Starch Synthesis in Arabidopsis. Granule Synthesis, Composition, and Structure[1]

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The aim of this work was to characterize starch synthesis, composition, and granule structure in Arabidopsis leaves. First, the potential role of starch-degrading enzymes during starch accumulation was investigated. To discover whether simultaneous synthesis and degradation of starch occurred during net accumulation, starch was labeled by supplying $^{14}CO_2$ to intact, photosynthesizing plants. Release of this label from starch was monitored during a chase period in air, using different light intensities to vary the net rate of starch synthesis. No release of label was detected unless there was net degradation of starch during the chase. Similar experiments were performed on a mutant line (*dbe1*) that accumulates the soluble polysaccharide, phytoglycogen. Label was not released from phytoglycogen during the chase indicating that, even when in a soluble form, glucan is not appreciably degraded during accumulation. Second, the effect on starch composition of growth conditions and mutations causing starch accumulation was studied. An increase in starch content correlated with an increased amylose content of the starch and with an increase in the ratio of granule-bound starch synthase to soluble starch synthase activity. Third, the structural organization and morphology of Arabidopsis starch granules was studied. The starch granules were birefringent, indicating a radial organization of the polymers, and x-ray scatter analyses revealed that granules contained alternating crystalline and amorphous lamellae with a periodicity of 9 nm. Granules from the wild type and the high-starch mutant *sex1* were flattened and discoid, whereas those of the high-starch mutant *sex4* were larger and more rounded. These larger granules contained "growth rings" with a periodicity of 200 to 300 nm. We conclude that leaf starch is synthesized without appreciable turnover and comprises similar polymers and contains similar levels of molecular organization to storage starches, making Arabidopsis an excellent model system for studying granule biosynthesis.

The Arabidopsis leaf is an excellent system in which to study starch granule biosynthesis for several reasons. First, starch accumulates in large amounts over a short period; up to one-half of the carbon assimilated through photosynthesis is stored as starch during the light period. As a consequence, it is possible to analyze the composition and structure of starch made over a period of a few hours by a defined set of enzymes. In contrast, starch synthesis in storage organs occurs over a long developmental period, during which there are usually considerable changes in the complement of starch-synthesizing enzymes (Smith and Martin, 1993; Burton et al., 1995) and in overall cellular conditions. Second, the rate of starch synthesis in leaves can be controlled by altering the irradiance and measured accurately by supplying $^{14}CO_2$. Third, our knowledge of the complete genome sequence of Arabidopsis and the availability of transposon and T-DNA-tagged populations enables specific knockout mutations to be obtained for all of the putative enzymes of starch synthesis and degradation (Thorneycroft et al., 2001). Despite the suitability of leaf starch as a model system, relatively little is known about its synthesis, composition, and structure, compared with starches from storage organs. To address this, we have studied three major aspects of the synthesis of Arabidopsis starch where differences between leaves and storage organs have been reported, or might be expected.

First, we investigated whether leaf starch is subject to turnover during its synthesis. Turnover (the simultaneous occurrence of synthesis and degradation) may be expected to affect both the amount and nature of the starch. However, it is not known whether such turnover occurs. In storage organs, where starch synthesis and starch degradation usually occur in different developmental phases, the enzymes of starch degradation may not be present during the phase of starch accumulation. The only reported example of turnover in storage organs is in transgenic potatoes (*Solanum tuberosum*) in which the flux of carbon into starch was increased 6-fold by elevating ADP-Glc pyrophosphorylase activity (Sweetlove et

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al., 1996). However, little, if any, turnover was observed in the wild-type tubers. This is consistent with earlier findings (Dixon and ap Rees, 1980). In contrast to storage organs, leaf starch is remobilized each night and the enzymes responsible for starch degradation are present and may be active in the chloroplast during starch synthesis in the day. Under some conditions, starch degradation has been shown to occur in leaves during the light (Fondy et al., 1989; Servaites et al., 1989; Hausler et al., 1998). However, it is not clear whether any degradation occurs simultaneously with synthesis. To study this, we conducted ^{14}C pulse-chase labeling experiments to determine whether label incorporated during the pulse was released during the subsequent chase period.

Second, we examined factors that influence amylose content in leaf starch. Estimates of amylose content for leaf starch are scarce. Typically, values of approximately 15% or less have been found, whereas most storage starches contain between 20% and 30% amylose. In wild-type Arabidopsis leaves, the amylose content of the starch is low (Zeeman et al., 1998b), but in starch-excess mutants, increased amylose contents have been reported (Critchley et al., 2001; Yu et al., 2001). We have established a robust method for the measurement of amylose content of Arabidopsis starch and used this method to investigate the conditions of amylose synthesis in wild-type leaves and in starch-excess mutant lines.

Third, we investigated starch granule size, shape, and structure in leaves. Granules from leaves are generally reported to be very small (Badenhuizen, 1969) and discoid, whereas those from many storage organs are larger (typically 15–100 μm; Jane et al., 1994) and roughly spherical or oval in shape. Granules of storage starches possess two main levels of internal structure, created by the organization of amylopectin molecules (French, 1984; Jenkins et al., 1993). Alternating, concentric crystalline and amorphous lamellae with a periodicity of 9 nm make up semicrystalline zones. These alternate with amorphous zones with a periodicity of a few hundred nanometers. Although there are indications that leaf starch granules contain at least some crystalline structures (Buttrose, 1963; Waigh, 1997), it is not known to what extent they possess the levels of organization seen in storage starches.

RESULTS

Starch Turnover

Starch Synthesis Is Not Accompanied by Significant Turnover

To discover whether starch turnover occurs during periods of starch accumulation in Arabidopsis leaves, we performed pulse-chase experiments. A pulse of $^{14}CO_2$ was supplied to photosynthesizing wild-type plants and the incorporation of label into starch measured. The $^{14}CO_2$ was then removed and the plants maintained for a chase period of 5 h in the light in air. After the chase, the label in starch was measured again to determine whether any of the starch made during the pulse had been degraded. We found that none of the ^{14}C incorporated during the pulse was released during the chase (Table I). However, the rate of starch synthesis during the chase was high. We reasoned that the starch labeled during the pulse might rapidly be buried by newly synthesized starch during chase, perhaps rendering it inaccessible to degradative enzymes. This would restrict the release of ^{14}C during the chase if turnover occurred only on newly synthesized starch. To reduce the rate of burial of labeled starch during the chase, and thus increase the chances of detecting any turnover, the experiment was repeated but with a large reduction in light intensity at the end of the pulse to limit the rate of starch synthesis during the chase. Although the rate of starch synthesis was reduced by this treatment, there was still no detectable release of ^{14}C from starch during the pulse (Table I). In a further experiment, light intensity after the pulse was lowered to a point at which starch content during the chase showed a decline rather than an increase. In this case, as expected, there was a significant loss of ^{14}C from starch during the chase (Table I).

Table I. *The distribution of ^{14}C in Arabidopsis leaves during pulse and chase experiments*

Plants were supplied with $^{14}CO_2$ (400–600 μL L^{-1}, 1.25–1.88 MBq $mmol^{-1}$, and 170 μmol photons m^{-2} s^{-1}) for either 0.5 or 1 h. The $^{14}CO_2$ was then removed and the plants allowed to photosynthesize in air for a further 5 to 6.5 h. Samples were harvested and killed in boiling 80% (v/v) ethanol (wild type) or frozen in liquid N_2 (*dbe1*). Starch content and the distribution of label were determined as described in "Materials and Methods." Values are the mean ± SEs of four replicate samples, each comprising the leaves of a single plant (n.d., not determined).

Plant Material	Light Regime during Chase	Rate of Starch Synthesis during Chase	Length of Pulse + Chase	^{14}C Glucan after Pulse	^{14}C in Glucan after Chase
	μmol photons m^{-2} s^{-1}	*mg h^{-1} g^{-1} fresh wt*	*h*	*dpm × 1,000*	
Wild type	170	0.52	1 + 5	562 ± 26	566 ± 44
Wild type	80	0.36	0.5 + 6.5	275 ± 12	375 ± 38
Wild type	40	−0.18	0.5 + 6.5	394 ± 38	193 ± 46
*dbe*1	170	n.d.	1 + 5	Starch: 44 ± 5; phytoglycogen: 352 ± 5	Starch: 47 ± 5; phytoglycogen: 361 ± 26

Phytoglycogen Synthesis Is Not Accompanied by Significant Turnover

Failure to observe loss of label from starch granules during a chase period does not necessarily imply that starch-degrading enzymes are inactive during the light period. It is possible that once in a semicrystalline, granular form, the glucan is no longer susceptible to attack from most enzymes. We reasoned that a soluble α-1,4-, α-1,6-linked glucan might be more sensitive to the actions of starch-degrading enzymes during its synthesis than starch. Therefore, we performed similar pulse-chase experiments on the Arabidopsis mutant *dbe1*, which lacks an isoform of the debranching enzyme isoamylase (Zeeman et al., 1998b). This mutant accumulates the soluble, highly branched glucan phytoglycogen, which does not form semicrystalline granules but remains soluble in the stroma of the chloroplast. It is accumulated together with small amounts of starch during photosynthesis and degraded during the subsequent dark period. During the pulse, starch and phytoglycogen were labeled in the ratio 5:1, reflecting the relative rates of synthesis of the two glucans in *dbe*1 leaves (Zeeman et al., 1998b). No ^{14}C was lost from either starch or phytoglycogen during the chase (Table I).

Amylose Content

Measurement of the Amylose Content of Leaf Starch

To investigate the amylose content of leaf starch, solubilized starch was fractionated by gel permeation chromatography (GPC) on a column of Sepharose CL2B (Fig. 1). Starch from wild-type plants eluted as two peaks: an initial amylopectin-containing peak, with a wavelength of maximal absorbance when complexed with iodine (λ_{max}) of 550 nm, and a second amylose-containing peak with a λ_{max} of 585 nm. The λ_{max} value for the amylose peak is substantially lower than that reported for amylose from other species (λ_{max} usually greater than 600 nm), suggesting either that amylose from Arabidopsis leaves is more branched than that from other species, or that the amylose peak contains branched glucans in addition to amylose. Two approaches were taken to distinguish between these possibilities. First, fractions from the amylose peak from wild-type starch were pooled and subjected to butanol precipitation, a treatment that precipitates linear but not branched glucans. The λ_{max} of the precipitated material was 620 nm. Second, the fractionation was repeated with starch from the *sex1* mutant of Arabidopsis, a starch-accumulating mutant in which the starch has a high amylose content (Yu et al., 2001). The λ_{max} of the amylose peak from this mutant was 620 nm.

These results suggest that the amylose from Arabidopsis starch is similar to that found in storage starches. The amylose-containing peak in the wild type consists of both amylose with a λ_{max} of 620 nm and branched glucans with a λ_{max} similar to that of amylopectin, leading to an overall λ_{max} of 585 nm. The higher λ_{max} of the amylose-containing peak from *sex1* starch reflects the fact that most of the material in the peak is amylose. In both samples, the λ_{max} of the glucan tail following the amylose peak fell to values approaching that of amylopectin, further indicating the presence of small amounts of branched glucan in these fractions.

Using GPC to examine the amylose content of starch yields useful qualitative information. However, due to the presence of the small amounts of branched material in the amylose-containing fractions, it was not possible to use this method to quantify accurately the amylose content, particularly in samples containing little amylose. Therefore, we established a separate method for determining the amylose content based on the different iodine-binding capacities of the two polymers (Hovenkamp-Hermelink et al., 1988). Pure amylose and amylopectin were prepared from a bulk preparation of starch, derived from the wild type and starch-excess mutant lines, using Sepharose CL2B chromatography followed by butanol precipitation.

Standard curves of the absorbance of the iodine-polymer complexes were used to generate the following equation to calculate amylopectin to amylose ratios from mixed samples:

$$\text{Percentage amylose} = (3.039 - 7.154(A_{700}/A_{525}))/(3.048(A_{700}/A_{525}) - 19.129)$$

The wavelengths 700 and 525 nm were used to give a wider range of ratios than possible when using the λ_{max} for amylose and amylopectin. The calculated relationship between amylose content and the ratio of A_{700} to A_{525} is shown in Figure 2. Mixtures of purified amylose and amylopectin gave the predicted A_{700} to A_{525} ratios. We then used this method and GPC to investigate factors influencing the amylose content of Arabidopsis starch.

The Amylose Content of Starch Is Related to Leaf Starch Content

To discover how the amylose content of starch related to the pattern of starch synthesis and the starch content of the leaf, we investigated the amylose contents of starches from leaves with different starch contents—either wild-type leaves kept in the light for extended periods, or leaves from mutant plants with lesions affecting the pathway of starch degradation. First, we measured the amylose content of starch from batches of wild-type plants grown in controlled conditions. At the end of a 12-h photoperiod, the amylose content of the starch was 6% ± 1.7% (n = 4, mean ± SE). When a batch of wild-type plants was transferred from normal light-dark con-

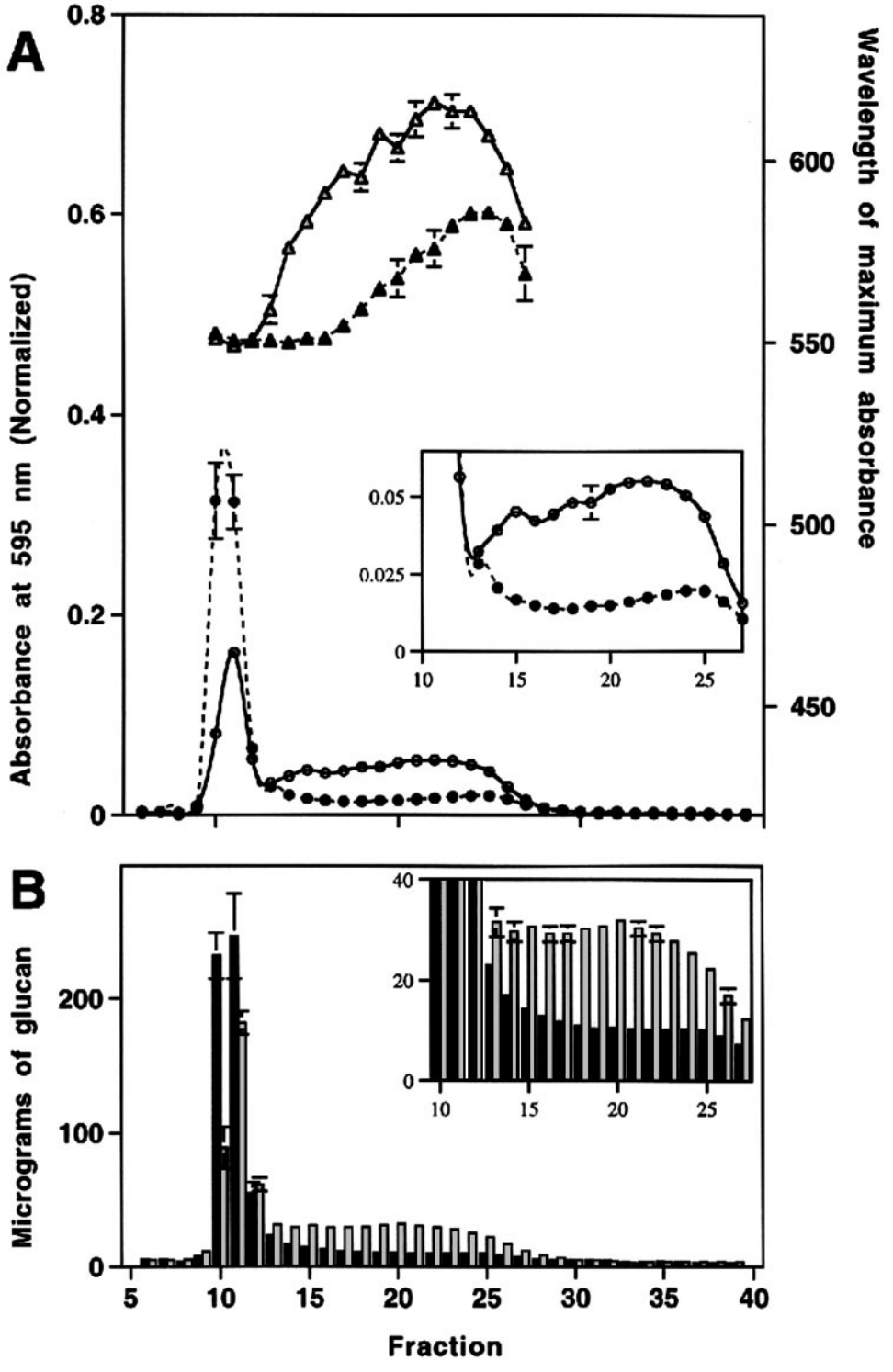

Figure 1. Separation of amylose and amylopectin fractions of Arabidopsis starch using Sepharose CL2B chromatography. Starch from the wild type (black symbols) and the mutant line *sex1* (white symbols) was isolated from batches of 200 plants harvested at the end of the photoperiod. Samples of this starch were solubilized and applied to the column. Values are the means ± SEs of three samples. A, Fractions were analyzed to determine the absorbance of the glucan-iodine complex at 595 nm (circles; inset; *y* axis enlarged for clarity). The absorbances were summed and each then divided by the total to give a normalized trace. The wavelength of maximum absorption of the glucan-iodine complex (λ_{max}; triangles) was also determined for each sample. B, The glucan content of each fraction was determined by treatment with amyloglucosidase and α-amylase and measurement of released Glc (inset; *y* axis enlarged).

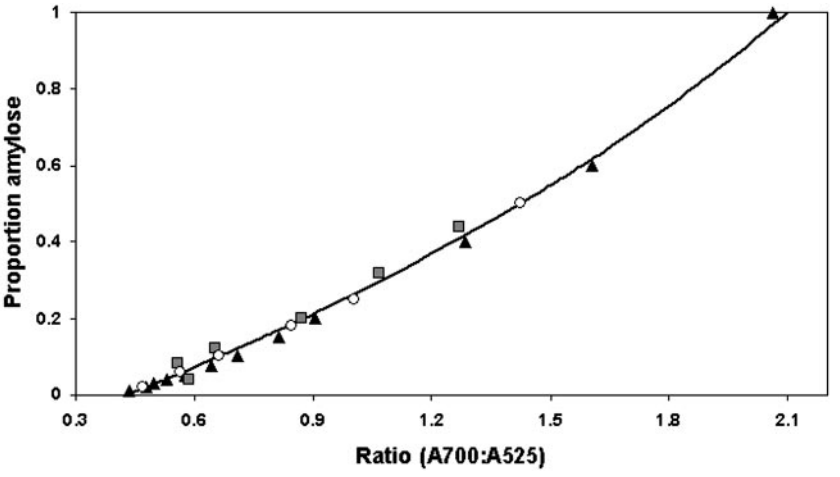

Figure 2. Relationship between the percentage of amylose and the ratio of the absorbance of the glucan-iodine complex at 700 and 525 nm. Known amounts of purified amylose or purified amylopectin from Arabidopsis were dissolved, mixed with iodine solution, and the absorption spectra for the polymer-iodine complex established. The theoretical relationship between the absorbance at 700 and at 525 nm was calculated for mixtures of the two polymers (solid line). This relationship was tested by measuring the absorbance ratios of different mixtures of amylose and amylopectin containing 5 (squares), 10 (triangles), and 20 (circles) μg of total glucan.

ditions to continuous light, they accumulated starch to very high levels (Fig. 3A). The amylose content rose from 4% after 12 h (the start of the extended light period) to 13% after 84 h, 20% after 180 h, and 25% after 220 h in the light. GPC analysis confirmed the increase in the low-M_r, amylose-containing fractions (Fig. 3B).

Two starch degradation mutants were also used in this study. The *sex1* mutant lacks a homolog of the potato R1 protein, involved in the phosphorylation of starch (Yu et al., 2001), whereas the *sex4* mutant is deficient in chloroplastic endoamylase (Zeeman et al., 1998a). The starch content of leaves of *sex1* and *sex4* is much higher than in the wild type (5- and 3-fold, respectively; Trethewey et al., 1994; Zeeman et al., 1998a). When harvested at the end of a normal photoperiod, the starch from *sex1* and *sex4* contained 21% $\pm$ 2.5% (n = 3) and 33% $\pm$ 7% (n = 3) amylose, respectively (see also Fig. 3C).

There is a gradual accumulation of starch in *sex1* and *sex4* leaves during development (Zeeman and ap Rees, 1999). In wild-type plants, leaves of all ages contain a similar amount of starch at the end of the day; in the mutants, the oldest leaves contain the most starch, whereas the youngest, developing leaves contain little or no more than the wild type. To determine whether the high-amylose starch is synthesized in all tissues in the *sex* mutants, we extracted starch from different-aged leaves of wild-type and *sex4* plants and determined the amylose content (Table II). The amylose content of the starch from the wild type was low in all leaves irrespective of age, whereas in *sex4* the amylose content increased as the leaves aged, correlating with the increase in starch content.

Granule-Bound Starch Synthase (GBSS) Content of Leaf Starch

We investigated whether the different amylose contents of the starches described above may be attributable to different contents of the starch synthase isoform responsible for amylose synthesis, GBSS. The identity of the GBSS protein on SDS-polyacrylamide gels of granule-bound proteins from leaf starch was established by matrix-assisted laser-desorption ionization (MALDI)-time of flight mass spectroscopy. Tryptic fragments of a major protein of 59 kD (the predicted molecular mass of the mature GBSS protein encoded in the Arabidopsis genome, chromosome locus At1g32900) were analyzed. Comparison of the pattern of peptides using the MASCOT search engine (Matrix Science; http://www.matrixscience.com/cgi/index.pl?page=/search_intro.html) confirmed that this protein was GBSS (probability-based Mowse score 215, coverage of fragments 33%).

Coomassie Blue-stained gels of granule-bound proteins revealed that the GBSS content of starch from *sex1* was slightly greater than that of the wild type, whereas that of *sex4* was slightly lower (Fig. 4A). However, to determine the GBSS content of the leaves on a fresh weight basis, proteins derived from the insoluble material of leaves were separated and analyzed by immunoblotting, using an antibody raised to the pea embryo GBSS. This antibody recognized a single, 59-kD band on the blots and densitometry measurements of the blot revealed a linear relationship between the intensity of the band amount of sample loaded (Fig. 4B). Immunoblots of replicate samples of insoluble material from wild-type, *sex1*, and *sex4* leaves were then performed, revealing that the GBSS content of both *sex1* and *sex4* was greater than the wild type on a fresh weight basis (Fig. 4C). Densitometry readings of this blot revealed that compared with the wild type, *sex1* and *sex4* leaves had 5- and 2-fold increase in GBSS content, respectively.

Granule Size, Shape, and Structure

Scanning electron microscopy of starch granules from wild-type leaves showed that they were irregularly discoid in shape and increased significantly in size when plants were kept in continuous light for long periods (Fig. 5). At the end of a normal photo-

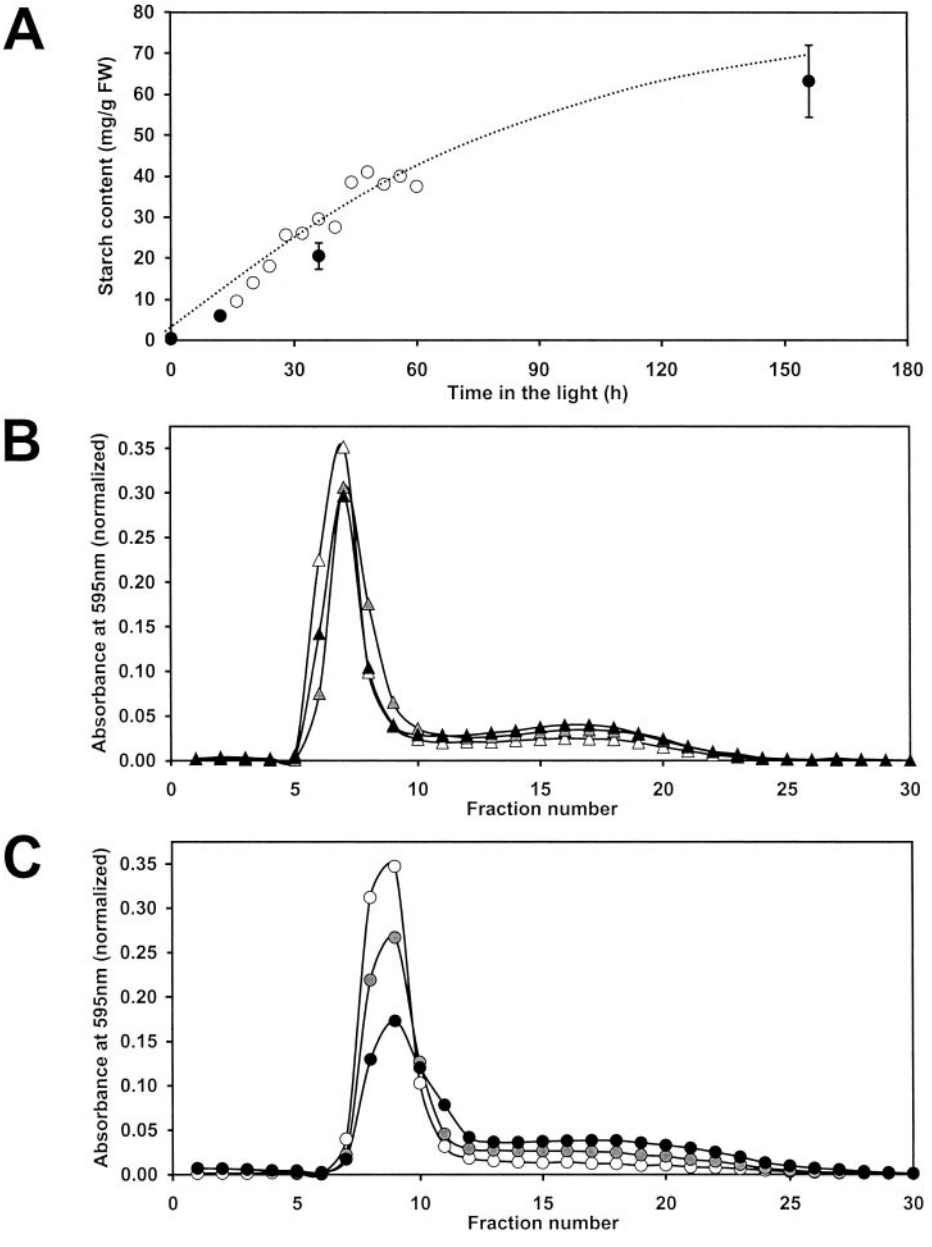

Figure 3. Influence of conditions of leaf starch synthesis on the amylose content of the starch. A, Wild-type plants were transferred from a diurnal light regime to continuous light and the starch content measured at intervals. Four plants were harvested and treated as one sample (white symbols). The results correspond well to data from a similar experiment conducted previously (black symbols; Critchley et al., 2001). B, Amylose and amylopectin were separated by Sepharose CL2B chromatography from starch extracted from plants after 12 (white triangles), 84 (gray triangles), and 180 (black triangles) h in the light. The absorbance of the glucan-iodine complex at 595 nm was determined. C, Amylose and amylopectin from starch extracted from wild-type (white circles), *sex1* (gray circles), and *sex4* (black circles) plants at the end of a normal photoperiod, as described in B.

period, granules were approximately 1 to 2 μm in diameter and 0.2 to 0.5 μm thick. After 180 h in continuous light, they had increased to approximately 2 to 3 μm in diameter and 0.4 to 0.6 μm thick.

To look for factors that influence granule size and shape, we examined starch from the starch-excess mutants *sex1* and *sex4*. Granules from *sex1* were larger, but similar in shape to the wild type (Fig. 5D). However, granules of *sex4* were strikingly different from wild-type granules in that they were much larger in both diameter (up to 6 μm) and thickness (1–4 μm) and more were more regular in outline (Fig. 5E). In this respect, the *sex4* granules resembled starch from storage organs. We investigated whether the alteration

Table II. *The amylose content of leaves of different ages of wild-type and sex4 leaves*

Four plants of the wild type and four of *sex4* were harvested at the end of the day and the leaves divided into six fractions. Fraction 1 comprised the three youngest leaves (not analyzed); fraction 2, the next three youngest leaves; and so on. Fraction 6 contained all the remaining, oldest leaves of the plant. Starch was extracted from each fraction and the amylose content determined using the iodine-based method described in "Materials and Methods."

Fraction	Amylose	
	Wild type	*sex4*
	%	
2	1	4
3	1	8
4	<1	6
5	<1	17
6	<1	34

in the size and shape of granules in the *sex4* mutant was correlated with changes in the chain length distribution of amylopectin. The shorter chains of amylopectin from Arabidopsis and pea leaf starch show a much more pronounced polymodal distribution of lengths than those of storage starches (Tomlinson et al., 1997; Zeeman et al., 1998a). We found that amylopectin from *sex4* had increased numbers of chains between six and 11 Glc residues in length and fewer between 19 and 29 residues compared with wild-type amylopectin (Fig. 6). However, these differences were small and the chain length distribution still showed the characteristic leaf-type profile.

When viewed under polarized light, large starch granules from Arabidopsis were birefringent, giving a typical "Maltese cross" pattern (Fig. 7). This indicates a high degree of radial molecular orientation within the granule and is a well-documented feature of storage starches. We used small-angle x-ray scattering (SAXS) to determine whether, as in storage starches, Arabidopsis amylopectin is organized within the granule into alternating crystalline and amorphous lamellae (French, 1984; Jenkins et al., 1993). Figure 8 shows the scattering profile for wild-type Arabidopsis starch with a peak in scattering intensity at a q value of 0.06, indicating a crystalline structure with periodicity of 9 nm (Jenkins et al., 1993).

We investigated whether leaf starch granules consist of alternating semicrystalline and amorphous zones (growth rings) using a technique developed to visualize these zones in storage starches (Pilling, 2001). Granules were cracked open by mechanical grinding of starch suspensions frozen in liquid nitrogen and incubated with α-amylase to preferentially digest amorphous regions. No growth rings were visible in granules from wild-type leaves, though the granules were partially digested during the incubation (Fig. 9, A and B). However, the treatment revealed growth rings in granules from *sex4* leaves (Fig. 9, C and D). These had a periodicity of about 0.2 to 0.3 μm, a distance almost the same as the total thickness of wild-type granules.

DISCUSSION

Starch Is Accumulated without Turnover

We found no evidence for turnover during starch accumulation despite conducting experiments designed specifically to reveal such a process. Radiolabel incorporated into starch during a pulse of $^{14}CO_2$ was not subsequently released during a chase in the light in air. A similar result was observed in pea leaves (Kruger et al., 1983). There are several possible explanations for this result. First, label released by degradative enzymes (as Glc or Glc-1-P) may be reincorporated into starch. This seems unlikely because released Glc would be transported to the cytosol and it is doubtful that the label would reenter the plastid for starch synthesis (Weber et al., 2000). Glc-1-P released through the action of starch phosphorylase could be reincorporated, but phosphorolytic activity in Arabidopsis chloroplasts is low (Lin et al., 1988) and it is unlikely that Glc-1-P would be a major product of degradation. Alternatively, malto-oligosaccharides released by turnover might be transferred to nascent amylopectin molecules by disproportionating enzyme (D-enzyme) as suggested for *Chlamydomonas reinhardtii* by Colleoni et al. (1999). This also seems unlikely because in Arabidopsis leaves, D-enzyme does not participate in starch synthesis in this way (Critchley et al., 2001). Second, the radiolabeled starch may not be accessible to the degrading enzymes due to the deposition of unlabeled starch on top of it. Reducing the light intensity during the chase to slow the deposition of unlabeled material did not result in detectable loss of label from the starch. If appreciable turnover were occurring, it should be more readily detectable using these conditions. However, label was released from the starch when the light was reduced to the extent that starch synthesis stopped and breakdown occurred. Third, the starch-degrading enzymes may not be active. This seems most likely because there is good evidence that the process of starch mobilization in leaves is regulated (Trethewey and Smith, 2000). For example, starch degradation in leaves at night often commences only after a lag, rather than on the light-to-dark transition (Gordon et al., 1980; Fondy and Geiger, 1982).

It is possible that the control of starch degradation is exercised at the point where the starch granule is attacked to liberate soluble glucans. This step is most likely catalyzed by α-amylase because no other enzyme has been convincingly shown to attack intact starch granules. However, our results with the phytoglycogen-accumulating mutant *dbe1* show that even when glucan is accumulated in a soluble form, no turnover is detectable, suggesting that other degradative enzymes may also be tightly regulated.

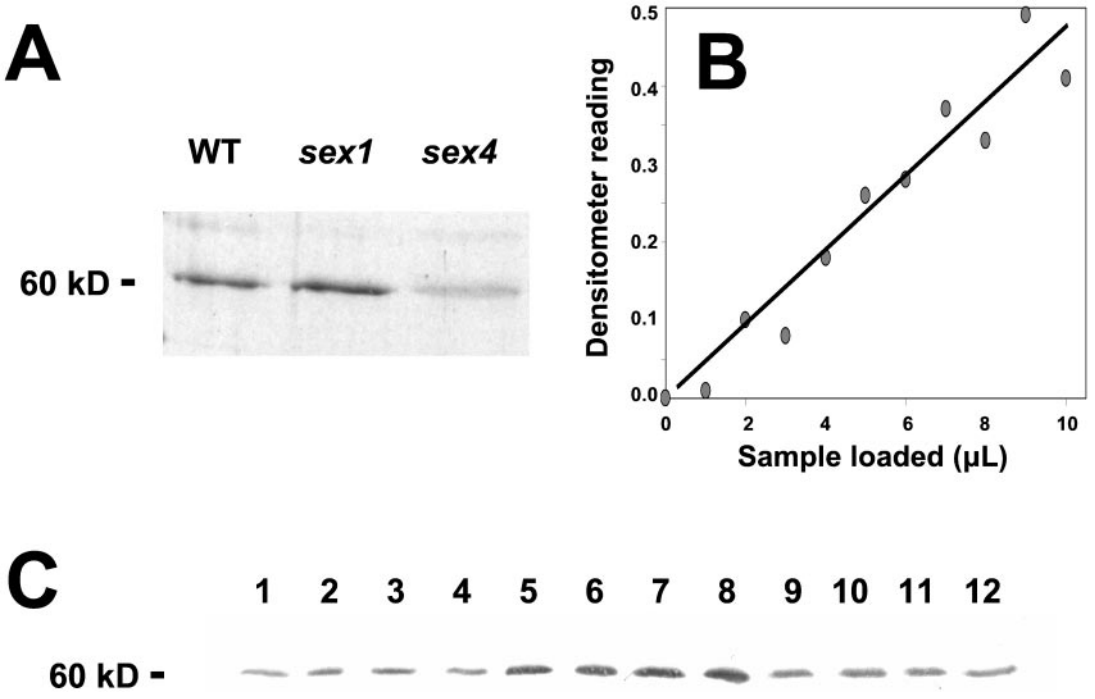

Figure 4. GBSS content of starch and leaves of wild-type, *sex1*, and *sex4*. A, Starch was isolated from wild-type, *sex1*, and *sex4* leaves at the end of the photoperiod. Granule-bound proteins were separated by SDS-PAGE and stained using colloidal Coomassie Blue. B, Proteins were extracted from the insoluble fraction of *sex1* leaves harvested at the end of the photoperiod. An immunoblot was performed using an antibody raised against the pea (*Pisum sativum*) embryo GBSS and the relationship between the amount of sample loaded and densitometry measurements plotted. C, Immunoblot of proteins extracted from the insoluble fraction of leaves from wild-type, *sex1*, and *sex4* leaves harvested at the end of the photoperiod. Each sample comprised the leaves of a single plant.

Amylose Content of Leaf Starch

We confirmed the earlier observation that Arabidopsis leaf starch has a very low amylose content when grown in a normal diurnal cycle. This contrasts with a study of leaf starch composition in tobacco, in which an amylose content of between 15% and 20% was found (Matheson, 1996). However, tobacco differs from Arabidopsis because, in addition to cycling in a diurnal fashion, a background level of storage starch accumulates in leaves as they mature (Matheson and Wheatley, 1962). When Arabidopsis plants were transferred from a diurnal cycle to continuous light, far more starch was synthesized and this starch had a higher proportion of amylose. Thus, the balance of synthesis shifts from almost exclusively amylopectin toward a significant proportion of amylose over time. In addition to this increased amylose synthesis in wild-type plants, high-amylose starch is also synthesized in the mutants *sex1* and *sex4*, which accumulate appreciably more starch than wild-type plants.

Although it is not clear from our current results what determines the amylose content of leaf starch, a number of factors may be important. The increase in amylose in *sex1* and *sex4* was accompanied by an increase in the GBSS content of the leaf, whereas in both mutants, soluble starch synthase activity is similar to (or lower than) that of the wild type (Caspar et al., 1991; Zeeman et al., 1998a). Therefore, it is possible that the higher ratio of GBSS to soluble starch synthase activity may cause the increased amylose in these lines. A similar change in the ratio of GBSS to soluble starch synthase could explain why wild-type plants transferred to continuous light accumulate starch with high amylose. Furthermore, in the mutant lines, starch is synthesized during the day but not completely degraded during the night. As a consequence, starch builds up over a number of diurnal cycles (Zeeman and ap Rees, 1999). It is plausible that GBSS trapped within the undegraded starch may remain active and may synthesize more amylose during each light period, none of which would be degraded during the dark. This would also lead to an accumulation of amylose correlating with the accumulation of starch. This hypothesis is supported by the amylose content of the starch from *sex4* leaves of different ages. The amylose content in young leaves is only 5%, whereas in the oldest leaves, which have experienced many diurnal cycles, the starch contains 34% amylose. In the wild type, all of the starch is degraded each

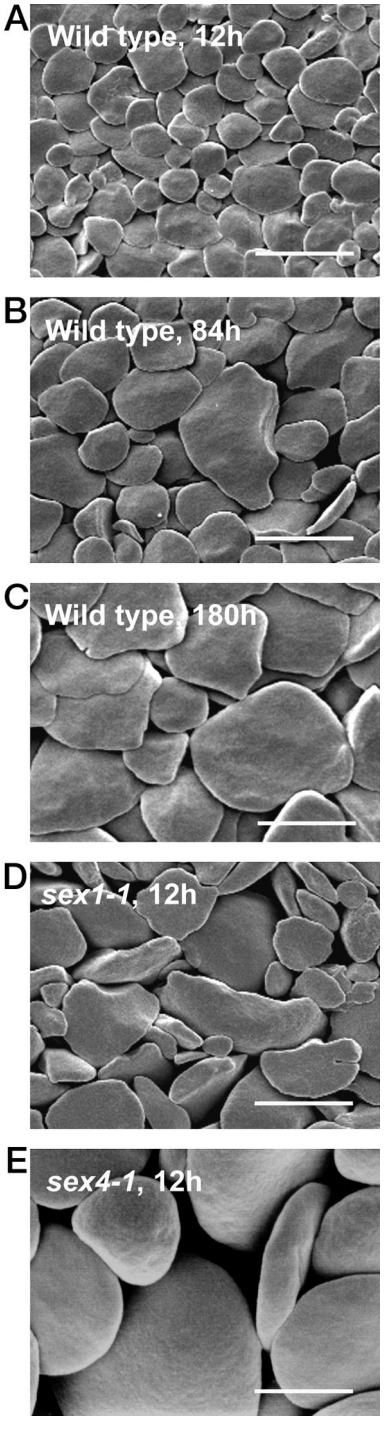

Figure 5. Scanning electron micrographs of starch granules isolated from plants at the end of the photoperiod (A, D, and E) or after a period of continuous light (B and C). The bar represents 2 μm.

night, including amylose, so the amylose content would not increase in this way unless the diurnal conditions were altered.

The increase in the GBSS content in the mutants cannot account in full for the increase in amylose content. Starch from *sex4* had the highest amylose content but the increase in the GBSS content in this mutant is not as marked as in *sex1*, starch from which has a lower amylose content. The explanation may lie in the difference in granule morphology between the two lines. It has been suggested that amylose is preferentially synthesized in the amorphous zones of starch granules (Blanshard, 1987). Granules from *sex4* are large and contain alternating semicrystalline and amorphous zones similar to storage starches (see below), whereas wild-type and *sex1* granules may be too small to contain these amorphous zones. Thus, amylose may be more readily synthesized in *sex4* granules than in wild-type or *sex1* granules. However, other factors such as the supply of substrates are also known to influence amylose synthesis (Van den Koornhuyse et al., 1996; Clarke et al., 1999) and may also contribute to the observed differences.

Structure and Morphology of Leaf Starch Granules

Starch granules of wild-type plants were flat and discoid. Even when plants were transferred to continuous light to promote further starch synthesis, the granules increased in size but did not alter radically in appearance. The granules from the *sex1* plants, which accumulate up to 5-fold more starch than the wild type, were also flat and discoid. It is tempting to speculate that the shape of the granules is defined by the spaces within the chloroplast, between layers of thylakoid membranes. However, *sex4* granules were much larger and thicker than all the other granules, even though this mutant only accumulates 3 times as much starch as the wild type. The cause of the different granule morphology, and how it relates to the enzymatic deficiency in this mutant (reduced plastidial endoamylase), is not yet clear.

The fundamental structures and layers of organization in starch granules of Arabidopsis leaves are similar to those found in storage starches. The birefringence of the granules indicates radial orientation of the constituent polymers and the amylopectin forms a repeated crystalline structure with 9-nm periodicity. The large granules from *sex4* also have an internal growth ring structure similar to granules from storage organs. Our results demonstrate that amylopectin with a chain length distribution characteristic of leaves can form granules with striking similarities in appearance, structure, and amylose content to starches from storage organs.

We conclude that, despite the presence of starch-degrading enzymes in chloroplasts, no degradation of starch was detected during periods of net starch synthesis. The starch granules themselves were found to contain varying amounts of amylose, depending on the conditions of synthesis, and exhibited very similar levels of structural organization to granules from non-photosynthetic tissues. We suggest that the mechanisms underlying the synthesis of Ara-

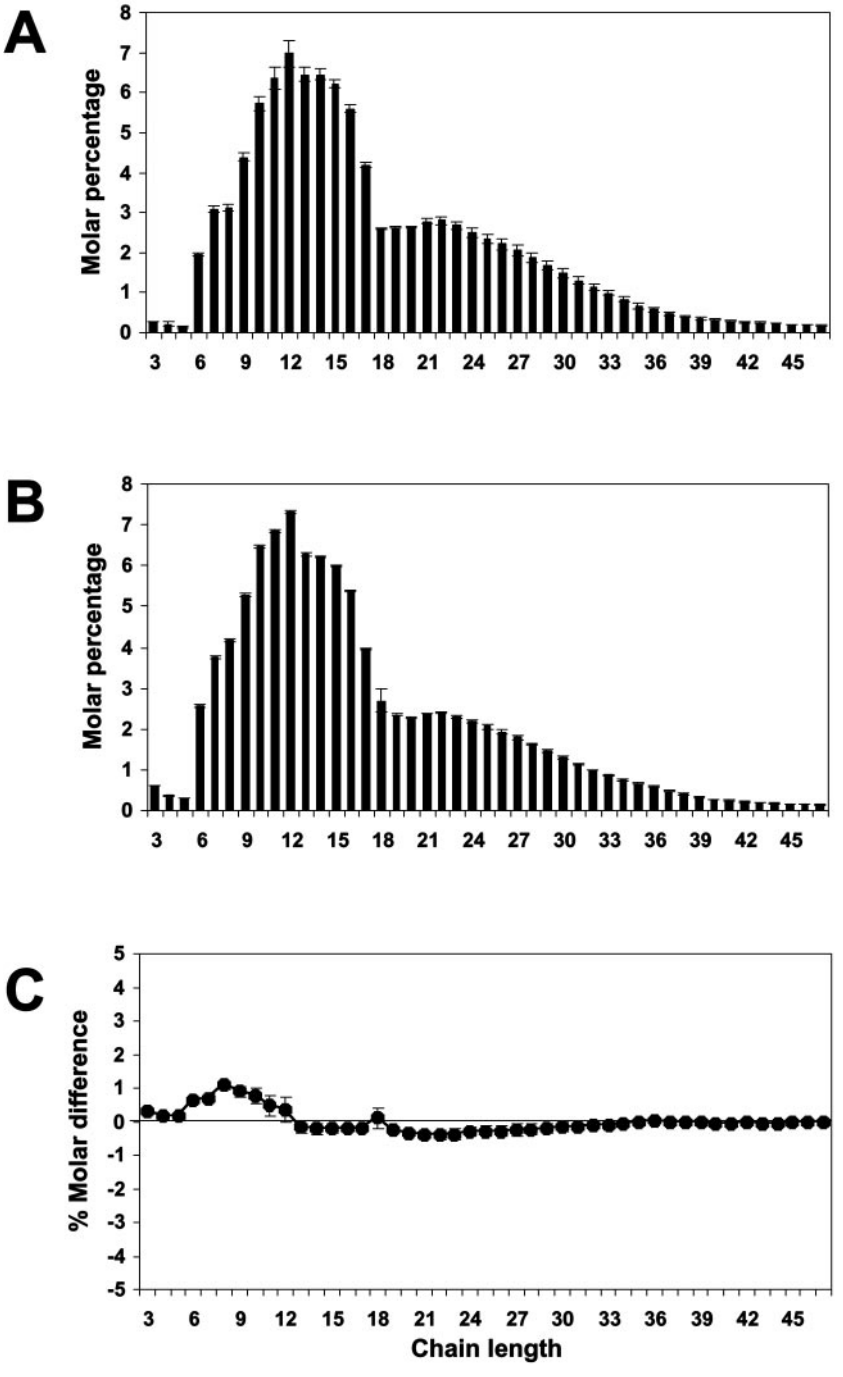

Figure 6. Analysis of the chain length distribution of amylopectin from the wild type and from *sex4* using fluorophore-assisted PAGE. Starch samples were solubilized, debranched with isoamylase, and derivatized with the fluorophore 8-amino-1,3,6-pyrenetrisulphonic acid. Chains of different lengths were separated by gel electrophoresis in a DNA sequencer (PE-Applied Biosystems, Foster City, CA) and the data analyzed using GeneScan 672 software (PE-Applied Biosystems). Peak areas of chains between three and 47 Glc residues in length were summed and the individual peak areas expressed as a percentage of the total. Three replicate samples of debranched, derivatized material were prepared from bulk starch extracted from batches of 200 wild-type (A) and *sex4* (B) plants. The values are the means ± SES of measurements made on these samples. To obtain a percentage molar difference plot (C), wild-type values were subtracted from those of *sex4*. The SES were added together.

bidopsis starch granules are broadly similar to those of seeds, tubers, and the leaves of other higher plants. These findings show that the analysis of starch biosynthesis in Arabidopsis may have valuable implications for understanding starch in commercially important crop species. Furthermore, because the factors that determine granule size, shape, and number are not known in any species, Arabidopsis mutants such as *sex1* and *sex4*, in which granule morphology and number are altered, represent useful tools with which to investigate these questions.

MATERIALS AND METHODS

Materials

All chemicals were obtained from Sigma Chemical Co. (Poole, Dorset, UK). Radioisotopes were supplied by Amersham Pharmacia Biotech (Amersham, Bucks, UK).

Plants and Growth Conditions

Wild-type Arabidopsis plants (ecotype Columbia) and the mutants *sex1-1* (Caspar et al., 1991; Zeeman and ap

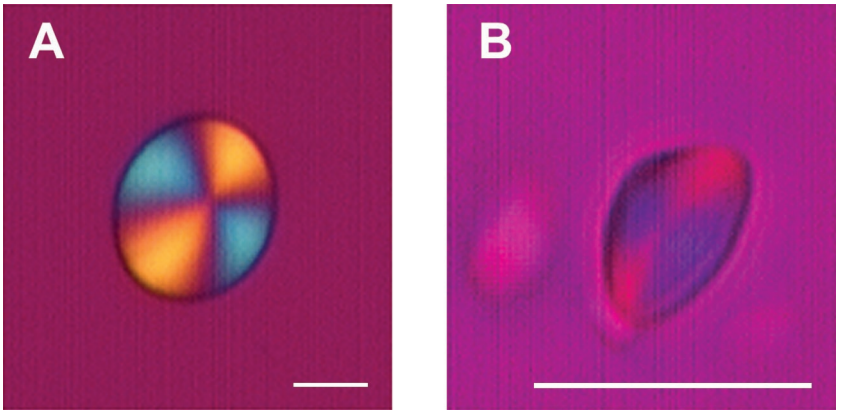

Figure 7. Light micrographs of starch granules viewed under polarized light. Starch granules from potato cv Desiree tuber (A) and from wild-type Arabidopsis plants after 180 h of continuous light (B) were suspended in water and digital images captured using Image-Pro Plus software (Media Cybernetics Inc., Silver Spring, MD). The bar represents 5 μm.

Rees, 1999; Yu et al., 2001), *sex4-1* (Zeeman et al., 1998a; Zeeman and ap Rees, 1999) and *dbe1-1* (Zeeman et al., 1998b) were grown in peat-based compost in a growth chamber with a 12-h-light/12-h-dark cycle. The irradiance was 170 μmol photons m^{-2} s^{-1}, the temperature 20°C, and the humidity 75%, unless otherwise specified. Wild-type and *dbe1-1* plants were used after 4 to 5 of weeks growth, whereas *sex4-1* plants were used after 5 to 6 weeks of growth and *sex1-1* plants after 6 to 7 weeks. At these ages the plants were at equivalent developmental stages.

In Vivo Labeling

To label starch with ^{14}C in vivo, photosynthesizing plants (total shoot mass of approximately 5 g) were exposed to $^{14}CO_2$ with a specific activity between 1.25 MBq $mmol^{-1}$ and 1.88 MBq $mmol^{-1}$ and a CO_2 concentration of either 400 μL L^{-1} (30-min pulses) or 600 μL L^{-1} (1-h pulses). The plants were sealed in a Perspex chamber (12.1-L volume) and $^{14}CO_2$ liberated by acidification of sodium [^{14}C]bicarbonate. The light intensity was the same as that used to grow the plants, unless specified, and the heat load was alleviated using a water trap. Considering the rate of photosynthesis of Arabidopsis plants growing under these conditions (Zeeman and ap Rees, 1999), less than 50% of the CO_2 supplied would have been incorporated during a 1-h pulse. As a consequence, the CO_2 concentration would have remained above 300 μg mL^{-1} in all of the experiments. At the end of the pulse period, the $^{14}CO_2$ was removed, the chamber opened, and pulse samples harvested. In the pulse and chase experiments, chase samples were left in the chamber, through which air was

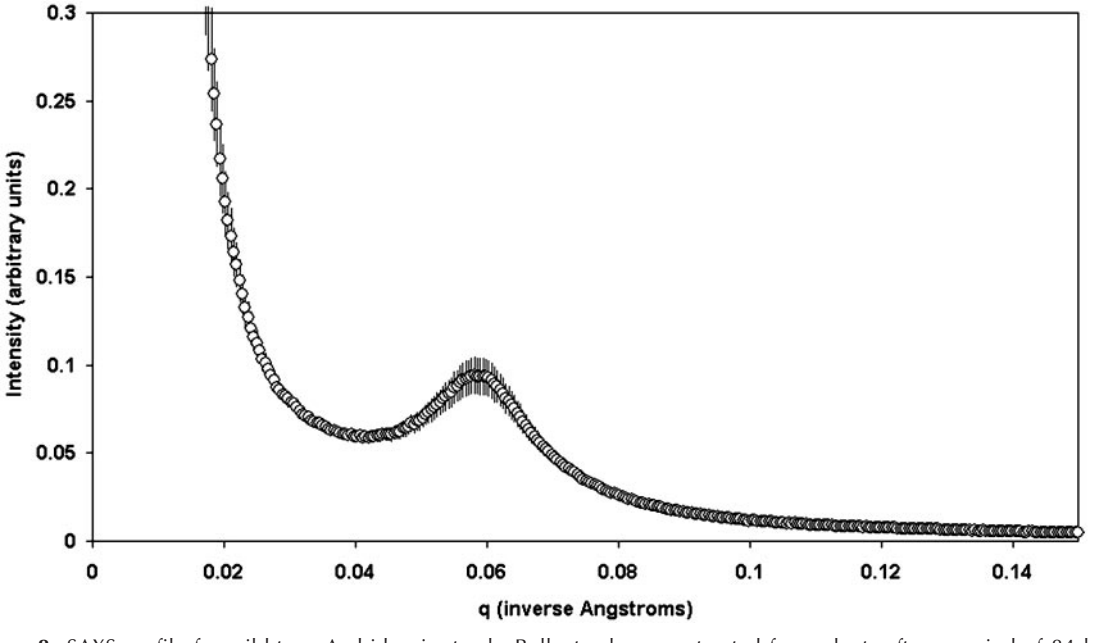

Figure 8. SAXS profile for wild-type Arabidopsis starch. Bulk starch was extracted from plants after a period of 84 h of continuous light. A low-divergence, high-intensity beam of radiation (λ = 1.5 Å) was focused onto starch samples, which were in the form of a 50% (w/w) slurry with water. Three replicate samples were analyzed and the results are the means ± SES.

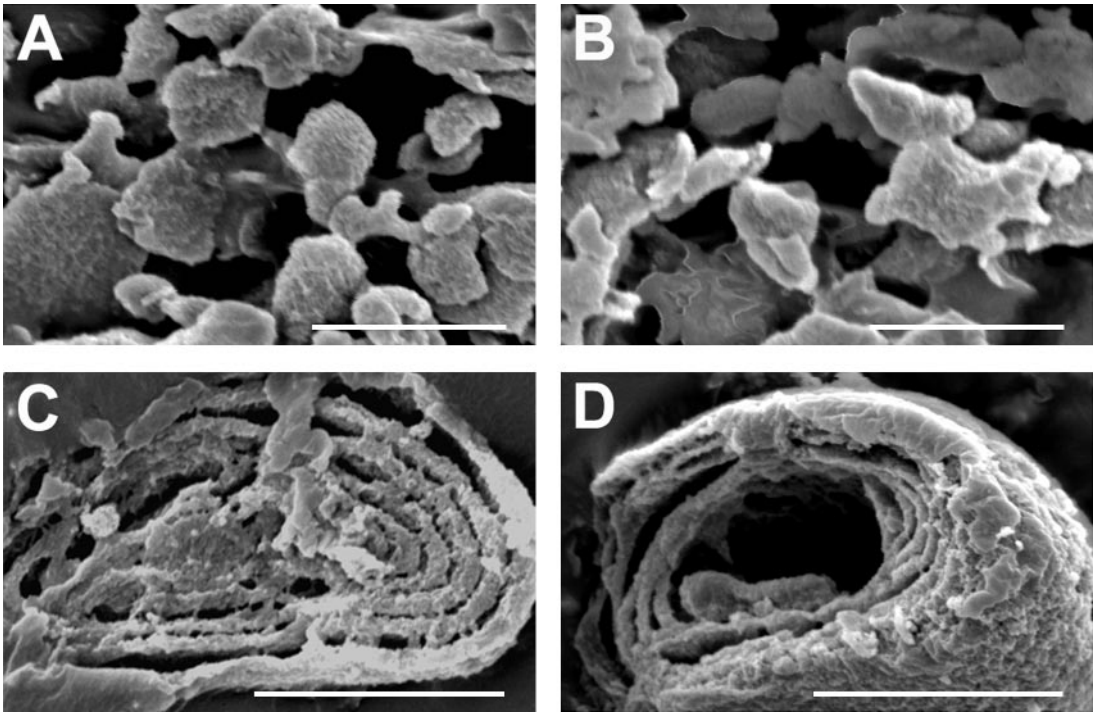

Figure 9. Scanning electron micrographs of partially digested starch granules from the wild type (A and B) and *sex4* (C and D). Granules were cracked by grinding in liquid nitrogen and partially digested with α-amylase to reveal internal growth ring structures.

pumped at a rate of 1.2 L min^{-1}. Wild-type plants were killed in boiling 80% (v/v) aqueous ethanol, whereas *dbe1* plants were frozen in liquid N_2. Starch content was determined by hydrolyzing the starch with α-amylase and amyloglucosidase and assaying released Glc as described by Zeeman et al. (1998a).

The ^{14}C in starch in wild-type plants was determined as described in Zeeman et al. (2002). Starch and phytoglycogen in *dbe1* plants were extracted by homogenizing leaves in an ice-cold aqueous medium because phytoglycogen, although soluble in water, is insoluble in 80% (v/v) ethanol (Zeeman et al., 1998b). The water-insoluble material, including starch, was removed by centrifugation and washed twice with ice-cold extraction medium. The soluble material and the washes were pooled and adjusted to 75% (v/v) methanol and 1% (w/v) KCl to precipitate the phytoglycogen. This precipitate was collected by centrifugation, redissolved in water, and stored at −20°C. The insoluble material was washed twice further with 80% (v/v) ethanol, resuspended in water, and stored at −20°C (Zeeman et al., 1998b). The ^{14}C content of the starch, and of phytoglycogen, was determined in the same way as starch in the wild type.

Analysis of Starch Composition and Amylopectin Structure

Starch granules were isolated from leaves as described in Zeeman et al. (1998a). Routine separation of amylose and amylopectin using a 9-mL Sepharose CL2B column was performed as described in Denyer et al. (1995) except that 0.35-mL fractions were collected at a rate of one fraction per 2 min. For improved separation, a larger column (90-mL volume, 115-cm length, and 0.78-cm^2 cross-sectional area) was used. Starch (1 mg) was dissolved in 100 μL of 0.5 M NaOH, applied to the column, and eluted with 10 mM NaOH. The flow rate was 0.185 mL min^{-1} and 2.78-mL fractions were collected every 15 min. Each fraction was divided in two and one-half used to determine the absorbance (at 595 nm) and the wavelength of maximal absorbance (λ_{max}) of the polymer-iodine complex by mixing with 10% (v/v) Lugol's solution (Sigma). The other half was adjusted to pH 5 by the addition of a small volume of 0.1 M HCl, and then lyophilized. The resultant material was dissolved in water and the glucan content measured as described above for the determination of starch content.

For the preparation of pure amylopectin and amylose fractions, 10 to 20 mg of starch was dissolved in 1 mL of 0.5 NaOH, applied to the 90-mL Sepharose CL2B column, and eluted with 100 mM NaOH. The two peak fractions containing amylopectin were pooled, neutralized by the addition of a small volume of 2 M HCl, and the glucan content of a sample determined after digestion to Glc (described above). The six to 10 peak fractions containing amylose were pooled, neutralized, and the amylose precipitated as follows. After boiling for 1 h in a sealed vessel, one-quarter volume of butan-1-ol was added to the sample. The mixture was boiled for 1 h and then cooled gradually. The amylose-butanol precipitate was collected by centrifugation and the amylose redissolved by boiling in water. To determine the

absorption spectrum of the polymer-iodine complex, samples were mixed with 10% (v/v) Lugol's solution.

The analysis of the distribution of chain lengths using fluorophore-assisted PAGE was performed exactly as described by Edwards et al. (1999).

Scanning Electron Microscopy of Starch Granules

Starch granules were viewed using a scanning electron microscope (model XL 30 FEG; Phillips Electronics NV, Eindhoven, The Netherlands). To visualize the internal structure of the starch granules, starch preparations were washed with acetone, dried in air, and ground in a liquid N_2-cooled mortar to crack the granules. Cracked granules were then treated with α-amylase (5 units for 30 min in 0.5-mL reaction medium containing 100 mM MES-NaOH, pH 6.0) to preferentially digest amorphous regions of the starch granules (Pilling, 2001). The granules were collected by centrifugation, washed three times in cold acetone (−20°C), dried, and then viewed under the scanning electron microscope.

Light Microscopy and X-Ray Diffraction

Light micrographs were obtained using a Microphot microscope (Zeiss, Jena, Germany). Images were captured using Image-Pro Plus software. SAXS profiles were obtained at the Daresbury Laboratory (Daresbury, Cheshire, UK) as described by Jenkins and Donald (1995).

Gel Electrophoresis, MALDI-Mass Spectroscopy, and Immunoblotting

Starch granule-bound proteins were extracted by boiling starch in SDS sample buffer (Laemmli, 1970; 100 mg starch mL^{-1}) for 10 min. Gelatinized starch was removed by centrifugation and the proteins in the supernatant resolved by SDS-PAGE as described by Denyer et al. (1995). To determine GBSS content of fresh tissue, leaves (200 mg) were homogenized in ice-cold medium containing 100 mM Tris, pH 7.2; 5 mM EDTA; and 1% (w/v) SDS. The insoluble material was removed by centrifugation and washed twice in extraction medium. The pellet was resuspended in 0.5 mL of SDS sample buffer and boiled for 10 min. Insoluble material was removed by centrifugation and proteins in the supernatant resolved by SDS-PAGE. GBSS was detected by immunoblotting using a polyclonal antibody raised against the pea (*Pisum sativum*) embryo GBSS (Smith, 1990) according to the method described by Bhattacharyya et al. (1990).

MALDI-mass spectroscopy was performed using a Bruker Reflex III (Bruker Daltonics, Coventry, UK). Protein bands were cut from the gel, digested with trypsin, and prepared for mass spectroscopy using the optimal conditions established by Speicher et al. (2000).

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