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Circadian Clock Regulation of Starch Metabolism Establishes GBSSI as a Major Contributor to Amylopectin Synthesis in *Chlamydomonas reinhardtii*^{1[W][OA]}

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Chlamydomonas reinhardtii displays a diurnal rhythm of starch content that peaks in the middle of the night phase if the algae are provided with acetate and CO₂ as a carbon source. We show that this rhythm is controlled by the circadian clock and is tightly correlated to ADP-glucose pyrophosphorylase activity. Persistence of this rhythm depends on the presence of either soluble starch synthase III or granule-bound starch synthase I (GBSSI). We show that both enzymes play a similar function in synthesizing the long glucan fraction that interconnects the amylopectin clusters. We demonstrate that in log phase-oscillating cultures, GBSSI is required to obtain maximal polysaccharide content and fully compensates for the loss of soluble starch synthase III. A point mutation in the GBSSI gene that prevents extension of amylopectin chains, but retains the enzyme's normal ability to extend maltooligosaccharides, abolishes the function of GBSSI both in amylopectin and amylose synthesis and leads to a decrease in starch content in oscillating cultures. We propose that GBSSI has evolved as a major enzyme of amylopectin synthesis and that amylose synthesis comes as a secondary consequence of prolonged synthesis by GBSSI in arrhythmic systems. Maintenance in higher plant leaves of circadian clock control of GBSSI transcription is discussed.

Reports of circadian clock control in plant starch metabolism are presently limited to the observation of growth ring structures in potato (*Solanum tuberosum*) tuber starch granules (Buttrose, 1962). These ring structures are made of a diurnal succession of semicrystalline and amorphous polysaccharide layers, which persist under constant illumination. This issue was re-

cently revisited by Pilling and Smith (2003), who confirmed Buttrose's results, but found that the ring formation depended on interplay between physical and biological factors that include the clock. Indeed, in cereal endosperm granules, similar structures are independent of the clock and seem to be due directly to diurnal variations in light and temperature (Buttrose, 1962). In *Arabidopsis* (*Arabidopsis thaliana*), transcript abundance of 12% of the genes has been shown to be under circadian clock control, and it may well be that, when more sensitive methods are used, a majority of nuclear genes respond to the clock at the mRNA abundance level (Harmer et al., 2000). Not surprisingly, these include genes of the starch synthesis and degradation pathway (Smith et al., 2004). However, circadian oscillations of transcript abundance of only a minority of these genes are expected to yield physiologically relevant responses. A recent report conclusively demonstrated that abundance of the mRNAs coding enzymes of starch degradation (glucan water dikinase [GWD], amyloamylase, and plastidial β -amylase) were under circadian clock control. However, protein levels were shown to remain more or less constant throughout the circadian cycle. Starch metabolism itself might be under tight clock control, but this is only indirectly suggested by the observation of clock control of maltose metabolism

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(Lu et al., 2005). In fact, starch content in plant tissues is not currently thought to be under circadian clock control, and diurnal oscillations are believed to result simply from alternating periods of illumination of the leaf. Most of the results currently published on the Arabidopsis leaf are consistent with this view. However, it must be stressed that other leaf systems, such as maize (*Zea mays*) or sugar beet (*Beta vulgaris*), have been reported to anticipate the arrival of darkness, suggesting the presence of a circadian component of carbohydrate metabolism regulation (Li et al., 1992; Jeannette and Prioul, 1994). On the other hand, oscillations of starch content in roots have been clearly documented, but experiments designed to probe the maintenance of such oscillations under constant illumination were not performed (Henson et al., 1986; for review, see Henson and Duke, 1990). The presence of recurrent rhythms of starch granule degradation in leaves or roots suggests that those components of the starch-synthesizing machinery that operate within the granule will experience recurrent destruction of their physical environment. One such enzyme, granule-bound starch synthase I (GBSSI), the major protein bound to starch, is responsible for amylose synthesis (Recondo and Leloir, 1961; Nelson and Rines, 1962). Amylose can be defined as a poorly, yet significantly, branched polymer of comparatively smaller size (for a review of starch structure, see Buléon et al., 1998; for a review of starch biosynthesis, see Ball and Morell, 2003). This activity transfers Glc from ADP-Glc to the nonreducing end of an α -1,4-linked polymer. Mutants defective for this enzyme were reported in many different plant systems, including unicellular green algae (for review, see Ball et al., 1998). These mutants selectively lose the ability to synthesize amylose with little or no impact on amylopectin synthesis and starch content. A significant, yet modest, impact of the absence of GBSSI on the synthesis of amylopectin was later reported for unicellular green algae (Delrue et al., 1992). It was subsequently found that the function of GBSSI became important for amylopectin synthesis in a particular mutant background defective for some unidentified soluble starch synthase (Maddelein et al., 1994).

We have previously demonstrated through in vitro experiments that, in *Chlamydomonas reinhardtii*, GBSSI synthesizes amylose through extension of amylopectin chains (Van de Wal et al., 1998). However, a minimum of several hours seem to be required to achieve the synthesis of fully grown amylose molecules, even when using very high substrate concentrations (5 mM ADP-Glc). Leaf starch in plants is known to contain little, if any, amylose, despite the presence of high amounts of GBSSI activity and protein. It is therefore possible that leaf GBSSI is destroyed through starch catabolism before it has the time to synthesize amylose. In line with the observation of recurrent degradation of starch granules in leaves, transcription of the granule-bound starch synthase gene has been shown to be under circadian clock control in snapdragon (*Antirrhinum majus*; Merida et al., 1999), Arabidopsis (Tenorio et al.,

2003), as well as sweet potato (*Ipomoea batatas*) leaves (Wang et al., 2001).

In this report we chose to cover two distinct, yet related, issues in *Chlamydomonas*. First, we chose to investigate the patterns of starch synthesis and degradation in cultures subjected to diurnal rhythms of light and darkness. Second, we wished to investigate the impact of polysaccharide degradation rhythms on GBSSI activity and function. GBSSI was chosen because it is the only enzyme solely active within the granule and is therefore bound to be sensitive to the rhythmic disappearance of its physical environment. *C. reinhardtii* is ideally suited for investigations dealing with circadian clock control and starch metabolism. Many useful mutants have been reported in this organism, and the cells can be naturally synchronized in liquid medium by simply applying a 12-h-light/12-h-dark photoperiod (for review, see Ball, 2002).

In this article, we report that starch content is under circadian clock control in the monocellular alga *C. reinhardtii*. We provide evidence that the control of starch synthesis is exerted at the level of ADP-Glc synthesis. We demonstrate that circadian clock control of starch metabolism requires the presence of either soluble starch synthase III (SSIII) or GBSSI. We show that both enzymes play similar roles in the biosynthesis of amylopectin and are required for the synthesis of the long B-chains that interconnect the amylopectin clusters. We further prove that the function of GBSSI on both amylopectin and amylose synthesis requires elongation on an amylopectin primer. We provide evidence for the proteolytic attack of GBSSI during starch catabolism, thereby explaining the requirement for circadian clock regulation of GBSSI mRNA abundance. The importance of circadian clock regulation of starch metabolism in vascular plants is discussed.

RESULTS

Starch Content Is under Circadian Clock Control in the Monocellular Alga *C. reinhardtii*

It has been previously noted that photoautotrophic cultures of *C. reinhardtii* grown either in low or high CO₂ displayed a diurnal rhythm of starch content that did not correlate with lights off and on (Klein, 1987; Thyssen et al., 2001). Because the minimal starch content is reached at the middle of the light phase, we investigated the possibility that starch metabolism as a whole (as reflected by starch content) could be under tight circadian clock control in the monocellular alga *C. reinhardtii*. To be under circadian clock control, a particular rhythm should comply with the following three criteria: persist under constant conditions with a period of about 24 h; be entrained to 24 h by environmental cues (such as day and night shifts or cycles of varying temperatures); and display the same period and rate over a wide range of temperatures, a property known as temperature compensation (for review of

circadian rhythms in *Chlamydomonas*, see Mittag et al., 2005).

Persistence of the rhythm under constant illumination (LL) and constant darkness (DD) were monitored over three consecutive cycles (Fig. 1, B and C). To examine the long-term persistence of this rhythm, we inoculated a culture at low density in the light and monitored starch content 10 d later (Fig. 1D). It is clear that robust circadian oscillations could still be detected

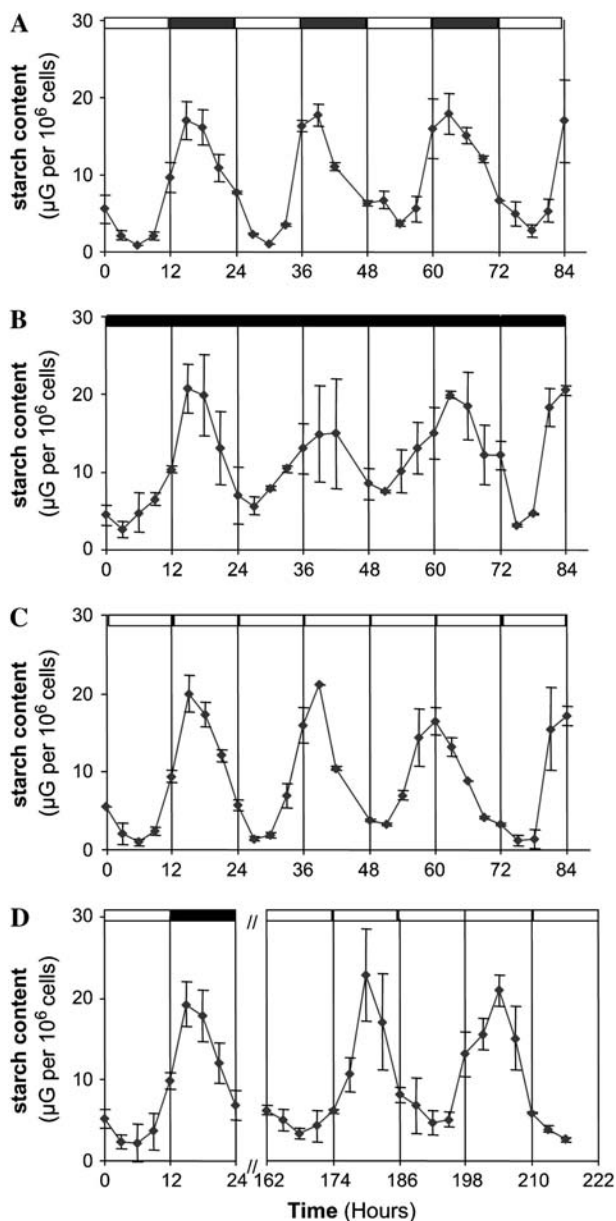


Figure 1. Accumulation of starch during a 12-h-light/12-h-dark cycle. Cells were synchronized for 6 d under a 12-h-light/12-h-dark cycle in the presence of acetate. The culture was then kept under a 12-h-light/12-h-dark cycle (LD; A), a continuous dark phase (DD; B), or grown under a continuous light phase (LL; C). The bar at the top of the graph indicates the corresponding day (white box)/night (black box) periods. The rhythmic accumulation of starch in the *C. reinhardtii* LL culture is demonstrated to remain present after more than a week under continuous light (D).

after 10 d of constant illumination. Entrainment could be evidenced when 4- to 7-d-old cultured (inoculated at 5×10^5) cells subjected to nitrogen starvation in the presence of acetate and constant light were subjected to a switch to darkness. Nitrogen starvation abolishes the presence of rhythms after 24 h (Fig. 2, A and B) when cell divisions are blocked. Despite the fact that, under normal conditions, starch degradation is delayed for at least 4 h after nightfall, the switch to constant darkness experienced by the starved cells triggered immediate degradation despite the presence of acetate (Fig. 2C). Twenty-four hours after the switch to darkness, circadian rhythms of starch content comparable to those displayed in Figure 1C could be maintained in constant darkness if the cultures containing acetate were resupplied with nitrogen. Temperature compensation was observed on DD cultures grown at 15°C and 30°C, thereby establishing that starch content is indeed under circadian clock control in the monocellular alga *C. reinhardtii*.

Starch Synthesis Is Tightly Correlated to ADP-Glc Pyrophosphorylase Activity

To investigate the nature of the major steps responsible for the oscillation of starch content in these cultures, we assayed ADP-Glc pyrophosphorylase. A correlation was observed between this activity and the rate of starch biosynthesis when assaying the enzyme in the synthesis direction (Fig. 3A). This enzyme constitutes the major rate-controlling step of the biosynthetic pathway and, consequently, is subjected to tight regulation (for review, see Ballicora et al., 2003). Recently, a redox regulatory mechanism involving thioredoxin has been shown to activate AGPase in vascular plants (Ballicora et al., 2000; Tiessen et al., 2002; Hendriks et al., 2003). The oxidized inactive form of the enzyme consists of a dimer of small subunits linked through a Cys bridge involving Cys-12. The *Chlamydomonas* enzyme does not contain this residue (Zabawinski et al., 2001). In line with this observation, we found no evidence of enzyme dimerization in western-blot experiments performed under oxidizing conditions (data not shown), and AGPase was not found as a thioredoxin target in large-scale screens aiming at establishing the *Chlamydomonas* reductome (Lemaire et al., 2004).

We have previously reported through northern-blot analysis that an abundance of mRNA encoding the small subunit of ADP-Glc pyrophosphorylase was under circadian clock control. In plants, over 12% of the genes of *Arabidopsis*, including AGPase genes, are known to encode transcripts displaying circadian oscillations (Harmer et al., 2000). However, previous experiments investigating the abundance of several different transcripts in *Chlamydomonas* demonstrated that transcript abundance rhythms were not necessarily followed by similar modifications in protein amount or activities (Lemaire et al., 1999, 2002; Gibon et al., 2004).

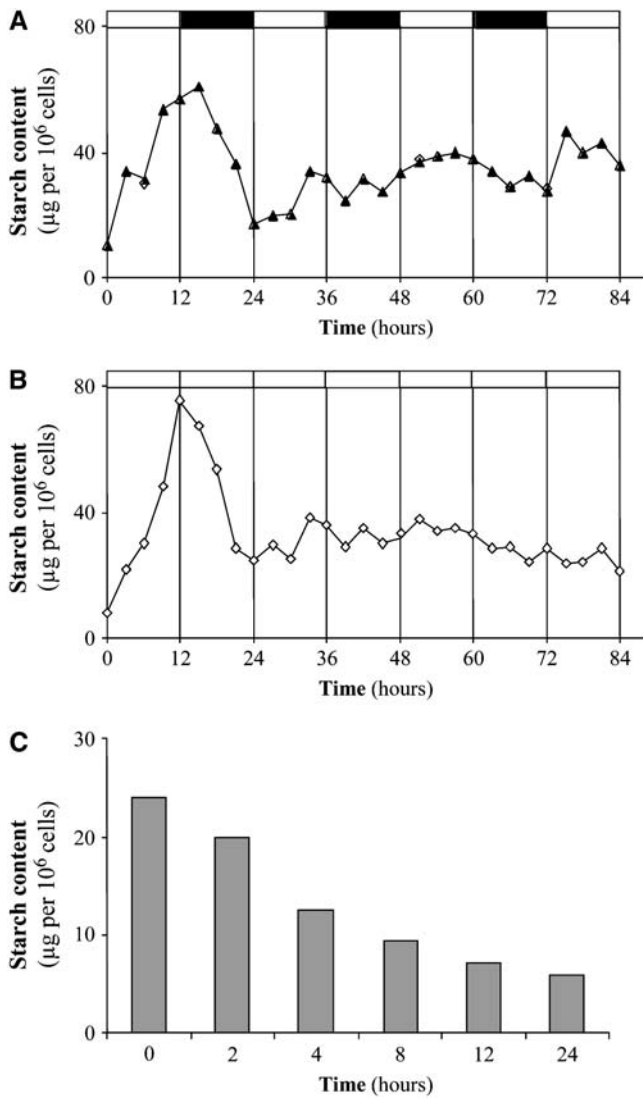


Figure 2. Nitrogen starvation abolishes the rhythms. Precultures were synchronized for 6 d under a 12-h-light/12-h-dark cycle in the presence of acetate. A and B, The cells were harvested at the end of the dark phase by a low-speed spin and resuspended in a nitrogen-depleted medium containing acetate under a 12-h-light/12-h-dark cycle (▲; A) or under LL (◇; B). C, Cells were cultured under nitrogen-starved medium under LL during 5 d as in A and B and directly switched to DD in the presence of acetate. The bar at the top of the graph indicates the corresponding day (white box)/night (black box) periods. The x axis corresponds to the hours after the switch to darkness.

To explain the rhythmicity detected in AGPase activity, a quantitative assessment using an antibody raised against the small subunit of AGPase (kindly provided by C. Hannah) in western-blot experiments was performed (Fig. 3B). Quantification of chemiluminescence using the bioimaging system (Syngene) suggests that the amounts of AGPase protein oscillate in line with the enzyme activity (Fig. 3B).

To reinforce this hypothesis, we decided to investigate the maximal catalytic properties of AGPase. Indeed, it was previously noted that maximal catalytic

properties correlated well with protein levels in Arabidopsis (Gibon et al., 2004). Persistence of the activity rhythm under substrate-saturated concentration was monitored in two distinct synchronized cultures (Fig. 3A). The experiments clearly show that the activity in this case did correlate with the quantified protein levels.

Oscillation of Starch Content Requires Either *STA2* or *STA3*

We tested all our characterized mutants of starch metabolism with respect to their starch content oscillation patterns in nitrogen-supplied cultures. Among the strains tested, only IJ2, a double-mutant strain containing a defect in both the *STA2* and *STA3* genes encoding, respectively, GBSSI and an unidentified soluble starch synthase that was initially called SSII, failed to display circadian rhythms of starch content (Fig. 4).

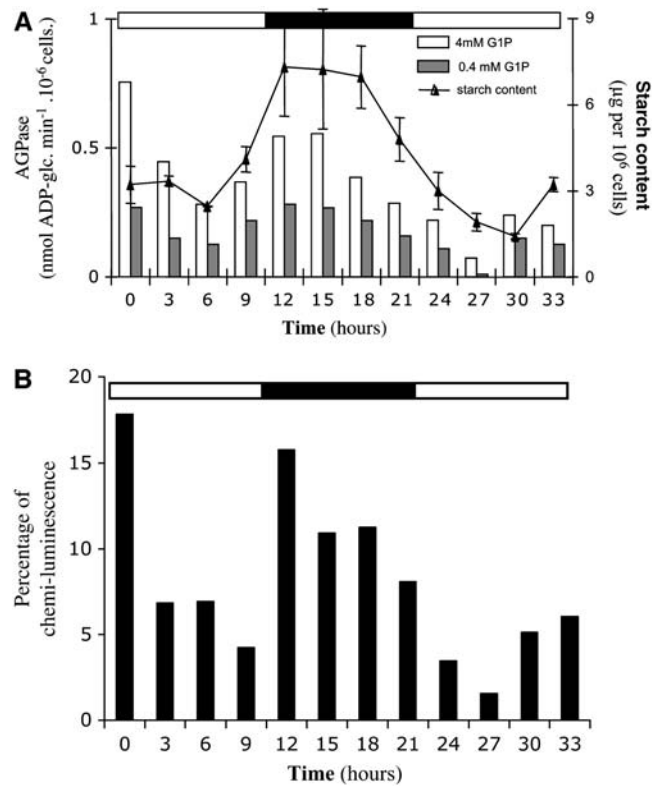


Figure 3. Starch synthesis is correlated to ADP-Glc pyrophosphorylase activity. A, Starch accumulation (▲) and ADP-Glc pyrophosphorylase activities were measured on wild-type cells grown under a 12-h-light/12-h-dark cycle in the presence of acetate. The ADP-Glc pyrophosphorylase activities were assayed in the direction of ADP-Glc synthesis in the presence of 4 mM Glc-1-P (white area) and 0.4 mM Glc-1-P (shaded area). Units are expressed in nanomoles of ADP-Glc synthesized per minute and per million of cells from the means of two distinct measures. B, Harvested samples were used to determine the amounts of AGPase protein through diurnal cycle. The chemiluminescence produced by the immunocomplexes was quantified by using the Chemi-Genius bioimaging system (Syngene). The means of two distinct measures are expressed as a percentage of chemiluminescence.

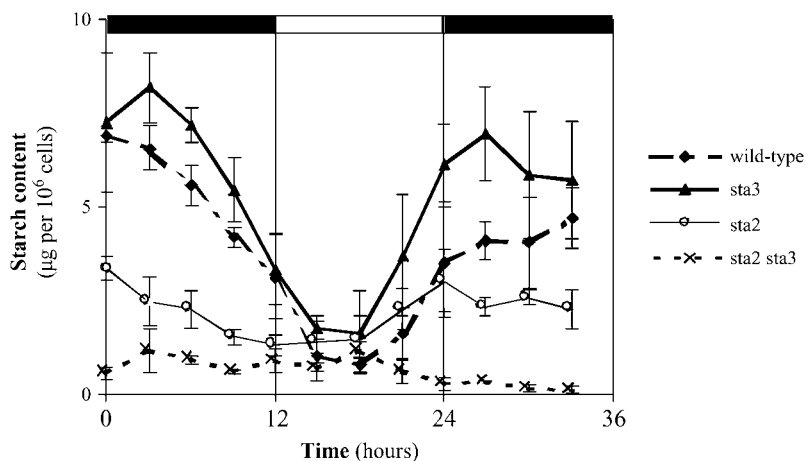


Figure 4. Accumulation of starch during a 12-h-light/12-h-dark cycle in various *Chlamydomonas* mutants. Cells were synchronized by growing for 6 d under a 12-h-light/12-h-dark cycle in the presence of acetate and then the culture was grown under a 12-h-light/12-h-dark cycle. Results displayed are the mean \pm SD of three distinct and independent experiments performed on the wild-type strain (\blacklozenge), the SSIII-defective *sta3-2* mutant (\blacktriangle), the GBSSI-defective *sta2-1* mutant (\circ), and the double *sta2-28::ARG7 sta3-2* mutant (\times). The single mutants and wild-type reference have isogenic genetic backgrounds.

This failure was not due to the low amounts of starch detected and therefore to the low amplitude of variation expected. Indeed, mutants of *STA11* encoding a D-enzyme and accumulating low amounts of polysaccharide never failed to display the typical diurnal rhythm of starch accumulation (Wattebled et al., 2003). Similar results were obtained with the low starch *sta1* mutant (see Supplemental Material 1) defective for the large subunit of the ADP-Glc pyrophosphorylase, further proving that low starch content per se does not prevent oscillation of starch amounts. To check that ADP-Glc pyrophosphorylase activity was not modified in the double mutant, we assayed this enzyme in the wild type and the double mutant. The ratio of double-mutant to wild-type activities in the middle of the day and night were determined at 1.1 and 0.95, respectively, suggesting that there is no difference with the corresponding wild-type 137C strain. We then turned our attention to circadian rhythms of starch accumulation in the corresponding starch synthase single mutants. We were surprised to find that mutants of *STA3* accumulated levels that seemed slightly higher than the wild-type isogenic reference, whereas isogenic pairs of wild type and disrupted mutants of *STA2* displayed a strong reduction ($50\% \pm 10\%$; three separate experiments) of starch amount in GBSSI-defective strains (Fig. 4). Because significant reductions in starch content had not been reported previously in mutants defective for GBSSI, we confirmed this decrease in another strain carrying a point mutation affecting GBSSI activity. A pair of isogenic strains differing by the presence of the *sta2-2* mutation confirmed the reduction in starch amounts. In addition, cosegregation analysis was performed on 12 wild type and 12 recombinants and established that a significant reduction in starch amount (30%) resulted from the presence of the *sta2-2* mutation. This reduction, however, is only seen in nitrogen-supplied oscillating cultures. Indeed, all mutants of *STA2* examined to date accumulate wild-type levels of starch in nitrogen-depleted media, where starch adopts a structure very

similar to that found in the storage organs of vascular plants (Libessart et al., 1995).

STA3 Encodes SSIII

Because the precise nature of the defective soluble starch synthase had not been previously determined, we resorted to identifying which of the four distinct soluble starch synthases had become defective in the *sta3* mutants. Several starch synthase expressed sequence tags (ESTs) were detected through homology searches. Two different ESTs with homology to the higher plant SSIII were used to amplify a 1.5-kb cDNA through reverse transcription (RT)-PCR. This partial cDNA sequence showed the closest homology to other plant SSIIIs and was used to build our previously published phylogenetic trees of plant starch synthases (Ral et al., 2004). The full SSIII sequence was deduced by comparing this cDNA to the available gDNA sequences (see Supplemental Material 2). Additional cDNA sequences were thus generated through a combination of RT-PCRs that produced the full SSIII sequence (see "Materials and Methods"). SSIII in *Chlamydomonas* is a protein with the characteristic long N-terminal arm that typifies this class of protein. This is confirmed by previously published dendrograms (Ral et al., 2004). The size of the *Chlamydomonas* enzyme is compatible with that of the starch synthase that was reported missing in all *sta3* mutants analyzed to date. Because the four independent *sta3* alleles were generated either by x-ray mutagenesis (known to yield large rearrangements) or by insertional mutagenesis, we searched for the presence of allele-specific modifications at the *STA3* locus by Southern-blot analysis. Using the 1.5-kb partial cDNA as a probe, we found one such modification in the *sta3-2* allele carried by the I154 strain. This modification was seen to cosegregate with the *sta3-2* allele upon crossing strain I154 with a wild-type strain ($n = 20$). It is remarkable that all four distinct *sta3* alleles display identical phenotypes with respect to the amount of starch produced and with respect to the

chain-length (CL) distribution of the remaining amylopectin (Fig. 5). All four alleles lead to the absence of a 120-kD protein that cross-reacts against an antibody designed against a conserved peptide of the C-terminal end of all starch synthases (Buléon et al., 1997). In addition, they all lack a slow migrating band of similar molecular mass as evidenced by denaturing zymogram gels. The *sta3-2* allele only failed to amplify an RT-PCR fragment covering a portion of the enzyme common to all starch synthases (see Supplemental Material 3). This defect cosegregated with *sta3-2* (see Supplemental Material 4). The absence of the corresponding message sequences explains the absence of protein and enzyme activity in the *sta3-2* mutants. Because a detailed analysis of all other enzyme activities had been previously carried out (Fontaine et al., 1993) and because all mutant alleles display the same phenotype, we can safely conclude that this phenotype is selectively due to the absence of SSIII. We therefore propose to change the name of the *Chlamydomonas* SSII to SSIII to conform to the higher plant nomenclature.

SSIII and GBSSI Are Selectively Involved in the Synthesis of Long B-Chains in Amylopectin

The presence of normal or slightly higher amounts of starch in nitrogen-supplied oscillating cultures of the *sta3* mutants contrasted with results obtained under nitrogen starvation. We measured the λ_{max} of the iodine-polysaccharide complex of both the wild type and *sta3* mutant strains and found the latter to display strong diurnal variations, whereas the wild-type reference exhibited similar patterns but with a much smaller amplitude (Fig. 6A). High λ_{max} of the iodine-polysaccharide complex reflects the presence of long glucans in amylopectin because very little or no amylose (<5%) can be detected under these conditions. Because GBSSI is known to be responsible for the

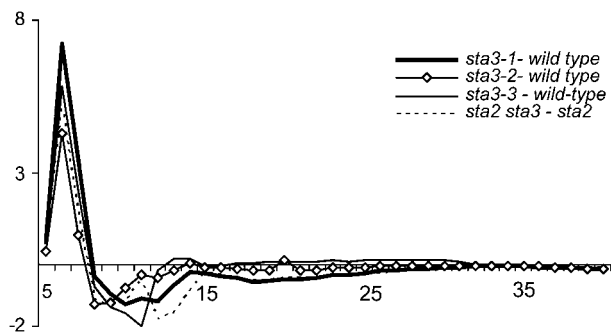


Figure 5. CL distribution differences of amylopectin from SSIII-defective mutants. CL distributions were obtained after isoamylase-mediated enzymatic debranching through capillary electrophoresis of APTS-labeled fluorescent glucans. The x axis displays DP scales (DP5–40) and the y axis represents the relative frequency of chains expressed as percentages. CL distribution differences of purified amylopectin from *sta3-1* (bold black line), *sta3-2* (\diamond), and *sta3-3* (thin black line) mutant alleles minus wild-type isogenic strain. Dash points represent CL distribution difference from *sta2-sta3* minus *sta2* isogenic strain.

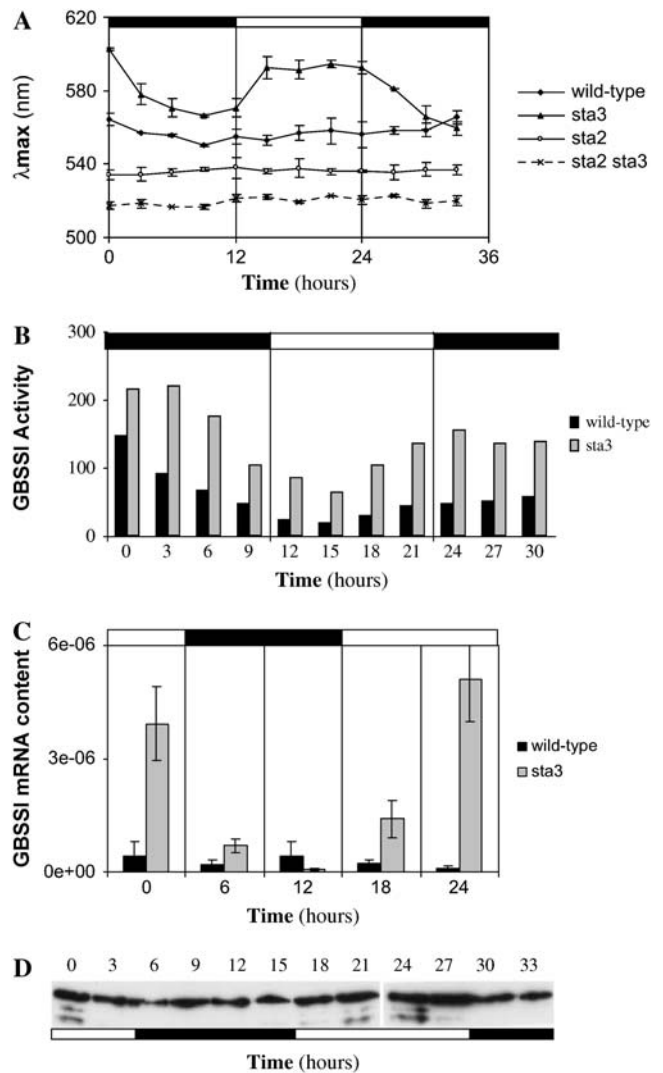


Figure 6. SSIII and GBSSI are selectively involved in amylopectin synthesis. Cells were synchronized for 6 d under a 12-h-light/12-h-dark cycle in the presence of acetate and then the cultures were kept under a 12-h-light/12-h-dark cycle. A, The λ_{max} of the iodine-polysaccharide complex displayed is the mean \pm SD of three distinct and independent experiments performed on the wild-type strain (\diamond), the SSIII-defective (*sta3-2*) mutant (\blacktriangle), the GBSSI-defective (*sta2-1*) mutant (\circ), and the double *sta2-sta3* mutant (\times). B, GBSSI activity measured on both the wild-type (black bars) and the *sta3-2* (gray bars) cells grown under a 12-h-light/12-h-dark cycle while units are expressed in nanomoles of ADP-Glc incorporated per minute and per million of cells. C, GBSSI mRNA content (measured relative to 18S RNA content) in both the wild-type strain (black bars) and the *sta3-2* mutant (gray bars). D, Western blots revealing the GBSSI protein. Note the presence of proteolytic fragments of GBSSI at 0 and 21 to 24 h.

synthesis of long chains in amylopectin (Delrue et al., 1992; Maddelein et al., 1994), we assayed GBSSI activity and corresponding transcript and protein levels in oscillating wild-type and *sta3* cultures as displayed in Figure 6, B to D. It is clear that GBSSI activity is significantly induced by the presence of the *sta3* defect and compensates for the absence of SSIII by synthesizing a

large amount of long glucans within the amylopectin fraction. The selective increase in enzyme activity in the *sta3* mutants results from an even larger increase (from 0.3- to 50-fold, depending on the circadian time) in GBSSI transcript abundance (Fig. 6C) measured by quantitative PCR for most of the circadian times tested (with the exception of 12 h; Fig. 6C). That the induction of GBSSI is responsible for compensation of the *sta3* defect in nitrogen-supplied oscillating cultures is further proved by the observation of a collapse of starch amount in the *sta3 sta2* double mutant (Fig. 4). At the protein level, we have been able to detect the appearance of smaller cross-reacting fragments at specific times of the circadian cycle (Fig. 6D). To confirm that cross-reactions are specific, trypsin digestions were performed on each proteolytic fragment detected by Coomassie Blue (see Supplemental Materials 5 and 6). Matrix-assisted laser-desorption ionization analysis shows clearly that all proteolytic fragments analyzed are covered by approximately 30% (16 matches) of trypsin digested peptides corresponding to the GBSSI protein. This result is agreement with the disappearance of those proteolytic fragments in strains disrupted in the GBSSI structural gene. Because the detection methods used in the western blot displayed in Figure 6D cannot be used for quantification, we chose two time points (6 h after the onset of darkness and 6 h after lights on) to quantify the relative abundance of intact and proteolyzed fragments using the chemiluminescence detection procedure. The relative abundance of intact versus proteolyzed fragments detected by the antibody varied from 0.8 (middle of the day) to 1.6 (middle of the night). These results suggest that starch degradation is accompanied by release and partial proteolysis of GBSSI. Because the double mutant fails to display diurnal rhythms of starch amounts, we hypothesized that this was due to the absence of an essential and similar role in amylopectin synthesis played both by GBSSI and SSIII. As previously noted, GBSSI is responsible for the synthesis of long chains in amylopectin (Delrue et al., 1992; Maddelein et al., 1994). However, SSIII was not previously noted to play such a function. Indeed, the CL distribution of the *sta3* mutants shows a selective increase of small glucans around degree of polymerization 6 (DP6) and, therefore, a relative reduction in the medium size (DP10–40) chains within amylopectin (Fig. 5). It must be stressed, however, that both techniques used at the time for analyzing the debranched chains (high-performance anion exchange with pulsed amperometric detection) were inadequate to analyze the very long glucan fraction of amylopectin. In addition to this, compensation due to the induction of GBSSI would prevent the detection of any decrease of long glucans due to the absence of SSIII. To examine the potential function of SSIII in long glucan synthesis, we therefore resolved to compare this fraction in the presence or absence of SSIII in a genetic background lacking GBSSI. We chose gel filtration on a TSKHW50 column to detect chains above (average) DP50. The chromatogram results are sum-

marized in Table I and clearly show that SSIII is chiefly responsible for the synthesis of chains above (average) DP50.

GBSSI Is a Major Enzyme of Amylopectin Synthesis in Oscillating Cultures

We had previously noted that GBSSI is required for normal amylopectin synthesis in the presence of a *sta3* defect (Maddelein et al., 1994). This work further shows that, despite the presence of SSIII, GBSSI mutants that experience diurnal rhythms of starch synthesis and degradation display a substantial decrease in starch amounts. Under these conditions, little or no amylose is synthesized and the role of GBSSI seems to be confined to amylopectin synthesis. The *Chlamydomonas* GBSSI displays a high specific activity that has enabled us to observe synthesis of amylose in vitro from purified granules (Van de Wal et al., 1998). In this system, we were able to show that this fraction is synthesized through extension of amylopectin followed by cleavage and release into the amylose fraction. The whole process at pH 8 and 3.2 mM ADP-Glc takes a minimum of 4 h at 20°C. It is reasonable to speculate that at physiological concentrations of ADP-Glc (below 1 mM) amylose synthesis does not have time to occur before GBSSI is released by recurrent starch granule degradation, establishing GBSSI as an enzyme of amylopectin synthesis in physiological conditions leading to recurrent starch degradation. These speculations are only valid if the maltooligosaccharide (MOS)-mediated priming of amylose synthesis that is observed in vitro does not contribute significantly to polysaccharide synthesis in vivo. Indeed, MOS-mediated synthesis of amylose is an instant process in all systems tested (Denyer et al., 1996; Van de Wal et al., 1998). To probe the impact of MOS-primed polysaccharide synthesis by GBSSI, we searched in our previously isolated mutant collection for a defective *sta2* allele that produced a GBSSI with a marked difference in its ability

Table I. Wild-type and mutant amylopectin CL distribution

Amylopectins were separately pooled after CL2B gel permeation chromatography, dialyzed, debranched, and subjected to TSK-HW50 chromatography. Starch was extracted from nitrogen-starved cells. Four milligrams of amylopectin fractions purified by gel filtration were loaded on each column after debranching. The optical density of the iodine polysaccharide complex was monitored for each 2-mL fraction at λ_{\max} . The (average) DP scale was generated by using the λ_{\max} values of the debranched glucans as internal standards (Banks et al., 1971). The values listed in the table are Glc weight percentages estimated by the amyloglucosidase assay by summing Glc amounts corresponding to those fractions corresponding to the average DP scale.

	Average DP of Debranched Amylopectin Chains			
	>90	90–40	40–20	<20
Wild type	5.5	18	33.5	43
<i>sta2</i>	3	15.5	22	60.5
<i>sta3</i>	6.5	13	20.5	60
<i>sta2 sta3</i>	0.5	1	35	63.5

to prime synthesis from amylopectin or MOSs. The *sta2-2* mutant allele proved to define such a mutation. The apparent K_m of the *sta2-2* GBSSI for ADP-Glc was measured at 0.5 mM with a V_{max} at 2% of the wild-type enzyme (see Table II). However, the V_{max} is 80% of the wild-type level when assayed in the presence of 50 mM maltotriose. The *sta2-2* mutation was localized at position 28 of the mature protein and consisted of a missense from Pro to Leu. The sensitivity of the enzyme to activation by MOS was qualitatively similar to that previously reported for the wild-type enzyme (Van de Wal et al., 1998). To further characterize the *sta2-2* defect, we purified starch granules from both wild-type and *sta2-2* mutant strains and subjected them to *in vitro* synthesis with labeled ADP-Glc in the presence or absence of 50 mM maltotriose. The mutants accumulated identical amounts of GBSSI protein in comparison to the wild-type references. A typical experiment is presented in Figure 7. These experiments show first that the high levels of incorporation witnessed in the amylopectin fraction are inhibited by the presence of 50 mM maltotriose in the wild-type strain (Fig. 7, A and B). Second, the mutant fails to incorporate high levels of label into amylopectin in the absence of maltotriose and synthesizes the same low amount of amylopectin in the presence or absence of maltotriose (Fig. 7, C and D). Third, these experiments also clearly show that the mutant is able to synthesize near wild-type amounts of amylose in the presence of MOSs. We have calculated according to our previous report (Van de Wal et al., 1998) that the small, but significant, difference in amylose synthesis witnessed between the wild type and *sta2-2* mutant in the presence of MOSs is due to the additional ability of the former to synthesize amylose by extension and cleav-

age from an amylopectin chain. These results prove that *sta2-2* mutants have lost their ability to synthesize amylose by extension of amylopectin but respond normally to MOSs. The mutant contains normal amounts of GBSSI protein bound to starch and does not display a lower affinity for ADP-Glc (on the contrary). We therefore conclude that the decrease in amylopectin content witnessed in the *sta2-2* mutants comes as a consequence of its inability to extend amylopectin chains. We can also safely conclude that amylose synthesis through MOSs does not account for synthesis of this fraction *in vivo* in *Chlamydomonas*. This observation holds true for all conditions tested. Indeed, *sta2-2* mutants fail to accumulate amylose both under nitrogen starvation and under nitrogen-supplied media.

DISCUSSION

The Unusual Pattern of Starch Synthesis and Degradation in *Chlamydomonas*

In *C. reinhardtii*, the phases of starch synthesis and degradation are not correlated with lights on and off. Indeed, in the presence of acetate, starch content peaks in the middle of the night and reaches a minimum in the middle of the light phase. At first glance, this seems to suggest that starch metabolism regulation is very different in *Chlamydomonas* when compared to vascular plants. However, we do not believe this to be true. Indeed, the tissues that are studied in vascular plants consist either of mature leaves, mature cereal kernel endosperm, resting legume seed embryos, or potato tubers. In most cases, these tissues contain mostly cells that have either stopped or restricted their cell division cycles. We believe this to define the major differences between the systems. Storage polysaccharide metabolism in yeast (*Saccharomyces cerevisiae*) has indeed been demonstrated to be regulated by cell cycle progression through the combined action of several signaling cascades involving kinases and phosphatases (for review, see François and Parrou, 2001).

We thus expect that the unusual pattern of starch synthesis and degradation described for *Chlamydomonas* or, more likely, other unusual patterns will be found in actively dividing cells of vascular plants, such as meristem cells or *in vitro*-cultured cells. The exact nature of the pattern will mostly depend on the nature and timing of the energy requirements that are imposed by the ongoing cell cycles.

On the other hand, if cell divisions are slowed and stopped by nutrient starvation, the unusual oscillation patterns stop and starch accumulation in *Chlamydomonas* proceeds in a fashion similar to those typifying polysaccharide synthesis in storage organs.

The Nature of Circadian Clock Control of Starch Metabolism

This work proves that starch metabolism is under tight control of the circadian clock in the monocellular

Table II. Enzymatic properties of *sta2-2* allele mutant and wild-type GBSSI

Kinetic parameters, apparent K_m and V_{max} , were determined through the Hanes and Woolf procedure after measuring initial velocities (nmol min^{-1}) at 1, 2, 4, 8, 12, 16, and 24 mM ADP-Glc and 2.2 μM ADP- ^{14}C -[U]-Glc. Maltotriose effect on specific activity of GBSSI was measured by incubating starch granules in the absence and in the presence of 50 mM maltotriose at 3.2 mM ADP-Glc and 2.2 μM ADP- ^{14}C -[U]-Glc final concentration. Percentage of activation is expressed as (specific activity measured in the presence of maltotriose divided by the specific activity measured in the absence of maltotriose) \times 100. In these experiments, starch granules (100 μg) were incubated for 15 min at 30°C. Reactions were stopped by four washing steps with 95% ethanol. The incorporation of ^{14}C -[U]-Glc was estimated by liquid scintillation counting.

	Kinetic Parameters	
	STA2	<i>sta2-2</i>
K_m (ADP-Glc) mM	4.8	0.5
V_{max} (nmol min^{-1} mg^{-1})	10	0.2
	Specific Activities (nmol min^{-1} mg^{-1} of Starch)	
0 mM maltotriose	1.0	0.3
50 mM maltotriose	4.8	4
Percentage of activation	480	1,300

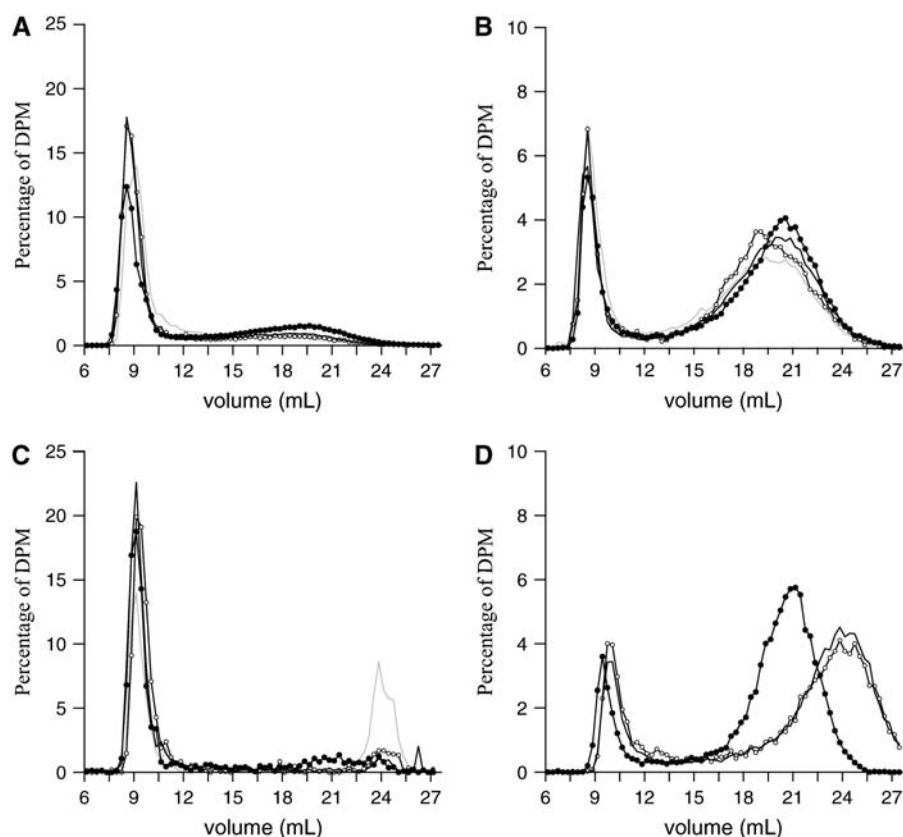


Figure 7. Kinetics of amylose in vitro synthesis. Starch from the wild type (A and B) and the mutant *sta2-2* defective for GBSSI (C and D) was subjected to in vitro synthesis in the presence of ^{14}C -labeled ADP-Glc, or both ^{14}C -labeled ADP-Glc and 50 mM maltotriose. After in vitro synthesis, the amylopectin and amylose were separated by CL2B-Sepharose chromatography. Incorporation of ^{14}C from ADP-[U- ^{14}C]Glc was monitored after 1 h (solid gray line), 4 h (○), 8 h (solid black line), and 24 h (●). A and B, Starch purified from wild-type 137C reference strain assayed in the absence of maltotriose (A) and in the presence of 50 mM maltotriose (B). C and D, Starch purified from the *sta2-2* GBSSI mutant strain assayed in the absence of maltotriose (C) and in the presence of 50 mM maltotriose (D). For the sake of clarity, data are expressed as percentage of the total label eluted from the column. Total disintegration per minute (dpm) values at the amylopectin peak fraction for the 8-h time point were, respectively, 15,000 (A), 2,700 (B), 700 (C), and 750 (D) dpm. The column used for analysis of the *sta2-2* samples displayed a two-thirds reduction in the recovery yield (C and D) by comparison to that used for the wild-type starch samples. The absolute amounts of amylopectin synthesized in B, C, and D are thus comparable and 5- to 6-fold inferior to that which was achieved in A.

alga *C. reinhardtii*. The oscillations witnessed in *Chlamydomonas* are tightly correlated to those of ADP-Glc pyrophosphorylase activity. Analysis of transcript or protein abundance and of enzyme activity suggests that the control exerted by the circadian clock could be at the transcriptional level and not through redox regulation, as in higher plants (see above). It must be stressed that, in *Chlamydomonas*, the cell cycle is clearly gated by the circadian clock (Goto and Johnson, 1995) and it is presently very difficult to distinguish direct control by the clock from circadian clock control exerted through the cell cycle. Our AGPase enzyme assays correlate nicely with the quantification of enzyme amounts. This result suggests that clock regulation in this case does operate at the level of enzyme synthesis probably through the previously evidenced clock control of mRNA abundance (Zabawinski et al., 2001).

Whereas ADP-Glc pyrophosphorylase activity explains the observed rate of starch synthesis, other en-

zyme activities are bound to explain the degradation rates. By analogy with the recent progress made in understanding higher plant leaf starch degradation, GWDs and β -amylases are obvious candidates to explain the observed rates of starch degradation (Ritte et al., 2002; Scheidig et al., 2002). Interestingly, GWDs and a plastidial β -amylase have recently been postulated to be regulated by redox control in plants (Mikkelsen et al., 2005; Sparla et al., 2006), whereas transcript abundance corresponding to plastidial β -amylase, GWD, and amyloamylase were demonstrated to be under circadian clock control in *Arabidopsis* (Chandler et al., 2001; Lu et al., 2005).

The Importance of GBSSI and SSIH in Controlling Synthesis of Amylopectin's Long Chains

While investigating the impact of mutations on starch content oscillations, we discovered a tight functional

link between GBSSI and SSIII. In fact, quantitative PCR experiments strongly suggest that the absence of SSIII triggers a signal that is relayed to increase dramatically GBSSI mRNA abundance. This regulation ensures full compensation of the absence of SSIII by GBSSI. How the regulation of starch metabolism is perceived and relayed out of the chloroplast remains to be determined. The functional link between GBSSI and SSIII is now demonstrated to consist of an unexpected shared function in the synthesis of long glucans (above DP50) within amylopectin. According to the cluster model proposed for amylopectin structure (Hizukuri, 1986), amylopectin chains above DP50 are responsible for hooking together the clusters of amylopectin into large-size macromolecules. We therefore hypothesize that the double mutant failed to build large-size amylopectin molecules, leading to a collapse of granule architecture. We have previously described the separation of amylopectin into several fractions of distinct size on TSK HW75S columns (this material is no longer commercially available). These peaks are not resolved by chromatography on a Sepharose CL-2B column. The mutants lacking both GBSSI and SSIII displayed a continuum of small-size amylopectin molecules, which are absent from the GBSSI and SSIII single mutants (Maddelein et al., 1994). We believe that these result from the limiting numbers of long glucans available for building large-size polysaccharide molecules. We therefore propose that both GBSSI and SSIII are responsible for the synthesis of the long B-chains of amylopectin that in turn are required for the integrity of high mass amylopectin. In this respect, we believe that the typical small-chain increase seen in the SSIII mutant amylopectin results from the accumulation of the preferred SSIII substrate. These small glucans (with a maximum at DP6) are also found in amylose chains synthesized *in vitro* from GBSSI in purified starch granules and are likely to define the transfer products of the branching enzyme, which is also present at the surface or within purified granules.

The Selective Impact of Circadian Rhythms of Starch Degradation on Amylose Synthesis

Amylose synthesis in our system requires a period of time that often exceeds that available to GBSSI before it is released from the granules through recurrent starch degradation. That GBSSI is subjected to proteolysis after recurrent starch degradation is suggested by the presence of proteolytic fragments at defined times during the circadian cycle. Because of this proteolytic attack due to the release of GBSSI from the granules by starch catabolism, GBSSI synthesis *per se* must be tightly regulated by the circadian clock. In *Chlamydomonas*, this control may operate at the transcriptional level by mechanisms analogous to those proposed for circadian clock control of Arabidopsis GBSSI expression (Tenorio et al., 2003). Alternatively, the control may be exerted at the translational level through the action of the RNA-binding protein Chlamy

1 (Mittag, 1996). This work shows that, in addition to the presence of high substrate concentrations (Van den Koornhuysse et al., 1996), amylose synthesis depends on the extent and timing of starch degradation, which itself depends on the rate of starch degradation and on granule numbers.

MATERIALS AND METHODS

Materials

ADP[U-¹⁴C]Glc and α [³²P]dCTP were purchased from Amersham; ADP-Glc was obtained from Sigma. The CL2B Sepharose column and Percoll were obtained from Amersham-Pharmacia Biotech, and the starch assay kit was obtained from Roche.

Chlamydomonas Strains, Growth, and Media

The three wild-type strains of *Chlamydomonas reinhardtii* used in this study were 137C (*mt- nit1 nit2*), 37 (*mt+ pab2 ac14*), and 330 (*mt+ nit1 nit2 arg7 cw15*). I152, I154, and I39 are independent mutants directly derived from strain 137C by x-ray mutagenesis and carry, respectively, the *sta3-1*, *sta3-2*, and *sta3-3* mutant alleles as described elsewhere (Fontaine et al., 1993). TERBD20 (*nit-1 nit2 sta2-1 cw15 arg7*) is a recombinant from a cross involving 330 and 18B (*mt- nit1 nit2 sta2-1*). The 25B strain is derived from 137C by UV mutagenesis and carries the *sta2-2* mutation (Delrue et al., 1992). Finally, the JPR5 strains were generated through insertional mutagenesis of the pMS188 (kindly provided by J.D. Rochaix) plasmid in the TERBD20 genome background. It contains a point mutation in the *STA2* gene and an insertion in the *STA3* locus (*sta3-4*).

Standard media have been fully described (Harris, 1989) and growth conditions and nitrogen-starved media have been described previously (Ball et al., 1991; Delrue et al., 1992). For the study concerning enzyme activity, mRNA level, and starch measurement in a 12-h-light/12-h-dark cycle, pre-cultures were synchronized for a minimum of 4 d under alternating light and dark conditions. Under these conditions, release of mobile daughter cells occurred at the beginning of the light phase. Cultures were inoculated at 5×10^4 cells mL⁻¹. One hundred milliliters of each early log-phase culture (10^6 cells mL⁻¹) were sampled every 3 h, and 200 mL of fresh medium were added every 6 h to compensate medium volume reduction. Experimental design and culture volumes and numbers were calculated to require a maximum of 10% volume addition of fresh medium every 6 h.

Starch Purification

Pure native starch from *C. reinhardtii* was prepared from nitrogen-limited culture and harvested after 5 d of growth under continuous light (Ball et al., 1991). Cells always prepared at the early log-phase step (10^6 cells mL⁻¹) were harvested (4,000g for 20 min) and resuspended in 300 μ L of 10 mM Tris acetate, pH 7.5, 1 mM EDTA. Algal suspensions were disrupted by sonication on ice. 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 with sterile distilled water, centrifuged at 10,000g, and kept dry at 4°C. Starch amounts were measured by amyloglucosidase assay (Delrue et al., 1992).

CL Distribution Analysis

Ten milligrams 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 amylofermosa* isoamylase (Hayashibara Biochemical Laboratory) at 45°C during 4 h. The reaction was stopped by boiling for 10 min. After neutralization with 10 M NaOH, the samples were lyophilized and subjected both to TSKHW-50 chromatography using a 90-cm-long column (i.d. 1.6 cm) and to fluorophore-assisted carbohydrate electrophoresis using the procedure described previously (Morell et al., 1998).

Apparent K_m Determination of GBSSI

The initial velocities of incorporation of ^{14}C -[U]-Glc were measured from freshly purified starch granules (100 μg) at 30°C for 15 min in a 200- μL final volume of incubation buffer (50 mM Glyc-Gly, pH 9.0, 100 mM $(\text{NH}_4)_2\text{SO}_4$, 0.4% β -mercaptoethanol, 5 mM MgCl_2 , 0.05% bovine serum albumin with various concentrations of ADP-Glc (1, 2, 4, 8, 12, 16, 24 mM) containing 2.2 μM ADP- ^{14}C -[U]-Glc at 10.5 GBq/mmol). Reactions were terminated by four washing steps in 95% ethanol. The incorporation of ^{14}C -[U]-Glc was measured by liquid scintillation counting. K_m and V_{max} parameters were determined through the Hanes and Woolf procedure from a series of three datasets.

Enzyme Assays

GBSSI was assayed as described previously (Delrue et al., 1992). In vitro synthesis of amylose was performed by using the previously described method (Van de Wal et al., 1998). ADP-Glc pyrophosphorylase was assayed in the direction of ADP-Glc synthesis presence of 1 mM of 3-phosphoglyceraldehyde. Crude extracts (40 μL) were incubated for 15 min at 30°C in 210 μL of the incubation buffer containing 74 mM HEPES/KOH, pH 7.5, 0.5 mM ATP, 3.5 mM MnCl_2 , 25 $\mu\text{g}/\text{mL}$ bovine serum albumin, and 4 mM or 0.4 mM of Glc-1-P containing 6 nM ^{14}C -Glc-1-P. The reaction was stopped by boiling for 10 min. Unincorporated Glc-1-P was dephosphorylated using 60 units of calf intestine alkaline phosphatase for 1 h at 30°C (Roche). The reaction mixture was then filtered on DE81 ion-exchange paper (Whatman) and washed five times with 10 mL of distilled water. The radioactivity on the dried filters was then monitored. Enzyme activity in the direction of ADP-Glc synthesis was monitored both at 0.4 and 4 mM Glc-1-P final concentration.

Western-Blot Analysis and Quantification of ADP-Glc Pyrophosphorylase Protein

Western-blot analyses were performed on GBSSI and AGPase proteins through day-night cycles. GBSSI protein was extracted by denaturing 10 μg of starch granules in 80 μL of denaturing buffer (2% SDS and 5% β -mercaptoethanol) for 10 min. The samples were centrifuged for 20 min at 10,000g. The supernatants were loaded onto 7.5% SDS-PAGE. The immunocomplexes were detected by a colorimetric method as described by Wattedled et al. (2002). In addition, GBSSI enzyme quantities were determined for a restricted number of samples (two) through the techniques outlined below for AGPase.

The amounts of AGPase protein through day-night cycles were determined by quantifying the chemiluminescence of the immunocomplex. The polyclonal antibody raised against the maize (*Zea mays*) small subunit of AGPase (bt2) was a kind gift from Dr. Curtis Hannah (Giroux and Hannah, 1994). Bradford assays (Bio-Rad) were carefully performed on each harvested sample. The equivalent of 50 μg of total protein was separated on 10% SDS-PAGE according to established procedures (Laemmli, 1970). SDS-PAGE was electroblotted onto a polyvinylidene difluoride membrane (Hybond-P; Amersham Biosciences) in transfer buffer (48 mM Tris, 39 mM Gly, 0.0375% [w/v] SDS, and 20% methanol) for 2 h at room temperature at 100 mA using the mini trans-blot cell (Bio-Rad). The washing and hybridization steps were performed according to the manufacturer's recommendations. Primary antibody and secondary antibody (anti-rabbit conjugated with horseradish peroxidase; Amersham Biosciences) were diluted at 1:5,000 and 1:10,000 in blocking buffer, respectively. Blots were incubated for 5 min at room temperature in the Immobilon western horseradish substrate (Millipore). The chemiluminescence output was easily followed (every 2 min up to 10 min) by a cooled CCD camera from the ChemiGenius² bioimaging system (Syngene). The signals of chemiluminescence were quantified using GeneTools software (Syngene).

Cloning the Full-Length SSIII cDNA

Protocols for extraction of algal RNA and DNA have been reported (Rochaix et al., 1991). A partial cDNA clone corresponding to algal SSIII was isolated by RT-PCR amplification. Primers corresponding to the first two ESTs bearing strong homology with SSIII from higher plants (GenBank accession nos. AV641107 and BF995362) were designed as follows: primer I, 5'-GCC GGC GCC TAC GAG AAC AAC AAC; and primer II, 5'-CAA AGC GCA CCT CGT CGT GCT AGC. Total fractions of RNA on both wild-type strains and the *sta3-2* strain were reverse transcribed using oligo(dT) primer. PCR amplifications of wild-type DNA gave a specific fragment of 1,467 bp. This specific fragment was similar to that of SSIII already cloned from higher plants (Gao

et al., 1998). Incomplete gDNA sequences were then retrieved from the Chlamydomonas genome databank (<http://genome.jgi-psf.org/chlamy>). Four different primers were designated from this gDNA sequence: primer III, 5'-CTG AAG GCG CTG CGT AAG GAG; primer IV, 5'-GCA GCG CCG GCG TCA CCT TGT AGA; primer V, 5'-GTT CAT CGT GGC GGT GGT GTC G; and primer VI, 5'-ACC TGC GCG TGT TCT TCA TC. RT-PCR amplification gave us a 3.7-kb SSIII cDNA that was completed by using RACE-PCR with oligo(dT) primer and a specific 5'-RACE primer, 5'-CTC CTT ACG CAG CGC CTT CAG. This sequence was cloned into the TOPO-cloning vector.

Real-Time Quantitative PCR

Total RNA was extracted from the wild-type 137C and *sta3-2* I154 strain with the RNeasy plant mini kit (Qiagen) following the manufacturer's instructions. cDNA synthesis was performed using the Invitrogen first-strand synthesis kit with oligo(dT) primer. Specific amplifications were detected using Brilliant SYBR Green QPCR master mix (Stratagene). The specific fluorescence was detected at 520 nm and analyzed with MX4000 analysis software by comparison with specific standard curves.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers DQ019314 (SSIII cDNA) and AC148879 (SSIII gDNA).

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