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Toward an Understanding of the Biogenesis of the Starch Granule

DETERMINATION OF GRANULE-BOUND AND SOLUBLE STARCH SYNTHASE FUNCTIONS IN AMYLOPECTIN SYNTHESIS*

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Plant starch synthesis can be distinguished from those of bacterial, fungal, and animal glycogen by the presence of multiple elongation (starch synthases) and branching enzymes. This complexity has precluded genetic assignment of functions to the various soluble starch synthases in the building of amylopectin. In *Chlamydomonas*, we have recently shown that defects in the major soluble starch synthase lead to a specific decrease in the amount of a subset of amylopectin chains whose length ranges between 8 and 40 glucose residues (Fontaine, T., D'Hulst, C., Maddelein, M.-L., Routier, F., Marianne-Pepin, T., Decq, A., Wieruszeski, J. M., Delrue, B., Van Den Koornhuysse, N., Bossu, J.-P., Fournet, B., and Ball, S. G. (1993) *J. Biol. Chem.* 268, 16223–16230). We now demonstrate that granule-bound starch synthase, the enzyme that was thought to be solely responsible for amylose synthesis, is involved in amylopectin synthesis. Disruption of the *Chlamydomonas* granule-bound starch synthase structural gene establishes that synthesis of long chains by this enzyme can become an absolute requirement for amylopectin synthesis in particular mutant backgrounds. In the sole presence of soluble starch synthase I, *Chlamydomonas* directs the synthesis of a major water-soluble polysaccharide fraction and minute amounts of a new type of highly branched granular material, whose structure is intermediate between those of glycogen and amylopectin. These results lead us to propose that the nature of the elongation enzyme conditions the synthesis of distinct size classes of glucans in all starch fractions.

Starch is a huge complex quaternary structure made of α glucans that accumulate in the chloroplast of the leaf (transient starch) or the amyloplast of the plant storage tissue cell. The polysaccharide is usually defined as a mix of two distinct fractions: amylopectin and amylose. Amylopectin, the major compound, is composed of intermediate size $\alpha(1\rightarrow4)$ -linked glucans (the A and B1 chains) that are clustered together and hooked to longer spacer glucans (the B2, B3, and B4 chains) by $\alpha(1\rightarrow6)$ linkages (Hizukuri, 1986). Amylose is often referred to as a smaller linear molecule with very few $\alpha(1\rightarrow6)$ branches (for

review, see Manners (1989)). ADP-glucose is now firmly established as the sole precursor for starch synthesis and ADP-glucose pyrophosphorylase (EC 2.7.7.27) as the rate-limiting step in the pathway (Tsai and Nelson, 1966; Lin *et al.*, 1988; Müller-Röber *et al.*, 1992). The finding of enzyme activities in plants capable either of transferring glucose from the nucleotide sugar to the non reducing end of glucans in $\alpha(1\rightarrow4)$ position (starch synthases) (EC 2.4.1.21) or of enzymes capable of transferring whole segments of the linear molecules in $\alpha(1\rightarrow6)$ position (branching enzymes) (EC 2.4.1.18) is another historical landmark in the study of starch biosynthesis (for review, see Preiss (1991)). In all plant systems analyzed to date, from *Chlamydomonas* to maize, the synthesis of amylose has been shown both *in vivo* (Nelson and Rines, 1962; Visser *et al.*, 1991; Delrue *et al.*, 1992) and *in vitro* (Leloir, 1961; Baba *et al.*, 1987) to rely on the action of granule-bound starch synthase (GBSS),¹ the major protein associated with the granule. Waxy mutants defective for this activity lack the amylose fraction, and by default the remaining polysaccharide was assumed to be pure wild-type amylopectin. In *Chlamydomonas* we have shown that mutants carrying defects in the *ST2* (waxy) gene lack not only amylose but also a fraction of high molecular weight material that displays a level of branching approaching that of the wild-type amylopectin (Delrue *et al.*, 1992). Meanwhile, biochemical and genetic studies performed on *st-3*-carrying mutants defective in one of the two *Chlamydomonas* soluble starch synthases showed that this enzyme (SSII) is necessary for the maintenance of the intermediate size glucans of the amylopectin clustered chains (Fontaine *et al.*, 1993). In absence of this activity the amylopectin is enriched in long glucans and in very short glucose stubs (chain length < 9). In this paper we report that GBSS is responsible for the building of the long glucan backbone in this amylopectin. This backbone includes a substantial number of B4 and B3 chains, according to the nomenclature adopted by Hizukuri (1986) further demonstrate that these long chains are not necessary to obtain full amylopectin synthesis. We show that in absence of the two other synthases, the residual activity (soluble starch synthase I) builds a new type of granular structure intermediate between glycogen and starch.

EXPERIMENTAL PROCEDURES

Materials—[U-¹⁴C]Glucose 1-phosphate and [*D*-glucose-U-¹⁴C]adenosine diphosphate glucose were purchased from Amersham Corp.

¹ The abbreviations used are: GBSS, granule-bound starch synthase; SSII, soluble starch synthase II; SSI soluble starch synthase I; DP, degree of polymerization; HPAE-PAD, high performance anion-exchange chromatography with pulsed amperometric detection; λ_{max} , maximal absorbance wavelength of the iodine polysaccharide complex; WSP, water-soluble polysaccharide; GPC, gel permeation chromatography.

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ADP-glucose, maize amylopectin, and *Pseudomonas amyloclavata* isoamylase were from Sigma. Glucose 1-phosphate, rabbit muscle glycogen, and rabbit muscle phosphorylase were obtained from Boehringer (Mannheim, Germany). DEAE-Trisacryl type M was from IBF Biotechnics (Villeneuve la Garenne, France).

Chlamydomonas Strains, Growth Conditions, and Media—The reference strains of *Chlamydomonas reinhardtii* used in this study wild-type with respect to starch were 137C (*mt⁺ nit-1 nit-2*), 37 (*mt⁺ pab-2 ac-14*), and 330 (*mt⁺ cw-15 arg-7.7 nit-1 nit-2*), which was used for transformation. I152, 18B, and 25B are independent mutants directly derived from strain 137C by mutagenesis and carry the *st-3-1* (I152), the *st-2-1* (18B), and the *st-2-2* (25B) mutant alleles. B9 is a meiotic recombinant strain of *mt⁺ pab-2 ac-14 st-3-1* genotype. 44 low starch mutant strains (ML1–ML44) out of 80,000 colonies screened were selected using the iodine stain after mutagenizing strain I152 with UV light (5% survival) as described in Delrue *et al.* (1992). Genetic techniques used have been described by Harris (1989a). All the strains used to generate Fig. 2 are meiotic products obtained by crossing the various strains listed above. Quantifications and structural analyses were always performed on this progeny in order to avoid interference of background mutations. Standard media have been fully detailed by Harris (1989b), while growth conditions, nitrogen-starved media, and diploid clone selection have been described by Ball *et al.* (1990, 1991) and Delrue *et al.* (1992).

Electron Microscopy—Fixation and embedding protocols are those 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 λ_{\max} measures can be found in 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 ($2 \cdot 10^6$ cells·ml⁻¹) grown in HSA (High Salts Acetate) under continuous light (80 microeinsteins·m⁻²·s⁻¹). The detailed description of the differential (NH₄)₂SO₄ precipitation of SSI and SSII together with the anion exchange purification on DEAE-Trisacryl type M of those enzyme activities can be found in Fontaine *et al.* (1993). Soluble starch synthase 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 mg of bovine serum albumin·ml⁻¹, 10 mg of rabbit liver glycogen·ml⁻¹, 4 mM ADP-glucose containing 1 nmol of [U-¹⁴C]ADP-glucose (specific activity 200 μCi·μmol⁻¹). After 15 min of incubation at 30 °C, the reaction was stopped by adding 2 ml of ice-cold ethanol. GBSS was assayed as described by Delrue *et al.* (1992). Branching enzymes were always assayed on the same DEAE chromatograms by incubating up to 40 μl of sample in a 0.2-ml final volume of 0.1 M sodium citrate (pH 7.0), 1 mM AMP, 40 μg of rabbit liver phosphorylase containing 50 mM [U-¹⁴C]glucose 1-phosphate (final specific activity 0.22 μCi·μmol⁻¹). After a 30-min incubation at 30 °C, the reaction was stopped by 10% trichloroacetic acid precipitation on ice. The resulting precipitate was filtered, rinsed, dried, and counted in a liquid scintillation counter (assay A) as described by Thon *et al.* (1992). The two partially (from 35- to 80-fold) purified branching enzyme peak fractions were further characterized by monitoring the branching of amylose (1 mg·ml⁻¹) in 0.1 M sodium citrate (pH 7), after adjusting the amount of enzyme activity to equal amounts using assay A (Krisman *et al.* (1985)). Under these conditions, the first peak eluting from the column decreased the amylose λ_{\max} from over 620 nm down to 570 nm in 18 h of incubation, while the second peak branched the polysaccharide down to a λ_{\max} of 530 nm. None of these properties were altered in strains IJ2 and I152 relative to the wild-type 137C strain. The absence of amylose activity in the peak BE fractions was checked by using the standard assay described by Ball *et al.* (1991). For zymograms, soluble crude extracts were prepared as described above and were further cleared by centrifugation at 20,000 × g for 30 min at 4 °C. Proteins were measured immediately using the Bio-Rad protein assay kit. From 20 to 60 μg of total proteins were loaded on a 30:1, 7.5%, 0.75-mm-thick, native polyacrylamide gel containing 4 mg of rabbit liver glycogen·ml⁻¹. Electrophoresis was carried out in the cold (4 °C) at 30 V/cm for 90 min, using the Mini-PROTEAN II cell (Bio-Rad) and 2.5 mM Tris-glycine (pH 8.3) buffer. The gel was incubated between 4 and 12 h at room temperature in a minimal volume of the starch synthase assay mixture described above but without glycogen and with 2 mM final cold ADP-glucose. An equally sensitive zymogram assay can be performed by using amylose as external primer and extra thin Phast-System native gels as described by Fontaine *et al.* (1993). However, amylose-primed zymograms allow the detection of SSII only.

Starch Fractionation and Methylation—Separation of starch fractions on TSK HW-75(S) columns (Merck) was as previously detailed (Delrue *et al.*, 1992). Methylation of total starch and of fractions dialyzed and freeze-dried after TSK HW-75 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 Glc either to those of α(1→4) and α(1→6)-linked Glc or to those of Glc in terminal non-reducing position (Delrue *et al.*, 1992; Fontaine *et al.*, 1993).

NMR—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 mono- and disubstituted glucose (δ ~5.2 and 4.85 parts/million, respectively) (Gidley, 1985).

Debranching Analysis—Isoamylase-mediated debranching of GPC-purified fractions was achieved as described previously (Fontaine *et al.*, 1993) with the following modifications. After completion of the debranching reaction, samples were kept at 80 °C in 10% Me₂SO to avoid retrogradation of the long glucans into insoluble material. Half of the debranched polysaccharide was subjected to TSK HW-50(F) chromatography using a 50-cm-long column (inner diameter, 1.6 cm) fed by 10% Me₂SO (30 ml/h flow rate). The other sample half was subjected to desalting using a TSK-HW-40(S) chromatographic step as described previously (Fontaine *et al.*, 1993). The pooled fractions were subjected to high performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) on a Carbo-Pac PA-100 column as fully described by Fontaine *et al.* (1993).

Water-soluble Polysaccharide: Assays and Sizing—As for starch purification, WSP was prepared from nitrogen-limited (8 mg of (NH₄)₂SO₄·liter⁻¹) cultures, inoculated at 10⁶ cells·ml⁻¹, harvested after 5 days of growth under continuous light (80 microeinsteins·m⁻²·s⁻¹) in otherwise HSA medium. Algae were ruptured by sonication at a density of 10⁸ cells·ml⁻¹ in 10 mM Tris (pH 7.5). A crude starch pellet was obtained by spinning the lysate at 2000 × g for 20 min. The pellet could then be further purified as described by Delrue *et al.* (1992) to yield pure starch while the supernatant was further cleared by spinning at 10,000 × g for 30 min at 4 °C. The crude soluble extract was immediately frozen and thawed after 24 h of storage. This treatment yielded an abundant membrane, chlorophyll, and cellular debris precipitate that could be conveniently spun out by two high speed (20,000 × g) centrifugations without loss of WSP. This supernatant was assayed with the starch determination kit from Boehringer after taking the hexose levels into account. In order to size this material, the crude soluble fraction yielded by 5 liters of culture was evaporated down to 1 ml, adjusted to 10% Me₂SO, and loaded on a TSK HW-50 column as described above. A DP scale was generated on the column by using the glucan ladders Glucidex 12, 19, and 6. The controlled α-dextrins Glucidex were a gift from Roquette Frères (Lestrem, France). Tables describing the amount and size distribution of the dextrin mixtures can be obtained from Roquette Frères (Lestrem, France). The glucan ladder scale was in perfect accordance with that generated by the λ_{\max} of the debranched glucans described above. The amount of glucan in each fraction was determined using the amyloglucosidase assay.

Transformation-mediated Gene Disruption—Standard glass bead-mediated transformation (Kindle, 1990) was performed on the cell wall-deficient, arginine-requiring strain 330 with 1 μg of pARG7.8 carrying the wild-type arginosuccinate lyase gene. Transformants were selected by complementation of the arginine auxotrophy present in the recipient strain. The transformants were screened for starch structure and amount by spraying iodine directly on replica plates (Fontaine *et al.*, 1993). The frequency of mutant selection in the starch pathway was in the order of 10⁻³.

Genetic Analysis of the Disrupted Strains—Strain BAFR1 (*mt⁺ cw-15 arg-7.7 st-2-Δ1 nit-1 nit-2*), containing an unknown number of functional ARG7⁺ copies due to pARG7.8 integration, was crossed with 37E17 (*mt⁺ pab-2 ac-14 st-3-1*). The segregants were picked at random and checked for all markers included in the cross. Only those crosses that yield impeccable segregations of all known markers are used. In such a cross the presence of one, two, or three functional unlinked ARG⁺ loci of plasmid integration would lead, respectively, to 25, 12.5, or 6.25% ARG⁺ recombinants.

RESULTS

Selection of Starch-defective Mutants—In an attempt to identify the genes responsible for amylopectin synthesis in a background where the major soluble starch synthase is defective, 44 strains were selected that accumulated less than 8 μg of starch·10⁻⁶ cells after UV mutagenesis of an *st-3-1*-carrying

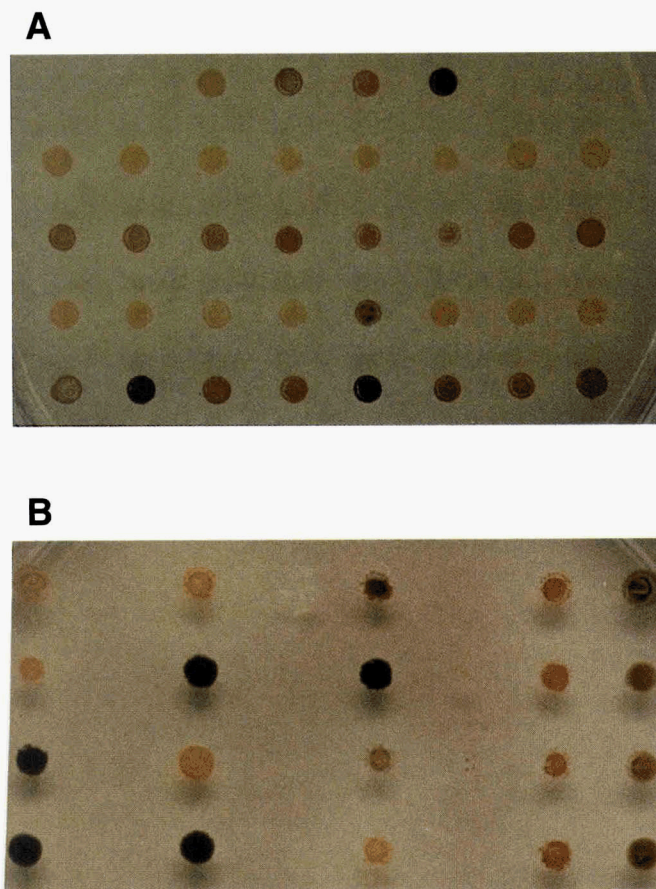


FIG. 1. Genetic analysis of *Chlamydomonas* double mutants. Iodine stain of cell patches incubated for 5 days in $-N$ medium in the presence of acetate and light. **A**, row 1 displays our reference strains with (from left to right): I7, a low starch (5%) mutant with defective ADP-glucose pyrophosphorylase (Ball *et al.*, 1991); I152, the high amylose (olive staining) mutant with defective soluble starch synthase, the amyloseless 18B (red staining) carrying a defect in the GBSS structural gene and the wild-type blue staining 137C reference. Rows 2 and 4 represent the phenotype of 16 (ML1–ML16) of the 44 mutants selected after UV mutagenesis of the high amylose I152 strain. Rows 3 and 5 display the diploids obtained after crossing the same 16 mutants with a suitable strain carrying the *st-2-1* mutation. This complementation test clearly shows that 14 out of 16 strains carry a defect in the *ST-2* gene. **B**, a cross was made between ML1 and wild-type strain 37. From left to right, columns 1 (tetrad 1), 2 (tetrad 2), and 3 are four-spore tetrads; columns 4 and 5 display a single eight-spore tetrad. Tetrads 1 and 2 are parental ditypes, while tetrads 3 and 4 represent, respectively, a tetratype and a non-parental ditype. The phenotype due to the new defect can thus be seen as the red staining colonies in a haploid wild-type background in tetrads 3 and 4.

strain (I152). We were surprised to find (Fig. 1) that 36 out of the 44 selected strains carried a single additional defect in the previously characterized GBSS structural gene (*ST-2*). Since 26 of these are independent mutants, we had thus defined a minimum of 26 new *st-2* alleles. Moreover we constructed strains carrying both *st-3-1* and the previously characterized *st-2-1* or *st-2-2* mutations by crosses (Fig. 2). In all cases the double mutants had less than 20% of wild-type starch amounts (Fig. 2), in most cases the downfall was spectacular ($<10\%$), and in some cases we recorded the lowest values ever observed for *Chlamydomonas* in nitrogen-limited cells ($0.9 \pm 0.4 \mu\text{g}\cdot 10^{-6}$ cells). We then subjected the starch of strains carrying either the single new *st-2* alleles or, in a few cases, the double *st-3 st-2* mutations to fractionating by gel filtration (Fig. 3). All recombinants tested carrying only the new *st-2* defects displayed analogous if not identical starch structures, that is, absence of both amylose and a high molecular weight branched material

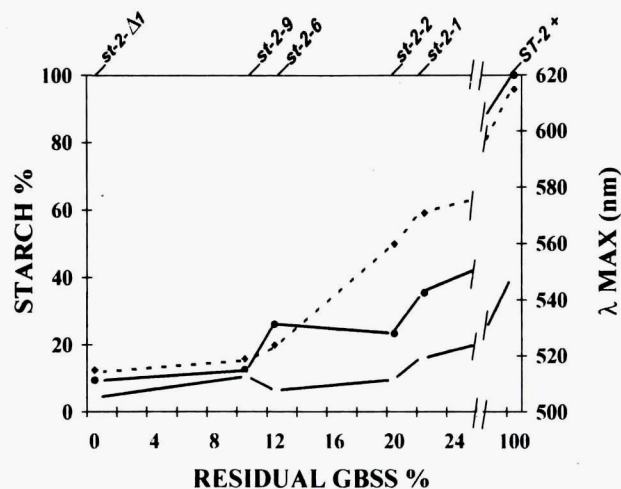


FIG. 2. Starch content and amylopectin λ_{\max} of mutants defective for both granule-bound and soluble starch synthase II. Figure displays starch amounts and amylopectin λ_{\max} of double mutants with the same *st-3-1* defect (leading to the absence of SSII activity) and with different *st-2* alleles leading to progressively more severe GBSS defects. Severity of the GBSS defect was reflected by the residual GBSS activity measured for each mutant allele. These residual activities are expressed as percentages of the wild-type GBSS V_m on the x axis. The position of each mutant allele on the V_m axis is indicated at the top of the figure. Starch levels and amylopectin λ_{\max} were averaged in each case from four different strains of identical genotype. These are expressed as percentages both of the wild-type (\circ) and of the SSII-defective strain containing *st-3-1* (\bullet). Amylopectin λ_{\max} of double mutants is displayed as a broken line (\bullet). V_m and of K_m of GBSS were measured for each of the mutant *st-2* alleles in an *ST-3+* background. V_m is expressed as percentage of strain 137C (4 nmol of ADP-glucose incorporated into glucan/min/mg of starch). The wild-type K_m is 4 mM ADP-glucose (Delrue *et al.*, 1992). The K_m measured in the *st-2-1*, *st-2-2*, *st-2-6*, and *st-2-9* were found, respectively, at 24, 6, 19, and 43 mM.

that we have named amylopectin type II (Fig. 3, A and B). The double mutants displayed no detectable amylose and an amylopectin of variable structure (as witnessed by the λ_{\max}) ranging over a wide size range (Fig. 3, C and D). This epistasis of waxy-like mutations over other defects has also been observed in maize (Creech, 1965) (for review, see Shannon and Garwood (1984)). All of the new *st-2*-defective strains were impaired in granule-bound starch synthase, thus confirming our genetic data. However, the most defective strain isolated by UV mutagenesis still had 10% of the wild-type activity. This prompted us to use molecular techniques to disrupt the GBSS structural gene (see below). We thus obtained strains carrying the *st-2-Δ1* allele yielding no residual enzyme activity. Water-soluble glucans (WSP) were also assayed in wild-type and single or double mutant *Chlamydomonas* strains. Significant amounts ($0.5 \mu\text{g}\cdot 10^{-6}$ cells) of WSP accumulated in all *st-3-1*-carrying strains. Interestingly, this level rose to $1.5 \mu\text{g}\cdot 10^{-6}$ cells in strains carrying an additional defect in the GBSS gene. Wild-type strains accumulated less than $0.1 \mu\text{g}\cdot 10^{-6}$ cells. It is worth stressing that Fig. 2 shows a clear correlation between the decrease in residual GBSS activity displayed by strains carrying distinct *st-2* alleles and that of the λ_{\max} of the double mutant starch. The latter is in all double mutants composed solely of amylopectin. This result thus suggests that the amount of GBSS activity correlates with that of the long glucan content in the amylopectin fraction.

Structural Characterization of a Novel Amylopectin Subfraction—Type II amylopectin accounts for 2–10% of the total wild-type starch. It is significantly branched and has been shown to be under GBSS control (Delrue *et al.*, 1992). We investigated the fine structure of this material both by NMR and by debranching the polysaccharide using *P. amyloclavata*

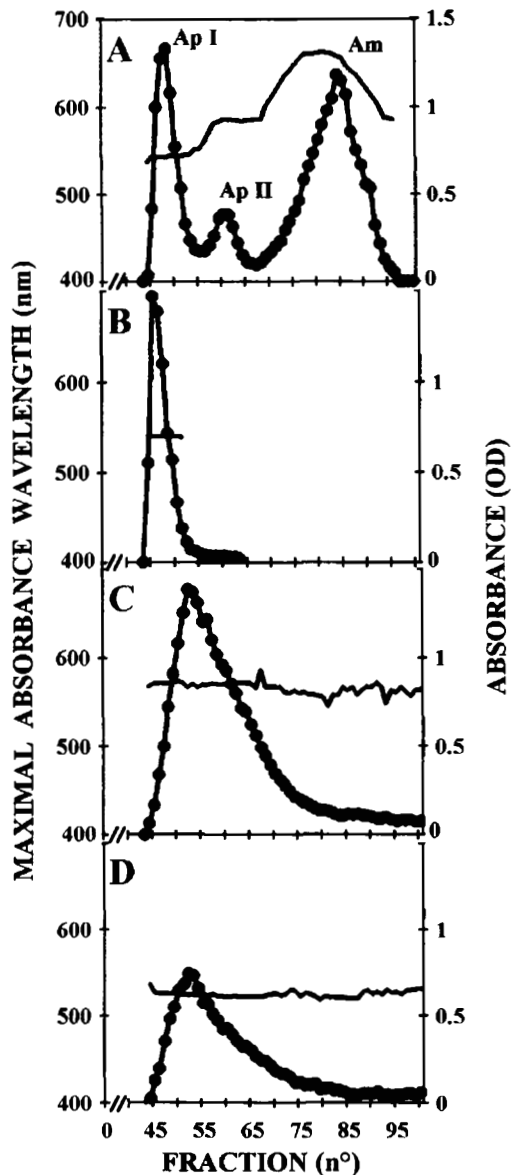


FIG. 3. Separation of amylopectin and amylose on TSK-HW75 chromatography. The optical density (●) was measured for each 3-ml fraction at λ_{max} , while λ_{max} (expressed in nm) is displayed as an *unbroken thin line*. All samples were loaded on the same column set-up as described previously (Delrue *et al.*, 1992). The wild-type haploid 137C strain (*panel A*) can be seen to carry the standard type I (*ApI*) and type II (*ApII*) amylopectins and amylose (*Am*). *Panel B* displays starch from strain BAFR1 disrupted for the GBSS structural gene (*ST-2*). *Panels C and D* are starch chromatograms from double mutant strains S8 (*st-2-2 st-3-1*) and IJ2 (*st-2-Δ1 st-3-1*). The starch from these two strains displays a marked difference in its iodine interaction (λ_{max} of 560 and 520 nm, respectively).

isoamylase (Table I; Figs. 4C and 5B). Proton and ^{13}C NMR confirm the presence of abundant branches typical of amylopectin that we had initially detected using methylation analysis. On the other hand, the ^{13}C spectrum places this material even closer to amylose than that of the previously characterized *st-3* mutant amylopectin (Fontaine *et al.*, 1993). The level of branching detected by proton NMR is higher (5.8%) in type I polysaccharide than in type II amylopectin (3.6%). High performance anion exchange chromatography and gel filtration of the debranched chains (Table I) show that amylopectin type II contains both very long amylose-like glucans and intermediate size (chain length 8–40) chains. Moreover, type II material displays abundant small glucans (chain length < 8), which are

TABLE I
Distribution of glucose weight percentages with respect to size of the isoamylase debranched chains prepared from GPC-purified starch fractions

Polysaccharide ^a	>90 ^b	90–70	70–50	50–30	30–22	<22 ^c
137C ApI	1	2	8	19	14	55
137C ApII	32	5	5	12	14	32
1152 (+ <i>st-3-1</i>) Ap	15	8	3	14	7	53
137C Am	53	12	3	12	4	16
1152 (+ <i>st-3-1</i>) Am	59	6	9	10	7	9
BAFR1 (<i>st-2-Δ1</i> +) Ap	0	3	4	22	17	54
IJ2 (<i>st-2-Δ1 st-3-1</i>) Ap	0	0	3	18	7	72
Rabbit liver glycogen	0	0	0	11	7	82

^a Ap (amylopectin) and Am (amylose) were separately pooled after TSK-HW75 GPC, dialyzed, and freeze-dried. They were then debranched and subjected to TSK-HW50 GPC as described under "Experimental Procedures." The genotypes of the starting strains are listed between brackets. Starch was extracted from nitrogen-limited cells as described by Delrue *et al.* (1992). The chromatograms that enabled us to compute this table are shown in Fig. 7.

^b >90 stands for all chains with DP over 90. 90–70 stands for chains whose DP are inferior to 90 and superior to 70. The values listed in the table are glucose weight percentages estimated by the amyloglucosidase assay.

^c A more detailed chain length distribution for the small glucans can be seen in Fig. 5.

typically decreased in the major amylopectin species (Fontaine *et al.*, 1993) or in the amylopectin from granule-bound starch synthase-defective mutants (Fig. 5D). These small glucans are very similar to those found in single or double mutants defective for the *Chlamydomonas* soluble starch synthase II (Fig. 5, C and H) and can also be clearly seen in amylose (Fig. 5, A and F). It is worth stressing that they all have a characteristic peak at DP 6. It is obvious, however, that the amylose branching pattern in wild-type and soluble starch synthase-defective strains remains very similar (Fig. 5, A and F) (see also Table I and Fig. 7, panels E and F). In our debranching experiments, the amount of reducing ends generated by amylose purified by GPC is a mere 3-fold inferior to that generated by type II material and 5-fold less than type I amylopectin. These indirect estimates confirm the direct measures that we had made previously using starch methylation (Fontaine *et al.* 1993).

Gene Disruption Experiments—Results obtained to date in *Chlamydomonas* can be interpreted in terms of two sequential steps in a single pathway. One can assume, for instance, that the long chains of amylopectin synthesized by GBSS are absolutely required in order to branch, then elongate, the intermediate size glucans. In fact we can speculate that the residual GBSS activity seen in all our mutants might be sufficient to fulfill this function for amylopectin synthesis. This seems to contradict observations made in higher plants, where insertion or point mutations leading to the absence of GBSS do not lead to a decrease in the quantity of starch. However, it is now known that plants have duplicate genes, often under different tissue-specific controls, for at least some if not most of the enzymes of the starch pathway. Moreover, in maize two distinct granule-bound starch synthases have been reported (Macdonald and Preiss, 1985). The absence in our 28 UV-generated mutations of a single null allele prompted us to solve this problem in *Chlamydomonas* by creating deletions in the GBSS structural gene. We have used the plasmid tagging technique that was recently developed for *Chlamydomonas* in an effort to generate a tagged *st-2* mutant. Non-homologous recombination events leading to integration of plasmids in the alga are now known to generate deletions several kilobase pairs long (Tam and Lefebvre, 1993). On a total of 11,000 independent integration events, 15 mutants affected in the starch pathway were selected; two of these turned out to carry new *st-2* alleles as was initially shown by complementation analysis and then by stan-

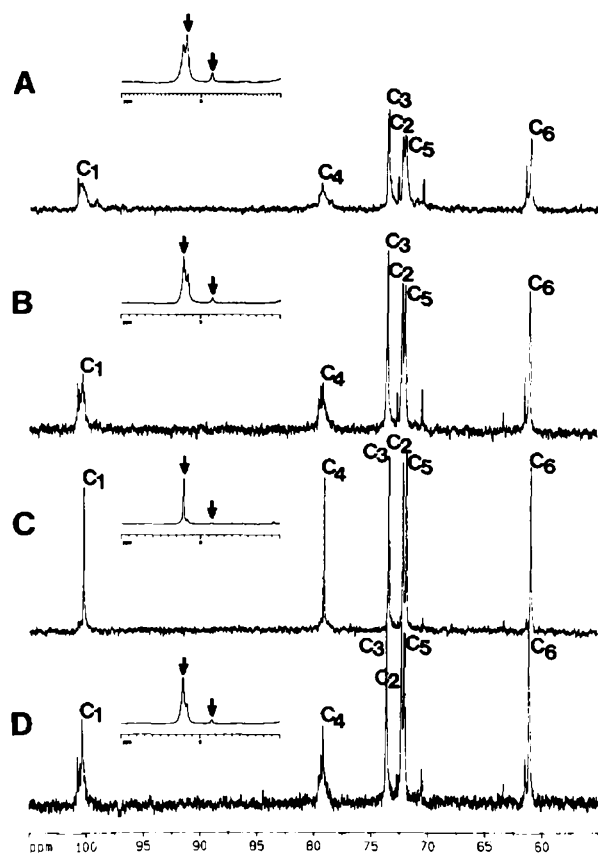


FIG. 4. NMR analysis of wild-type and mutant polysaccharide. ^1H and ^{13}C spectra of purified wild-type and mutant amylopectins in dimethyl sulfoxide- $d_6/\text{D}_2\text{O}$ (80:20). The ^{13}C resonances of all Glc carbons are displayed and numbered from 1 to 6, while only those proton resonances that were used to estimate the amount of branching are boxed and shown by arrows. Panels A and B display, respectively, the rabbit liver glycogen and the amylopectin from the IJ2 double mutant (*st-2-Δ1 st-3-1*). Panels C and D show wild-type (strain 137C) amylopectin II and I. The broadening of the signals that are clearly seen in panels A and B and, to a lesser extent, in panel D reflects a greater heterogeneity of the carbon environments due to an increase in branching. The NMR spectra from *Chlamydomonas* amylose and from the amylopectins from the single *st-2*- or *st-3*-containing mutants were previously displayed (Fontaine *et al.*, 1994).

dard crosses. Both displayed identical phenotypes. Strain BAFR1 was chosen for further investigations because it readily gave fertile crosses. While all genetic markers involved segregated as expected, no recombination between the defective starch and the *ARG*⁺ phenotypes could be observed in these crosses (153 meiotic products observed). None of the 17 *ARG*⁻ recombinants harbored the *st-2-Δ1* mutation. This result establishes linkage between the mutation and one of the functional *pARG7.8* loci of integration. In addition Southern analysis clearly witnesses two integration loci (Fig. 6A), which coincides with the most probable theoretical distribution fitting the observed segregation for the arginine requirement (see methods). It is worth noting that both loci harbor truncated versions of the original plasmid. In contrast with results obtained on all mutants obtained by UV mutagenesis, there was a complete loss of both the major granule-bound 76-kDa protein (Fig. 6B) and of all detectable enzyme activity. We therefore conclude that we have isolated *bona fide* null alleles of *st-2*. We then characterized the starch of the mutants and found the levels to be normal. The structure of this polysaccharide closely resembled that of the starches from GBSS-defective strains and contained only type I-like amylopectin (Fig. 3D, Table I). We therefore conclude that despite the obvious involvement of

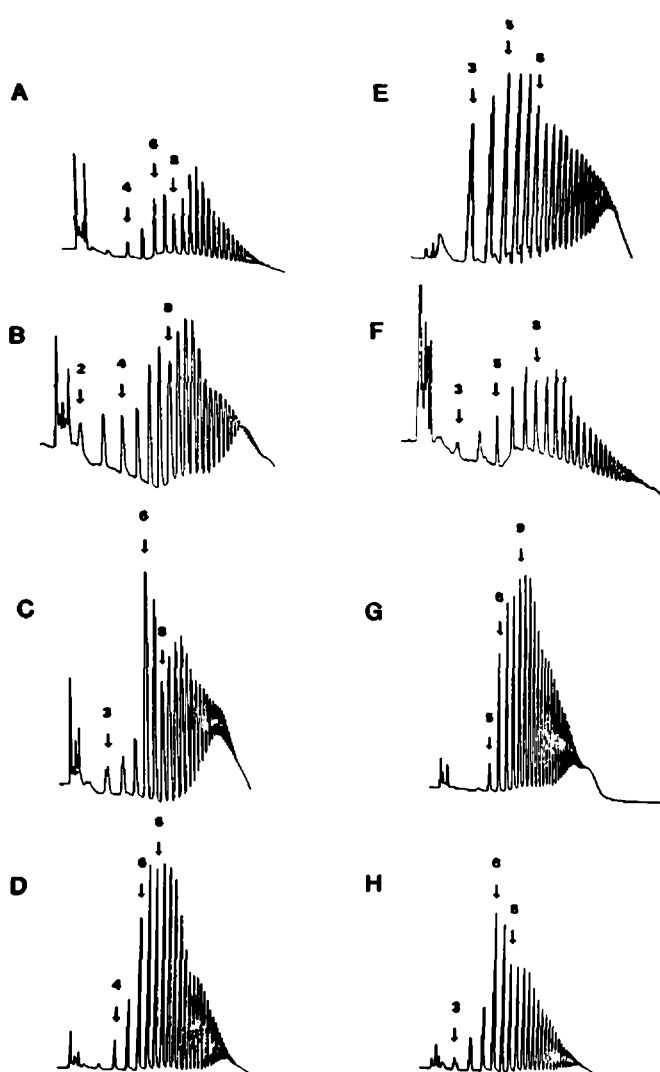


FIG. 5. HPAE-PAD chromatography of water-soluble debranched glucans from wild-type and mutant GPC-purified polysaccharide fractions. TSK-HW75-purified starch fractions are shown. Glucans differing by only one Glc residue are clearly separated up to DP 25. The DP of some of the chains are given on top of each chromatogram. The figure can be directly read as an histogram of amylopectin chain size since the detection procedure is proportional to the number of molecules and not to the amount of Glc present. Panel A displays wild-type amylose (strain 137C). Panels C and D are amylopectin from mutants carrying the gene disruption *st-2-Δ1* in a wild-type (strain BAFR1) (panel D) and in an *st-3-1* mutant background (strain IJ2) (panel C). Panel B displays amylopectin type II from the wild-type strain 137C, while panel G shows amylopectin type I from the same strain. Panel E displays rabbit liver glycogen. Panels F and H show, respectively, amylose and amylopectin from an *st-3-1* mutant strain (I152).

GBSS in the building of long glucans in normal amylopectin (Figs. 2 and 3), the latter is not required for full mutant amylopectin synthesis. The single "pathway" hypothesis can thus be rejected. The interaction between the GBSS disruption and our tightest soluble synthase defects (*st-3-1*) yields over a 90% decrease in granular starch with respect to the already low levels (40% of wild-type) of the reference *st-3-1*-containing strains. Since these high amylose strains still contain 40–60% amylopectin, one cannot escape the conclusion that GBSS controls *in vivo* most of amylopectin synthesis in this particular background.

Determination of the Structure of a New Polysaccharide under SSI Control—The little (<2% of the wild-type) granular polysaccharide left in the *st-2-Δ1 st-3-1*-carrying strain was

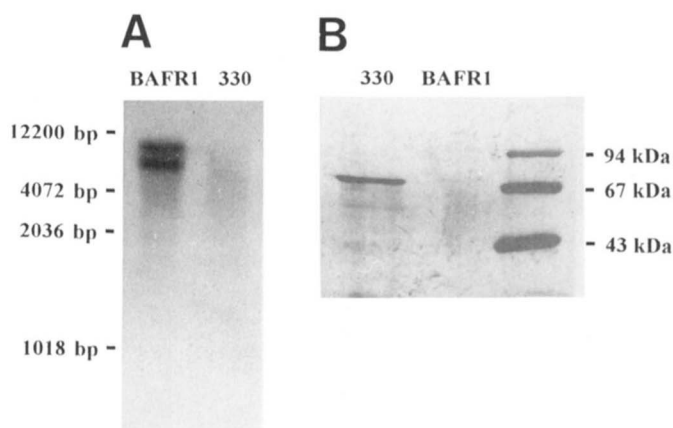


FIG. 6. Molecular characterization of the *st-2-Δ1* disruption. *Panel A*, 10 μ g of *Bam*HI-digested *Chlamydomonas* DNA were hybridized with an *Nru*I/*Sal*I 321-base pair fragment covering a small part of the bacterial tetracycline resistance gene contained in pARG7.8, which is proximal to the *ARG7* gene. *Bam*HI does not cleave pARG7.8, which was used in our mutagenesis experiments. Both *Bam*HI bands seen in BAFR1 display molecular weights of 9.6 and 7.2 kilobase pairs, consistent with a deletion of part of the 11.6-kilobase pair pARG7.8 plasmid at the site of integration. The untransformed strain 330, as expected, does not show any hybridization with the probe. *Panel B*, silver stain of a 5–15% SDS-acrylamide gel of total protein extracted from 3 mg of purified starch by the SDS-boiling procedure described by Delrue *et al.* (1992). *Lane 1* displays the wild-type 76 kDa GBSS, *lane 2* is from strain BAFR1, and *lane 3* displays molecular weight standards.

subjected to analysis by gel filtration on TSK-HW75 (Fig. 3D) columns followed by NMR (Fig. 4B) and methylation analysis. The pooled polysaccharide fractions were further debranched, and the size distribution of the chains was determined (Figs. 5C and 7C and Table I). The overall picture emerging from these studies is that of a very highly branched polysaccharide (9.3% branching according to NMR, 6.5% according to methylation analysis) whose structure is intermediate (Fig. 7C) between those displayed by both standard type I (Fig. 7A) or waxy amylopectin (6.1% branching according to NMR, 5.2% according to methylation analysis) and that of glycogen (Fig. 7D) (9.7% branching according to NMR, 6.7% according to methylation analysis). While this polysaccharide remains overall of very high molecular weight, it spans an unusually dispersed size range (Fig. 3D). It is worth noting that electron micrographs clearly show (Fig. 8) that this material still adopts a granular structure *in vivo*. In order to check that changes in branching enzyme activities due to complex regulatory feedback mechanisms could not explain the observed phenomena we partially purified the branching enzymes from the single and double mutants and compared them to the wild-type. The anion exchange chromatograms obtained were identical to those that we have previously displayed (Fontaine *et al.*, 1993) for wild-type and *st-3*-carrying strains with no evidence for branching enzyme alterations in the mutants using both assays for branching enzyme (see “Materials”). By comparison, the glycogen-primed soluble starch synthase zymograms display a complete wipe out of the major soluble starch synthase form in all *st-3-1*-carrying strains.

In the strains containing defects for both granule-bound and soluble synthases the WSP exceeded the amount of granular starch by 2–3-fold. The size of this fraction was further estimated by GPC on TSK-HW50 columns. The material was of small size and a significant fraction did not stain at all with iodine. The bulk (over 50% in weight) of the fraction was estimated to be in a range of DP 5–20. The sensitivity of this material to amyloglucosidase and its relative insensitivity and low sensitivity to, respectively, β and α amylase suggest the presence of a branched dextrin.

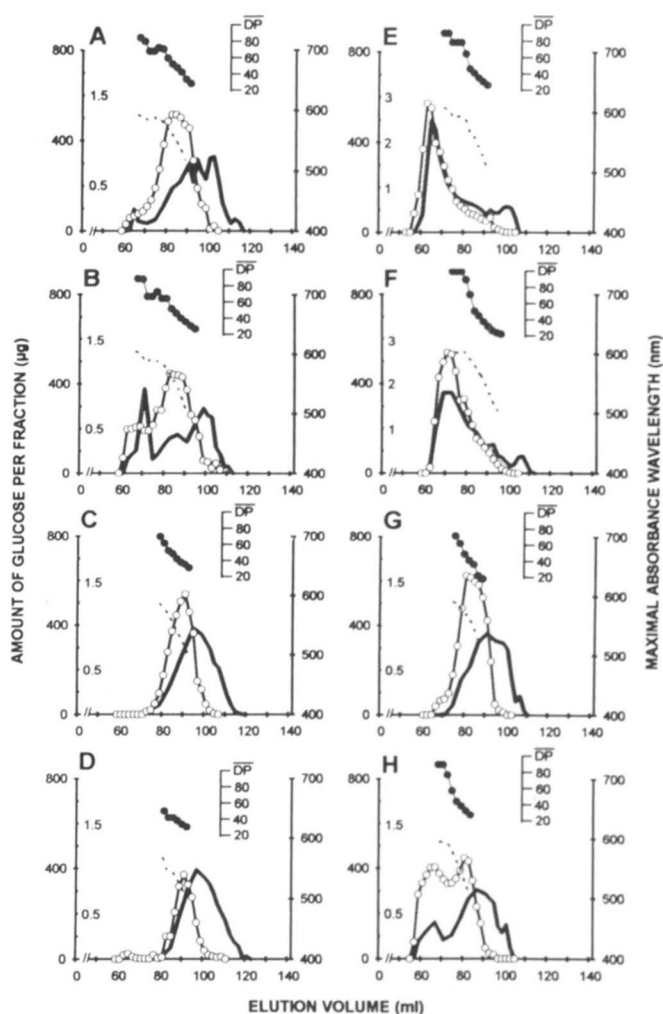


FIG. 7. Separation of isoamylase debranched glucans by TSK-HW50 chromatography. 4 mg of GPC-purified starch fractions were loaded on each column after debranching. The optical density (\odot) of the iodine polysaccharide complex was measured for each 2 ml fraction at λ_{\max} . The OD scale is shown on the left y axis at the inner side and was adjusted in panels E and D to accommodate for the high absorbance due to the long glucans of amylose. The total amount (μ g) of glucose per 2-ml fraction is scaled on the outer side of the left y axis, while the right y axis represents the wavelength (nm) scale. The x axis shows the elution volume scale (ml). λ_{\max} values are displayed for all fractions where they could be determined (broken line). The column set-up and debranching conditions are described under “Experimental Procedures.” The DP scale (\bullet) was generated by using the λ_{\max} of the debranched glucans as internal standards according to Banks *et al.* (1971). The accuracy of the scale and column set-up was double-checked by loading the defined glucan α dextrin ladder Glucidex. The amount of glucose (thick unbroken line) was used to generate Table I. *Panels A and B* display ApI and ApII from the wild-type reference strain 137C. *Panel C* shows the chromatogram of debranched amylopectin purified from the double mutant strain IJ2 (*st-2-Δ1 st-3-1*), while *panel D* shows that of debranched rabbit liver glycogen. *Panels E and F* show the chromatograms of debranched GPC-purified amylose from, respectively, the wild-type 137C reference and the mutant 1152 (*st-3-1*). *Panel G* displays the debranched amylopectin chromatogram from BAFR1 carrying the *st-2-Δ1* disruption. *Panel H* displays the debranched amylopectin chromatogram from 1152.

DISCUSSION

We have shown that in *Chlamydomonas* the granule-bound and the major soluble starch synthases carry out partly redundant functions for incorporation of glucose into high molecular weight branched α glucans. Both of these can function in complete absence of the other leading to two distinct mutant amylopectin structures. These mutant structures are analogous, although not identical, to the two types of amylopectin that can

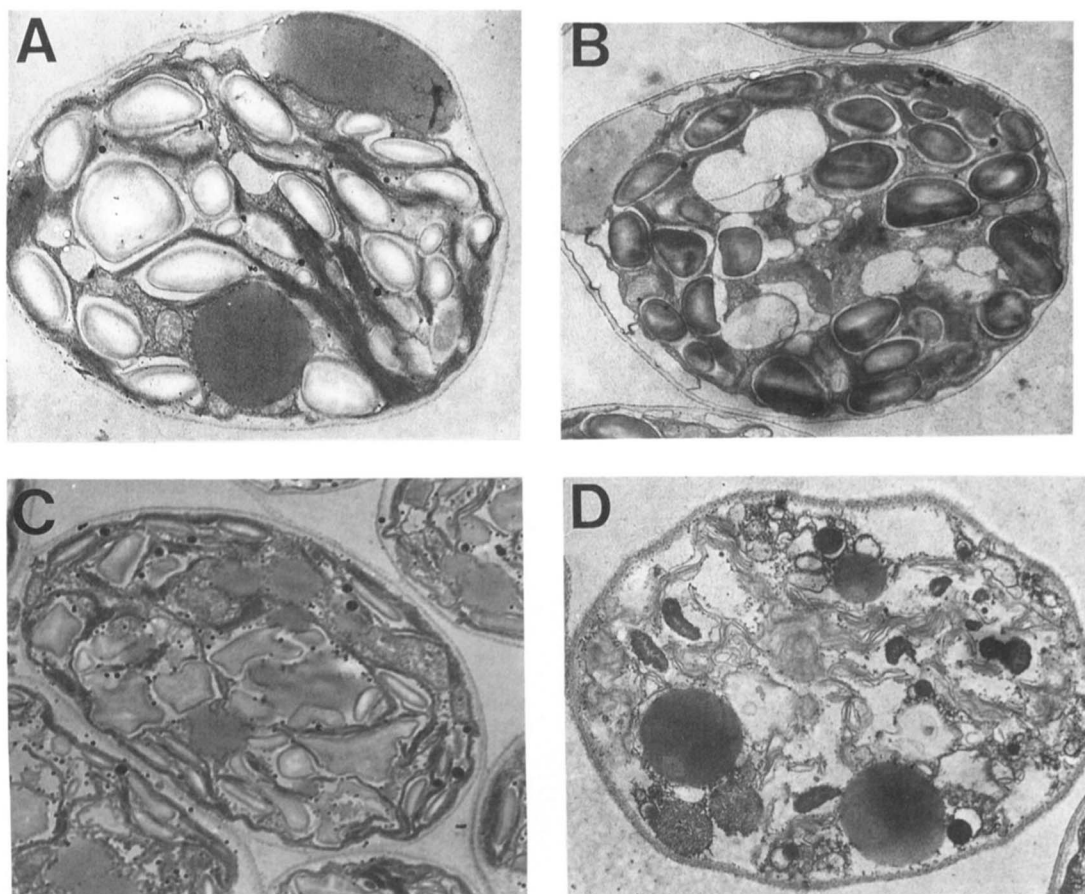


FIG. 8. **Electron microscopy of wild-type and nitrogen-starved cells.** Typical nitrogen-starved cells are virtually filled with starch and galactolipid droplets after 4 days incubation in $-N$ medium in the presence of acetate and light. Typical full-size starch granules can be seen in panels A and B displaying the wild-type strain IN24 and the mutant IR16 harboring the *st-2-Δ1* disruption for the GBSS structural gene. Strain IV33 (panel C), carrying a defect (*st-3-1*) for the major soluble starch synthase activity, harbors 40% of wild-type amounts and shows less regularly shaped starch granules. The double mutant strain IJ2 (panel D) (*st-2-Δ1 st-3-1*) shows a strong reduction in starch amounts (2% of wild-type amounts) and contains numerous smaller starch granules of various altered shapes and size (electron micrographs are of the same magnitude ($\times 11,000$)).

be found in wild-type algae. However, in that case, amylopectin type II can also be viewed as an intermediate toward normal (type I) amylopectin synthesis. We do not know at present exactly how both syntheses mix in wild-type cells. It seems very likely that some long chains synthesized by GBSS are embedded in type I amylopectin. Our data indeed imply that all three starch synthases are involved in amylopectin synthesis. On the other hand, the structure of wild-type amylose and from amylose purified from soluble starch synthase-defective strains do not differ significantly, confirming that this enzyme has no role to play in the synthesis of this starch fraction. The existence of amylose in this respect might be simply explained by the inaccessibility of the glucan synthesized inside the granule by GBSS to important soluble enzymes present at the surface. Thus GBSS at the surface of the granule would have access to the full set of enzymes necessary to obtain amylopectin, while the enzyme inside the starch granule would be missing some crucial component (such as a specific branching enzyme).

On its own *in vivo*, *Chlamydomonas* soluble starch synthase I seems to be quite inefficient in synthesizing granular starch in growth arrested cells (<2% of the wild-type levels). It is interesting to note that the increase in WSP recorded in the double mutants parallels that reported for the "waxy dull" double mutant of maize (Creech, 1965; Shannon and Garwood, 1984). To our knowledge no structural analyses of the water-soluble material contained in *du wx* maize have been reported. The high amylose dull mutant was reported to be defective for

both soluble starch synthase II and branching enzyme IIa (Preiss and Boyer, 1980). The double maize mutants also display a decrease in the level of granular starch. However, in *du* and *du wx* mutants the severity of the defects do not come near those that we report here for *Chlamydomonas*. One can speculate that in higher plants this might in turn be due to the presence of duplicated genes and functions.

In *Chlamydomonas* double mutants, despite the presence of normal amounts of both types of branching enzymes, the branching level of the polysaccharide synthesized by soluble starch synthase I becomes exceedingly high and yields a granular material that does not truly qualify as starch. The granular and soluble material are also very different from the structure and physical properties of both glycogen or phytoglycogen. Although one might argue that this is a mere reflection of a modification in the ratio of branching to synthase and debranching activities, we believe that the very nature of the starch synthase is responsible in establishing a particular size distribution of the glucan chains. There are at present two equally viable mechanisms through which this can be explained. One would be that the production of characteristic glucan lengths is an intrinsic property of the starch synthase. The second would be that granule-bound and soluble starch synthases all have distinct abilities to interact directly or indirectly with the starch branching enzymes. Direct interaction could be mediated through physical contact in the building of a multi-subunit complex. Indirect specific interaction could be achieved

either by both the selective use (as primers) or the production of structural motives which are, respectively, products or selective substrates of distinct starch branching enzymes.

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