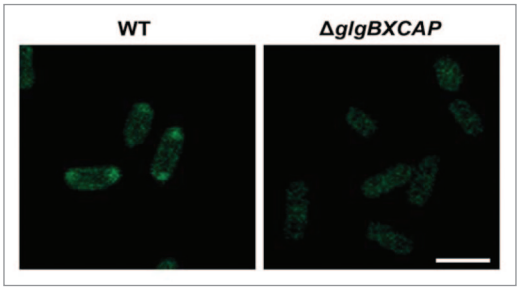


**Figure 2.** Confocal fluorescence microscopic observation of iodine stained glycogen granules in *E. coli*. *E. coli* cells were cultured in M9 minimal medium supplemented with glucose, harvested at the end of the exponential growth and stained as described in the main text. Note the presence of green fluorescence dots in the poles of WT cells, but not in the glycogen-less Δ*glgBXCAP* cells lacking the whole glycogen biosynthetic machinery. Bar = 2.5 μm

The method for confocal fluorescence microscopic observation of iodine stained starch and glycogen granules was valid not only for structures occurring inside the cell, but also for pure, isolated polymers. Potato starch, amylose and amylopectin were stained with iodine solution (see above), rinsed gently in distilled water for about 1 min, mounted on microscopic slides, and examined using a D-Eclipse C1 confocal microscope with Ar 488 nm excitation. As shown in **Figure 3**, in all cases green fluorescence emission was detected using BA515/30 filter (detector gain setting 7.2–7.5). No green fluorescence emission could be detected in samples that were not stained with iodine solution (not shown).

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.


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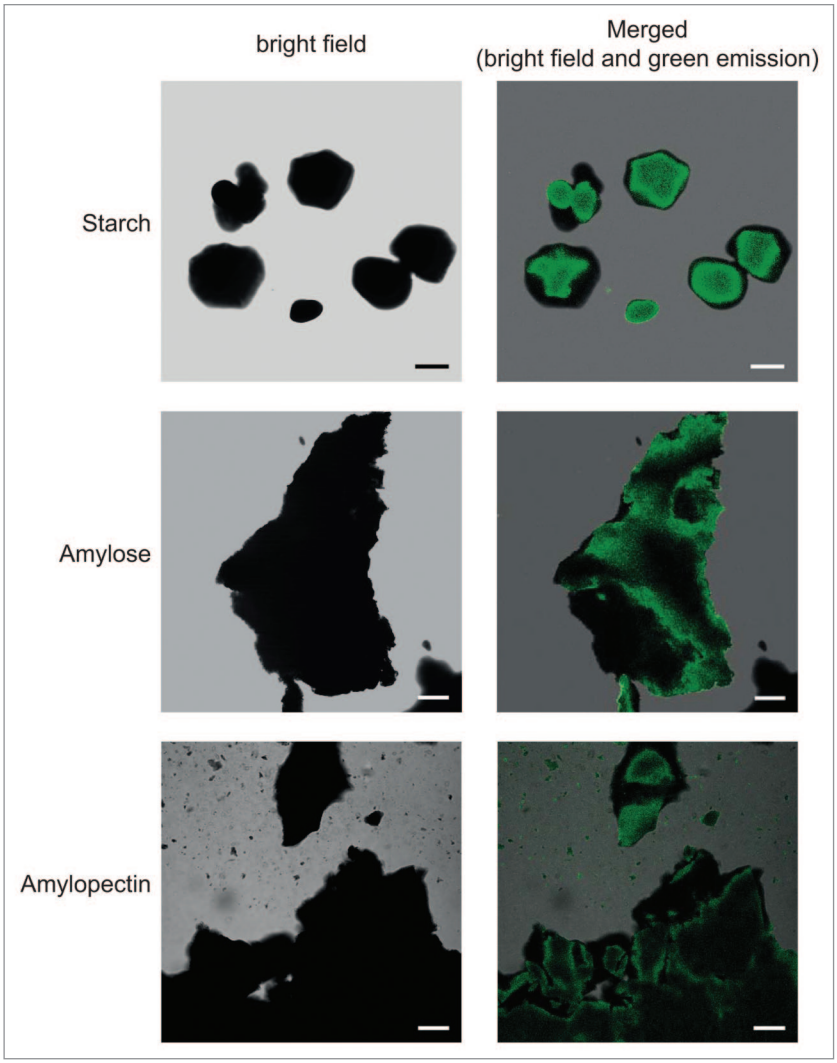


**Figure 3.** Confocal fluorescence microscopic observation of iodine stained pure potato starch, amylopectin and amylose. Pure, commercially available potato starch (Roche), amylose (Sigma A00512) and amylopectin (Sigma A8515) were stained with iodine solution, rinsed gently in distilled water for about 1 min, mounted on microscopic slides, and examined using a D-Eclipse C1 confocal microscope with Ar 488 nm excitation using BA515/30 filter (detector gain setting 7.2–7.5). Bar = 10 μm for starch granules and 20 μm for amylose and amylopectin.


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