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Waxy *Chlamydomonas reinhardtii*: Monocellular Algal Mutants Defective in Amylose Biosynthesis and Granule-Bound Starch Synthase Activity Accumulate a Structurally Modified Amylopectin

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Amylose-defective mutants were selected after UV mutagenesis of *Chlamydomonas reinhardtii* cells. Two recessive nuclear alleles of the *ST-2* gene led to the disappearance not only of amylose but also of a fraction of the amylopectin. Granule-bound starch synthase activities were markedly reduced in strains carrying either *st-2-1* or *st-2-2*, as is the case for amylose-deficient (waxy) endosperm mutants of higher plants. The main 76-kDa protein associated with the starch granule was either missing or greatly diminished in both mutants, while *st-2-1*-carrying strains displayed a novel 56-kDa major protein. Methylation and nuclear magnetic resonance analysis of wild-type algal storage polysaccharide revealed a structure identical to that of higher-plant starch, while amylose-defective mutants retained a modified amylopectin fraction. We thus propose that the waxy gene product conditions not only the synthesis of amylose from endosperm storage tissue in higher-plant amyloplasts but also that of amylose and a fraction of amylopectin in all starch-accumulating plastids. The nature of the *ST-2* (waxy) gene product with respect to the granule-bound starch synthase activities is discussed.

Our knowledge of starch synthesis and degradation, while having developed mostly from observations made in higher-plant storage tissues, has benefited from investigations performed on a number of model microbial systems. *Chlorella pyrenoidosa*, for instance, has been the subject of several of the pioneer studies dealing with the enzymology of starch anabolism (16, 20). Prokaryotic organisms such as *Escherichia coli* have yielded a number of relevant genetic and biochemical studies, mostly because of the parallel that can be drawn between the regulation of plant and bacterial ADP-pyrophosphorylases (reviewed in reference 18). However, the very nature of the storage polysaccharide (glycogen) has prevented the use of model bacterial systems to investigate the biogenesis of the starch granule itself. Thus, our knowledge of the intricate pathways of amylose and amylopectin biosyntheses stems solely from those elegant genetic investigations performed on pea or cereal mutations which express themselves only in the endosperm (reviewed in references 15 and 22). Even in that case, we do not yet know precisely which of the starch synthases and starch branching enzymes are responsible for amylose or amylopectin biosynthesis, let alone how they act coordinately to produce a complex structure such as the starch granule. One of the best and most studied endosperm mutants, waxy maize, seemed until very recently to shed at least some light on the biosynthesis of amylose. Waxy mutations have been identified in most cereals (22, 32) and more recently in storage tissues of dicots such as potato (9) or in the perisperm of the amaranth (11). They all lead to the decrease or absence of both amylose and granule-bound starch synthase. While there is no doubt that one of the main proteins associated with the starch granule is the product of the waxy gene, the identity of this waxy protein with the major granule-bound starch synthase has been recently questioned (24). Thus, the widespread view that this enzyme is solely responsible for amylose synthesis is, in fact, a matter of controversy. In the hope to bring additional insights in our understanding of the biogenesis of the starch granule, we have embarked in a systematic screen for mutants altered in the structure of starch in the monocellular alga *Chlamydomonas reinhardtii*. We report here the isolation and characterization of waxy mutations expressed in photosynthesizing plastids of unicellular green algae. We show that, as in higher-plant endosperm tissue, the absence of amylose is correlated with a marked decrease of granule-bound starch synthase and is accompanied by the decrease or loss of one of the main proteins associated with the starch granule. Allele-specific variation of K_m for ADP-glucose cosegregates in crosses with molecular weight modifications of the main granule-associated protein, suggesting that granule-bound starch synthase is the product of the waxy (*ST-2*) gene in *C. reinhardtii*. An in-depth structural investigation of mutant and wild-type starch shows that waxy *Chlamydomonas* strains have also lost an important component of the amylopectin fraction. We thus suggest that granule-bound starch synthase controls the biosynthesis not only of amylose but also of amylopectin in all types of photosynthesizing or starch-accumulating plastids.

MATERIALS AND METHODS

Materials. [U-^{14}C]ADP-glucose was purchased from Amersham (Amersham, United Kingdom). The starch determination kit was purchased from Boehringer (Mannheim, Germany). Rabbit liver glycogen was supplied by Sigma Chemical Co. (St. Louis, Mo.). Percoll was from Pharmacia LKB Biotechnology (Uppsala, Sweden).

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Strains, media, and incubation and growth conditions. Our reference strains are 137C (*mt nit-1 nit-2 y-1*), 37 (*mt*$^+$ *pab-2 ac-14*), and I7 (*mt nit-1 nit-2 y-1 st-1-1*) (2). 18B and 25B are strains derived by UV mutagenesis of 137C and carry the *st-2-1* and *st-2-2* mutant alleles, respectively. B20 (*mt*$^+$ *pab-2 ac-14 st-2-1*) and B5 (*mt*$^+$ *pab-2 ac-14 st-2-2*) are meiotic segregants obtained by crossing strains 18B and 25B with strain 137C. UV mutagenesis was performed by irradiating cells at 5% survival, using a TS-15 (254 nm) transilluminator (Ultra-Violet Products, Inc., San Gabriel, Calif.) displaying a peak intensity of 7.0 mW cm^{-2}. Irradiation was followed by overnight incubation in HS (high salts) medium. Media and culture conditions used in our starvation experiments were as described by Ball et al. (2). HS and HSA (high salts acetate) media were as described by Harris (6). *p*-Aminobenzoic acid was added to a final concentration of 50 μg ml^{-1}. For testing growth on nitrates, ammonium chloride was substituted by 250 mg of $NaNO_3$ per liter in HS or HSA medium.

Genetic techniques. Gametogenesis and crosses were as described by Harris (7). Vegetative diploids were always selected as microcolonies growing after 4 days on minimal medium with nitrate as a sole nitrogen source. Complementation tests were thus carried out by constructing starch-defective strains containing the same nuclear markers as strain 37. Meiotic segregants were isolated and analyzed at random.

Electron microscopy. Fixation and embedding protocols are those described by Harris (8).

Starch levels. An ethanol-washed pellet, harvested by centrifugation (1000 × *g*, 10 min) of a solution made of 5 volumes of ethanol per volume of cell suspension, was boiled for 10 min in water at a standard concentration of 10^8 cells ml^{-1} (10^7 cells ml^{-1} in starvation experiments). The extract was then submitted to complete amyloglucosidase digestion as described in the starch determination kit from Boehringer. After subsequent phosphorylation by hexokinase, glucose 6-phosphate levels were determined by the glucose 6-phosphate dehydrogenase assay.

Starch purification. Pure native starch was prepared from nitrogen-limited cultures containing 8 mg of $(NH_4)_2SO_4$ per liter, inoculated at 10^5 cells ml^{-1}, and harvested after 5 days of growth under continuous light (80 μE m^{-2} s^{-1}) in otherwise HSA medium.

Algae were ruptured by sonication at a density of 10^8 cells ml^{-1}. A crude starch pellet was obtained by spinning the lysate at 2,000 × *g* for 20 min. The pellet was rinsed in 10 mM Tris HCl (pH 8.0)–1 mM EDTA, resuspended in 1 ml of the same buffer per 10^8 starting cells, and passed twice through a Percoll gradient (9 ml of Percoll per ml of crude starch pellet). The purified starch pellet was rinsed by centrifugation at 2,000 *g* in distilled water and was kept at 4°C for immediate use or was freeze-dried for subsequent analysis.

Spectral properties of the iodine-starch complex. A 0.01% starch solution was made in 10% dimethyl sulfoxide (DMSO) containing 0.2% I_2 and 0.02% KI from a freshly prepared stock solution. The absorbance of the complex was monitored from 700 to 400 nm.

Enzyme assays. (i) Soluble starch synthase. Soluble crude extracts were always prepared from late-log-phase cells (2 × 10^6 cells ml^{-1}) grown in HSA under continuous light (80 μE m^{-2} s^{-1}). The cell pellet was suspended to a concentration of 10^8 cells ml^{-1} in 50 mM Tris HCl (pH 7.5)–2 mM dithiothreitol–2 mM EDTA and was ruptured by sonication. Lysis was monitored under the microscope, while starch and cell debris were collected by two rounds of centrifugation at 10,000 × *g* for 10 min.

The supernatant was further purified by Bio-Gel P2 chromatography. Crude extracts were kept at most for 1 hour on ice after stability was checked for each enzyme under these conditions. Proteins were determined by the Lowry method.

Total soluble starch synthase activity was assayed in 0.1 ml (final volume) of 50 mM glycine NaOH (pH 9)–100 mM $(NH_4)_2SO_4$–5 mM β-mercaptoethanol–5 mM $MgCl_2$–0.5 mg of bovine serum albumin per ml–10 mg of rabbit liver glycogen per ml–4 mM ADP-glucose containing 1 nmol of [U-^{14}C]ADP-glucose (specific activity, 200 μCi μmol^{-1}). After 15 min of incubation at 30°C, the reaction was stopped by addition of 2 ml of ice-cold ethanol. The resulting precipitate was filtered, rinsed, dried, and counted in a liquid scintillation counter.

Citrate-stimulated activity was monitored as described above except that 0.5 M citrate was added prior to incubation. A primer-independent starch synthase activity can be assayed in *Chlamydomonas* spp. only in the presence of 0.5 M citrate and was assayed as described above in the absence of glycogen.

(ii) Granule-bound starch synthase. The granule-bound activity was measured from freshly purified starch granules in the absence of glycogen in the conditions and buffer used for measuring the soluble activity except that the ADP-glucose concentration was raised to 10 mM with the same specific radioactivity of [U-^{14}C]ADP-glucose. K_m constants expressed in millimolar substrate were measured by using the least-squares fit to Hanes plots. Data are given as mean ± standard error from three separate experiments using seven substrate concentrations. K_m estimates from diploids originate from a single experiment.

Starch methylation. Methylation was performed as described by Paz Parente et al. (14), with the following modifications. One to 2 mg of polysaccharide was dissolved in 500 μl of DMSO at 80°C during 6 h; 0.5 ml of lithium methyl sulfonyl carbanion was added to the cooled sample, which was then sonicated for 2 h and left overnight at room temperature. The sample was then frozen (−20°C) and subjected to methylation by adding 0.5 ml of methyl iodide and was sonicated for 2 h. Methyl ether compounds obtained after hydrolysis, reduction, and acetylation were separated by gas chromatography (Girdel 300 apparatus; capillary column [25 m by 0.2 mm] filled with DB-1; column temperature, 120 to 240°C with a temperature gradient of 2°C/min; helium pressure, 40 kPa).

NMR Spectroscopy. The 100-MHz ^{13}C nuclear magnetic resonance (NMR) spectra were obtained on a BRUKER AM-400 WB spectrometer equipped with a 5-mm mixed ^{1}H/^{14}C probehead operating in the pulsed F.t. mode and controlled by an Aspect 3000 computer with an array processor. The ^{13}C NMR experiments were performed with the standard BRUKER pulse program POWGATE with ^{1}H broadband composite pulse decoupling. The products were analyzed in DMSO-D6 at 90°C (δ-CH_3 of DMSO = 39.6 ppm). A 90° pulse (4.8 μs) and a 1.0-s recycle delay were used in each experiment.

Starch fractionation. Five to 10 mg of pure native starch was solubilized in 0.5 ml of DMSO at 100°C for 30 min. The diluted sample was then adjusted to 10% DMSO and layered on a TSK HW-75(S) Fractogel column (1.6 by 145 cm; Merck); Fractions of 3 ml were collected after addition to the column of 10% DMSO containing 0.02% sodium azide at a flow rate of 10 ml/h. Similar separations were achieved by using a Sepharose CL2B (Pharmacia) column of the same

TABLE 1. Structural properties of wild-type and mutant starch

Strain	λ_{max} (nm[a])	Relative amt[b]	
		2,3,4,6-Glc	2,3,6-Glc
137C	590	0.6	31
18B	550	0.9	19
25B	550	0.7	21

[a] Rounded to the nearest decimal.

[b] 2,3,4,6-Glc and 2,3,6-Glc represent the methyl ether derivatives of a glucose in terminal nonreducing position and an $\alpha(1\rightarrow4)$-linked glucose, respectively. The amounts were compared by taking 2,3-Glc [a methyl ether derivative of an $\alpha(1\rightarrow4)$- and $\alpha(1\rightarrow6)$-linked glucose] as an arbitrary standard (assigned a value of 1).

size and starch sample dissolved at the same concentration in 0.01 M NaOH. Samples of 3 ml were collected after addition to the column of 0.01 M NaOH at the same flow rate.

Purification of granule-bound proteins. Granule-bound proteins were purified according to Vos-Scheperkeuter et al. (30). Briefly, 2 to 5 mg of pure native starch was suspended in 80 μl of 5% (vol/vol) β-mercaptoethanol–2% (wt/vol) sodium dodecyl sulfate (SDS) and extracted by boiling for 10 min. The supernatant obtained after a 5-min centrifugation at 10,000 × *g* was stored while the pellet was submitted to a second round of extraction. The pooled supernatants were subjected to polyacrylamide gel electrophoresis.

RESULTS

Selection of amylose-defective mutants. Over 4,000 colonies were isolated on minimal medium after UV mutagenesis of the wild-type *Chlamydomonas* strain 137C. Colonies were then replica plated on nitrogen-free medium with acetate and incubated in the light. *C. reinhardtii* was previously shown to accumulate large amounts of starch when starved for many different essential elements (2). Nitrogen starvation was chosen because it also leads to destruction of chlorophylls, enabling immediate colony staining by iodine vapors. Under these conditions, wild-type colonies were revealed by their typical dark blue color. Mutant phenotypes fell into one of two distinct phenotypic classes (red or yellow). They appeared stable and were defective under all conditions tested whether in phototrophic (light only), mixotrophic (light and acetate), or heterotrophic (dark and acetate) growth conditions. The suspected mutants were further characterized by measuring starch amounts and monitoring their λ_{max} on absorption spectra after complexation with iodine. Yellow mutants turned out to be extremely low in starch (<5% of wild-type amounts) but displayed a λ_{max} with iodine similar to that of the wild-type polysaccharide. They were phenotypically very similar to the *st-1-1* mutant (3) and are thus probably either directly or indirectly defective in the supply of ADP-glucose. Red mutants were characterized by, on average, a very small increase in starch amounts (10% of wild-type quantities) and a considerable shift in λ_{max} of the iodine-starch complex (Table 1) which appeared similar to that described for amylose-defective (waxy) mutants of higher plants (33). The two red mutants selected (18B and 25B) were then crossed to strain 37, and segregants obtained after a random spore type of analysis were subjected to phenotypic characterization. The mutations showed clean Mendelian segregations, with no detectable linkage to other markers included in the cross. Segregants of opposite mating type carrying the starch-defective allele *st-2-1* or *st-2-2* derived from 18B and 25B, respectively, together with other suitable genetic markers were thus obtained in order to perform complementation tests. Recessivity of the *st-2-1* and *st-2-2* mutations was checked both in the initial cross and by backcrossing with the wild-type strain 137C. The *st-2-1* and *st-2-2* mutations belonged to a single complementation class and showed less than 1% recombination after meiosis.

Structural characterization of wild-type and mutant starch. Starch purified from our wild-type reference (137C) and *st-2-1*- and *st-2-2*-carrying strains were subjected to both methylation and gel filtration analysis (Table 1; Fig. 1 and 2; Table 2). Results obtained by using the TSK HW-75 (25) and Sepharose CL2B (33) columns were compared, and the conclusions were essentially the same except that TSK repeatedly (five different experiments were done, two of which are shown in Fig. 1 and 2) resolved the single heterogeneous wild-type amylopectin into two distinct peaks characterized by different sizes (Fig. 1D) and structural properties (Table 2). The polysaccharide recovered from peak 2 obtained by TSK HW-75 chromatography was recovered by a 30-min centrifugation at 40,000 × *g* and loaded on a TSK HW-75 column, on which it eluted at the expected position. It is clear that *st-2-1*- and *st-2-2*-carrying strains are characterized not only by a severe defect in amylose biosynthesis (both mutants accumulated between 1 and 4% amylose) but also by a change in the structure of amylopectin, as evidenced by a global shift of the λ_{max} of the polysaccharide-iodine complex of the amylopectin peak separated by CL2B chromatography (λ_{max} 565 nm). Formally identical but more demonstrative results were obtained by using a TSK column, whereby the amylopectin peak 2 was virtually lost in the mutants. Glucose amounts were measured in each peak by the amyloglucosidase assay (Fig. 2 and Table 3). We thus conclude that *ST-2* controls the biosynthesis not only of amylose but also of a substantial portion of the amylopectin.

Diploids heterozygous for either the *st-2-1* or *st-2-2* mutation displayed a twofold decrease amount of amylose relative to the wild-type haploid (Fig. 2 and Table 3) and were characterized by the replacement of amylopectin peak 2 by a single amylopectin peak (λ_{max} 550 nm).

Smaller-size material that was intermediate between amylose and amylopectin was detected in wild-type haploid (λ_{max} 600 nm) and heterozygous diploid (λ_{max} 585 nm) starch. This fraction amounted to 5% of total starch for the *st-2-2*/+ heterozygote and the wild-type haploid and up to 9% in *st-2-1*/+ diploids (as estimated by the amyloglucosidase assay).

All possible combinations of homozygous mutant diploids were constructed and yielded structures indistinguishable from the haploid mutants (not shown).

Results obtained by methylation analysis were confirmed by high-field ^{13}C NMR of the total polysaccharide fraction (Fig. 3). The major chemical shifts at 100.00, 78.9, 73.2, 71.9, 71.6, and 60.6 ppm observed in all samples can be attributed respectively to C-1, C-4, C-3, C-2, C-5, and C-6 of an $\alpha(1\rightarrow4)$ internal glucose, while the neighboring shifts at 100.66, 70.25, and 61.09 are essentially due to C-1, C-4, and C-6 in terminal nonreducing position (4). Shifts at 100.52 and 79.24 ppm are due to C-1 and C-4, respectively, of glucose residues which are proximal to a branching point. The NMR spectra of strains 18B and 25B clearly confirm the presence of additional branching points by comparison with our wild-type reference (137C), which in turn displayed a spectrum identical to that described for higher-plant starch (4).

To ascertain the consequences of the *st-2-1* mutation on the starch granule shape and size, electron micrographs were

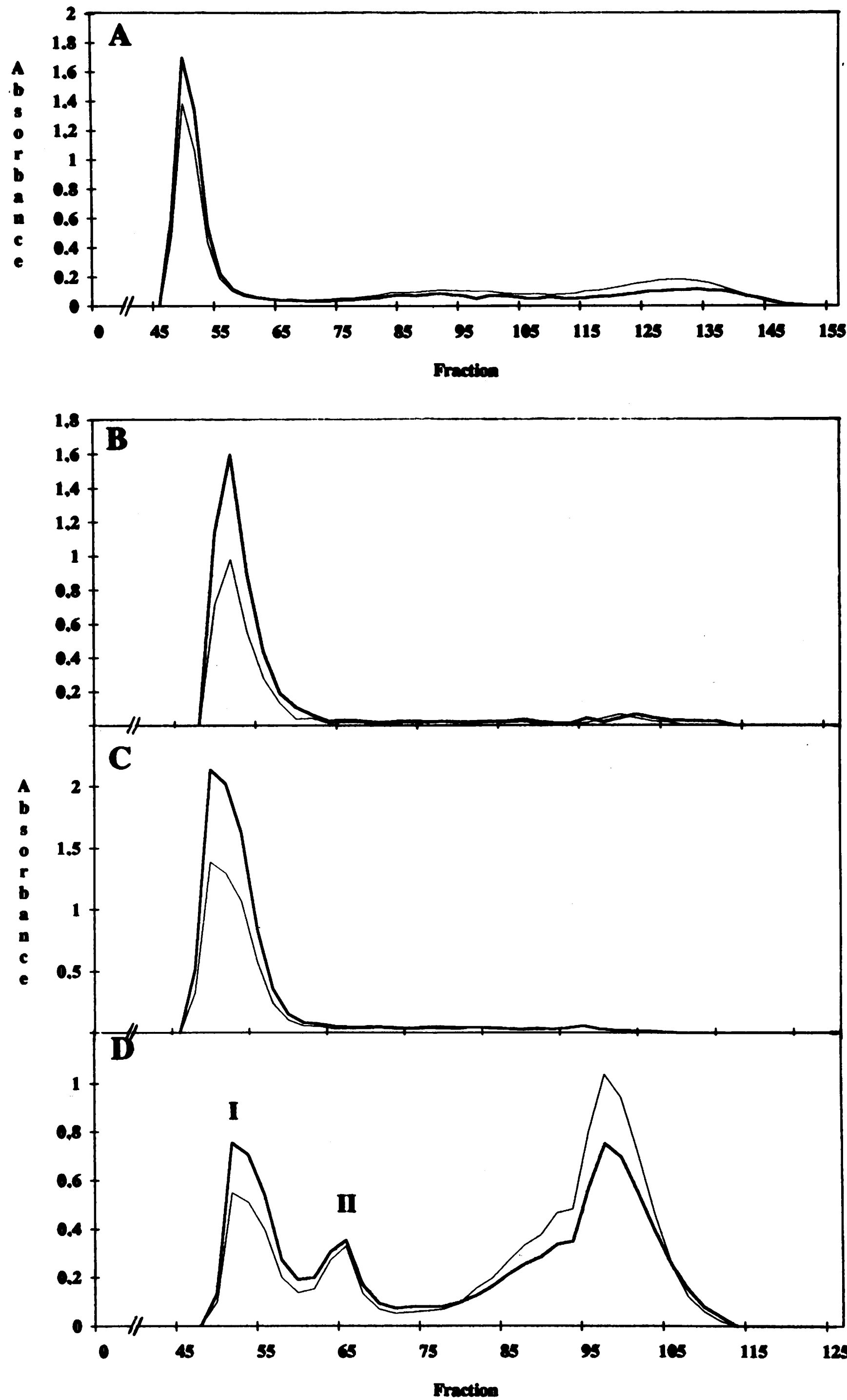

FIG. 1. Sepharose CL2B chromatography of wild-type starch (A) compared with TSK HW-75 (D) chromatography using columns of identical design (see Materials and Methods). The optical density was measured for each 3-ml fraction at 560 (thick line) and 630 nm (thin line) upon complexation with iodine. Amylose (λ_{max} > 610 nm) was dispersed on a total of 72 fractions of 3 ml, each using Sepharose CL2B (fractions 76 to 148), and amounted to 35% of the total amyloglucosidase digestible material. This value compares favorably with the 39% that was measured by using TSK HW-75 (Table 3) and that peaked in a total of only 20 fractions (87 to 107). Fractions 75 to 86 (λ_{max} ranging between 590 and 600 nm) cannot be classified as amylose. Experiments depicted in panels B to D were performed on the same TSK HW-75 column and show the chromatographic separation of starch extracted from *st-2-1* (strain 18B [B])- and *st-2-2* (strain 25B [C])-carrying mutants compared with the wild-type reference (strain 137C [D]).

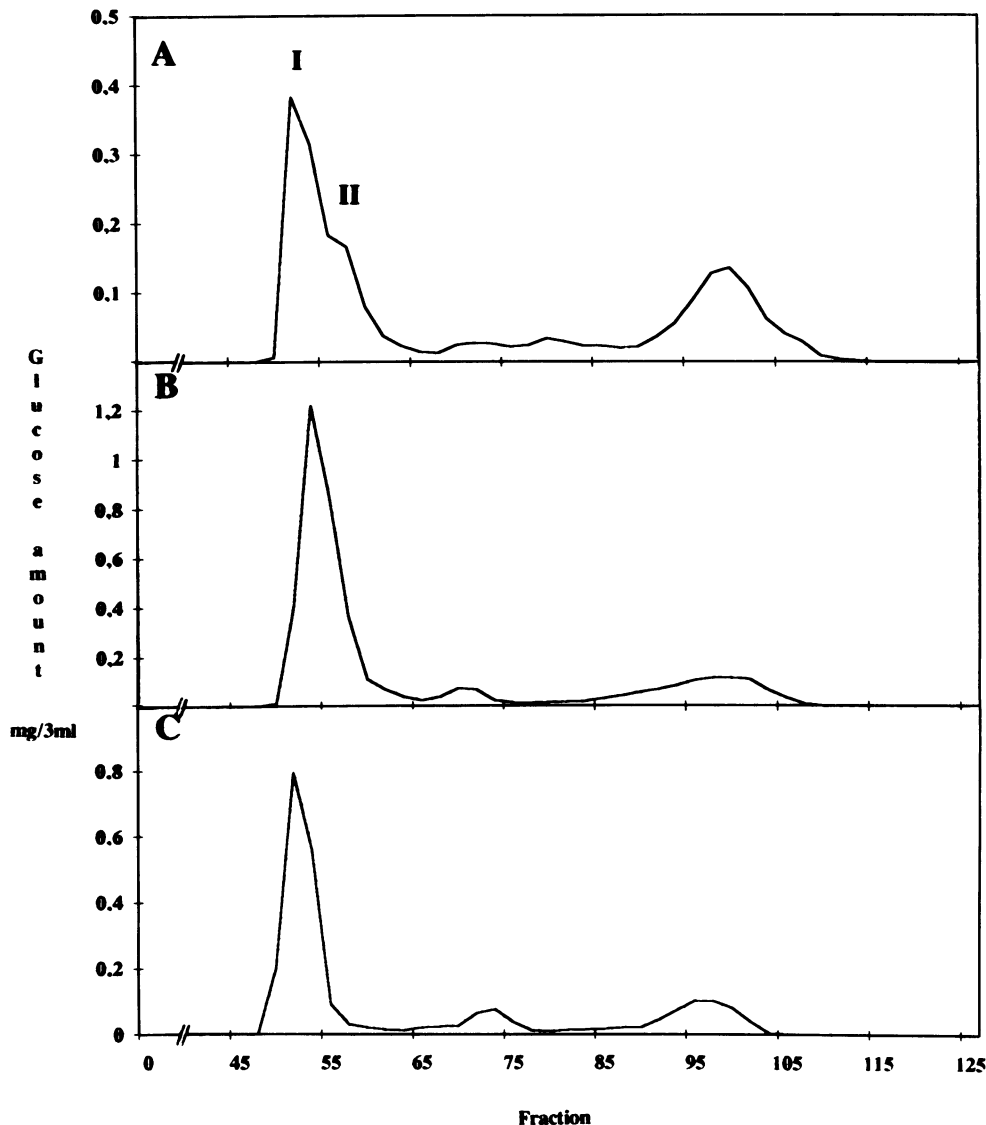

FIG. 2. TSK HW-75 chromatography of wild-type starch (A) compared with that of heterozygous *st-2-1*/+ (C) and *st-2-2*/+ (B) diploids. Glucose amounts were measured in each 3-ml fraction by the amyloglucosidase assay and are expressed in total milligrams contained per 3-ml fraction. All experiments were performed on the same column, which was different (although of identical design) from those used for the assay shown in Fig. 1.

TABLE 2. Structural properties of amylopectins and amylose purified by TSK HW-75 chromatography

Origin	Q^a	λ_{max} (nm)	Relative amt[b]	
			2,3,4,6-Glc	2,3,6-Glc
137C I[c]	35	555	0.9	21
137C II[c]	21	580	0.8	19
137C amylose	39	620	0.8	60
18B	>95	540	0.9	16
25B	>95	540	0.8	15

[a] Glucose amounts estimated as a weight percentage after fractionation of the starch by TSK HW-75 chromatography. Glucose was measured by the amyloglucosidase assay.
[b] See Table 1, footnote *b*.
[c] I and II represent the two amylopectin peaks present in strain 137C.

TABLE 3. Amylose content and characterization of granule-bound starch synthase from haploid and diploid wild-type and mutant strains

Strain	Amylose[a]	K_m ADP-glucose[b] (mM substrate)	V_{max}[c]
137C	39	3.7 ± 1.2	100
18B	<5	23.2 ± 1.7	24
25B	<5	4.1 ± 2.3	25
18B × 37	21	4.7	90
25B × 37	21	4.6	90

[a] Estimated as weight percentage from fractionation of the starch by TSK HW-75 chromatography. Glucose was measured by the amyloglucosidase assay.
[b] Mean ± SE from three separate experiments. K_m estimates from diploids originate from a single experiment.
[c] Percentage relative to the value for strain 137C. V_{max} of 137C is 3.7 nmol of ADP-glucose incorporated into glucan per min per mg of starch.

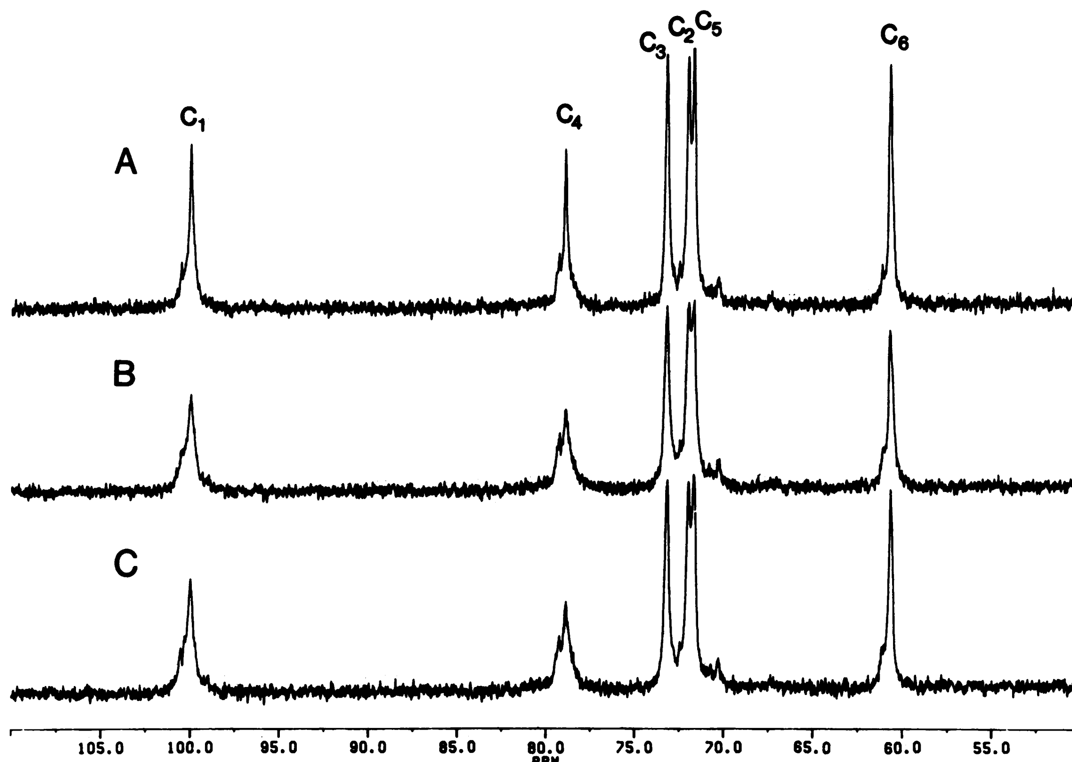

FIG. 3. ^{13}C NMR spectra at 100 MHz and 80°C in 90% $(CD_3)_2SO$ of 137C (A), 18B (B), and 25B (C) starches.

taken from the 18B and 137C strains (Fig. 4). Surprisingly, the morphology of pyrenoidal starch seemed to be dramatically affected by the defect, while stromal granules displayed an intermediate phenotype.

***st-2-1*- and *st-2-2*-carrying strains are characterized by a major defect in granule-bound starch synthase.** The analogy that could be drawn between *ST-2* and the higher-plant waxy mutants prompted us to assay granule-bound starch synthase in our amylose-defective strains. V_{max} and K_m values for ADP-glucose listed in Table 3 clearly confirm this analogy. Interestingly, we detect allele-specific variation of K_m values even for mutants with apparently identical polysaccharide structures. Soluble starch synthases were also assayed in mutant and wild-type cells. We were unable to detect any qualitative (activity in the absence of primer in the presence of 0.5 M citrate) or quantitative differences between the strains.

***st-2-1* and *st-2-2* can be correlated with allele-specific modifications of the main (waxy) 76-kDa protein associated with the starch granule.** Waxy mutants in higher plants are often correlated with modifications in the electrophoretic pattern of proteins associated with the starch granule. The major granule-bound protein present in wild-type starch has been named the waxy protein and is usually absent or greatly diminished in waxy mutants (5). In very few cases, minor alterations in molecular weight of the waxy protein have been reported and were taken as initial evidence that the waxy locus encoded this protein (21). Since then, cloning and sequencing of the maize locus (10, 23) have largely confirmed this hypothesis. Our experiments (Fig. 5) show that in *C. reinhardtii*, there is a major 76-kDa protein that is greatly diminished in *st-2-2*-carrying strains and is replaced in *st-2-1* mutants by a 56-kDa polypeptide which is present in amounts similar to that of the wild-type 76-kDa protein. Both proteins were characterized by identical N-terminal amino acid sequences (unpublished data). By analogy with higher plants, we will call these waxy proteins. Other minor proteins (at 55 and 66 kDa) could also be observed during these experiments and were not affected by the mutations.

Allele-specific K_m variations of granule-bound starch synthase cosegregate in crosses with the 76- and 56-kDa waxy proteins. Allele-specific variation of K_m values for mutants with apparently identical polysaccharide structures gives us a unique opportunity to test whether granule-bound starch synthase kinetic parameters and molecular weight of the waxy protein are linked phenotypes. On a total of 10 different meiotic products obtained in a cross between strains B20 and 25B, all five strains carrying the 56-kDa protein were characterized by K_m values ranging between 25 and 20 mM ADP-glucose, while the five segregants carrying the 76-kDa protein displayed K_m values ranging between 1.5 and 4 mM ADP-glucose, establishing that both phenotypes are genetically linked. The diminished amount of 76-kDa protein present in the *st-2-2*-carrying segregants (25% of the wild-type) was tightly correlated with the decrease in total granule-bound activity, suggesting that the apparently normal enzyme is present in lesser quantities in the starch of these mutants.

DISCUSSION

Waxy mutations have been described in higher plants that affect amylose biosynthesis only in starch accumulated in nonphotosynthesizing plastids: the amyloplasts. Only in one well-documented case, in potato, has an effect been scored in microspores (9), in columella cells of the root cap, and in

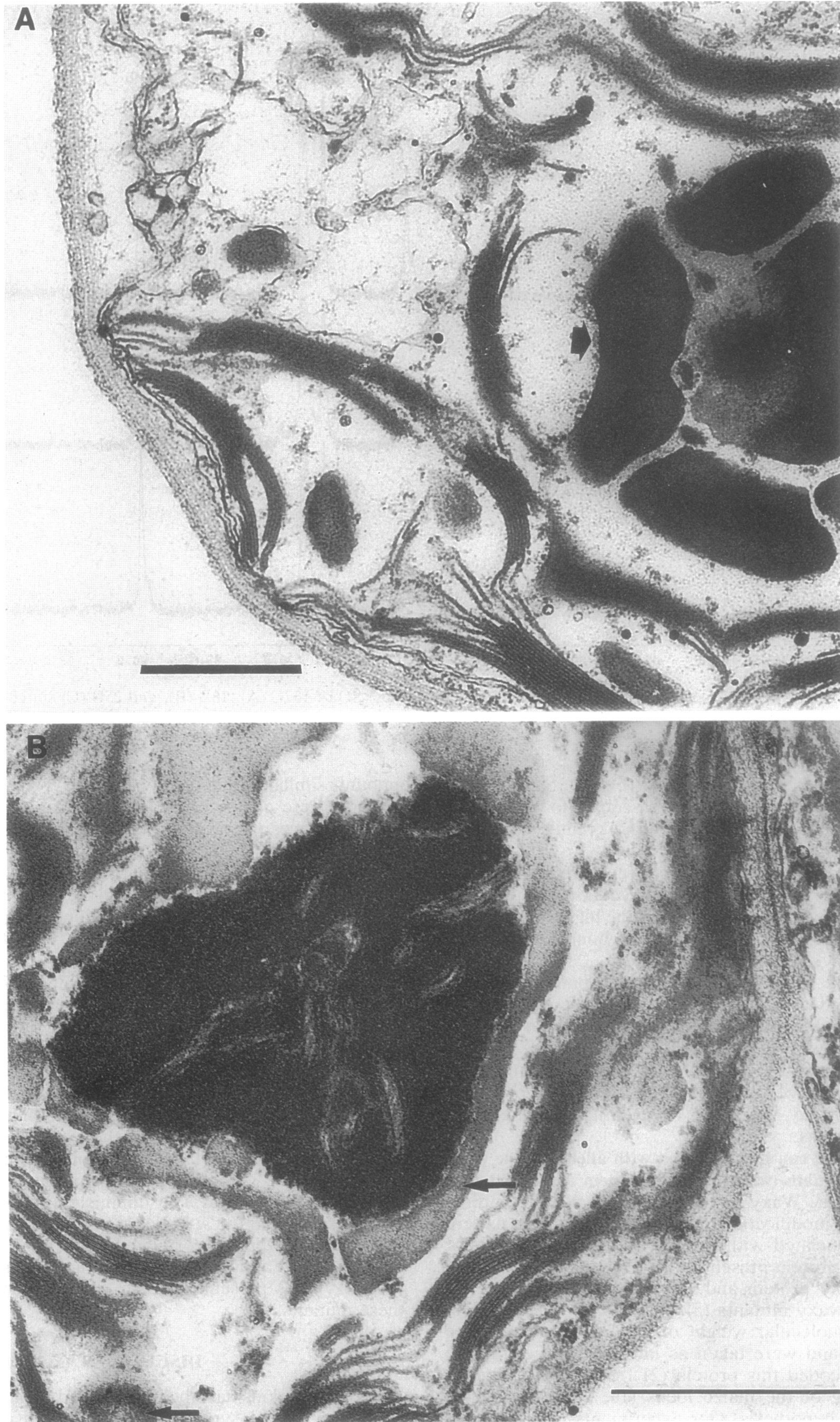

FIG. 4. Typical pyrenoid from wild-type strain 137C (A) compared with that of 18B, an *st-2-1*-carrying strain (B). The latter is surrounded by a highly disorganized crown of starch granules, in sharp contrast to the classical compact structure displayed by *Chlamydomonas* wild-type starch (2). The starch granules are shown by arrows. Bars = 1 μm.

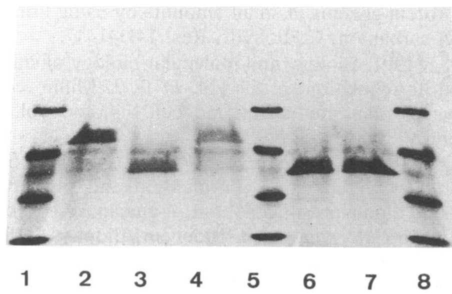

FIG. 5. Coomassie blue stain of an SDS-5 to 15% acrylamide gel of total proteins extracted from 5 (lanes 2 to 4) or 10 (lanes 6 and 7) mg of starch granule preparations. Lane 2 is a wild-type sample; lanes 3 and 6 (strain 18B) and lane 7 (B20) are samples from *st-2-1*-carrying strains; lane 4 (strain 25B) is a sample from an *st-2-2*-carrying strain. Lanes 1, 5, and 8 are molecular size standards (from top to bottom, 94, 67, 43, and 30 kDa).

some leaf cells which was not in all cases correlated with the phenotype present in the tubers (27, 29). However, all of these findings were based on simple cytological observations, with no purification of the starch. Our results for *C. reinhardtii* clearly suggest that waxy-related gene products controls the biosynthesis of amylose in all types of plastids and starch-accumulating eukaryotes during both autotrophic and heterotrophic growth. The absence of amylose which typifies waxy mutants has led to the widespread view that the remaining polysaccharide is pure amylopectin. However, when one takes a closer glance at the λ_{max} of the iodine-polysaccharide complex published for wild-type and waxy maize amylopectin after Sepharose CL2B chromatography (33), a dramatic shift (from 540 to 480 nm) can be seen together with a loss of apparent heterogeneity of the reported values. We have observed an analogous phenomenon in *C. reinhardtii* by using CL2B chromatography. Moreover, we have systematically observed a separation of the single heterogeneous amylopectin peak into two distinct peaks. Both of these peaks can be characterized as amylopectin by their size and number of branching points (as seen by methylation analysis). It is clear that amylopectin peak 2 is missing both in *st-2-1-* and *st-2-2*-carrying strains and in heterozygote diploids. The mutant amylopectin seems also to differ from wild-type peak 1. Thus, it appears that in addition to the synthesis of amylose, the waxy gene product controls, at least in *Chlamydomonas* spp. and more likely in all plants, that of an important fraction of the wild-type amylopectin. The waxy locus has been cloned from barley (19) and completely sequenced in maize (10, 23), and a waxy cDNA has also been obtained from *Solanum tuberosum* (28). All of these sequences have been cloned by using as the starting point the main granule-associated protein that is missing or altered in the mutants. That this waxy protein is the product of the waxy gene and that this locus is responsible for the presence or absence of amylose are thus beyond doubt.

That the waxy protein might harbor ADP-glucose glycosyltransferase activity was recently further suggested by comparing the *E. coli* glycogen synthase protein with the waxy products of maize and barley (15). However, the reported homology is on the whole very weak and confined to the N terminus. That this homology is still significant is suggested by conservation of the putative ADP-glucose binding site (15).

What at this point remains a matter of debate is whether this protein is or is not the main granule-bound starch synthase. In fact, the absence of activity in the mutants and the proportional increase in activity with respect to the wild-type allele dose in gene dosage experiments (26) can be easily explained if one assumes that the main granule-bound starch synthase binds to amylose without being the product of the waxy gene. This argument, first suggested by Preiss and Levi (17), remains valid even in face of the recently reported complementation of the *amf* (an equivalent of waxy) mutation of potato by the wild-type gene encoding the major granule-bound protein (27). The problem with testing this hypothesis is that even modifications in the kinetics of the activity can be attributed to structural environment modifications in the granule and not necessarily to the enzyme itself. It seemed that the only way to get around this argument would be to solubilize the activity and determine whether it copurifies with the waxy gene product. This has been achieved in only three cases and with uncertain or conflicting results (13, 24, 30). In maize, two starch synthases were solubilized from the granule and displayed distinct immunological and biochemical properties, one of which had a molecular mass (60 kDa) similar to that of the waxy protein. Eighty percent inhibition was scored on this solubilized starch synthase activity with use of an antibody elicited against whole granule-bound proteins (13), while 20 to 40% inhibition was reported on the native soluble enzymes. However, both granule-bound activities appeared diminished in waxy mutants. Results obtained by Vos-Scheperkeuter et al. (30) are even more confusing since these authors were unable to solubilize the activity from potato starch granules and relied on partial inhibition of the amaranth solubilized enzyme by an antibody prepared, this time, against the purified waxy protein from potato (and not whole granule-bound proteins). In the latest report (24), a 59-kDa waxy protein was identified in pea by cross-reaction with the same antibody elicited against the waxy protein of potato. No inhibition of the activity was found with use of this antibody, which recognized the main 59-kDa granule-bound protein from pea. However, an antibody raised against a 77-kDa protein associated with pea starch granules that copurified with the major and possibly only solubilized activity did not recognize any of the waxy proteins.

In this paper, we report allele-specific variation of K_m for ADP-glucose of granule-bound starch synthase not only between wild-type and mutant granules (which could be accounted for by a modification in structure of the starch) but also between strains carrying mutant alleles, leading to similar starch structures. The latter could be seen to cosegregate in crosses with waxy proteins of distinct molecular weights. Since K_m for ADP-glucose is an intrinsic property of granule-bound starch synthases and since waxy proteins display some similarity to the *E. coli* enzyme (15), our observations cannot be easily accounted for by any hypothesis other than that stating that the waxy or *ST-2* genes encode the major granule-bound starch synthase.

The absence of the amylopectin peak 2 fractions in our *st-2-1-* and *st-2-2*-carrying mutants suggests that granule-bound starch synthase is able to elongate chains from a nonreducing end available on an amylopectin molecule to generate peak 2. Leloir et al. (12) initially showed that granule-bound starch synthase is able in vitro to incorporate glucose into the amylopectin fraction under high concentrations of the nonphysiological substrate UDP-glucose. These results were confirmed by Baba et al. (1) in their in vitro synthesis experiments using the natural substrate ADP-

glucose at concentrations closer to what is generally thought as physiological.

Our results establish a major role for granule bound starch synthase in vivo in the biosynthesis of what is usually defined as amylopectin either by direct synthesis or by branching of amylose. That all amylose is synthesized directly from amylopectin remains in this respect an interesting working hypothesis.

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