Storage, Photosynthesis, and Growth: The Conditional Nature of Mutations Affecting Starch Synthesis and Structure in *Chlamydomonas*

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Growth-arrested *Chlamydomonas* cells accumulate a storage polysaccharide that bears strong structural and functional resemblance to higher plant storage starch. It is synthesized by similar enzymes and responds in an identical fashion to the presence of mutations affecting these activities. We found that log-phase photosynthetically active algae accumulate granular α(1→4)-linked, α(1→6)-branched glucans whose shape, cellular location, and structure differ markedly from those of storage starch. That synthesis of these two types of polysaccharides is controlled by both a common and a specific set of genes was evidenced by the identification of a new *Chlamydomonas* (STA4) locus specifically involved in the biosynthesis of storage starch. Mutants defective in STA4 accumulated a new type of high-amylose storage starch displaying an altered amylopectin chain size distribution. It is expected that the dual nature and functions of starch synthesis in unicellular green algae will yield new insights into the biological reasons for the emergence of starch in the eukaryotic plant cell.

**INTRODUCTION**

Starch accumulates as a complex granular structure made of α glucans in the leaf cell chloroplast (transient starch) and in the amyloplast of the plant storage tissue cell (storage starch) (for review, see Preiss, 1991). To date, all published structural characterizations of starches deal with storage starch, and very little remains to be known about the fine structure and composition of leaf (transient) starch. That both kinds of polysaccharides are synthesized in plastids by similar enzymes from ADP-glucose is evident from biochemical experiments performed mainly with spinach leaves (Ghosh and Preiss, 1966; Ozbun et al., 1972) and from the genetic and biochemical work more recently performed with Arabidopsis (Caspar et al., 1985; Lin et al., 1988), *Nicotiana sylvestris* (Hanson et al., 1988), and potato (Müller-Röber et al., 1992). The storage polysaccharide is usually defined as a mix of two distinct fractions: amylopectin and amylose. Amylopectin is by far the major component. It is composed of intermediate-sized α(1→4)-linked glucans that are clustered together by α(1→6) linkages (for review, see Manners, 1989). Segments of these chains intertwine to form parallel arrays of double helices responsible for the crystallinity of starch. These arrays are separated one from another by longer spacer glucans. The exact conservation in the case of storage starch of the amylopectin cluster size (9 nm) throughout the plant kingdom suggests the existence of a highly ordered, precise, and well-conserved biosynthetic pathway (Jenkins et al., 1993). Amylose is often referred to as a smaller linear molecule with very few α(1→6) branches, whose association with amylopectin inside the granule remains to be determined.

In growth-arrested *Chlamydomonas* cells, we have been able to show that starch adopts, by many criteria, a structure reminiscent of maize endosperm storage starch (Delrue et al., 1992; Fontaine et al., 1993; Maddelein et al., 1994). Nitrogen starvation in particular has enabled us to screen very effectively for mutants affected in starch structure or amounts. Under these particular physiological conditions, the destruction of chlorophylls and massive accumulation of starch allowed us to visualize the pure iodine polysaccharide interaction directly on colonies. Moreover, the large amounts of starch synthesized in these cells made routine structural characterizations of starches feasible for a single-cell organism. This genetic approach, coupled with carbohydrate biochemistry, has enabled us to assign specific functions to granule-bound and soluble starch synthases in the building of different size classes of glucans of the amylopectin clusters (Maddelein et al., 1994).
Here, we turn our attention to starch synthesis and structure in actively photosynthesizing and dividing algal cells. We show not only that the amounts, shape, and cellular location of starch are changed but also that the polysaccharide is dramatically impoverished or devoid of a distinct amylose fraction, despite the presence of massive granule-bound starch synthase (GBSS) activities. We further show that the structure of amylopectin is modified, that the balance of starch synthases is affected, and that at least one specific additional gene (STA4) is needed for normal storage starch synthesis, which is not required for building starch during growth. This new locus is involved in building normal storage amylopectin clusters. We speculate that different structural needs for starch function in photosynthesis and storage may help to explain the emergence of starch as a distinct entity of the photosynthetic eukaryotic plant cell.

RESULTS

Log-Phase Wild-Type Chlamydomonas Starch Is Devoid of Amylose

The starch content of Chlamydomonas under unrestricted growth conditions ranges from 0.5 to 4 µg per 10^6 cells. This can be compared with the 30 to 80 µg of starch accumulated under growth arrest (in nitrogen-, phosphate-, or sulfur-starved media). Most but not all of the starch typically surrounds the pyrenoid (Figure 1) and adopts a morphology dictated by the shape of this ribulose-1,5-biphosphate carboxylase/oxygenase-containing cell structure. In nutrient-starved cells, starch accumulates mostly in the stroma during 48 hr. This accumulation correlates with a scavenging mechanism that yields a nonphotosynthetic cell with disorganized thylakoid membranes (Bulte and Wollman, 1992). Moreover, pyrenoid-like structures, when visible, often lack their typical surrounding starch sheath. The disappearance or rearrangements of both the pyrenoid and chloroplast membranes coincide with the appearance of massive lipid droplets. Our preliminary lipid composition studies pointed to the presence of galactolipids, suggesting that these bodies originate from preexisting thylakoid membranes. These growth-arrested cells are virtually filled with starch whose shape and cell distribution are altered. The composition and structure of the polysaccharide from the growth-arrested cells were compared with those of the actively growing algae. Figure 2 clearly shows that log-phase photosynthesizing algae build starches with either a drastic decrease in or disappearance of amylose. The amylopectin also seems to be modified, as shown by a 20-nm increase (from 550 ± 5 nm to 570 ± 5 nm) in the λ_max of the iodine–polysaccharide complex of the Sepharose CL2B chromatograms (Figure 2).

Similar results were obtained using TSK HW-75(S) columns in 10% DMSO. Different researchers have named intermediate material a series of starch fractions of variable structures.
and sizes whose branching levels are intermediate between those of amylose and amylopectin. In *Chlamydomonas*, we have named amylopectin type II such an intermediate but homogeneous high molecular mass fraction that contains >3% branches (Delrue et al., 1992). Interestingly, in this case, although starch-storing cells never fail to separate the previously characterized type I and II amylopectins, the latter was very poorly separated from type I polysaccharide in log-phase *Chlamydomonas*. This suggests either the disappearance of type II material or, more likely, a significant increase in molecular mass of this fraction. We designated storage starch as the polysaccharide that accumulated during nitrogen starvation. The polysaccharide synthesized by light-grown log-phase (unstarved) *Chlamydomonas* cells was called photosynthetic (rather than transient) starch. Figure 2 also shows that *Chlamydomonas* storage starch has a composition and a $\lambda_{\text{max}}$ of the purified amylopectin and amylose fractions that are very similar to those of maize endosperm starch. This analogy extends further to the distribution of chain length sizes that typifies *Chlamydomonas* storage starch.

**GBSS Is Present and Active under Unrestricted Growth but Remains Unable To Synthesize Amylose**

The absence of the low molecular mass amylose fraction in storage starch has been associated with defects in GBSS activity in *Chlamydomonas* and in many higher plant systems (Nelson and Rines, 1962; Visser et al., 1991; Delrue et al., 1992). To ascertain whether the enzyme responsible for amylose synthesis was present, we assayed both GBSS and proteins extracted from the starch granule. Results shown in Figure 3 indicate that the absence or decrease of amylose in the nitrogen-supplied cultures was not due to a decrease in GBSS activity and protein. However, the in vivo contribution of GBSS was still visible, but solely in the amylopectin fraction, confirming the involvement of GBSS in amylopectin biosynthesis (Maddelein et al., 1994; see later discussion).

**Conditional and Unconditional Expression of Mutants Defective for Starch Biosynthesis**

Three genes (*STA1*, *STA2*, and *STA3*) to date have been shown to control starch amounts and/or structure in *Chlamydomonas*. Strains carrying a *sta1-1* defect harbor reduced ADP-glucose pyrophosphorylase activity through desensitization of the enzyme to 3-phosphoglycerate activation (Ball et al., 1991). We confirmed that the severe low-starch phenotype of this mutant was unconditional and expressed itself in both storage and photosynthetic starches in a similar if not identical fashion (Ball et al., 1991). *STA2* is the GBSS structural gene (Delrue et al., 1992). We have shown that this enzyme is not only involved in amylose synthesis, as is generally believed, but is also responsible for synthesis of long chains in amylopectin (Maddelein et al., 1994). The starch chromatogram of a *sta2* disruption under unrestricted growth also lacked the amylose fraction. However, the amylopectin displayed a 25-nm drop in the $\lambda_{\text{max}}$ of the iodine–polysaccharide complex (Table 1). This correlates with a significant decrease in the amount of long chains we detected after debranching the purified amylopectin. These observations add additional support to the involvement of GBSS in the synthesis of the long glucans of amylopectin, as was previously demonstrated (Maddelein et al., 1994).

*sta3*-carrying mutants are defective for the major soluble starch synthase (SSS) activity (Fontaine et al., 1993). They displayed a very significant reduction (60%) in storage starch accumulation and a specific decrease in amylopectin of those chains whose size ranged from 8 to 40 glucose residues in
Figure 3. GBSS Activities and Proteins during Storage or Growth.

(A) Histogram of GBSS-specific activity of wild-type strain 137C expressed in nanomoles of ADP-glucose incorporated into glucan per milligram of starch per minute. Means are given for three separate measurements.

(B) A Coomassie Brilliant Blue R 250-stained 5 to 7.5% SDS-acrylamide gel of starch-bound proteins. Lane 2 contains the starch-bound protein fraction from the nitrogen-starved wild-type 137C strain; lane 3 contains starch-bound proteins extracted from the same strain grown in undepleted medium. Lane 4 contains proteins extracted from undepleted cultures of BAFR1, a strain carrying a gene disruption (sta2-29::arg7) for the GBSS gene. The molecular size standards in lane 1 are given in kilodaltons. Proteins simultaneously extracted from equal amounts of polysaccharide (1 mg) were loaded on the gel. The major 76-kD band corresponds to the GBSS protein and has the typical GBSS N-terminal sequence (Delrue et al., 1992).

+N, nitrogen supplemented; −N, nitrogen starved.

length (Fontaine et al., 1993; Maddelein et al., 1994). These SSS-defective strains displayed their structural deficiencies on both storage and photosynthetic starches (Table 1). The double mutants defective for both GBSS and SSSI (sta2 and sta3) displayed similar behavior. They are characterized in both cases (storage and photosynthetic starch) by the presence of very small amounts of a polysaccharide whose structure is intermediate between those of amylpectin and glycogen. Yet, in log-phase cells, the deficiency in starch amounts of all sta3 sta2 double mutants was much less pronounced relative to the wild-type or mutant strains (Table 1). Also, although in all wild-type or single mutant genotypes there was a fourfold (for SSSI-defective strains) to 20-fold increase (for wild-type or GBSS-defective strains) in starch during storage, the strains containing SSSI only (sta3 sta2 double mutants) were unable to trigger the increase in polysaccharide synthesis that normally takes place under these conditions. Storage starch synthesis thus requires either SSSI, GBSS, or both. These observations could be explained if one assumes a change in the ratio between the two types of soluble starch synthases activities during storage. Although we were able to detect some modifications in both our purification (by anion exchange) and zymogram (on native PAGE) assays, these changes in balance, when detectable, never exceeded a twofold relative decrease in SSSI. The SSSI/SSSI activity ratio measured in the presence of glycogen decreased from 0.6 to 0.3 when the cells switched to storage. However, the total SSS activity also decreased in nitrogen-deprived medium from 40 (during growth) to 9 (during storage) nmol of Glc incorporated into starch per hour per 10⁶ cells. It remains to be shown how these small shifts detected in soluble extracts affect the precise balance of starch synthase activities at the very surface of the granule where synthesis is occurring.

STA4, a Novel Chlamydomonas Locus Necessary for Storage Amylopectin Synthesis, Is Not Required for Starch Synthesis during Growth

To determine whether modifications in structure localization and amounts could be solely explained either by physiological modifications of carbon fluxes or by the selective action of specific genes, we undertook a systematic screen for mutants expressing a clear-cut conditional phenotype. The latter consisted of strong alterations in both structure and amounts of storage starch that would not affect polysaccharide synthesis during growth. Of a total of 2 × 10⁴ cells surviving after x-ray treatment, we found seven mutants altered for storage starch synthesis. Six of these carried mutations that mapped to the previously characterized STA2 and STA3 genes. As mentioned previously, they expressed their phenotypes on starch structure under all growth conditions. Strain I73 exhibited a severe high-amylose phenotype that is in fact due to a defect in amylpectin content and structure (see later discussion).
Table 1. Starch Amounts, Composition, and \( \lambda_{\text{max}} \) Values of the Iodine–Amylopectin Complex during Growth or Storage for Various Combinations of Mutations Decreasing Soluble and Granule-Bound Starch Synthases from *Chlamydomonas*

<table>
<thead>
<tr>
<th>Growth</th>
<th>Genotype</th>
<th>Ap (%)</th>
<th>Am (%)</th>
<th>( \lambda_{\text{max}} ) of Ap (nm)</th>
<th>Starch Amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td>+N</td>
<td>(+ +)</td>
<td>&gt;95</td>
<td>&lt;5</td>
<td>570</td>
<td>3.7</td>
</tr>
<tr>
<td>-N</td>
<td>(+ +)</td>
<td>65 to 85</td>
<td>15 to 35</td>
<td>555 (Ap I)</td>
<td>31</td>
</tr>
<tr>
<td>+N</td>
<td>(sta2::Δt +)</td>
<td>&gt;95</td>
<td>&lt;5</td>
<td>540</td>
<td>3.5</td>
</tr>
<tr>
<td>-N</td>
<td>(sta2::Δt +)</td>
<td>&gt;95</td>
<td>&lt;5</td>
<td>540</td>
<td>29</td>
</tr>
<tr>
<td>+N</td>
<td>(+ sta3-7)</td>
<td>&gt;90</td>
<td>5 to 10</td>
<td>590</td>
<td>2.5</td>
</tr>
<tr>
<td>-N</td>
<td>(+ sta3-7)</td>
<td>40 to 60</td>
<td>60 to 40</td>
<td>590</td>
<td>14</td>
</tr>
<tr>
<td>+N</td>
<td>(sta2::Δt sta3-7)</td>
<td>&gt;95</td>
<td>&lt;5</td>
<td>515</td>
<td>0.8</td>
</tr>
<tr>
<td>-N</td>
<td>(sta2::Δt sta3-7)</td>
<td>&gt;95</td>
<td>&lt;5</td>
<td>515</td>
<td>1.5</td>
</tr>
</tbody>
</table>

To estimate starch amounts, three mutant meiotic segregants for each genotype class were selected from crosses involving parents carrying *sta3-7* and *sta2::Al*. *sta2::Al* is an abbreviation for *sta2-29::ARG7*, a strain whose *STA2* locus has been disrupted by a functional *ARG7* gene. Means of starch accumulation were calculated for each class using the amylglucosidase assay for three distinct meiotic products. The amount of starch is expressed as micrograms of starch per \( 10^6 \) cells. Starch composition is given in weight percentages as assayed by the amyloglucosidase assay. Levels of amylose below 5% cannot be detected on starch chromatograms. Am, amylose; Ap, amylopectin; + N, during growth; - N, during storage.

The starch chromatograms displayed in Figure 4 show that the composition and \( \lambda_{\text{max}} \) values of the fractions are altered with respect to the wild type. However, these defects could not be scored during growth, during which the starch chromatogram and \( \lambda_{\text{max}} \) values of the fractions were identical to those displayed in Figure 2C. In addition, a significant decrease (60%) in starch amounts was scored only during storage.

Characterization of the *STA4* Defects

The *sta4-1* defect segregated as a single Mendelian defect through meiosis. Typically, it is incompletely dominant on amylose content and to a lesser extent on the \( \lambda_{\text{max}} \) values of the amylopectin fraction. Genetic analysis clearly demonstrated that this locus segregates independent from both the *STA2* and *STA3* defects. As with the previously characterized SSS-defective (*sta3*), high-amylose strains, we found significant decreases in storage starch amounts in the meiotic mutant progeny. By many other criteria, *sta4-1*–carrying strains behaved like *sta3* mutants. They were dramatically modified in the chain length distribution (Figure 5) of the purified amylopectin and showed no change in that of amylose. As was the case for the *sta3* defect, we found no decrease in the branching for the mutant amylopectin (which amounted precisely to 5%, as estimated by both methylation analysis and proton nuclear magnetic resonance [NMR]; Figure 6D). However, in this case the distribution of small glucans could be distinguished from both the wild-type and SSS-defective mutants and did not show as clearly the maximal frequency of chains containing six glucose residues (degree of polymerization 6) that was seen in the latter (Figure 6). The combination of heterozygous defects in both genes added up in diploids to a point at which the starch chromatograms and the \( \lambda_{\text{max}} \) values of the purified amylopectin

![Figure 4](https://example.com)  
**Figure 4.** Separation of Wild-Type and Mutant Amylopectin and Amylose by TSK HW-75(S) Chromatography.

The optical density (●) of the iodine–polysaccharide complex was measured for each 3-mL fraction at \( \lambda_{\text{max}} \), where \( \lambda_{\text{max}} \) is displayed as an unbroken thin line. All samples were loaded on the same column setup as described previously (Delrue et al., 1992). NO, number.  
(A) Wild-type haploid 137C starch purified from nitrogen-starved cultures.  
(B) Starch from nitrogen-starved 173 cells carrying the *sta4-1* mutation.
were indistinguishable from those of homozygous sta3 or sta4 diploid mutants (Figures 7B to 7F). They showed a different kind of genetic interaction when combined with the GBSS (sta2) defects (Figure 7A).

In both cases, the double mutant starch is lacking amylose, and the amylopectin becomes more highly branched (6 to 7%, according to proton NMR). However, and most importantly, sta4 sta2 double mutants did not show the spectacular decrease in polysaccharide content that was seen in sta3 sta2 mutants during storage. However, the decrease in starch amounts remained significant, as revealed by the yellow color of the iodine-stained cell patches of strains carrying both mutations (Figure 8). Decreases of 50 and 75% were measured in the single mutant (50% decrease) and the wild-type (75% decrease) strains (Figure 7G). This correlates with the absence of SSS defects that could be scored in sta4 strains. In fact, despite intensive attempts, we were unable to detect a modification in the amount of activity or kinetics either in crude extracts or after both anion exchange chromatography or native PAGE of all enzymes that could be scored and that are known to be involved in starch biosynthesis. We therefore concluded that the STA4 gene encodes an as yet unidentified product that is necessary for normal amylopectin cluster biosynthesis in storage starch only.

**DISCUSSION**

**The Decrease or Absence of Amylose in Starch from Growing Cells**

Wild-type starch is usually defined as a mix of two distinct fractions: amylose and amylopectin. Despite many attempts, we were able to score only trace amounts of a distinct amylose fraction in growing algae. It has been shown previously that GBSS is present in rate-limiting amounts for storage starch synthesis in higher plants (Tsai, 1974; Kuipers et al., 1994). Although this remains true for growth-arrested starch-storing Chlamydomonas, it is clearly not the case for growing algae. On the contrary, the specific activity of GBSS in its natural environment (the starch granule) displays a very spectacular increase. Moreover, under these conditions, GBSS seems to be fully active in vivo. Indeed, the contribution of this activity to the structure of amylopectin can be easily monitored in strains carrying deletions in the GBSS structural gene.

There are two simple explanations for these seemingly contradictory observations. The first is to hypothesize two distinct populations of GBSS encoded by a single locus. One of these would be surface bound on the granule and would have full access to the soluble enzymes necessary to obtain amylopectin. The second would be less accessible and involved in amylose biosynthesis. It is well known that GBSS is characterized by a Michaelis constant for ADP-glucose that is well above those displayed by the SSSs. However, when GBSS is solubilized, this Michaelis constant drops to a level comparable to the soluble enzymes (Macdonald and Preiss, 1985). It is thus reasonable to suppose that starch itself is responsible for these differences and that, according to its position, GBSS can display variable affinities for the substrate. We would thus assume quite simply that the availability of ADP-glucose could be more critical to the synthesis of amylose deeper inside than to that of amylopectin at the very surface of the granule. A second mechanism that could explain our results would involve the presence of a critical balance between elongation and
branching activities at the surface of the granule where synthesis is taking place. Any event that would affect that balance toward branching would impede amylose formation by GBSS. As has been observed for the relative expression of both SSS activities, a modification in enzyme balance is likely to occur when the plant cell switches from growth to storage. Both of these explanations can be put to the test by studying the structure and composition of starch in low ADP-glucose–containing mutants and by setting up cell-free starch-synthesizing systems from purified native granules.

**Different Structure-Function Relationships during Storage and Growth Might Explain the Appearance of Starch in Photosynthetic Eukaryotes**

Amylose synthesis is not the only event that distinguishes storage from recurrent starch synthesis coupled to photosynthesis and cell division. The fine structure of purified amylopectin from the growing algae is also different from that characterizing storage starch. This correlates with a modification in the cellular location of starch synthesis that occurs predominantly around the pyrenoid in low-CO$_2$ growing cultures and that is confined to the stroma during storage.

Storage synthesis in growing cells has also been reported to respond to the availability of CO$_2$ by switching location (Ramazanov et al., 1994). This could reflect changes in the distribution of the active Calvin cycle enzymes or the building of distinct multienzyme complexes. On the other hand, starch in its pyrenoidal form may be involved as an active component of the CO$_2$ concentration mechanism. It is striking that SSSI on its own is unable to deal with storage yet remains perfectly able to yield what appears to be a normal pyrenoidal sheath of polysaccharide. Thus, different biosynthetic enzymes might have evolved to deal with many distinct physiological constraints in plants. This might explain the multiplicity of enzymes catalyzing the same biochemical reaction. It is likely that specific enzyme forms would assume a predominant function under quite different circumstances. The latter include synthesis not only during photosynthesis in high and low CO$_2$ but also storage after growth arrest.

Storage in plants seems to have favored the appearance of a polysaccharide of high glucose-storing capacity with very

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(A) Wild-type I amylopectin.
(B) Amylopectin from the SSSI-deficient strain 1152.
(C) Amylopectin from the high-amylose 173 strain.
(D) An example of a chromatogram for strain 173 harboring the *sta4-1* defect. The degrees of polymerization of some of the chains are given above the chromatogram. Part of the proton NMR spectrum is also displayed, showing the signals used to quantitate the branching of the purified 173 amylopectin.
Figure 7. Genetic Analysis of the sta4-1 Defect.

In (A) to (F) are TSK HW-75(S) (in 10% DMSO) starch chromatograms from nitrogen-starved cells. The optical density (●) of the iodine–polysaccharide complex was measured for each 3-mL fraction at $\lambda_{\text{max}}$, where $\lambda_{\text{max}}$ is displayed as an unbroken thin line. All samples were loaded on the same column setup as described previously (Delrue et al., 1992). 60th heterozygotes ([C] and [O]) contain more amylose and have a slight significant increase (10 nm) in the $\lambda_{\text{max}}$ value of the major amylopectin species. NO, number.

(A) Starch chromatogram from the haploid recombinant strain TB3 (sta4-1 sta2-6) whose phenotype is shown in Figure 8.
(B) Starch chromatogram from a wild-type diploid reference (obtained by crossing 137C with strain 37).
(C) Starch chromatogram of a diploid heterozygous for STA3.
(D) Starch chromatogram of a diploid heterozygous for STA4.
(E) Starch chromatogram of a diploid simultaneously heterozygous for STA3 and STA4.
(F) Starch chromatogram of a homozygous mutant (sta3-1 sta3-1) diploid.
(G) and (H) Amounts of starch accumulated during growth (+N) or storage (−N) for the four genotype classes. The y-axis is expressed in micrograms of starch per $10^6$ cells. Values are means ($n = 3$) from three separate experiments.
low osmotic pressure (lower than that of animal, fungal, or bacterial glycogen). This in turn yields a very tight crystalline product. Sta4 should enable us to screen effectively for high-amylose mutants and thus allow us to determine the nature of the gene product.

Figure 8. Phenotype of Wild-Type and Mutant Strains.

Cell patches from a tetratype tetrad generated from a cross between strains I73 and 37E-8J. The genotype corresponding to each recombinant is shown in parentheses. Cells were incubated for 5 days on solid nitrogen-deprived medium and sprayed twice with iodine vapors.

METHODS

Materials

Glucose 1-phosphate-1,2-14C and D-glucose-1,2-14C–ADP-glucose were purchased from Amersham. ADP-glucose, maize amylopeptin, and Pseudomonas amyloderamosa isoamylase were purchased from Sigma. Glucose 1-phosphate, rabbit muscle glycogen, and rabbit muscle phosphorylase were obtained from Boehringer Mannheim.

Chlamydomonas reinhardtii Strains, Growth Conditions, Cytological Observations, and Media

The wild-type reference Chlamydomonas strain used in this study was 137C (mt2 nit1 nit2). Diploids were selected by complementation on minimal medium after crossing with either strain 37 (mt pab2 ac14), strain B9 (mt pab2 ac14 sta3-1), or 37E-17 (mt pab2 ac14 sta3-1). IR16 and LI1 strains are meiotic segregants of a cross between BAFR1 (mt nlt2 cw15 arg7 sta2-29; ARG7) and strain 37E-17. IR16 contains the sta2-29; ARG7 gene disruption; LI1 contains both sta2-29; ARG7 and stas-1. I73 was obtained from strain 137C by x-ray mutagenesis at 104 R, leading to 4% survival, and defined the sta4-1 defect.

To examine genetic interactions between the STA2 and STA4 loci, tetrads of a cross between I73 and 37E-8J (mt pab2 ac14 sta2-6) were dissected. TB1 (mt nlt1 and/or nlt2), TB2 (mt sta4-1 pab2 ac14 nlt1 and/or nlt2), TB3 (mt sta4-1 sta2-6 ac14 nlt1 and/or nlt2), and TB4 (mt sta2-6 pab2) are derived from the same tetratype tetrad.

All experiments were performed in continuous light (40 μE m−2 sec−1) in the presence of acetic at 24°C in liquid cultures that were shaken vigorously without air or CO2 bubbling. Late log-phase cultures were inoculated at 105 cells mL−1 and harvested at 2 × 106 cells mL−1. Nitrogen-starved cultures were inoculated at 5.104 cells mL−1 and harvested after 4 days at a final density of 1 to 2 × 106 cells mL−1. Genetic techniques are described by Harris (1989a). Standard Tris-acetate-phosphate (TAP) medium is fully detailed by Harris (1989b); nitrogen-starved medium (TAP-N) and diploid clone selection are described by Ball et al. (1990, 1991) and Delrue et al. (1992). Fixation and embedding protocols are as described by Harris (1989c).

Measures of Starch Levels, Starch Purification, and Spectral Properties of the Iodine-Starch Complex

A full account of amyloglucosidase assays, starch purification on Percoll gradients, and λmax measurements is provided by Delrue et al. (1992).

Crude Extract Preparation, Enzyme Assays, Partial Purification of Enzyme Activities, and Zymograms

Soluble crude extracts were always prepared from late log-phase cells (2 × 106 cells mL−1) grown in high-salts acetate medium under continuous light (80 μE m−2 sec−1). The detailed description of the differential (NH4)2SO4 precipitation of soluble starch synthase I (SSSI) and SSSII, together with the anion exchange purification on DEAE Trisacryl type M (IBF Biotechnics, Villeneuve la Garenne, France) of those enzyme activities, is provided by Fontaine et al. (1993). SSS activity was assayed in a 0.1-mL final volume of 50 mM glycine-NaOH, pH 9, 100 mM (NH4)2SO4, 5 mM β-mercaptoethanol, 5 mM MgCl2, 0.5% Trisacryl type M (IBF Biotechnics, Villeneuve la Garenne, France) of D-gluco-1-phosphate-1,2-14C and D-gluco-1,2-14C–ADP-glucose were purchased from Amersham. ADP-glucose, maize amylopeptin, and Pseudomonas amyloderamosa isoamylase were purchased from Sigma. Glucose 1-phosphate, rabbit muscle glycogen, and rabbit muscle phosphorylase were obtained from Boehringer Mannheim.
mg mL⁻¹ BSA, 10 mg mL⁻¹ rabbit liver glycogen, and 4 mM ADP-glucose containing 1 nmol of d-glucose-U-¹⁴C-ADP-glucose (specific activity 200 μCi μmol⁻¹). After a 15-min incubation at 30°C, the reaction was stopped by adding 2 mL of ice-cold ethanol. Granule-bound starch synthase (GBSS) was assayed as described by Delrue et al. (1992). Branching enzymes were always assayed on the same DEAE chromatograms as used for the SSSs by incubating up to a 40-μL sample in a 0.2-μL final volume of 0.1 M sodium citrate, pH 7.0, 1 mM AMP, 40 μg of rabbit liver phosphorylase containing 50 mM glucose 1-phosphate-4-¹⁴C (final specific activity of 0.22 μCi μmol⁻¹). After a 30-min incubation at 30°C, the reaction was stopped by addition of 10% trichloroacetic acid.

The resulting precipitate was filtered, rinsed, dried, and counted in a liquid scintillation counter (assay A). Amylase, phosphoglucomutase, ADP-glucose pyrophosphorylase, and phosphorylase activities were monitored by using the standard assays described by Ball et al. (1991). For SSSs, the analysis was completed by zymograms as described by Maddelein et al. (1994).

**Starch Fractionation, Methylation, Nuclear Magnetic Resonance, and Debranching Analyses**

Separation of starch fractions on TSK HW-75(S) columns (Merck) was performed as detailed previously (Delrue et al., 1992). In this study, we also used Sepharose CL2B chromatography with 10 mM NaOH as solvent on the same column setups. In this case, the starch sample (10 mg) was first dissolved at 100°C in 90% DMSO and precipitated with four volumes of pure ethanol for 48 hr at room temperature. The precipitate was harvested by spinning at 5000 g for 20 min and redissolved without overdrying in 5 mL of 10 mM NaOH. Methylation of total starch or of pooled fractions dialyzed and freeze-dried after TSK HW-75(S) chromatography was performed according to Paz Parente et al. (1985) and adapted to starch analysis (Delrue et al., 1992). The branching percentage was assayed as the ratio of methyl ether derivatives of α(1→4)-linked glucose either to those of α(1→4)- and α(1→6)-linked glucose or to those of glucose in terminal nonreducing position (Delrue et al., 1992; Fontaine et al., 1993). Nuclear magnetic resonance (NMR) analysis was performed as described by Fontaine et al. (1993). The level of branching was estimated by integration of the same regions of proton resonances of the monosubstituted and disubstituted glucose (δ=5.2 and 4.65 parts per million, respectively; Gidley, 1985). Isoamylase-mediated debranching of fractions purified by gel filtration was achieved as previously described (Maddelein et al., 1994).

**Genetic Analysis of the sta4-1 Defect**

The segregation of the high-amylose sta3 mutations in crosses can be easily followed by simply scoring the color of nitrogen-starved cell patches sprayed with iodine vapors. The sta4-1 mutation, on the other hand, yields a phenotype that systematically requires double checking by recording the full spectrum of the iodine–polysaccharide complex. Briefly, individual patches inoculated as 10-μL drops on solid TAP-N were grown for 4 to 7 days under continuous light. The cell patches were then sprayed twice at 24-hr intervals with iodine vapors. The sta4-1 mutation transformed all starch patches to a typical shoulder above 600 nm and had a λmax value generally (but not always) above 615 nm. The second class had a more regularly shaped spectrum with no shoulder above 600 nm and a λmax value generally below 605 nm. Selected members of the first class (even those with a low λmax value) never failed to display a starch chromatogram on TSK HW-75(S) columns identical to that of strain 173, whereas all members tested of the second class displayed starch chromatograms similar to those of wild-type strains. More than 200 colonies were analyzed using this technique and yielded a clean single-gene inheritance pattern. The same analysis was performed on the meiotic progeny (122 colonies analyzed) of a cross performed between strains 173 and B9 (carrying sta3-1). In this case, 27 wild-type recombinants were scored, and double checked by TSK HW-75(S) chromatography, demonstrating independent segregation of STA3 and STA4. Independent segregation of STA2 and STA4 was demonstrated by similar techniques using both random spores and tetrad analysis after crossing strain 173 with 37E-8J.

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