A Mutant of Arabidopsis Lacking a Chloroplastic Isoamylase Accumulates Both Starch and Phytoglycogen

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In this study, our goal was to evaluate the role of starch debranching enzymes in the determination of the structure of amylopectin. We screened mutant populations of Arabidopsis for plants with alterations in the structure of leaf starch by using iodine staining. The leaves of two mutant lines stained reddish brown, whereas wild-type leaves stained brownish black, indicating that a more highly branched polyglucan than amylopectin was present. The mutants were allelic, and the mutation mapped to position 18.8 on chromosome 1. One mutant line lacked the transcript for a gene with sequence similarity to higher plant debranching enzymes, and both mutants lacked a chloroplastic starch-hydrolyzing enzyme. This enzyme was identified as a debranching enzyme of the isoamylase type. The loss of this isoamylase resulted in a 90% reduction in the accumulation of starch in this mutant line when compared with the wild type and in the accumulation of the highly branched water-soluble polysaccharide phytoglycogen. Both normal starch and phytoglycogen accumulated simultaneously in the same chloroplasts in the mutant lines, suggesting that isoamylase has an indirect rather than a direct role in determining amylopectin structure.

INTRODUCTION

Amylopectin is the major glucan polymer of starch and is responsible for the semicrystalline structure of the granule. It is composed of chains of α-1,4–linked glucose residues connected by α-1,6 linkages (branch points). During amylopectin synthesis, the branch points are formed in a heterogeneous pattern, with regions of frequent branching alternating with relatively unbranched regions. This results in clusters of α-1,4–linked glucan chains of 12 to 20 residues (French, 1984). Neighboring chains in the clusters interact with one another to form higher order crystalline arrays (Jenkins et al., 1993). Branched α-1,4–linked glucans lacking this cluster structure, such as glycogen (synthesized in bacteria and animals), are soluble and do not form crystalline arrays. The manner in which the distribution of branch points is determined during amylopectin synthesis is not well understood, and the potential role of starch debranching enzymes in this process has recently received considerable attention (Ball et al., 1996; Manners, 1998).

Plant debranching enzymes catalyze the hydrolysis of α-1,6 linkages and fall into two classes: isoamylases and pullulanases (limit dextrinases; R enzymes). A major distinction between these two classes is the inability of isoamylase to

hydrolyze the α-1,6 linkages in the yeast polysaccharide pullulan (maltotriose units linked end to end by α-1,6 linkages; Nakamura, 1996). Although pullulanase activity has been identified in a range of plants, isoamylases have been reported in only a few. This probably reflects difficulties associated with measuring isoamylase in crude extracts rather than its rarity within the plant kingdom.

It is generally assumed that debranching enzymes play an important role in the degradation of starch (Beck and Ziegler, 1989). However, *sugary1* (*su1*) mutations of maize (Pan and Nelson, 1984; Doehlert et al., 1993) and rice (Nakamura et al., 1996) indicate that they also may be involved in starch synthesis. Both mutations reduce the debranching enzyme activity in the developing endosperm and result in a reduction in the synthesis of starch and in the accumulation of a more highly branched, soluble polysaccharide (phytoglycogen). A mutation at the *STA7* locus of the unicellular green alga Chlamydomonas also causes the loss of a debranching enzyme (Mouille et al., 1996). These mutants contain no starch but, like the *su1* mutants, accumulate phytoglycogen. Pan and Nelson (1984) proposed that the ratio of branching-to-debranching enzyme activity was important in determining the correct number of branch points in amylopectin, thus explaining the phenotype seen in the *su1* mutants of cereals. More recently, Ball et al. (1996) proposed the "glucantrimming" model, attempting to explain not only the frequency but also the pattern of branches in amylopectin via

the action of debranching enzymes. They propose a synthetic cycle involving the sequential action of starch synthase, branching enzyme, and debranching enzyme. In this model, starch synthases elongate short chains at the surface of the starch granule, forming new amylopectin "clusters." Branching enzymes branch the ends of these chains extensively to form "preamylopectin," and debranching enzymes subsequently trim these branches to produce short chains that are suitable substrates for starch synthases, which act again to form the next cluster, thus completing the cycle.

The factors directly responsible for the accumulation of phytoglycogen in the *su1* mutants of cereals have not been clearly established. Although the mutation in maize lies in a gene encoding an isoamylase (James et al., 1995), there are numerous secondary effects on other starch-metabolizing enzymes, including pullulanase (Pan and Nelson, 1984; Doehlert et al., 1993; Rahman et al., 1998) and soluble starch synthase (Singletary et al., 1997). Thus, it is not clear which change is primarily responsible for the accumulation of phytoglycogen. It also has been claimed that an isoform of branching enzyme is altered in *su1* maize kernels (Boyer and Preiss, 1978). However, these data were not reproducible (Preiss, 1988).

The synthesis of transitory starch in leaves is a better system in which to investigate the role of debranching enzymes than are the *su1* mutants of cereals. Leaf starch is synthesized during the course of a single photoperiod rather than over days or weeks, as in storage organs. This reduces the possibility of developmental changes in enzyme activities and isoform complements during granule formation. Transitory starch has a high amylopectin content compared with storage starches, and like storage starch, its amylopectin has a polymodal distribution of chain lengths (Matheson, 1996; Tomlinson et al., 1997). In this study, we characterize an isoamylase-deficient mutant *dbe1* (for debranching enzyme) of Arabidopsis. This mutant accumulates both starch granules and phytoglycogen in the same chloroplasts. We can detect no alterations in the other enzymes of starch metabolism. We argue that this phenotype cannot be explained by the glucan-trimming model as it stands and propose an alternative explanation for the role of isoamylase in starch synthesis.

RESULTS

### Isolation, Genetic Analysis, and Mapping of Mutants with Altered Starch Structure

A mutant screen based on that described by Caspar et al. (1991) was used to isolate mutants of Arabidopsis (ecotype Columbia) with altered starch structure. Two populations of mutagenized Arabidopsis plants, produced by x-ray and ethyl methanesulfonate (EMS) mutagenesis, respectively, were screened. The M_2 generation was grown for 3 to 4 weeks; at the end of the photoperiod, single leaves from each plant were detached, decolorized, and stained for starch with an iodine solution. The starch present in the leaves of wild-type plants stains dark brown to black. The leaves of two mutant lines, one from each mutant population, stained reddish brown, suggesting the presence of a starch polymer with a structure different from that of the wild type (Figure 1).

The two mutant lines were crossed to one another to determine whether the mutations lie within the same gene. Five independent, reciprocal crosses were performed. All of the 103 F_1 plants screened exhibited the mutant phenotype, suggesting that the mutations lie at the same locus. We have named this locus *DBE1*, and the mutant alleles *dbe1-1* (x-ray generated) and *dbe1-2* (EMS generated).

We investigated the genetic characteristics of the mutation by backcrossing both *dbe1-1* and *dbe1-2* to the wild type. Six crosses were performed for *dbe1-1*, and the 124 F_1 plants all exhibited a wild-type phenotype when stained for starch. These plants were allowed to self-pollinate, and the segregation of the mutant phenotype in the F_2 generation was observed. Of 146 F_2 plants, 39 had the mutant phenotype, whereas 107 had a wild-type phenotype (a ratio of 2.8:1). For *dbe1-2*, two crosses were performed that yielded 40 F_1 plants; all had a wild-type phenotype. The segregation ratio in the F_2 generation was 3.2:1 (283 plants analyzed). The segregation ratios are close to the 3:1 ratio predicted for a single, recessive, nuclear-localized mutation.

The *DBE1* locus was mapped. The mutant line *dbe1-2* was crossed to three multiple marker mapping lines (CS3078, CS3079, and CS3080), and the linkage of the mutant phenotype with the visible phenotypes of the marker lines was scored in the F_2 progeny. The marker *dis1-1* (position 18.7
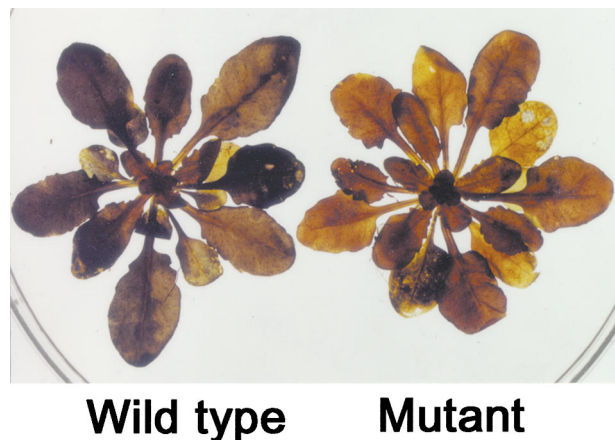
Figure 1. Leaves of Wild-Type and *dbe1* Plants Stained for Starch with Iodine.

Plants were harvested at the end of a 12-hr photoperiod, decolorized in hot 80% (v/v) ethanol, and stained with an iodine solution.

on chromosome 1) was found to be tightly linked to the mutant phenotype. We used restriction fragment length polymorphism (RFLP) markers to map the mutation further. The DNA from F_2 plants homozygous for *dbe1* was digested with EcoRI and used for DNA gel blot analysis. The blots were probed with the RFLP markers 241A (CD3-47 at position 32 on chromosome 1), 254A (CD3-50 at position 56.3 on chromosome 1), and ALC50 (*chl1-1* genomic clone at position 14.3 on chromosome 1). Analysis of the recombination frequencies indicated a map position of 18.8 on chromosome 1.

A Transcript for an Isoamylase-like Protein Is Missing from *dbe1-1*

The Arabidopsis expressed sequence tag (EST) 297385 (GenBank accession number H36690) was used to probe a gel blot of total RNA extracted from leaves of wild-type Arabidopsis and the mutant line *dbe1-1.* We used the x-ray–generated mutant line because mutants derived by x-ray mutagenesis are more likely to lack the transcripts of the affected gene. The sequence of this EST clone is similar to those of debranching enzyme genes, particularly that of the maize isoamylase gene *SU1* (James et al., 1995). The product of the full-length cDNA corresponding to the EST shows 52% similarity and 42% amino acid identity to the *SU1* gene product (T.-S. Yu and J. Chen, manuscript in preparation). The gel blot (Figure 2A) showed that mutant line *dbe1-1* lacks a 2.9-kb transcript corresponding to the EST. As a control, the gel blot also was probed with a fragment of the ubiquitin gene (from Antirrhinum). The ubiquitin transcript was present in both the wild-type and the mutant line (Figure 2B). We mapped the EST clone by using RFLP analysis for recombinant inbred lines and a segregating F_2 population of *dbe1-2* crossed with the Landsberg *erecta* wild type (T.-S. Yu and J. Chen, manuscript in preparation). The results showed that the *dbe1* mutation cosegregated with this clone.

A Debranching Enzyme Activity Is Missing from the *dbe1* Mutant Lines

We used native PAGE to investigate the starch-hydrolyzing enzymes in the *dbe1* lines and in the wild type. This approach has previously proved successful in identifying a mutant of Arabidopsis with altered endoamylase activity (Zeeman et al., 1998). Soluble proteins were separated in polyacrylamide gels containing amylopectin. The gels were incubated at pH 7, and the bands of starch-hydrolyzing activity were detected by iodine staining. Five major bands of starch-hydrolyzing activity were visible in wild-type extracts (Figure 3A). Tentative identification of the enzymes causing the bands is possible because the products of hydrolysis of amylopectin by different enzymes stain different colors with iodine (Kakefuda and Duke, 1984). Three were putative amy-
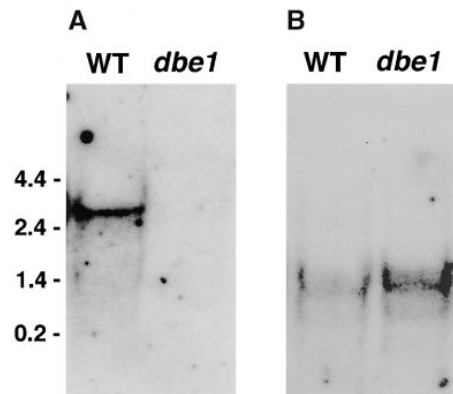
Figure 2. RNA Gel Blot Analysis for the Expression of an Isoamylase Gene in Wild-Type and *dbe1* Plants.
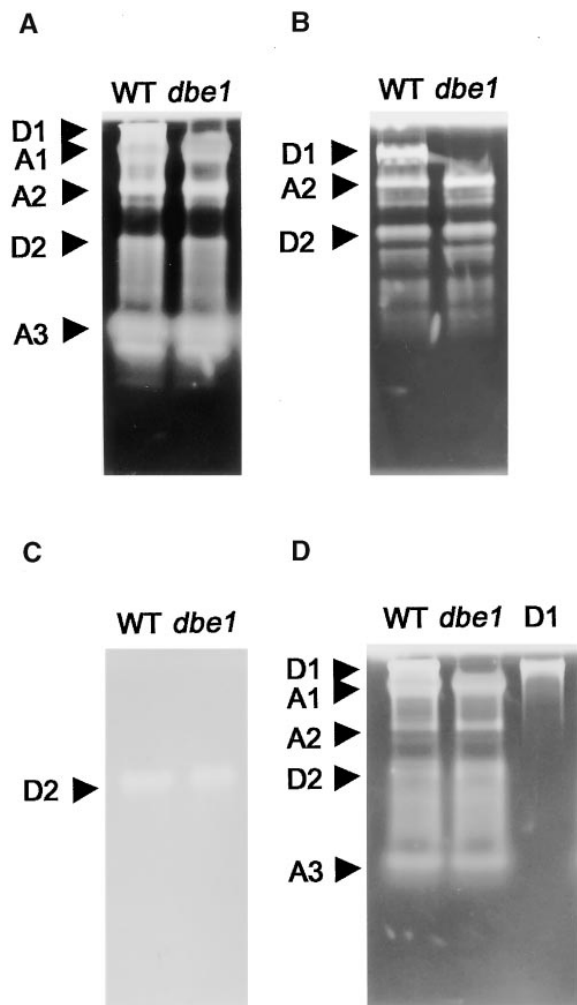
(A) Gel blots of total RNA (20 μg per lane) extracted from leaves of the wild type (WT) and the mutant line *dbe1-1* (*dbe1*) and probed with Arabidopsis EST 297385.
(B) Gel blots of total RNA (10 μg per lane) extracted from leaves of the wild type and the mutant line *dbe1-1* and probed with a fragment of the Antirrhinum ubiquitin gene.
Length markers are given in kilobases at left.

lases (clear or brown-staining bands; A1, A2, and A3), and two were putative debranching enzymes (blue-staining bands; D1 and D2). The enzyme activity causing the uppermost blue-staining band (D1) in the wild-type extract was always absent from extracts of both *dbe1* lines. This enzyme has been shown to be localized to the chloroplast of wild-type Arabidopsis plants (Zeeman et al., 1998). By extracting wild-type and mutant tissue together, we confirmed that the absence of this band was not due to the presence of inhibitors of this enzyme in the mutant leaves (data not shown).

To investigate the nature of this putative debranching enzyme, we used native gels containing β-limit dextrin (derived from potato amylopectin) or Red Pullulan (a specific chromogenic substrate for pullulanase-type debranching enzymes). The enzyme D1, missing in the *dbe1* lines, was able to hydrolyze the β-limit dextrin, again resulting in a blue-staining band (Figure 3B). To determine whether this enzyme could hydrolyze pullulan, proteins were separated in an amylopectin-containing gel and immediately electroblotted onto a second gel containing Red Pullulan. After incubation, clear bands indicating pullulanase activity appeared against the red background (Figure 3C). Only one band was detected in extracts of wild-type leaves. This band corresponded to the blue-staining band D2 on the amylopectin-containing gels, and the activity causing the band was also present in extracts of *dbe1-1* leaves. No pullulanase activity could be detected corresponding to the position of band D1. Together, these data suggest that the putative debranching enzyme D1 is an isoamylase. The intensity of band D2 in gels of *dbe1-1* extracts was similar to that of the

wild-type extracts in a range of gels, each loaded with different amounts of extract (data not shown). Although native gels are semiquantitative at best, these data suggest that the total pullulanase activity causing band D2 was not reduced in the mutant.

Partially Purified Enzyme D1 Has the Properties of an Isoamylase
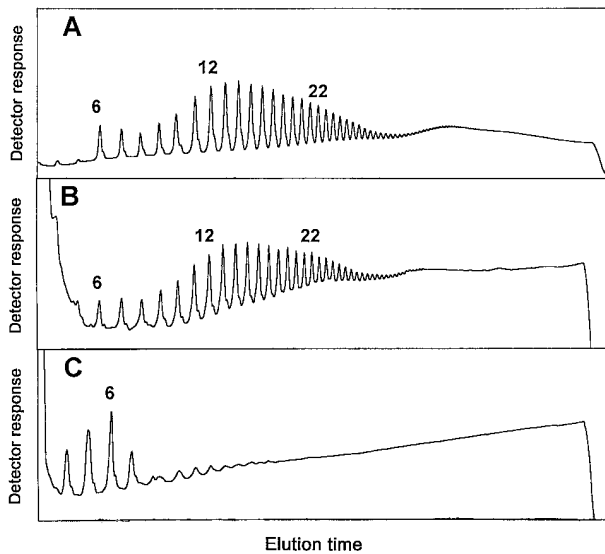
The putative isoamylase activity was purified away from other starch-hydrolyzing activities by the sequential use of polyethylene glycol (PEG) precipitation, preparative native PAGE, and Mono Q anion exchange chromatography (Figure 3D). Two preparations, each from a different batch of wild-type plants, yielded identical results. As in crude extracts, the partially purified enzyme was able to hydrolyze β-limit dextrin but not pullulan in native gels (data not shown). This showed that the enzyme was neither β-amylase nor pullulanase.

To confirm that enzyme D1 was a debranching enzyme and to exclude the possibility that it was an α-amylase, the products of its activity on potato amylopectin were analyzed. Liberated α-1,4-glucan chains were separated by high-performance anion exchange chromatography (HPAEC) and detected using a pulsed amperometric detector (PAD). The products released by enzyme D1 were compared with those liberated by *Pseudomonas amyloderamosa* isoamylase and porcine pancreas α-amylase. The *P. amyloderamosa* isoamylase and the Arabidopsis enzyme D1 yielded very similar distributions of chain lengths (Figures 4A and 4B), whereas the α-amylase produced only very short chains (Figure 4C). This proves that the enzyme purified from Arabidopsis has debranching activity.

Other Starch-Metabolizing Enzymes Are Unaffected by the Mutation in *dbe1*

We measured the activities of enzymes involved in the synthesis and degradation of starch (Table 1). All assays were performed under optimal conditions, and the linearity of the assays with respect to time and to the volume of extract was confirmed. The activities of the starch-degrading enzymes were similar in the wild type and the mutant, with the exception of pullulanase, which was 40% higher in the mutant.

No differences were detected in the total activity of starch synthase or branching enzyme between the wild type and *dbe1-1.* However, because these enzymes are usually present as multiple isoforms (Smith et al., 1997), we sought to discover whether there had been any change in the relative activities of the isoforms in *dbe1*. The isoforms of these enzymes were separated by native PAGE. Starch synthases were detected by incubation of glycogen-containing gels with ADP–glucose followed by iodine staining (Figure 5A). Branching enzyme activity was detected by incubating gels

Figure 3. Native PAGE of Starch-Degrading Enzymes in Extracts of Leaves of the Wild Type and *dbe1-1.*

(A) Soluble proteins were extracted from leaves and separated in gels containing 0.1% (w/v) amylopectin. The gels were incubated overnight at room temperature in 50 mM Tris, pH 7.0, 1 mM CaCl$_2$, 1 mM MgCl$_2$, and 1 mM DTT and stained with an iodine solution.
(B) As described for **(A)**, except that the gel contained 0.1% (w/v) β-limit dextrin in place of the amylopectin.
(C) Soluble proteins were separated in amylopectin-containing gels, electroblotted onto a gel containing 1% (w/v) Red Pullulan, and incubated as described in **(A)**. Clear bands appeared against the red background.
(D) Soluble proteins from the wild type and *dbe1-1* were separated as described in **(A)**, together with the partially purified enzyme D1. Bands of amylase activity (A1, A2, and A3) and debranching enzyme activity (D1 and D2) are indicated. WT, wild type; *dbe1, dbe1-1.*

Figure 4. Comparison of the Products of Digestion of Potato Amylopectin.

(A) Potato amylopectin was digested with isoamylase from *P. amyloderamosa* at pH 3.5. Liberated chains were separated and detected by HPAEC using a PAD.
(B) As described for **(A)**, except that amylopectin was digested with the partially purified enzyme D1 at pH 6.5.
(C) As described for **(A)**, except that amylopectin was digested with porcine pancreas α-amylase at pH 7.
The chain lengths of specific peaks are shown on the traces.

with glucose 1-phosphate and phosphorylase a. Glucans synthesized by phosphorylase a in the presence of branching enzyme were visualized by iodine staining (Figure 5B). Four bands of starch synthase activity and two of branching enzyme activity were detected in crude extracts of leaves of the wild type. We detected no changes in the relative abundance of any of these isoforms in *dbe1-1* relative to the wild type. None of the starch synthase or branching enzyme bands was seen in control gels incubated without either ADP–glucose or phosphorylase a, respectively.

The *dbe1* Mutant Accumulates Both Starch and Phytoglycogen

Mutations that affect debranching enzyme activity in the endosperms of maize (Pan and Nelson, 1984; James et al., 1995), rice (Nakamura et al., 1996), and the unicellular alga Chlamydomonas (Mouille et al., 1996) all result in the synthesis of a soluble, more highly branched polyglucan (phytoglycogen) and in a reduction in the synthesis of starch. Therefore, we established a method for the separate extraction of starch and soluble glucans from Arabidopsis leaves. Recoveries using these extraction methods were measured

by adding representative amounts of starch granules or glycogen to the extraction medium of one of two replicate samples before homogenization of the leaves. The recoveries of the added compounds, after the subtraction of the endogenous values, were 90.3 ± 14% for glycogen and 85 ± 8% for the starch granules (mean ±SE; $n = 3$).

The wild type accumulated starch at an essentially linear rate throughout the day (Figure 6A). This starch was degraded throughout the night, again at an almost constant rate. Small amounts of soluble glucans were detected in the wild type, equivalent to 0.6% of the starch content at the end of the day (Figure 6B). The mutant line also accumulated and degraded starch at a linear rate throughout the day and night, respectively, but the total starch accumulated was only one-tenth that made by the wild type. The mutant also synthesized appreciable amounts of soluble glucans, equivalent to half the amount of starch made by the wild type. During the night, most of the soluble glucan (90%) was degraded during the first 8 hr of darkness.

Analysis of Polyglucan Composition and Structure

The composition of the starch from the wild type and *dbe1-1* was analyzed by using Sepharose CL2B chromatography (Figures 7A and 7B). The wild-type and mutant starches were similar in that they were composed mostly of a high molecular weight compound with relatively little low molecular weight material. When complexed with iodine, the high molecular weight material had a wavelength of maximum absorbance (λ_{max}) between 550 and 560 nm, which is characteristic of amylopectin. The low molecular weight material had a λ_{max} between 575 and 600 nm, suggesting a glucan

Table 1. Comparison of the Maximum Catalytic Activities of Starch-Metabolizing Enzymes in Crude Extracts of Leaves of Wild-Type and *dbe1* Arabidopsis Plants

Enzyme	Activity (nmol min^{-1} g^{-1} Fresh Weight)	
	Wild Type	*dbe1-1*
α-Amylase	58 ± 4.3	68 ± 6.6
β-Amylase	1063 ± 19	1040 ± 22
Starch phosphorylase	130 ± 30	95 ± 12
Maltase	56 ± 6.0	61 ± 3.2
D-Enzyme	508 ± 29	536 ± 19
Pullulanase	34 ± 0.3	47 ± 1.7
Total starch synthase	203 ± 11	174 ± 5
Soluble starch synthase	169 ± 1	144 ± 5
Branching enzyme[a]	11 ± 0.9	11 ± 1.2

[a] Activity is measured as the stimulation of the incorporation of carbon-14 from ^{14}C-glucose 1-phosphate into glucan by phosphorylase a and is given as micromoles per minute per gram fresh weight of tissue.
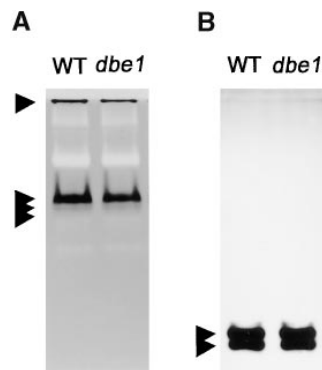
Figure 5. Native PAGE of Isoforms of Soluble Starch Synthase and Branching Enzyme in Extracts of Leaves of the Wild Type and *dbe1-1.*

(A) Soluble proteins were extracted from leaves, subjected to native PAGE in glycogen-containing gels, and then incubated at pH 8.6 with 1 mM ADP–glucose. Gels were stained with an iodine solution to reveal bands of soluble starch synthase activity, indicated by arrowheads.
(B) Soluble proteins were extracted from leaves, subjected to native PAGE, and then incubated at pH 7.2 with glucose 1-phosphate and phosphorylase a. Gels were stained with an iodine solution to reveal bands of branching enzyme activity, indicated by arrowheads.
WT, wild type; *dbe1*, *dbe1-1.*

more branched than amylose. Iodine–amylose complexes typically have a λ_{max} of 620 nm. The λ_{max} values of the iodine complexes of maize phytoglycogen, oyster glycogen, and the soluble glucan from *dbe1-1* were 442, 440, and 430 nm, respectively, suggesting that the soluble glucan is highly branched.

The distribution of chain lengths of the starches and of the soluble glucan from *dbe1-1* was investigated by debranching the glucans with isoamylase from *P. amyloderamosa* and separating and detecting the liberated chains by HPAEC using a PAD. The chain length distribution of the amylopectin from the wild type (Figure 8A) was strongly polymodal, with maxima at chain lengths of 12, 15, and 22 glucose residues. The amylopectin from *dbe1-1* starch had a distribution of chain lengths very similar to that from wild-type Arabidopsis (Figure 8B). This suggests that the structure of the amylopectin in *dbe1-1* is similar to that in the wild type, despite the elimination of the chloroplastic isoform of isoamylase.

The chain length profile of the soluble glucan from *dbe1-1* (Figure 8C) was markedly different from that of Arabidopsis amylopectin. The proportion of very short chains, particularly those of <10 glucose residues, was increased compared with that of amylopectin. This is a characteristic of glycogen-like glucans, and because short chains are unable to interact with neighboring chains to form higher order structures, it results in a soluble molecule rather than an in-

soluble granule (Gidley and Bulpin, 1987). We compared the chain length profile of the soluble glucan from *dbe1-1* with that of phytoglycogen from *su1* maize kernels (Figure 8D). The profiles were very similar, suggesting that the soluble glucan in *dbe1-1* is phytoglycogen. Interestingly, the polymodal distribution of short chains, evident in leaf amylopectin, was still visible in the soluble glucan from *dbe1-1.*

Starch and Phytoglycogen Accumulate in the Same Chloroplasts in the *dbe1* Mutant

The starch granules from the wild type and the mutant line *dbe1-1* (Figures 9A and 9B) were viewed by scanning elec-
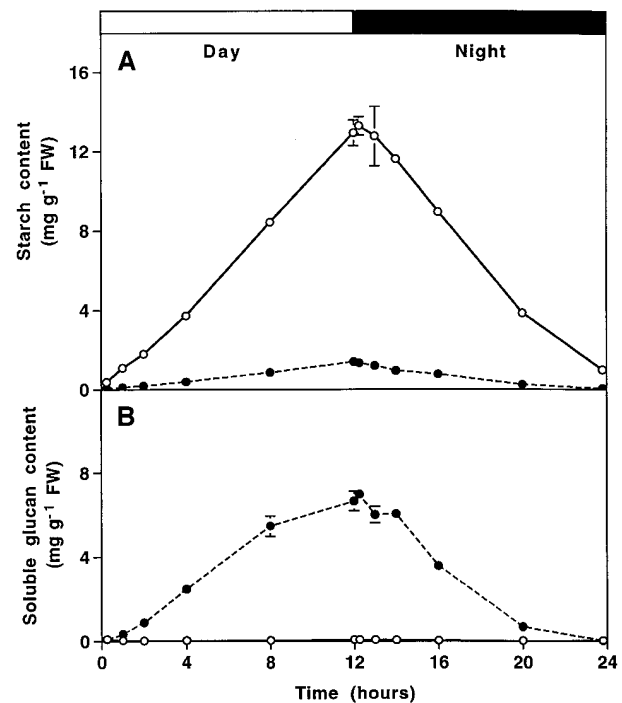

Figure 6. The Starch and Phytoglycogen Content of Wild-Type and *dbe1-1* Leaves during the Day and Night.

(A) Starch was extracted from the leaves of individual plants and digested with α-amylase and amyloglucosidase; the released glucose was then measured. Each point represents the mean ±SE of six replicate samples. Where absent, the error bars are smaller than the symbols.
(B) Phytoglycogen was extracted from the leaves of individual plants and digested and measured as described in **(A)**. Each point represents the mean ±SE of six replicate samples. Where absent, the error bars are smaller than the symbols.
Open symbols and solid lines represent the wild type. Closed symbols and dashed lines represent *dbe1-1.* FW, fresh weight.
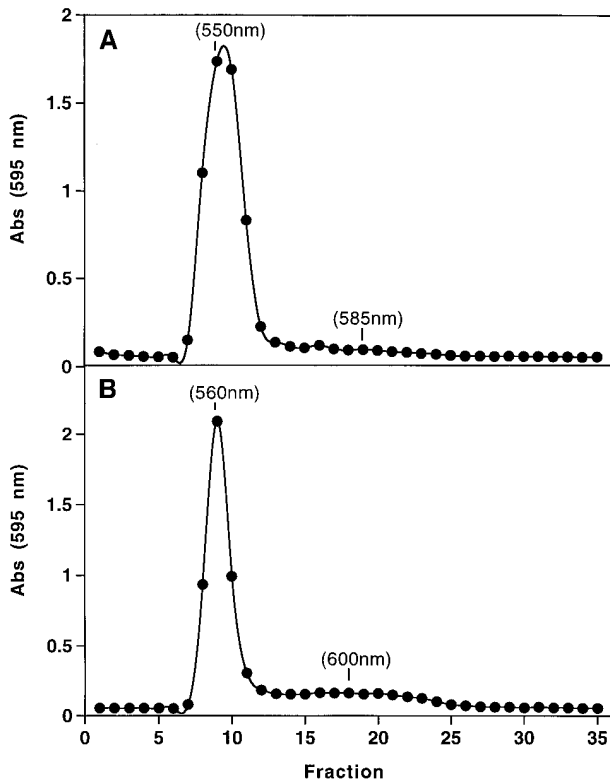
Figure 7. Sepharose CL2B Chromatography of Starch from Wild-Type and *dbe1-1* Plants.

(A) Wild-type starch (0.5 mg) was dissolved in 0.5 M NaOH, applied to a 5-mL column, and eluted with 0.1 M NaOH. The absorbance (Abs) of fractions after the addition of an iodine solution was measured at 595 nm. The λ_{max} values of the peak fractions are given within parentheses.

(B) Starch from *dbe1-1* was analyzed as described in **(A)**.

tron microscopy. The granules from wild-type leaves were irregular discoids with diameters of ∼1 to 2 μm and thicknesses of ∼0.2 to 0.5 μm. The granules isolated from the mutant were smaller, with diameters of ∼0.5 to 1 μm and thicknesses of ∼0.1 to 0.3 μm. By transmission electron microscopy, starch granules were clearly visible in the wild-type chloroplasts (Figure 9C). The chloroplasts from the mutant also contained small starch granules that were surrounded by numerous pockets of material (Figure 9D) not present in the wild type. These regions were less electron dense than the stroma and probably contained the soluble polysaccharide. To investigate whether this was the case, we pretreated similar sections by using a silver proteinate method to stain glucan polymers (Robertson et al., 1975). Microscopy (Figures 9E to 9G) revealed the presence of small glucan particles in the mutant that were ∼25 nm in size.

It is very likely that these are phytoglycogen molecules (Geddes, 1985).

DISCUSSION

The *dbe1* Mutant Lacks Isoamylase

In this study, we have provided evidence that mutations at the *DBE1* locus directly affect the expression of a chloroplastic isoform of isoamylase. First, we have shown that the x-ray–generated mutant line lacks the transcript of a gene with sequence similarity to an isoamylase gene from maize. Second, the mutation abolishes a starch-hydrolyzing enzyme activity in crude leaf extracts. This is a chloroplastic enzyme (Zeeman et al., 1998), and our analyses, both of crude leaf extracts and of the partially purified protein, are consistent with a debranching enzyme of the isoamylase type and not with any other kind of starch-hydrolyzing enzyme found in plants thus far. We have been unable to detect another isoamylase activity in leaf extracts. However, a second DNA sequence with sequence similarity to the isoamylase gene from maize, but distinct from *DBE1*, exists within the Arabidopsis genome (GenBank accession number AF002109).

Using a range of assays and native gel techniques to survey the other enzymes of starch synthesis and starch degradation, we found no other changes in the mutant compared with the wild type, with the exception of total pullulanase activity, which was 40% higher in the mutant. These data strongly suggest that a chloroplastic isoform of isoamylase is encoded at the *DBE1* locus.

Loss of Isoamylase Leads to the Accumulation of Both Phytoglycogen and Starch

The loss of the chloroplastic isoamylase has a considerable effect on the accumulation of glucan in the mutant leaves. The total amount of glucan accumulated in the mutant line is ∼40% less than that in the wild type, and >80% of this is accumulated in a soluble form. This soluble glucan is phytoglycogen. Its λ_{max} when complexed with iodine is 430 nm, compared with 440 nm for oyster glycogen and 442 nm for maize phytoglycogen. This contrasts with the λ_{max} of the iodine complex of amylopectin from Arabidopsis leaves, which is 550 to 560 nm. The distribution of chain lengths, analyzed by HPAEC using a PAD, closely resembles that of maize phytoglycogen, but it is distinct from that of Arabidopsis amylopectin. However, the *dbe1* mutant still accumulates some starch in the form of small granules that, in composition and amylopectin structure, are indistinguishable from the starch of the wild type. Transmission electron microscopy demonstrated that both the starch and the phytoglycogen accumulate in the same chloroplasts in *dbe1*.
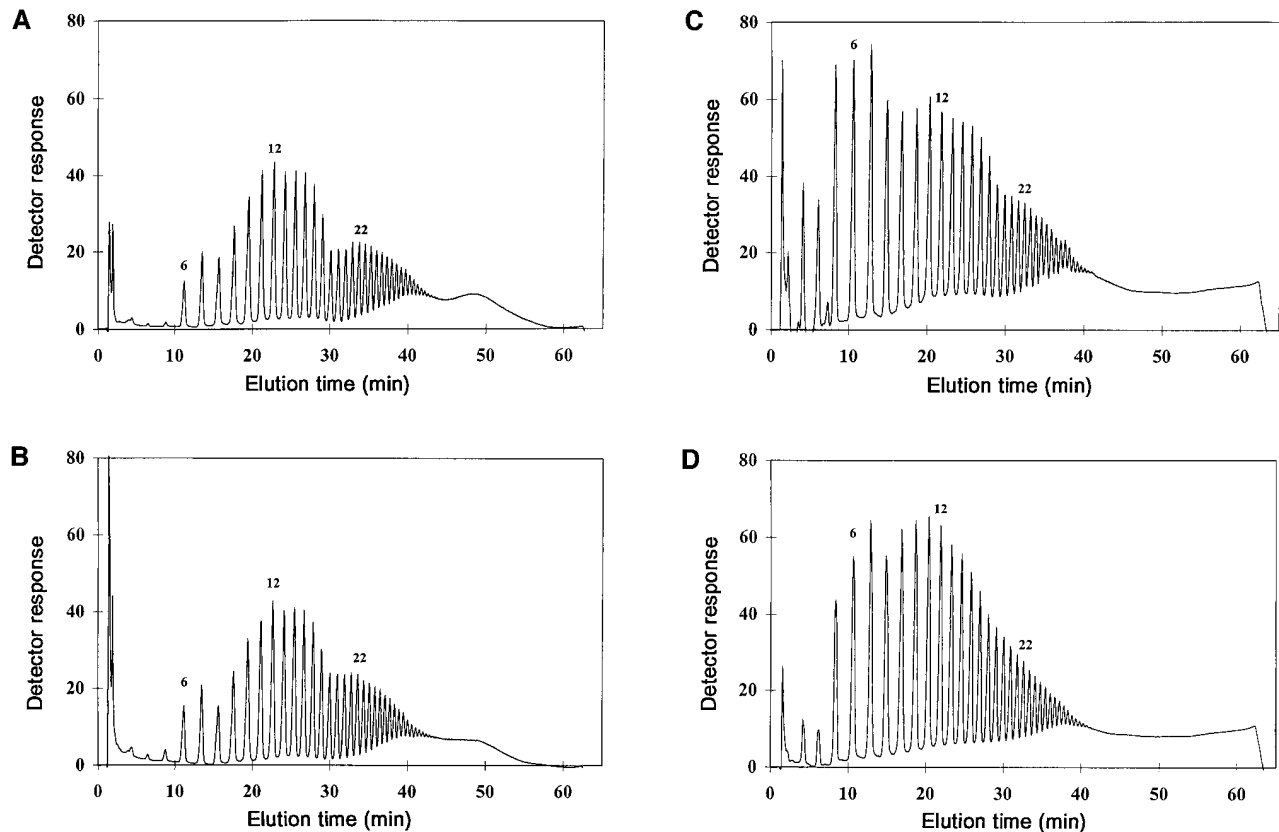
Figure 8. The Chain Length Distributions of Amylopectin from Wild-Type Arabidopsis and *dbe1-1* Plants, the Soluble Glucan from *dbe1-1*, and Phytoglycogen from *su1* Maize Kernels.

(A) Wild-type starch was debranched with *P. amyloderamosa* isoamylase at pH 3.5, and the liberated chains were separated and detected by HPAEC using a PAD.
(B) Starch from *dbe1-1* was analyzed as described in **(A)**.
(C) The soluble glucan from *dbe1-1* was analyzed as described in **(A)**.
(D) Phytoglycogen from *su1* maize was analyzed as described in **(A)**.
The chain lengths of specific peaks are shown on the traces.

Isoamylase Is Not Required for Starch Degradation

Until recently, debranching enzymes were considered to be involved primarily in starch breakdown. However, in the *dbe1* mutant, the cleavage of α-1,6 linkages during the night is not affected by the loss of the chloroplastic isoamylase D1. During the first 4 hr of the night, the wild type degraded its starch at a rate of 1.0 mg hr^{-1}, whereas in the mutant, starch and phytoglycogen together were degraded at a rate of 0.92 mg hr^{-1} (data from Figure 6). Considering that >80% of this was phytoglycogen and that phytoglycogen is more highly branched than amylopectin, this suggests that the rate of hydrolysis of α-1,6 linkages at night is actually higher in the mutant than it is in the wild type. Because no other isoamylase activity was detected, it is reasonable to assume

that pullulanase is responsible for the hydrolysis of α-1,6 linkages in *dbe1*. Pullulanase from most sources has a very low activity on glycogens, including phytoglycogen (Nakamura, 1996); hence, it is likely that the activity of other degradative enzymes is required first to generate a suitable substrate.

The Role of Isoamylase in Amylopectin Synthesis

Previous studies (Pan and Nelson, 1984; Mouille et al., 1996; Nakamura et al., 1996) have suggested that debranching enzymes are in some way involved in amylopectin synthesis. Our results are consistent with this view but provide more information about the nature of the involvement. In *dbe1*, the accumulation of phytoglycogen is caused specifically by the

loss of isoamylase activity, because there are no major changes in any of the other enzymes of starch metabolism. Although the mutation in *su1* maize has been shown to lie in an isoamylase gene (James et al., 1995), the considerable secondary effects on the activities of other starch-metabolizing enzymes (Pan and Nelson, 1984; Singletary et al., 1997; Rahman et al., 1998) have made it impossible to determine whether the loss of isoamylase alone is the cause of phytoglycogen accumulation. Our findings suggest that the loss of isoamylase could be the most important factor resulting in phytoglycogen accumulation in *su1* maize. In rice, the *su1* mutation has not yet been characterized, but it is clear that the mutation does not lie in the gene encoding pullulanase (Nakamura et al., 1996). It seems likely that the mutation will prove to be in an isoamylase gene.

In the glucan-trimming model of amylopectin synthesis (Ball et al., 1996; see Introduction), the synthesis of phytoglycogen in *su1* mutants of cereals and the *sta7* mutant of Chlamydomonas is interpreted as the accumulation of pre-amylopectin, a theoretical intermediate of amylopectin synthesis, which normally would be processed by debranching enzyme. The accumulation of both normal starch and highly branched phytoglycogen in *dbe1* chloroplasts cannot easily be explained by this model. Although an isoform of isoamylase activity is eliminated by the mutation, pullulanase activity remains. If pullulanase can participate in glucan trimming or if there is another isoform of isoamylase in leaves, then the logical consequence from the model would be either normal starch or, if the debranching enzyme activity were limiting, the accumulation of a single type of glucan with a branching pattern intermediate between amylopectin and phytoglycogen. Alternatively, if isoamylase is the only de-branching enzyme involved in the glucan-trimming process and its activity is abolished in *dbe1*, then the mutant should solely accumulate phytoglycogen. In neither case can the current glucan-trimming model explain why two discrete products—apparently normal amylopectin and the highly branched phytoglycogen—are synthesized in *dbe1*. The *su1* mutants of maize and rice also accumulate both starch and phytoglycogen in the endosperm (Boyer et al., 1977). However, it is not clear whether they accumulate at the same time or in the same plastids.

Our results suggest that if glucan trimming does occur, the process is more complex than that described by Ball et al. (1996). In *dbe1*, the debranching enzyme activity is clearly insufficient to allow a normal rate of starch synthesis, yet the remaining activity (pullulanase) must in some way be regulated so that it processes some preamylopectin to amylopectin while leaving most unprocessed.

An Alternative Model to Explain the Involvement of Isoamylase in Starch Synthesis

We propose an alternative explanation for phytoglycogen accumulation in isoamylase-deficient mutants. Rather than
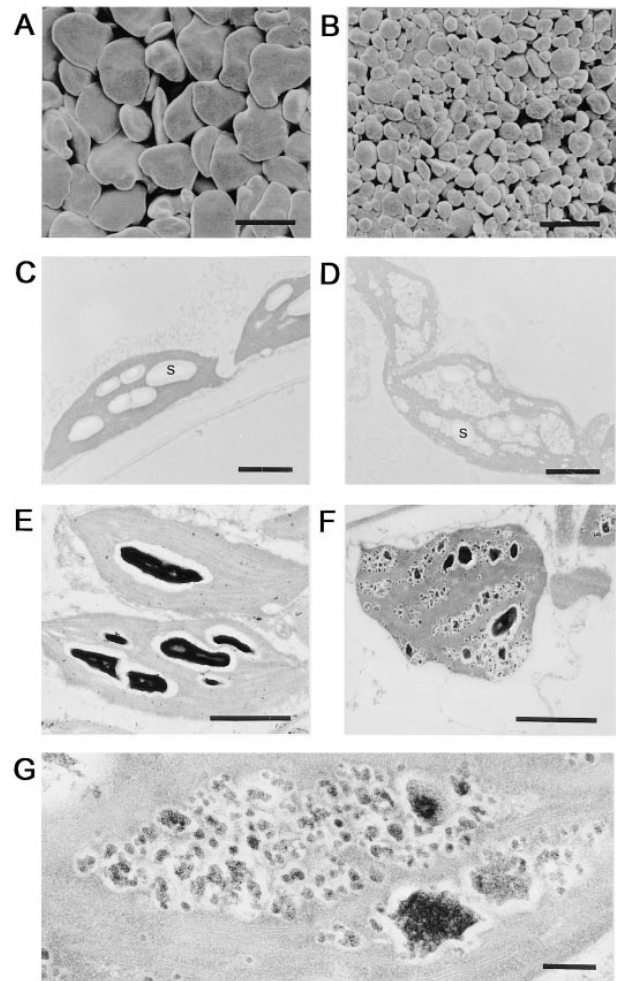
Figure 9. Starch Granule Morphology and Chloroplast Ultrastructure of Arabidopsis Wild-Type and *dbe1-1* Plants.

(A) and **(B)** Scanning electron microscopy of starch granules isolated at the end of the photoperiod from leaves of wild-type and *dbe1-1* Arabidopsis plants, respectively.
(C) and **(D)** Transmission electron microscopy of leaf sections of wild-type and *dbe1-1* Arabidopsis plants, respectively. Samples were taken two-thirds of the way through the photoperiod. Starch granules within the chloroplasts are labeled S.
(E) and **(F)** Sections similar to those shown in **(C)** and **(D)** that were pretreated using the silver proteinate method to stain glucan polymers. Starch granules in the wild type and mutant appear electron dense. Small glucan particles, likely to be phytoglycogen, are visible in the mutant chloroplasts.
(G) High magnification of a silver proteinate–treated leaf section of *dbe1-1* as described for **(F)**.
The bars in **(A)** to **(F)** = 2 μm; the bar in **(G)** = 200 nm.

acting in the manner proposed by Ball et al. (1996), isoamylase in wild-type plants may work together with other glucan-degrading enzymes to prevent phytoglycogen accumulation. We envisage that phytoglycogen is not an intermediate of amylopectin synthesis but rather a separate soluble product made in the stroma. We suggest that the action of starch synthases and branching enzymes on small maltooligosaccharides in the stroma may result in the synthesis of soluble branched glucans. This would potentially provide an abundance of alternative substrates for further elongation and branching, reducing the amount of the synthetic enzymes available to act at the surface of the starch granule. The action of the glucan-degrading enzymes in the stroma may prevent the accumulation of such soluble material, thus directing glucan synthesis to the surface of the granule. The reduction in isoamylase activity in *dbe1* and *su1* mutants would prevent or reduce the hydrolysis of any α-1,6 linkages formed in the soluble glucans, allowing their proliferation, as phytoglycogen, at the expense of starch synthesis.

There is evidence to support this model. First, the model could explain the starch- and phytoglycogen-accumulating phenotypes observed in isoamylase-deficient mutants. Second, small amounts of soluble glucans are detectable in wild-type Arabidopsis leaves (this study) and maize endosperm (Doehlert et al., 1993). These could be pools of soluble glucan that are rapidly turned over. Third, starch synthesis proceeds in the presence of considerable plastidial activities of α-1,4–glucan—degrading enzymes (such as starch phosphorylase, D-enzyme, and α-glucosidase), of which the preferred substrates are small soluble glucans (Steup and Schachtele, 1981; Lin and Preiss, 1988; Sun et al., 1995). It is likely that these enzymes would be active during starch synthesis because few possess discernable regulatory properties (Beck and Ziegler, 1989).

It is entirely plausible that a range of isoforms of starch synthases and branching enzymes, all acting together at the granule surface, could synthesize amylopectin without the direct participation of debranching enzyme (Manners, 1998). Recent work has shown that different isoforms of these enzymes have distinct specificities (Guan and Preiss, 1993; Craig et al., 1998) and that mutations that alter isoform complement result in changes in amylopectin structure (Craig et al., 1998; Gao et al., 1998).

METHODS

Plants

Two mutant populations of *Arabidopsis thaliana* ecotype Columbia were generated by x-ray mutagenesis and ethyl methanesulfonate (EMS) mutagenesis, respectively (the latter population was obtained from Lehle Seeds, Round Rock, TX). Unless otherwise stated, plants were grown in a growth room at 20°C with 75% relative humidity and a 12-hr photoperiod. The quantum irradiance was 50 μmol m^{-2} sec^{-1} for the first 3 weeks and 200 μmol m^{-2} sec^{-1} thereafter. The plants for enzyme assays and for analysis using native gels were grown in a greenhouse with minimum temperatures of 15 and 10°C during the day and night, respectively. The multiple marker mapping lines were obtained from the Arabidopsis Biological Resource Center (ABRC; Columbus, OH), and *sugary1* (*su1*) maize (*Zea mays*) kernels were a gift from I. Bridges (Zeneca Seeds, Bracknell, UK).

Materials

All of the enzymes were from Boehringer Mannheim, except for isoamylase, which was from Sigma, and β-amylase and maltase, which were from Megazyme (Bray, Ireland). Megazyme also supplied pullulan, Red Pullulan, and the Ceralpha and Betamyl kits. Radiochemicals were from Amersham International. Potato amylopectin was from Sigma.

Mapping

Conventional mapping was conducted by crossing the mutant plants to the mapping lines CS3078, CS3079, and CS3080. The F$_2$ progeny were scored for the mutation, and the visible phenotypes and the data were analyzed by using the Joinmap computer program (Stam, 1993). The extraction of DNA, gel blotting, and restriction fragment length polymorphism (RFLP) mapping were performed according to Wang et al. (1997). The RFLP probes were obtained from the ABRC.

Preparation of RNA and RNA Gel Blot Analysis

Total RNA was extracted from 2 to 3 g of mature Arabidopsis rosette leaves and blotted onto nitrocellulose filters, as described by Martin et al. (1985). Radioactive probes were made by labeling DNA fragments using the Klenow fragment of *Escherichia coli* DNA polymerase I primed with random hexanucleotide primers (Feinberg and Vogelstein, 1984).

Native PAGE

Separation of proteins on native amylopectin-containing gels (for the detection of starch-hydrolyzing enzymes) and electroblotting onto Red Pullulan–containing gels were performed as described by Zeeman et al. (1998). Gels containing 0.1% (w/v) β-limit dextrin in place of the amylopectin were treated the same way as those containing amylopectin. Experiments to detect isoforms of soluble starch synthase were performed as described by Edwards et al. (1995).

For the detection of the branching enzymes (Yamanouchi and Nakamura, 1992), the resolving gel contained 5% (w/v) acrylamide (30:0.8 acrylamide–bisacrylamide) and 375 mM Tris, pH 8.8. The stacking gel contained 3.3% (w/v) acrylamide and 63 mM Tris, pH 6.8. After electrophoresis with a 12 mA constant current at 4°C, the gel was washed twice with 35 mL of 50 mM Hepes, pH 7.0, and 10% (v/v) glycerol for 10 min and then incubated at room temperature in 30 mL of this medium plus 50 mM glucose 1-phosphate, 2.5 mM adenosine monophosphate, and 28 units of phosphorylase a (from rabbit muscle). The gels were stained with 0.67% (w/v) I$_2$ and 3.33% (w/v) KI.

Partial Purification and Characterization of Isoamylase

All of the purification steps were done at 0 to 4°C. Mature, healthy, wild-type Arabidopsis leaves (∼100 g) were harvested onto ice, washed with deionized water, homogenized using an electric blender, and then ground with a mortar and pestle in ice-cold extraction medium containing 100 mM Mops, pH 7.2, 1 mM EDTA, 1 mM DTT, and 10% (v/v) ethanediol. The sample-to-medium ratio (w/v) was 1:1. The homogenate was filtered through two layers of muslin, 0.1% (w/v) polyvinylpolypyrrolidone was added, and insoluble material was removed by centrifugation (24,000g for 30 min). The supernatant was adjusted to 3% (w/v) polyethylene glycol (PEG) 8000, the precipitate was removed by centrifugation, and the supernatant was adjusted further to 10% PEG. The 3 to 10% PEG precipitate was removed by centrifugation, dissolved in 10 mL of extraction medium, and subjected to preparative native electrophoresis at 4°C using a preparative electrophoresis apparatus (Prep Cell model 491; Bio-Rad). Six-centimeter resolving gels containing 7.5% (w/v) acrylamide (30:0.8 acrylamide–bisacrylamide), and 375 mM Tris, pH 8.8, and 1.5-cm stacking gels containing 3.75% (w/v) acrylamide and 63 mM Tris, pH 6.8, were used. Proteins eluted from the bottom of the gel were collected in 7-mL fractions of extraction medium.

Fractions containing the putative isoamylase (detected by native PAGE in amylopectin-containing gels) were pooled (∼100 mL), and the proteins were concentrated fourfold by filtration through a 10-kD filter (Jumbosep; Flowgen Instruments, Lichfield, UK). The filtrate was adjusted to contain 2 mM CaCl$_2$, 2 mM EDTA, 2 mM DTT, and 20 mM KCl (chromatography medium) and applied to a preequilibrated 1-mL Mono Q column (Pharmacia). Bound proteins were eluted with a linear gradient of KCl (from 0.02 to 0.5 M). Fractions containing the putative isoamylase were pooled, diluted with KCl-free chromatography medium, and subjected to a second round of Mono Q chromatography. Fractions containing the putative isoamylase but no other starch-hydrolyzing activities were pooled, frozen in liquid nitrogen, and stored at −80°C.

Enzyme Measurements

The enzymes maltase, D-enzyme, starch phosphorylase, and pullulanase were assayed using protocols described by Zeeman et al. (1998). Total and soluble starch synthases were assayed using the methanol–KCl precipitation method and resin method, respectively, as described by Jenner et al. (1994). Starch branching enzyme was assayed according to Smith (1988). The Ceralpha and Betamyl kits (Megazyme) were used to measure α-amylase and β-amylase activity, respectively. These assays are not liable to interference by isoamylase because they use specific, unbranched substrates and were performed according to the manufacturer's instructions.

Extraction and Measurement of Carbohydrates

Starch granules from healthy mature leaves of Arabidopsis were extracted as described by Zeeman et al. (1998). Phytoglycogen was extracted from su1 maize kernels by using a variation of the method described by Schoch (1957). Dry kernels (20 g) were ground to a powder using a coffee grinder. The powder was suspended in 100 mL of extraction medium (100 mM Mops, pH 7.2, 5 mM EDTA, and 2 mM DTT) and stirred at 4°C for 30 min. The suspension was filtered through a 45-μm nylon mesh, and the filtrate was centrifuged (3000g for 15 min

at 4°C). The supernatant was then heated to 95°C for 30 min and cooled, and the precipitated protein was removed by centrifugation. The phytoglycogen in the supernatant was precipitated by the addition of 75% (v/v) methanol and 1% (w/v) KCl, collected by centrifugation, and dehydrated by resuspension in hot methanol. The phytoglycogen was collected by centrifugation, dried, and stored at −20°C.

For the extraction of phytoglycogen from Arabidopsis, leaves were homogenized directly in extraction medium. All of the other procedures were identical to the procedure used for maize. To verify that the structure of the soluble glucan from the debranching enzyme mutant dbe1 was not altered during the extraction, phytoglycogen from su1 maize was added to dbe1-1 leaves at the start of the extraction process. The leaves were harvested at the end of an extended dark period and contained no endogenous phytoglycogen. The structure of the maize phytoglycogen (determined by high-performance anion exchange chromatography [HPAEC] using a pulsed amperometric detector [PAD]) was not altered during the extraction with dbe1-1 leaves (data not shown).

To measure both soluble and insoluble glucans in the same samples of Arabidopsis leaves, we established a protocol for the separate extraction of starch and phytoglycogen. All steps were performed at 0 to 4°C. Leaf tissue (250 to 500 mg) was homogenized using an all-glass homogenizer in 4 mL of extraction buffer (100 mM Mops, pH 7.2, and 5 mM EDTA). The homogenate was centrifuged at 3000g for 5 min. The pellet was washed twice by resuspension in 2 mL of extraction buffer and centrifuged. The starch-containing pellet was washed further by resuspension in 5 mL of 80% (v/v) ethanol. The insoluble material was collected by centrifugation, resuspended in 5 mL of water, and stored at −20°C. The aqueous supernatants were combined, and phytoglycogen was precipitated overnight at 4°C by the addition of 75% (v/v) methanol and 1% (w/v) KCl. The precipitate was collected by centrifugation, resuspended in 5 mL of water, and stored at −20°C.

Analysis of Polyglucan Structure

Sepharose CL2B analyses of starch preparations were performed as described by Denyer et al. (1995), except that 0.35-mL fractions were collected at a rate of one fraction per 2 min. HPAEC with a PAD was performed as described by Tomlinson et al. (1997), except that the gradient was adjusted to give an improved separation of shorter chains. The eluants were 150 mM NaOH, 500 mM Na acetate (eluant A), and 100 mM NaOH (eluant B). Gradients of the eluants were run according to the following values: 0 to 60 min, a linear gradient between 85% of eluant A/15% of eluant B and 15% of eluant A/85% of eluant B; 60 to 61 min, a linear gradient between 15% of eluant A/85% of eluant B and 85% of eluant A/15% of eluant B; and 61 to 65 min, constant 85% of eluant A/15% of eluant B.

Electron Microscopy

Starch granules were gold coated and viewed under a scanning electron microscope (model XL 30 FEG; Philips Electronics NV, Eindhoven, The Netherlands). Leaf sections for transmission electron microscopy were prepared as described by Barnes et al. (1996), except that samples were dehydrated in ethanol and embedded in London Resin White (Agar Scientific Ltd., Essex, UK). Sections were stained with uranyl acetate and lead citrate and viewed with a transmission electron microscope (model JEM-1200 EX; Jeol Ltd., London,

UK). Sections were stained for the presence of carbohydrate by using the silver proteinate method described by Robertson et al. (1975).

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