

Starch Division and Partitioning. A Mechanism for Granule Propagation and Maintenance in the Picophytoplanktonic Green Alga *Ostreococcus tauri*^{1[w]}

Jean-Philippe Ral, Evelyne Derelle, Conchita Ferraz, Fabrice Wattebled, Benoit Farinas, Florence Corellou, Alain Buléon, Marie-Christine Slomianny, David Delvalle, Christophe d'Hulst, Stéphane Rombauts, Hervé Moreau, and Steven Ball*

Laboratoire de Chimie Biologique Unité Mixte de Recherche 8576 du Centre National de la Recherche Scientifique, Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq cedex, France (J.-P.R., F.W., M.-C.S., D.D., C.D., S.B.); Observatoire océanologique, laboratoire Arago, Unité Mixte de Recherche 7628 Centre National de la Recherche Scientifique-Université Paris VI, BP 44, 66651 Banyuls-sur-mer cedex, France (E.D., B.F., F.C., H.M.); Institut de Génétique Humaine, Unité Propre de Recherche Centre National de la Recherche Scientifique 1142, 34396 Montpellier cedex 5, France (C.F.); Institut National de la Recherche Agronomique, Centre de Recherches Agroalimentaires, BP71627, 44316 Nantes cedex 03, France (A.B.); and Department of Plant Systems Biology, Ghent University, VIB, B-9052 Gent, Belgium (S.R.)

Whereas Glc is stored in small-sized hydrosoluble glycogen particles in archaea, eubacteria, fungi, and animal cells, photosynthetic eukaryotes have resorted to building starch, which is composed of several distinct polysaccharide fractions packed into a highly organized semicrystalline granule. In plants, both the initiation of polysaccharide synthesis and the nucleation mechanism leading to formation of new starch granules are currently not understood. *Ostreococcus tauri*, a unicellular green alga of the Prasinophyceae family, defines the tiniest eukaryote with one of the smallest genomes. We show that it accumulates a single starch granule at the chloroplast center by using the same pathway as higher plants. At the time of plastid division, we observe elongation of the starch and division into two daughter structures that are partitioned in each newly formed chloroplast. These observations suggest that in this system the information required to initiate crystalline polysaccharide growth of a new granule is contained within the preexisting polysaccharide structure and the design of the plastid division machinery.

Starch and glycogen define the most widespread form of Glc storage in living cells and consist of α -1,4 linked glucan chains with α -1,6 branches (Buléon et al., 1998; Ball and Morell, 2003). Hydrosoluble glycogen particles cannot grow greater than 40 nm in diameter because the structure becomes too crowded with Glc at the periphery of the particle to accommodate the presence of enzymes of glycogen synthesis or degradation (Meléndez et al., 1998). This limitation is due to both the higher branching level of the polymer and to its rather uniform branching pattern. There presently seems to be no other limit to the size of a starch granule than that afforded by the availability of substrate for continuing synthesis. Amylopectin, the major polysaccharide of starch, aggregates into insoluble semicrystalline material because of its asymmetrical

distribution of α -1,6 linkages generating clusters of branches responsible for formation of arrays of parallel double helical structures (Buléon et al., 1998). The appearance of starch coincides with the acquisition of photosynthesis by the eukaryotic cell. No such polymer can presently be found in bacteria, archaea, fungi, or animal cells. Although starch is clearly associated with the acquisition of photoautotrophy by eukaryotes, glycogen seems to be the predominant form of Glc storage within cyanobacteria and purple nonsulfur bacteria (for review, see Preiss and Romeo, 1989). In addition, nonphotosynthetic eukaryotes accumulating amylopectin-like polymers such as apicomplexa parasite heterotrophic dinoflagellates or others were always subsequently inferred to be derived from a photosynthetic eukaryote ancestor (McFadden et al., 1996). Starch has been found to accumulate in plastids in green algae and land plants, while red algae, glaucophytes, dinoflagellates, apicomplexa parasites, and cryptophytes accumulate an extraplastidial form of so-called floridean starch (Viola et al., 2001). Green plants and algae are known to use the bacterial ADP-Glc-based pathway of storage polysaccharide synthesis (Recondo and Leloir, 1961; Greenberg and Preiss, 1964). Although chloroplasts and cyanobacteria

¹ This work was supported by the French Ministry of Education, by the Centre National de la Recherche Scientifique, and by the Génomole Languedoc-Roussillon.

* Corresponding author; e-mail steven.ball@univ-lille1.fr; fax 33-3-20436555.

[w]The online version of this article contains Web-only data.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.104.044131.

share a common ancestor, the latter appear to synthesize glycogen, while starch as such may have appeared after endosymbiosis of the chloroplast. Plants and Chlorophycean algae such as *Chlamydomonas reinhardtii* have acquired multiple enzyme forms for each step of the metabolic pathway.

Ostreococcus tauri is a picophytoplanktonic species that belongs to the Prasinophyceae, a group of green algae thought to have diverged very early from the ancestor of all chloroplast-containing green plants and algae. *Ostreococcus*, with a 0.8- μm diameter, presently defines the tiniest eukaryotic cell and the smallest currently described genome for a photosynthetic eukaryotic organism (Chrétiennot-Dinet et al., 1995; Courties et al., 1998). A DNA-sequencing effort has recently yielded the full genome of this organism (H. Moreau and Y. Van de Peer, unpublished data). Because of its relative simplicity and because of its particular position with respect to the evolution of green plants, we have undertaken a detailed study of storage polysaccharide synthesis. We report that despite the genome simplification that took place in picophytoplanktonic species, a unique starch granule is synthesized at the center of the tiny plastid by using a pathway of similar complexity as that of higher plants or *Chlamydomonas*. We observe, however, that this granule elongates and divides into two daughter structures at the time of plastid division. We propose that localized synthesis and degradation regulate starch granule partitioning and maintenance in this species. The implications for the priming of starch granules in higher plants and algae are discussed.

RESULTS

Structural Characterization of *O. tauri* Storage Polysaccharide

The granule present at the center of each unique plastid was purified and shown to be starch by the following criteria. It contained an amyloglucosidase-sensitive polysaccharide that was shown by NMR to be composed of approximately 96% and 4% α -1,4 and α -1,6 glucosidic linkages, respectively. The polysaccharide dispersed in 10 mM NaOH could be fractionated by gel permeation chromatography (Fig. 1) on a sepharose CL2B column. A major polysaccharide fraction with an iodine interaction identical to that of plant amylopectin was always witnessed. A minor fraction whose relative importance depended on the growing conditions was purified with an average mass distribution and iodine interaction identical to those of cereal amyloses. Proton NMR performed on the amylopectin-like and amylose-like fractions yielded 6% and less than 1% α -1,6 glucosidic linkages, respectively, and thereby confirmed the identity of both fractions (see supplemental material, available at www.plantphysiol.org). The chain-length (CL) distribution of the *O. tauri* amylopectin fraction was compared to that of rabbit liver glycogen and of

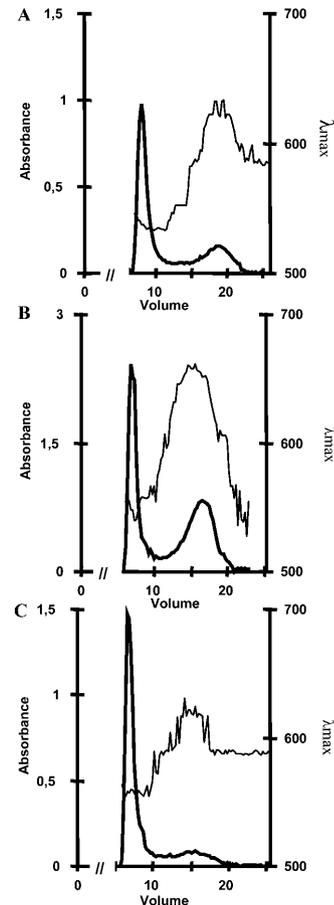


Figure 1. Separation of amylose and amylopectin CL2B gel permeation chromatography of starch from *O. tauri* (1 mg) dispersed in 10 mM NaOH (A) in comparison with *C. reinhardtii* (2 mg; B) and Arabidopsis (1 mg; C). The λ_{max} (wavelength of the maximal absorbance of the iodine-polysaccharide complex in nanometers) is scaled on the right axis. The absorbency of the complex at λ_{max} measured for each fraction is indicated on the left axis. The low λ_{max} fraction excluded from the column defines amylopectin, while the high λ_{max} amylose is separated throughout the column. The x axis shows the elution volume in milliliters.

Arabidopsis and *Chlamydomonas* amylopectin fractions (Fig. 2). The CL distribution of the material purified from *Ostreococcus* was typically trimodal and similar to those of the plant standards and differed noticeably from the unimodal CL distribution of the animal glycogens. The semicrystalline nature of the native starch granule was further checked by x-ray diffraction. Plant starches contain parallel double helical glucan chains that display either the A or B type of crystalline packing. The polysaccharide granules from *Ostreococcus* were shown to be predominantly of the B type, which further establishes this material as starch.

O. tauri Synthesizes Starch through the ADP-Glc Pathway

To identify the biochemical pathway of starch biosynthesis, we concentrated our efforts on two key

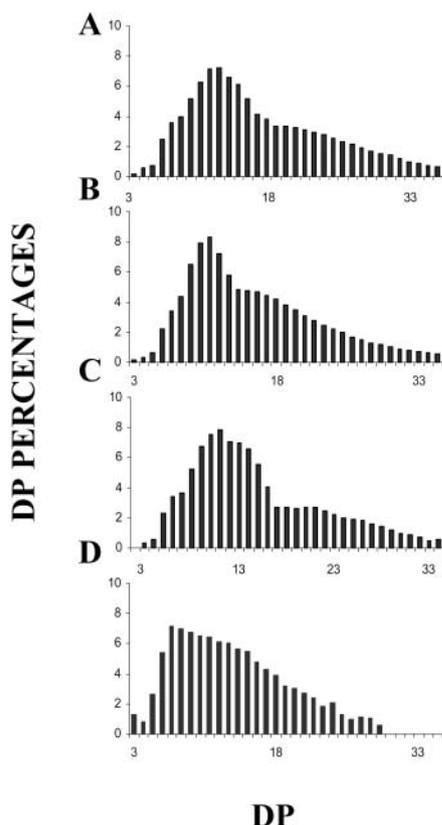


Figure 2. Polysaccharide CL distributions. Histograms of CL distributions obtained after isoamylase-mediated enzymatic debranching through capillary electrophoresis of 8-amino-1,3,6-pyrenetrisulfonic acid-labeled fluorescent glucans. The x axis displays degree of polymerization scales (DP 3–35) and the y axis represents the relative frequency of chains expressed as percentages. A, CL distribution of purified amylopectin from *O. tauri*. B and C, CL distribution of purified amylopectin from *Arabidopsis* (B) and *C. reinhardtii* (C) wild-type references. D, Bovine liver glycogen CL distribution.

activities. Bacteria and plants both synthesize their storage polysaccharides through the use of ADP-Glc (Recondo and Leloir, 1961; Greenberg and Preiss, 1964). ADP-Glc pyrophosphorylase defines an enzyme devoted to glycogen and starch biosynthesis (Ballicora et al., 2003). It has not been found in organisms such as

yeast (*Saccharomyces cerevisiae*) or humans, which use the UDP-Glc pathway of storage polysaccharide synthesis. We found an ADP-Glc pyrophosphorylase activity in *O. tauri* crude extracts through the use of two distinct enzyme assays (Fig. 3). The enzyme was activated most efficiently by 3-phosphoglycerate (PGA) and responded to inhibition by orthophosphate (see supplemental material) in a fashion similar to that described for land plants and cyanobacteria (Ballicora et al., 2003). To further confirm that *Ostreococcus* was using the ADP-Glc pathway, we turned our attention to granule-bound starch synthase (GBSSI), the most abundant enzyme bound to the starch granule in plants (Delrue et al., 1992). This enzyme has been shown to be selectively responsible for amylose synthesis. The major protein bound to starch was analyzed by matrix-assisted laser-desorption ionization-mass spectrometry (MALDI-MS) following tryptic digestion and shown to be identical to an expressed *Ostreococcus* genome sequence bearing strong resemblance to the plant GBSSI class of enzymes (Fig. 4A). As was demonstrated in *Chlamydomonas*, we were able to synthesize a significant amount of amylose in vitro by labeled ADP-Glc (Fig. 4B). In addition, the *Ostreococcus* GBSSI was shown to prefer markedly ADP-Glc (K_m , 1.3 mM), while the enzyme could not be saturated at 10 mM with UDP-Glc in a fashion similar to that which was originally shown in maize (*Zea mays*; Fig. 4C). Taken together, these results suggest strongly that prasinophytes synthesize starch through the ADP-Glc pathway.

Probing the *Ostreococcus* Genome for Enzymes of Starch Metabolism

We probed the full *Ostreococcus* genome sequence for genes of storage polysaccharide metabolism (see supplemental material for *Ostreococcus* accession numbers list). Each sequence was checked for expression by reverse transcription (RT)-PCR and was found to be expressed and to yield the expected RT-PCR fragment sizes. The results are displayed in Table I, and an example of the phylogenetic tree is given in Figure 5 for the starch synthases. The *Ostreococcus*

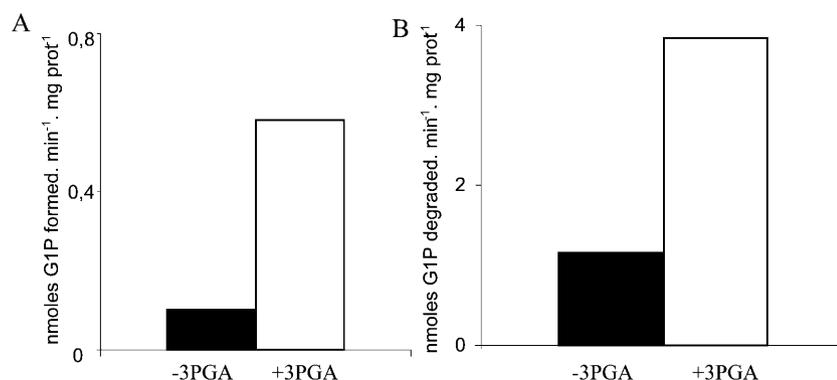


Figure 3. Histograms of ADP-Glc pyrophosphorylase activity. A displays the assay in the non-physiological direction of pyrophosphorylation in the presence (white box) or absence (black box) of 1 mM 3PGA. Units are expressed in nanomoles of Glc-1-P formed per minute and per milligram of protein. B represents the activity of ADP-Glc pyrophosphorylase in the direction of ADP-Glc synthesis in the presence or absence of 1 mM activator. Units are expressed in nanomoles of Glc-1-P degraded per minute and per milligram of protein.

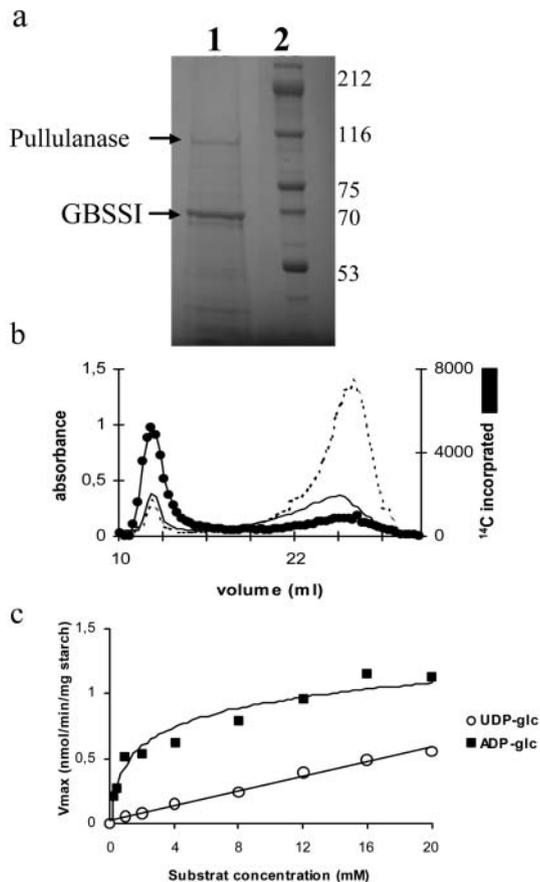


Figure 4. Analysis of GBSSI activity and protein from *O. tauri* starch granules. A, Coomassie Brilliant Blue R-250 stained 7.5% SDS-acrylamide gels of starch-bound proteins. Lane 1 represents starch-bound proteins extracted from 2 mg of polysaccharides purified after nitrogen starvation. Lane 2 displays the molecular-mass marker with the corresponding masses expressed in kilodaltons. B, Kinetics of in vitro synthesis of amylose. Starch from *O. tauri* was subjected to in vitro synthesis in the presence of ^{14}C -labeled ADP-Glc. After 16 h (black line) and 40 h (dotted line) of in vitro synthesis, the amylopectin and amylose were separated by CL2B-sepharose chromatography. C, ADP-Glc kinetics of the GBSSI from *O. tauri*. A total of 100 μg of polysaccharides were incubated with different concentrations of ADP-Glc (■) or UDP-Glc (○).

small-genome size had initially raised our hopes that this organism may have streamlined its metabolic pathways and reduced the number of enzyme forms required at each step of the pathway, thereby facilitating functional studies dealing with starch metabolism. Table I demonstrates that this is not the case and that *Ostreococcus* displays the same level of complexity as that of vascular plants with respect to starch biosynthesis and degradation. It is evident from the tree displayed in Figure 5 that the ancestor of Chlorophyceae, Prasinophyceae, and plants contained a minimum of four sequence families of starch synthases and that these have been conserved throughout evolution. The same observation holds for most other enzymes of starch metabolism despite the obvious general simplification of the *Ostreococcus* genome.

It is worth noting that the *Ostreococcus*, unlike *Arabidopsis* and other plants, do not appear to contain sequences related to glycogenins of the yeast or mammalian type. Glycogenin, a protein capable of autoglucosylation from UDP-Glc, was proven to be involved in the priming of yeast glycogen synthesis (Cheng et al., 1995).

Starch Granules Elongate and Divide during the *Ostreococcus* Cell Cycle

We proceeded to examine cytologically the pattern of starch synthesis and degradation by using transmission electron microscopy (TEM) of cells harvested at various stages of their diurnal rhythm of cell division and starch metabolism. We were surprised to see by TEM that cells were apparently engaged in a process of elongation, division, and partitioning of their unique starch granule into two daughter structures at the time of plastid division at the end of the light phase (Fig. 6, A–D). To check that the granule morphology was indeed modified and not only the subject of constriction through the plastid division machinery, starch granules purified from synchronized cultures engaged in plastid division and were subjected to scanning electron microscopy (SEM). The purified granules still displayed these division figures (Fig. 6, E and F), demonstrating that the starch itself was subjected to these modifications. The TEM observations show that in *O. tauri*, the number of starch granules is restricted to one and suggest that most if not all granules derive from a parental granule by a localized process of elongation and degradation. Interestingly, attempts to cure *O. tauri* of starch through prolonged incubation in darkness failed as viability was lost before the granules were entirely degraded. In an effort to understand the basis of this mechanism, we subjected minor proteins associated to starch to trypsin digestion followed by MALDI-MS. We were thus able to find pullulanase, the most abundant of the minor (non-GBSS) proteins associated to starch. Pullulanase has not been reported to date as associated to starch granules, and its presence may reflect a particular function of this enzyme in the partitioning process.

DISCUSSION

Prasinophytes Display the Full Complexity of Higher Plant Starch Metabolism

The results reported in this paper suggest that starch metabolism has appeared at the earliest stage in the green lineage with the full complexity that characterizes storage polysaccharide metabolism in vascular plants. This complexity seems to be a requisite to build starch through the ADP-Glc pathway. Indeed, it has not been subjected to any simplification to accommodate small-genome size despite the obvious reduction

Table 1. Storage polysaccharide metabolism in plants and bacteria

The protein-deduced from four different species was generated using Bioedit (Hall, 1999) and Forcon (Van de Peer et al., 1998). The species included were *E. coli*, *O. tauri*, Arabidopsis, and *O. sativa*. Amino acid sequence of *O. tauri* genome provided from the *Ostreococcus* blast research Web site (<http://194.167.19.196/>). *Ostreococcus* accession numbers for all reported sequences are given as supplemental material. Expression of all *Ostreococcus* sequences involved in starch metabolism was checked by RT-PCR and double checked by analysis of the Expression Sequence Tag Database. Amino acid sequences from other organisms were provided from the Arabidopsis Information Resource Web site (www.arabidopsis.org) and the RiceBlastDB Web site (<http://www.tigr.org/tdb/e2k1/osa1/osa1.shtml>); GenBank accession numbers are given as supplemental material). The following abbreviations were used: AGPase, ADP-Glc pyrophosphorylase; GS, glycogen synthase; SS, soluble starch synthase; GBE and SBE, respectively, glycogen and starch branching enzyme; GPHO and MPHO, glycogen and maltodextrin phosphorylase.

Genes in <i>E. coli</i> Genome	Genes in <i>O. taurii</i> Genome	Genes in Arabidopsis Genome	Genes in <i>O. sativa</i> Genome
AGPase	AGPase small subunit AGPase large subunit A and B	AGPase small subunit AGPase large subunit 1, 2, 3, and 4	AGPase small subunit 1 and 2 AGPase large subunit 1 and 2
α -Amylase A and B	α -Amylase A, B, and C	α -Amylase 1, 2, and 3	α -Amylase 1 to 9
0	β -Amylase A and B	β -Amylase 1 to 8	β -Amylase 1 to 7
GBE	SBE I, II-1, and II-2	SBE, 2-1, and 2-2	SBE 1 to 4
4- α -Glucanotransferase	4- α -Glucanotransferase 1 and 2	4- α -Glucanotransferase 1 and 2	4- α -Glucanotransferase 1 and 2
Glycogen debranching enzyme	Isoamylase A and B	Isoamylase 1, 2, and 3	Isoamylase 1, 2, and 3
GPHO and MPHO	Pullulanase	Pullulanase	Pullulanase
0	PHO A, B, and C	PHO1 and PHO2	PHO1 and PHO2
GS	R1 protein A and B	R1 protein	R1 protein
	GBSS I	GBSS I	GBSS I
	SS I, II, IIIa, IIIb, and IIIC	SS I, II, III, IV, and V	SSI, Iia, I Ib, III, III-2, IVa, and IVb
0	0	Glycogenin 1 to 7	Glycogenin 1 to 4

of complexity that has occurred in *Ostreococcus* concerning a variety of important processes such as those exemplified by cell cycle control (Khadaroo et al., 2004). It is, however, not obvious that this multiplicity of enzyme forms is required to build crystalline polysaccharides, as apicomplexan parasites are able to synthesize starch from UDP-Glc through the use of one enzyme form for each biochemical step defined in this pathway (Coppin et al., 2004).

Prasinophytes Lack Glycogenin-Like Proteins

Glycogenins were found as proteins associated to glycogen particles that are able to glucosylate themselves through the transfer of Glc from UDP-Glc to specific Tyr residues present on the protein. Several Glc residues are then elongated to form a protein-bound oligosaccharide. They have been suspected to be involved in the priming of glycogen synthesis in yeast and mammalian cells by supplying the primer for glycogen synthesis in these systems (for review, see Alonso et al., 1995). That this glycogenin function is indeed required for glycogen accumulation was demonstrated by selectively disrupting both glycogenin genes present in the yeast genome (Cheng et al., 1995). The yeast double mutants lost their ability to store glycogen. In plants, several proteins capable of auto-glucosylation were reported. However, in most instances, these were proven to be quite different with respect to the nature both of the sugars transferred and of glycosyl-linkage formed (Singh et al., 1995;

Langeveld et al., 2002). The extraplasmidial localization of such proteins also argues that they may not be involved in the priming of starch synthesis. Despite this, other genes encoding glycogenin proteins much more similar to the fungal-mammalian type of protein can be detected through analysis of the Arabidopsis and rice (*Oryza sativa*) genomes. Some of these contain predicted transit peptide sequences and may be involved in both polysaccharide and granule priming. We were unable to find, using standard bioinformatic tools, *Ostreococcus* genes that are related to the yeast or Arabidopsis glycogenin or glycogenin-like sequences. It remains possible, however, that proteins with similar functions but with divergent primary structures have escaped detection.

The Apparent Elongation and Division of *Ostreococcus* Starch Granules

The most surprising aspect revealed by *Ostreococcus* is no doubt defined by the apparent elongation and division of the unique starch granule at the center of the plastid. Elongation could be explained by a modification of the geometry of the photosynthetic membranes prior to plastid division. In fact, in this system, starch is centrally located with respect to the algal thylakoids and could result from the appearance of novel thylakoids supplying additional substrate during the cycle of plastid division. Division of the starch could be explained by a localized starch degradation process targeted at the plastid constriction

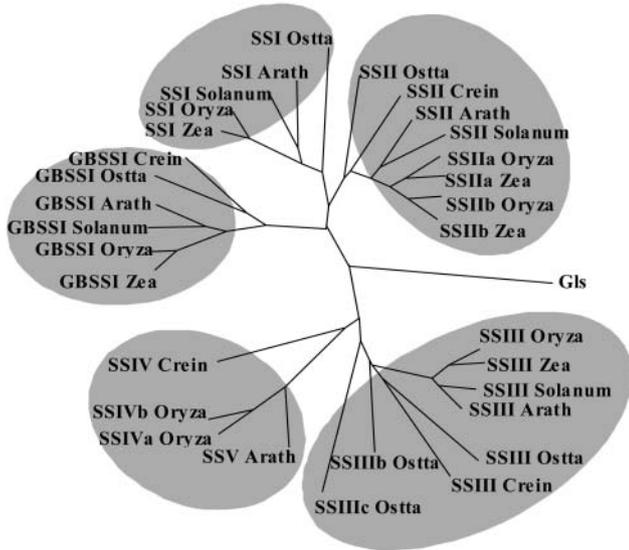


Figure 5. Dendrogram of starch synthases in plants and algae and glycogen synthases from *Synechocystis*. Sequences were aligned with ClustalW (Thompson et al., 1994). The sequence alignment was manually improved using BioEdit (Hall, 1999) and ForCon (Van de Peer et al., 1998). TreeCon (Van de Peer and De Wachter, 1997a, 1997b) was used for constructing the neighbor-joining (Saitou and Nei, 1987) tree based on Poisson-corrected distances, only taking into account unambiguously aligned positions (600 amino acids). Bootstrap analysis with 500 replicates was performed to test the significance of the nodes. The amino acid sequences used were as follows: *C. reinhardtii* (Crein) GBSSI (AF26420), SSII (AAC17970), SSIII, SSIV (TO7926); maize (Zea) GBSSI (M24258), SSI (AF036891), SSIIa (Harn et al., 1998), SSIIb (Harn et al., 1998); rice (Oryza) GBSSI (X62134), SSI (D16202), SSIIa (AF419099), SSIIb (Harn et al., 1998), SSIII (Gao et al., 1998), SSIVa (AY100470), SSIVb (AY100471); *Solanum tuberosum* (Solanum) GBSSI (X58453), SSI (Y10416), SSII (X87988), SSIII (X94400); Arabidopsis (Arath) GBSSI (AC006424), SSI (AF121673), SSII (AC008261), SSIII (AC007296), SSV (A021713), and *Synechocystis* glycogen-synthase Gls (NP441947). Accession numbers for *Ostreococcus* sequences are given as supplemental material.

site. The net result would be the creation of two starch granules of more or less equal size ready for another round of synthesis elongation and division. Such a behavior would render granule and possibly polysaccharide priming unnecessary. However if starch is completely degraded, one has to envision the existence of a mechanism regenerating novel granules. Interestingly, we have never been able to have *Ostreococcus* degrade its starch to completion through prolonged incubation in darkness. Along similar lines and at variance with results reported for Arabidopsis (Critchley et al., 2001), we have never been able to get conditions where *Chlamydomonas* cells degrade its starch completely. Interestingly, both of these systems seem to lack glycogenin.

We do not know if our results in *Ostreococcus* relate to the propagation of starch granules in vascular plants. In addition, we do not know if glycogenin-like sequences have any relevance to the mechanisms of granule seeding and polysaccharide priming in higher

plants. It is indeed evident that the volume occupied by starch in the tiny *Ostreococcus* chloroplast could present a serious physical problem and challenge to the plastid division machinery in this alga only. This may have prompted the evolution of a sophisticated polysaccharide partitioning machinery and could offer an explanation for the absence of starch in prokaryotes in general and cyanobacteria in particular. Alternatively, it remains possible that localized starch degradation may be at work in other species to provide the seeds of new starch granules. That starch catabolic enzymes are involved in controlling the number of seeds for novel granules in dividing plant cells remains an interesting possibility.

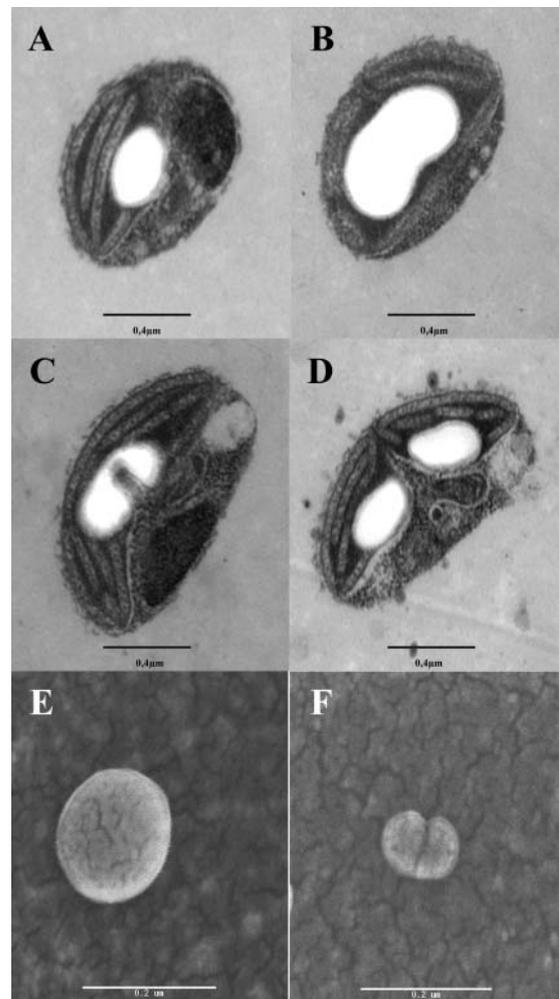


Figure 6. Micrographs of dividing starch granule in *O. tauri*. A to D, TEM of dividing *O. tauri* cells. Poorly synchronized cultures contain a mixture of cells at different stages of plastid division (bar = 0.4 μm). E and F, Field emission type SEM images of dividing starch granule purified from synchronized cultures. Starch granules isolated from G1 cells (E) and G2/M-phase (F). Cultures were synchronized by 12-h-day/12-h-night growth cycles. G1 granules always appear spherical with a somewhat smoother surface, while G2/M starch granules are small often compounded with a rough surface (bar = 0.2 μm).

MATERIALS AND METHODS

Strains and Growth Conditions

The *Ostreococcus tauri* strain OTTHO595 (Chrétiennot-Dinet et al., 1995) was grown in K medium prepared on the base of 36% seawater and sterilized by 0.2 μm filtration (Keller and Selvin, 1987). The nitrogen-limited medium defines K medium where NH_4Cl was substituted by equivalent concentration of NaCl. Experiments were performed under 12-h day/12-h night growth cycles or continuous light (100 $\mu\text{E m}^{-2}\text{s}^{-1}$). The wild-type *Arabidopsis* Wassilewskija reference strain was grown in the greenhouse (Zeeman et al., 1998). Media and culture conditions for growing the wild-type *Chlamydomonas* 137C strain were previously detailed (Delrue et al., 1992).

Starch Purification

Pure native starch from *O. tauri* was prepared from nitrogen-limited culture and harvested after 4 d of growth under continuous light. Cells were centrifuged (10,000g for 20 min) with 0.2% Pluronic. The pellet was resuspended in 300 μL of 10 mM Tris acetate, pH 7.5, 1 mM EDTA. Algal suspensions were disrupted by sonication. A crude starch extract was obtained by spinning down the lysate at 10,000g for 15 min. The pellet obtained from 1-L cultures was resuspended in 1 mL of 90% Percoll. The gradient was self-formed by centrifugation at 10,000g for 30 min. The starch pellet was collected and resuspended in 1 mL of 90% Percoll. After a 30-min spin at 10,000g, the purified starch pellet was rinsed in sterile distilled water, centrifuged at 10,000g, and kept dry at 4°C. Starch yields through this purification procedure were greater than 80%. Starch amounts were measured by the amyloglucosidase assay (Delrue et al., 1992). Starch granule purification from *Chlamydomonas reinhardtii* and *Arabidopsis* were previously described (Ball et al., 1991; Zeeman et al., 1998).

Separation of Starch Polysaccharide by Gel Permeation Chromatography

A total of 1 to 2.5 mg of starch dissolved in 500 μL of 10 mM NaOH was applied to a Sepharose CL2B column (0.5 cm [i.d.] \times 65 cm) equilibrated in 10 mM NaOH. Fractions of 250 to 300 μL were collected at a rate of 1 fraction/1.5 min. Glucans in each fraction were detected through the iodine-polysaccharide interaction.

CL Distribution Analysis

A total of 500 μg of dialyzed and lyophilized amylopectin purified after gel permeation chromatography were suspended in 55 mM sodium acetate, pH 3.5, and debranched by 10 units of *Pseudomonas amyloclavata* Isoamylase (Hayashibara Biochemical Laboratory, Okayama, Japan) at 45°C during 4 h. The reaction was stopped by boiling 10 min. After neutralization with 10 M NaOH, the samples were lyophilized and analyzed by fluorophore-assisted carbohydrate electrophoresis using the procedure previously described (Morell et al., 1998).

Enzyme Assays

GBSSI was assayed as described previously (Delrue et al., 1992). In vitro synthesis of amylose was performed by using the method detailed by Van de Wal et al. (1998).

ADP-Glc pyrophosphorylase was assayed both in the direction of pyrophosphorolysis and in the direction of ADP-Glc synthesis by using the protocols set up for *C. reinhardtii* (Ball et al., 1991). Activation by 3-PGA was measured at a concentration of 1 mM activator.

K_m Determination of GBSSI

The GBSSI was measured from freshly purified starch granules after 15 min of incubation at 30°C in the presence of 50 mM Gly, pH 9.0, 100 mM $(\text{NH}_4)_2\text{SO}_4$, 0.4% β -mercaptoethanol, 5 mM MgCl_2 , and 0.05% bovine serum albumin with various concentrations of ADP- or UDP-Glc containing 62 nCi of glycosyl-nucleotide at 10.5 GBq/mmol (for both UDP-Glc and ADP-Glc). K_m constants were measured through the Hanes and Woolf procedure from a series of two datasets.

In Vitro Synthesis of Amylose

A total of 500 μg of starch was incubated with 3.2 mM ADP-Glc in the presence of 22 mM Tris-HCl, pH 8.0, 0.47% β -mercaptoethanol, 5.5 mM MgCl_2 , and 2.2 μM ADP[U- ^{14}C]Glc at 10.5 GBq/mmol in a total volume of 2 mL at 30°C for 16 and 40 h. The reaction was terminated by adding 3 volumes of 96% ethanol. The ethanol-rinsed pellet was resuspended in 1 mL of 90% dimethyl sulfoxide and dispersed through boiling. The dispersed polysaccharide was precipitated by 3 volumes of 96% ethanol and stored overnight at -20°C .

After centrifugation at 15,000g for 20 min, the polysaccharide was resuspended in 500 μL of 10 mM NaOH and subjected to gel permeation on a Sepharose CL2B column (Sigma-Aldrich, Steinheim, Germany) as described above.

X-Ray Diffraction, NMR, SDS-PAGE, and Matrix-Assisted Laser-Desorption Ionization Time of Flight Analysis of GBSSI

Powder x-ray diffractograms were collected from Percoll purified starches as described previously (Buléon et al., 1997). Proton NMR spectra of dispersed polysaccharides were produced according to the protocol developed for *C. reinhardtii* (Fontaine et al., 1993). To analyze granule-bound proteins, purified starches (2 mg) were boiled for 10 min in 80 μL of 2% SDS and 5% β -mercaptoethanol and centrifuged for 20 min at 10,000g. Supernatants were loaded onto 7.5% SDS-PAGE and stained with Coomassie Brilliant Blue R250. Isolated proteins were extracted from the gel and bleached using acetonitrile/100 mM ammonium bicarbonate (v/v) at 30°C. After acetonitrile dehydration, dried proteins were subjected to trypsin digestion in 50 mM ammonium bicarbonate buffer for 8 h. Peptides were eluted twice by a 45% acetonitrile/10% formic acid bath at 30°C and a 95% acetonitrile/5% formic acid at 30°C. Samples were analyzed by matrix-assisted laser-desorption ionization time of flight.

TEM and SEM

O. tauri cells were collected by centrifugation and fixed in paraformaldehyde/glutaraldehyde/PIPES buffer, then postfixed in osmium tetroxide in PIPES buffer and dehydrated and embedded in Epon (Soyer, 1977). Sections were stained with uranyl acetate and lead citrate and examined in a Hitachi (Tokyo) H-600 transmission electron microscope. *O. tauri* starch granules were concentrated during centrifugation on a plastic Thermanox (Naperville, IL) coverslip. Coverslips were then mounted on a stub and coated with gold. Starch granules were viewed with a field emission scanning electron microscope Hitachi S-4500.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY570699 to AY570722.

ACKNOWLEDGMENTS

We thank Frédéric Chirat and Emmanuel Maes for their excellent technical assistance.

Received April 5, 2004; returned for revision May 11, 2004; accepted May 11, 2004.

LITERATURE CITED

- Alonso MD, Lomako J, Lomako WM, Whelan WJ (1995) A new look at the biogenesis of glycogen. *FASEB J* 12: 1126–1137
- Ball S, Marianne T, Dirick L, Fresnoy M, Delrue B, Decq AA (1991) *Chlamydomonas reinhardtii* low-starch mutant is defective for 3-phosphoglycerate activation and orthophosphate inhibition of ADP-glucose pyrophosphorylase. *Planta* 185: 17–26
- Ball SG, Morell MK (2003) From bacterial glycogen to starch: understand-

- ing the biogenesis of the plant starch granule. *Annu Rev Plant Biol* **54**: 207–233
- Ballicora MA, Iglesias AA, Preiss J** (2003) ADP-glucose pyrophosphorylase, a regulatory enzyme for bacterial glycogen synthesis. *Microbiol Mol Biol Rev* **67**: 213–225
- Buléon A, Colonna P, Planchot V, Ball S** (1998) Starch granules: structure and biosynthesis. *Int J Biol Macromol* **23**: 85–112
- Buléon A, Gallant D-J, Bouchet B, Mouille G, D'Hulst C, Kossman J, Ball SG** (1997) Starches from A to C. *Chlamydomonas reinhardtii* as a model microbial system to investigate the biosynthesis of the plant amylopectin crystal. *Plant Physiol* **115**: 949–957
- Cheng C, Mu J, Farkas I, Huang D, Goebel MG, Roach PJ** (1995) Requirement of the self-glucosylating initiator proteins Glg1p and Glg2p for glycogen accumulation in *Saccharomyces cerevisiae*. *Mol Cell Biol* **12**: 6632–6640
- Chrétiennot-Dinet MJ, Courties C, Vaquer A, Neveux J, Claustre H, Lautier J, Machado MC** (1995) A new marine picoeucaryote: *Ostreococcus tauri* gen. Et sp. Nov. (Chlorophyta, Prasinophyceae). *Phycologia* **34**: 285–292
- Coppin A, Varré J-S, Liénard L, Dauvillée D, Guérardel Y, Soyer-Gobillard M-O, Buléon A, Ball S, Tomavo S** (2004) Evolution of plant-like crystalline storage polysaccharide in the protozoan parasite *Toxoplasma gondii* argues for red alga ancestry. *J Mol Evol* (in press)
- Courties C, Perasso R, Chrétiennot-Dinet MJ, Gouy M, Guillou L, Troussellier M** (1998) Phylogenetic analysis and genome size of *Ostreococcus tauri* (Chlorophyta, Prasinophyceae). *J Phycol* **34**: 844–849
- Critchley JH, Zeeman SC, Takaha T, Smith AM, Smith SM** (2001) A critical role for disproportionating enzyme in starch breakdown is revealed by a knock-out mutation in *Arabidopsis*. *Plant J* **26**: 89–100
- Delrue B, Fontaine T, Routier F, Decq A, Wieruszski J-M, Van den Koornhuysen N, Maddelein ML, Fournet B, Ball S** (1992) Waxy *Chlamydomonas reinhardtii*: monocellular algal mutants defective in amylose biosynthesis and granule-bound starch synthase activity accumulate a structurally modified amylopectin. *J Bacteriol* **174**: 3612–3620
- Fontaine T, D'Hulst C, Maddelein M-L, Routier F, Marianne-Pepin T, Decq A, Wieruszski JM, Delrue B, Van Den Koornhuysen N, Bossu JP, et al** (1993) Toward an understanding of the biogenesis of the starch granule. Evidence that *Chlamydomonas* soluble starch synthase II controls the synthesis of intermediate size glucans of amylopectin. *J Biol Chem* **268**: 16223–16230
- Gao M, Wanat J, Stinard PS, James MG, Myers AM** (1998) Characterization of dull1, a maize gene coding for a novel starch synthase. *Plant Cell* **10**: 399–412
- Greenberg E, Preiss J** (1964) The occurrence of adenosine diphosphate glucose:glycogen transglucosylase in bacteria. *J Biol Chem* **239**: 4314–4315
- Hall TA** (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41**: 95–98
- Harn C, Knight M, Ramakrishnan A, Guan H, Keeling PL, Wasserman BP** (1998) Isolation and characterization of the zSSIIa and zSSIIb starch synthase cDNA clones from maize endosperm. *Plant Mol Biol* **37**: 639–649
- Keller MD, Selvin RC** (1987) Media for the culture of oceanic ultra-phytoplankton. *J Phycol* **23**: 633–638
- Khadaroo B, Robbins S, Ferraz C, Derelle E, Eychenie S, Cooke R, Peaucellier G, Delseny M, Demaille J, Van De Peer Y, et al** (2004) The first green lineage cdc25 dual-specificity phosphatase. *Cell Cycle* **3**: 513–518
- Langeveld SMJ, Vennik M, Kottenhagen M, van Wijk R, Buijk A, Kijne JW, de Pater S** (2002) Glucosylation activity and complex formation of two classes of reversibly glycosylated polypeptides. *Plant Physiol* **129**: 278–289
- McFadden GI, Reith ME, Munholland J, Lang-Unnasch N** (1996) Plastid in human parasites. *Nature* **381**: 482
- Meléndez R, Meléndez-Hevia E, Mas F, Mach J, Cascante M** (1998) Physical constraints in the synthesis of glycogen that influence its structural homogeneity: a two-dimensional approach. *Biophys J* **75**: 106–114
- Morell MK, Samuel MS, O'Shea MG** (1998) Analysis of starch structure using fluorophore-assisted carbohydrate electrophoresis. *Electrophoresis* **19**: 2603–2611
- Preiss J, Romeo T** (1989) Physiology, biochemistry and genetics of bacterial glycogen synthesis. *Adv Microb Physiol* **30**: 183–238
- Recondo E, Leloir L** (1961) Adenosine diphosphate glucose and starch biosynthesis. *Biochem Biophys Res Commun* **6**: 85–88
- Saitou N, Nei M** (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**: 406–425
- Singh DG, Lomako J, Lomako WM, Whelan WJ, Meyer HE, Serwe M, Metzger JW** (1995) Beta-Glucosylarginine: a new glucose-protein bond in a self-glucosylating protein from sweet corn. *FEBS Lett* **376**: 61–64
- Soyer MO** (1977) Une modification de la technique de Karnosky pour la préservation optimale des structures nucléaires chez les dinoflagellés. *Biol Cell* **30**: 297–300
- Thompson JD, Higgins DG, Gibson TJ** (1994) ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**: 4673–4680
- Van de Peer Y, Caers A, De Rijk P, De Wachter R** (1998) Database on the structure of small ribosomal subunit RNA. *Nucleic Acids Res* **26**: 179–182
- Van de Peer Y, De Wachter R** (1997a) Construction of evolutionary distance trees with TREECON for Windows: accounting for variation in nucleotide substitution rate among sites. *Comput Appl Biosci* **13**: 227–230
- Van de Peer Y, De Wachter R** (1997b) Evolutionary relationships among the eukaryotic crown taxa taking into account site-to-site rate variation in 18S rRNA. *J Mol Evol* **45**: 619–630
- Van de Wal M, D'Hulst C, Vincken J-P, Buléon A, Visser R, Ball S** (1998) Amylose is synthesized *in vitro* by extension of and cleavage from amylopectin. *J Biol Chem* **273**: 22232–22240
- Viola R, Nyvall P, Pedersen M** (2001) The unique features of starch metabolism in red algae. *Proc R Soc Lond B Biol Sci* **268**: 1417–1422
- Zeeman S, Northrop F, Smith AM, ap Rees T** (1998) A starch-accumulating mutant of *Arabidopsis thaliana* deficient in a chloroplastic starch hydrolyzing enzyme. *Plant J* **15**: 357–365