The formation of intermediary glucans, mature starch, and phytoglycogen was studied using leaves of Arabidopsis thaliana wild type and dbe mutant, which lacks plastidic isoamylase (Zeeman, S. C., Umemoto, T., Lue, W. L., Au-Yeung, P., Martin, C., Smith, A. M., and Chen, J. (1998) Plant Cell 10, 1699–1711). A new approach to the study of starch biosynthesis was developed based on “very short pulse” labeling of leaf starch through photosynthetic fixation of 14CO2. This allowed selective analysis of the structure of starch formed within a 30-s period. This time frame is shorter than the period required for the formation of a single crystalline amylpectin lamella and consequently permits a direct analysis of intermediary structures during granule formation. Analysis of chain length distribution showed that the most recently formed outer layer of the granules has a structure different from the mature starch. The outer layer is enriched in short chains that are 6–11 glucose residues long. Side chains with 6 glucose residues are the shortest abundant chains formed, and they are formed exclusively by transfer from donor chains of 12 glucose residues or longer. The labeling pattern shows that chain transfer resulting in branching is a rapid and efficient process, and the preferential labeling of shorter chains in the intermediary granule bound glucan is suggested to be a direct consequence of efficient branching. Although similar, the short chain intermediary structure is not identical to phytoglycogen, which is an even more highly branched molecule with very few longer chains (more than 40 glucose residues). Pulse and chase labeling profiles for the dbe mutant showed that the final structure is more highly branched than the intermediary structures, which implies that branching of phytoglycogen occurs over a longer time period than branching of starch. Thereby, starch serves as a buffer for assimilates, which enable leaves to continue the export of sugars during the night period. This is essential for plant performance, as demonstrated by the poor growth mutants, which are deficient in starch formation (1, 2) or starch remobilization (3, 4).

Starch, deposited as well defined granules inside plastids, is composed of two types of polysaccharides, amylose and amylopectin. Amylose consists of predominantly linear α-1,4-glucans with a molecular mass in the range of 10⁷ to 10⁸. Amylopectin is a highly branched structure containing shorter chains of α-1,4-glucans and frequent α-1,6 branch points (5). The molecular mass of amylopectin is 10⁷ to 10⁸ (6). Typically amylopectin constitutes 70–80% of the starch. However, transitory starch appears to contain almost exclusively amylopectin (4, 7).

Starch granules are highly organized structures, with densely (1.4 g ml⁻¹) packed molecules. At the supramolecular level, large granules are clearly ordered in layers, so-called growth rings, readily visible by light microscopy. The granules are semicrystalline, and the amylopectin molecules account for this crystallinity. According to the current understanding, the amylopectin glucan chains are radially arranged and ordered in alternating amorphous and crystalline lamellae. The amorphous lamellae are regions with more abundant α-1,6 branch points, whereas the crystalline lamellae consist of the linear α-1,4-glucan chains organized in parallel double helices congregated into clusters, which form crystalline lattices (8). The width of the repeated crystalline and amorphous layers is 9 nm, a feature that is highly conserved for starches from many botanical sources (9). By specific hydrolysis of the α-1,6 branch point using isoamylase (debranching) and chromatographic separation of the derived linear glucan chains, it is possible to obtain a branch length distribution profile of the amylopectin. No matter what the botanical source or organ, these profiles are polymodal, which is a direct reflection of the repeated lamellar structure of the starch granule. The profile may vary with the botanical source but is highly conserved for specific types of starch (10).

The molecular structure of starch directly affects its functionality, and because starch is of immense agronomic importance as a major constituent of many crops, there has been great interest in understanding the details of the biosynthesis and structure of starch. A large body of research has been directed at investigating starch biosynthesis in major crops including cereal seeds and potato tubers, as well as in important model systems such as Arabidopsis and the green algae Chlamydomonas. The major enzymes responsible for starch biosynthesis and its regulation have been identified (6, 11, 12), and the formation of the precise structure has proved to be a complex process that requires the concerted action of several isozymes of starch synthases and branching enzymes and most
likely also other modifying enzymes. Despite considerable progress in understanding the molecular basis of starch formation, we still do not know precisely the parameters defining the specific molecular structures and the three-dimensional organization of amylopectin molecules. A specific aspect that has prompted recent re-evaluation of how the final starch structure may be achieved is the apparent contribution of isoamylase. Mutants of maize (13), rice (14, 15), Chlamydomonas (16), and Arabidopsis (4), which all lack plastidic isoamylase activity, accumulate a more highly branched polysaccharide, phytoglycogen, which is not organized in well-defined granules. This suggests a close relationship between the packaging of the amylopectin molecules and the branching and chain length distribution; that debranching may be essential in determining the final amylopectin structure. Isoamylase has been proposed to remove excessive short chains, and two models of its action have been proposed. According to the first model, based on work with Chlamydomonas, debranching is an integrated element in formation of the final amylopectin structure. The model suggests the formation of highly branched “preamylopectin” on the outer surface of growing granules (16), and the final structure requires trimming of excessive short chains followed by elongation of the remaining chains. This “trimming” model would predict the structure of the outermost layer of a starch grain to be different from the final structure. An alternative model was proposed for the Arabidopsis system, where data suggest that starch and phytoglycogen represent two independent polysaccharides, which may accumulate simultaneously (4). It has been suggested that isoamylase is required to prevent accumulation of intermediary soluble highly branched glucans (17). This model does not directly predict a specific structure of the outer amylopectin layer but suggests that smaller phytoglycogen-like molecules may exist as a separate short-lived glucan pool.

To understand starch biosynthesis it would therefore be valuable to know specifically the structure of the outermost amylopectin layer and to investigate the potential formation of intermediary pools of glucan polymers. Because of the technical difficulties, there are as yet no direct observations of the structure of the most recently formed layer of amylopectin. We embarked on developing a method to reveal immature amylopectin structures, which would provide novel information on possible intermediate steps in achieving the final amylopectin structure. Based on pulse labeling of leaf starch through photosynthesis in 14CO2 we examined herein the structures of starch and phytoglycogen formed within a 30-s period. This corresponds to a time frame shorter than the period required for the formation of a single crystalline amylopectin lamella. The labeling pattern reveals that the outer layer of the granules has a different structure from the final starch, showing preferential formation of shorter chains (DP6–11). This new approach to the study of starch biosynthesis provides novel detailed information on starch and phytoglycogen biosynthesis.

**EXPERIMENTAL PROCEDURES**

**Plant Material**—Wild type (WT)1 and a mutant line of Arabidopsis thaliana (L.) Heyn. ecotype Columbia were grown in pot soil in a controlled climate chamber with mercury halide lamps supplemented with light from incandescent lamps at a photosynthetic flux of 120 μmol of photons s−1 m−2 in an 8-h photoperiod. Seeds of mutant line dbe1-1 (4) were kindly provided by Dr. Samuel Zeeman and Professor Alison Smith.

14C Labeling—Leaves were radiolabeled by photosynthesis in a 14CO2-containing chamber. For each labeling four uniform leaves (each with a lamina area of about 1–2 cm2) were excised from young vegetative plants at the end of the photoperiod. The leaves were kept in a transparent box on a humidified paper towel in light for about 10 min before the experiment was initiated. The leaves were then placed on a stainless steel grid in a 15-ml labeling chamber made of a polystyrene Petri dish. 14CO2 was evolved from 0.5 ml of a [14C]bicarbonate solution by the addition of 10-fold excess acid (HCl) and shaking the final solution with 1 ml of air contained in a 5-ml syringe fitted with a injection needle. The resulting radioactive air was injected into the labeling chamber, and the air in the chamber was immediately agitated efficiently for 2–3 s by an electric fan fitted beneath the grid. In total, 57 MBq of 14CO2 was injected resulting in a specific activity of 0.16 GBq μmol−1 carbon in the chamber. During both labeling and pre-incubation, 200 μmol s−1 m−2 light was provided by fluorescent light tubes. Labeling was continued for periods of 30 s to 10 min. The labeling was initiated by injection and terminated by opening the chamber and immediately transferring the leaves to hot extraction medium as described below. Other leaves were transferred to a humid transparent box on a wet paper towel and left in light for 2–20 min before extraction (chase by photosynthesis in nonlabeled air).

**Extraction Procedure**—The labeled leaves were each extracted first in 3× 5 ml of 90% (v/v) ethanol at 80 °C, then in 4× 5 ml of 80% (v/v) ethanol 40 °C, and finally in 5× 5 ml of 50 mM sodium acetate–HCl (pH 5.0) at 40 °C.

The extracted insoluble leaf material was transferred to a 1.5-ml screw-cap microtube with 300 μl of 50 mM sodium acetate–HCl (pH 5.0), and the starch in the sample was gelatinized by heating it for 10 min at 90 °C. The sample was then cooled to room temperature, added to 0.7 units of Pseudomonas isoamylase (2 μl of enzyme in 3.2 μl of NH4SO4) (Megazyme), incubated at 42 °C 1 h, and heated for 2 min to 95 °C to inactivate remaining enzyme. Anionic compounds were removed by applying 270 μl of the solution to a 100-μl anion-exchange column (OH− form of AG 2-X8; BioRad) and eluting the neutral glucans with an additional two more times with 100 μl of distilled water. All fractions containing neutral glucans (470 μl) were collected together and stored at ice until further analysis.

**Isolation of Starch Granules**—Five 14C-labeled leaves were homogenized for 30 s at 0 °C in 30 ml of a solution containing 50 mM Tris–HCl, pH 7.5, 0.1% sodium dodecyl sulfate (SDS), and 5 mM EDTA using a Polytron PT 3000 (Kinematica AG) homogenizer. Cell debris was filtered off through glass wool and the starch granules collected by centrifugation. The starch granules were washed three times with distilled water before being dried at room temperature.

**High Performance Anion-exchange Chromatography (HPAEC)—** HPAEC separation with on-line pulsed amperometric detection (PAD) of neutral glucans was performed as described (18). An aliquot (100 μl) of isoamylase-debranched starch or phytoglycogen was used for each chromatographic analysis. Fractions were collected either by automated collection (0.25- or 2-ml fractions) or by manual collection of individual peaks, each representing a defined degree of polymerization (DP, number of glucose residues in chain). The fractions were made acidic by addition of 200 μl of 2 M HCl to 0.8 ml of each fraction, and 3 ml scintillation liquid (Ecoscint A, National Diagnostics) was added to the mixture. Radioactivity in the samples was determined with a liquid scintillation counter (Wallac). For manually collected fractions, which varied in size, the whole fraction was counted after acidification and the addition of 10 ml of scintillation liquid.

**Degradation with β-Amylase**—Leaves were extracted, washed, and then gelatinized in 270 μl of buffer as indicated above. Radioactivity released to the buffer by heating the leaf samples was negligible (below 0.1% of label released by the following enzyme treatments). Outer chains were degraded by the addition of 2 μl (4 units) of barley β-amylase (Megazyme) and incubation for 1 h at 40 °C; radioactivity released to the solution was determined by counting the total radioactivity in five successive washes with 2 ml of buffer. The radioactivity of the fifth wash was in all cases below 0.3% of the total label released by the enzyme treatments. The remaining starch was then degraded and released from the leaf sample by incubating it for 1 h with an additional 2 μl (2 units) of Aspergillus niger amyloglucosidase (Megazyme) at 40 °C. The radioactivity released was determined in three successive washes with 2 ml of buffer, and total released activity was calculated from these data.

## RESULTS

**Very Short Pulse Labeling of Starch**—A procedure was developed for in vivo pulse labeling of starch in Arabidopsis...
leaves during photosynthesis in \( ^{14} \text{CO}_2 \). The procedure was optimized to allow very short pulses (30 s or less), rapid quenching of the labeled plant material, and incorporation of sufficient radioactivity for structural analysis. This was achieved by using ample \( ^{14} \text{CO}_2 \) and a minimized labeling chamber volume with efficient stirring. Direct extraction of whole leaves in hot ethanol (80 °C, 90% v/v) secured immediate quenching. To facilitate handling of the leaf material during extraction, samples were not homogenized or frozen before extraction. As we observed that freezing of the debranched starch samples occasionally influenced the HPAEC separation, the debranched samples were kept on ice but never frozen. For the very short pulses, the major fraction of the fixed \( ^{14} \text{C} \) was recovered in few metabolites, presumably in the Calvin cycle, disturbing the glucan HPAEC profiles. To completely remove these compounds the extensive washing procedure was followed by ion-exchange purification of each sample.

The Distributions of Radiolabel in Soluble Fractions and Starch—Large amounts of radioactivity were fixed in the leaves during 15–60 s of pulse labeling (Fig. 1A). Essentially all of the radioactivity was found in ethanol-soluble, water-soluble, or starch fractions, and only insignificant amounts remained in the insoluble fraction (cell walls, data not shown). Radioactivity incorporated per leaf increased with pulse time (Fig. 1A). Leaves were chosen to be of similar size (≈1 cm\(^2 \)), but to minimize the disturbance of the leaves after harvest the area was not determined. With increasing pulse time the fraction of \( ^{14} \text{C} \) found in starch increased from 3 (15 s) to 18% (60 s). During the following 10-min chase period (photosynthesis in nonradioactive atmosphere for 10 or 20 min before quenching), the radioactivity was present over the entire elution profile (Fig. 3C) but the labeling profiles did not reflect the size of the molecules. The labeling profiles show a similar and characteristic distribution (Fig. 3, B and C). This is reminiscent to the PAD response, which corresponds to the total starch fraction and is similar for all leaves (only shown in Fig. 3A). There was a peak plateau in radioactivity around 19–27 min (at approximately DP11–15), a valley starting at 32–33 min (DP 18) and a second peak at 40–44 min (around DP 23). On average, the radioactivity peaked later (longer chains) as compared with the PAD response. This difference is due to the fact that the PAD response primarily detects the glucose residue at the reducing end and therefore does not reflect the size of the molecules. The labeling profiles demonstrate that a uniform label of the chains was obtained after a 10-min chase period. Leaves quenched immediately after a 30 s pulse had a different distribution of label. Radioactivity was present over the entire elution profile (Fig. 3A) but

![Fig. 1. Distribution of radioactivity in pulse- and pulse-chase-labeled Arabidopsis leaves. Small whole leaves (1–1.5 cm\(^2 \)) were radiolabeled and extracted with 80% ethanol and acetate buffer. The remaining starch was released by isoamylase and purified over an anion-exchange column to remove residual contamination. The relative distribution of radioactivity recovered in the ethanol, buffer, and purified starch fractions was calculated. The leaves were labeled by photosynthesis in the presence of \( ^{14} \text{CO}_2 \) for 15, 20, 30, or 60 s and immediately quenched in hot ethanol (Pulse) or left to photosynthesize in a nonradioactive atmosphere for 10 or 20 min before quenching (Chase).](image)

![Fig. 2. Release of radiolabel from extracted leaves by β-amylase. Arabidopsis leaves were pulse-radiolabeled for 30 s, chased for 0–20 min, and extracted as described in Fig. 1. The starch-derived \( ^{14} \text{C} \) was released from the extracted leaves first with β-amylase. The leaf sample was then washed extensively, and the remaining β-limit dextrin was released by amyloglucosidase. The measured values are indicated as radioactivity released by β-amylase as % of total radioactivity released by β-amylase and amyloglucosidase.](image)
Pulse Labeling Analysis of Starch Intermediary Structures

with a preferential labeling of the shorter chains (DP 6–12) and a generally lower incorporation in the longest chains.

To evaluate the $^{14}$C distribution in individual unit chains, fractions were collected at short intervals for a pulse-labeled sample in which the amount of $^{14}$CO$_2$ used during labeling was increased (Fig. 4). The refined fractions reveal that radioactivity elutes as peaks corresponding exactly to the PAD-detected unit chains. A high incorporation in short chains was observed in agreement with the results presented in Fig. 3. The reason for the more extreme peak in the short-chain area in Fig. 3A is that the smaller unit chains elute close to each other and are collected in fewer fractions than the longer chains. Collection of smaller fractions prevents this effect (Fig. 4). The first large peak of radioactivity corresponds exactly to DP6. Only minute amounts of radioactivity are found at DP3, DP4, and DP5.

Detailed Analysis of WT and dbe Mutant—For a more robust comparison of the starch structure and distribution of radiolabel, peaks corresponding to individual unit chains were collected separately (Fig. 5). Consistent with the refined automatic sampling (Fig. 4), this analysis showed that the shortest chains, which contained a considerable amount of $^{14}$C after 30 s, were DP6 (Fig. 5A). After a 10-min chase period the label distribution shifted to longer chains (Fig. 5, B and C), and the distribution was similar to the relative corrected weight distribution of carbohydrate (Fig. 5D). Collecting individual unit chains permits calculation of the differential distribution between the pulse and pulse-chase samples (Fig. 5C). This clearly shows that during the 30-s pulse there is a relatively higher label in chains DP6–11 and a lower label in chains above DP12, with the largest difference at DP15–16. The very long chains (>DP40) showed little difference in relative intensity of label.

To gain more information on a possible phytoglycogen-like fraction and to validate that the labeling procedure will identify differences in glucan structures formation, we applied the procedure to the dbe mutant of Arabidopsis, which accumulates phytoglycogen (4). The labeling profiles showed a markedly different distribution of label in the dbe mutant, with higher density of label in the short chains, DP6–11, and a lower radioactivity in longer chains, especially above DP40, than in the WT plants (compare Fig. 5, A and F). This difference became more pronounced after the 10-min chase period (compare Fig. 5, B and G). These data reflect the fact that accumulated glucans in the dbe mutant are composed of shorter chains and a very low abundance of the longest chains as demonstrated for the corresponding distributions of total carbohydrate (Fig. 5, I and J). The calculated relative molar distributions of individual unit chains (Fig. 5, E and J) clearly illustrate that the short chains (DP3–10) are more abundant in the dbe mutant. The difference in distribution of radiolabel between pulse and pulse-chase for the dbe mutant (Fig. 5H) was less pronounced than for the WT plants, but we consistently observed a shift in labeling pattern toward shorter chains during the chase period. Although higher in the dbe mutant than in WT plants, the carbon accumulated in the very short chains (DP2–5) was still neither abundant (Fig. 5I) nor heavily labeled (Fig. 5, F and G).

The preferential pulse labeling of short chains could represent either soluble or granule-bound glucans. To investigate this possibility, pulse-labeled leaf batches were divided in halves, and starch grains were isolated by mild centrifugation after homogenization of a half-sample, whereas the other half-sample was analyzed as described above (nonhomogenized leaves). The samples were each analyzed for distribution of radiolabel in unit chains. The recovery of radiolabel was considerably lower in the isolated starch grains, but distribution was almost identical to the nonhomogenized samples (data not shown). The ratio of label in chains ≤11 and chains ≥12 is diagnostic for the chase shift in distribution (Fig. 5C). This

**Fig. 3. Distribution of radioactivity in pulse- and pulse-chase-labeled starch.** Leaves were pulse-labeled for 30 s, chased for 0 (A), 10 (B), or 20 min (C), and extracted as described in Fig. 1. The glucans released by isoamylase and purified over an anion-exchange column were separated by HPAEC. Fractions (2 ml) were collected, and radioactivity was determined in each fraction (open connected points, shaded area). The **solid continuous curve** represents the PAD detection of glucans in the sample. The time for PAD detection has been corrected for the small delay in fraction collection due to tubing volume.

**Fig. 4. Detailed analysis of radioactivity in smaller glucans derived from pulse-labeled starch.** Leaves were pulse-labeled for 30 s (no chase period). Glucans released by isoamylase were separated by HPAEC as described for Fig. 3, but smaller fractions (0.25 ml) were collected, and radioactivity was determined in each fraction (open connected points, shaded area). The **solid continuous curve** represents the PAD detection of glucans in the sample. The time for PAD detection has been corrected for the small delay in fraction collection due to tubing volume.
ratio was very similar for the two types of samples, as 29 and 30% of label in chains was found in isolated starch granules and extracted leaf samples, respectively. For a comparison, 17% of label was found in chains in the 10-min chased leaf sample.

**DISCUSSION**

During the last decade, considerable progress has been made in the understanding of starch biosynthesis. In general, the biochemical mechanisms responsible for starch formation are conserved from algae to higher plants (19). However, starch structure varies with tissue and species, and our understanding of the formation of the precise structures remains incomplete. Different models have been proposed to account for the highly organized structures (17, 20), and work done with mutants of *Chlamydomonas*, *Arabidopsis*, maize, and rice suggests that isoamylase may play a general role in determining starch structure. These studies have clearly demonstrated the value of specific mutants, and targeted selection of further mutants will be important in promoting a comprehensive understanding of starch formation. However, new approaches such as our very short pulse labeling technique will be essential to the realization of this research potential.

The current models for amylopectin biosynthesis predict that intermediary structures are formed during starch biosynthesis. We decided to develop a method that would specifically radio-label the most recently deposited starch, corresponding to only one 9-nm lamella. The intensity should be sufficient to allow determination of radioactivity in each unit chain after debranching. Transitory starch is formed during the photoperiod and degraded during the following night. *Arabidopsis* starch granules reach a diameter of around 1 μm (4), which corresponds to about 110 lamellae. Most likely, new granules are initiated over the entire photoperiod and average growth time for the individual granule would then be a fraction of the photoperiod. Assuming a 2-h growth period for the individual granule, it can be calculated that one 9-nm layer is formed in about 1 min. Our procedure allowed efficient labeling and quenching within considerably shorter time frames. Within 30 s about 10% of the incorporated label was found in the starch fraction (Fig. 1). Sample volumes used for starch debranching and ion-exchange purification were minimized to allow efficient loading of the sample on the HPAEC column. Typically, 5,000–10,000 dpm were loaded on the column for the 30-s pulse-labeled samples, enabling reliable analysis even in fractions with less than 0.5% of the applied radioactivity. As an additional benefit, our procedure allows for PAD analysis of the starch unit chains from just a single small leaf (see Fig. 3A). The ion exchange purification, introduced to remove residual...
Liberation of radiolabel by β-amylose degradation verifies specific pulse labeling of the outermost 9-nm lamella. The β-amylose does not bypass the α-1,6 branch points and therefore only degrades the outer chains. The release of 94% of the radiolabel (Fig. 2) after a 30-s pulse shows that essentially all of the radiolabel represents the outermost lamella. On the contrary, a uniform labeling of the starch glucose residues will result in retaining a fraction of radioactivity corresponding to the β-limit dextrin. Accordingly, β-amylose liberated only 50% of the radioactivity after a 10–20-min chase period. We conclude that a 30-s pulse will label only the outermost lamella chains, whereas after a 10–20-min chase period, the label will also represent internal lamellae chains. The distributions of label after chase periods of 10 and 20 min (Fig. 3, B and C) were very similar and reflected the distribution as observed by PAD, showing that the chased samples represent final starch structure. Thus, we have verified that pulse- and pulse-chase-labeled samples represent immature and mature starch structures, respectively.

Both radioactivity and PAD-detected carbohydrate (Figs. 4 and 5) demonstrate that there are few chains shorter than DP6. Occasionally, larger PAD-detected peaks that eluted earlier than DP6 were observed (see e.g. Fig. 3A). These compounds contained little or no radioactivity and do not represent starch-derived small-chain malto-oligosaccharides. The nature of these compounds remains to be identified. The profile of PAD-detected peaks alone does not allow us to draw conclusions with respect to chain formation, because these data reflect only the final starch structure, and potential short-chain intermediates may not accumulate to notable levels. The pulse labeling solves this problem; DP6 and longer chains are efficiently labeled during the 30-s pulse period, whereas very little label is incorporated in chains shorter than DP6 (Figs. 4 and 5). Because there are hardly any DP5 chains, the DP6 chains cannot originate from elongation of DP5 chains. DP6 chains must therefore be formed by transfer from longer chains, catalyzed by branching enzyme. Furthermore, the donor chains must be DP12 or longer; otherwise the branching reaction would result in stubs of DP5 or shorter. In vitro studies show that starch branching enzyme (SBEI) will primarily bind to chains DP6 and longer (21), and both SBEI and SBEII from potato will produce chains with a minimal length of DP6 (22). This finding fits well with the labeling pattern during starch biosynthesis as observed in this study, and with the preferential formation of DP6 in glycogen from Escherichia coli expressing the maize SBEI and SBEII (23).

During the pulse period a preferential labeling of short chains was observed. Generally, the distribution of a total population of polymeric unit chains may be represented as molar or weight-based distributions as shown in Fig. 5, D, E, I, and J. However, for transitory starch granules consisting of radially oriented amylpectin chains the distribution of surface-exposed chains must be considered. Although an even label of all nonreducing chains in the entire starch granule would show a molar distribution, label restricted to the nonreducing surface exposed ends will display a pattern similar to the weight-based distribution of the entire population of the chains if it is assumed that the surface structure is identical to the entire starch granule (calculated as a hypothetical concentric surface exposed by a section of a granule). Because a starch granule grows primarily by synthesis at the surface, it would then be expected that a very short pulse resulted in a label distribution similar to the weight-based distribution. Any significant deviation from this distribution would indicate either labeling of buried chains or that lamellar maturing processes occur that are slower than the pulse. In the first case, no shift in chain length profile would be expected with a pulse-chase experiment. In the second case, a shift can occur as an effect of molecular rearrangements. Our results favor the second of these options. Two contrasting scenarios could explain a time-dependent change in distribution. 1) Newly formed chains are efficiently branched and excessive branches are then elongated or trimmed off again, resulting in preferential pulse labeling of short chains. 2) Chains need first to be elongated to reach a length above average before being branched resulting in the mature chain length. This might result in preferential pulse labeling of longer chains. The pulse label being enriched in shorter chains suggest that the first option is correct. It should also be noted that about 2% of the label was recovered alone in DP6 chains. As discussed above, the DP6 chains are radiolabeled by elongation of chains (to DP12 or longer) followed by transfer of the six terminal glucose units to form a new branch. The final degree of branching is about 5% of all glucosidic bonds, and after 30 s already 2% of the label is found in only DP6 chains (each represents one new branch point formed). This suggests that within 30 s most of the branch points of the final starch have already been formed. Thus, branching is a highly efficient process. It is striking to note the shift in labeling profile from pulse- to pulse-chase-labeled samples that occurs at DP12 (Fig. 5C). This indicates a direct connection to the branching process, because DP12 are deduced to be the shortest unit chains used for branching. Chains shorter than DP12 originate from branching and elongation, and on a short time frame this fraction gain total carbohydrate and thereby radioactivity by both processes. Chains of DP12 and longer also lose carbon, and thereby radioactivity, by the branching process. In the short term, this may result in the observed preferential incorporation of radiolabel in short unit chains. The major “loss” of radioactivity is seen for DP12–19 (Fig. 5C), which may suggest that these medium length chains are the preferred (or just the most abundant) substrates for the branching process. Starch branching isoforms SBEI and SBEII have different preferences to substrate structure (24) and also make different products (22, 23). We further suggest that the true in vivo substrates and products are also determined by accessibility and timing of substrate formation.

The dbe mutant, which accumulates phytoglycogen, showed a significantly shorter average chain length as compared with the WT plants (Fig. 5, E and J). As expected from this structural difference, the pulse label of the dbe mutant was found preferentially in shorter chains as compared with the WT (Fig. 5, A and F). This probably reflects a more uniform labeling of the phytoglycogen molecule than of starch granules. Phytoglycogen is a noncrystalline polymer and therefore potentially has a much larger accessible area than granular starch. Therefore, more short chains can be assumed to be accessible for elongation.

The pulse-chase analysis of phytoglycogen revealed a smaller, but consistently observed change in distribution in favor of shorter chains during the chase period, i.e. opposite the pattern observed for WT plants. Thus, in the dbe mutant a more branched glucan is formed over the chase period. We suggest this to reflect that the more open internal structures of phytoglycogen remain accessible to branching enzymes. The radiolabeling data also show more clearly than PAD that the glucans in the dbe mutant have very few long chains (above DP40). This represents a marked difference from the WT starch.

The distribution of label in the chased dbe samples is similar
but does not exactly match the total carbohydrate distribution profile, indicating the formation of a more branched structure during the labeling experiment (Fig. 5, G and I) compared with glucans accumulated over the entire light period (Fig. 5, G and I). It is quite likely that the structure varies with the environment and metabolic activity of the leaf tissue. The *dbe* mutant accumulates both normal starch and phytoglycogen (4), and it is reasonable to expect that the distribution between these two pools may change under different conditions.

It has been suggested that soluble pre-amyllopectin or phytoglycogen (17) may be formed as starch biosynthetic intermediates. The short chain glucans observed in the pulse-labeled WT plants could represent such soluble glucans with a structure similar to phytoglycogen. Our analysis suggests that this is not the case. Despite a preferential labeling of short chains, the average chain length is considerably higher in the pulse-labeled WT samples than in *dbe* samples. Furthermore, the incorporation into the very long chains (above DP40) is significantly higher than in phytoglycogen (Fig. 5). Calculation of the label distribution in the pulse-labeled WT as a sum of phytoglycogen and starch did not result in a good description of the observed label distribution. Furthermore, pulse-labeled starch granules that were separated from soluble material showed a distribution very similar to the unhomogenized whole leaf data, suggesting that the pulse label distribution observed in our “in leaf” debranched samples represents granule-bound glucans.

At this point, we can draw the following conclusions. 1) Chains of DP6 are the shortest chains formed in considerable amounts. 2) Chains of DP6 are made from branching with donor chains of DP12 and longer. 3) Branching is highly efficient and occurs mainly within the time period required for synthesis of one 9-nm layer. 4) The efficient branching results in an intermediary glucan structure enriched in DP6–11 chains. 5) This short chain enriched intermediary structure is granule bound. 6) Although similar, the short chain intermediary structure is not identical to phytoglycogen.

Our data do not exclude that small soluble branched glucans are formed in the WT plants as short-lived intermediates. These would be eluted during the extraction procedure. Preliminary analysis did not reveal considerable incorporation of label in branched soluble glucans, but further analysis is required to reach conclusion. We can also not conclude whether branches are trimmed off of starch by isoamylase in the WT plants. However, if trimming is taking place, it appears to be occupied primarily with removing DP6–11 chains. Phytoglycogen contains more very short chains (DP3–5) than starch (Fig. 5, E and J). However, even in phytoglycogen the amount of carbon and label incorporated in these chains is low.

Neither trimming nor the existence of soluble branched glucans is strictly required to explain the observed labeling patterns. Still, these features may be important for initiation of correct starch structure, and the accumulation of phytoglycogen in the *dbe* mutant suggests that isoamylase activity is indeed required for correct starch biosynthesis. It is reasonable to speculate that the formation of phytoglycogen may be a self-enhancing reaction because the formation of an open and highly branched structure will provide even more nonreducing ends for further elongation and branching. The large accumulation of phytoglycogen in mutants may therefore over-emphasize the direct contribution of isoamylase. To reach a final conclusion about the potential contribution of trimming, the methodology will need to be further developed to allow for analysis of radiolabeling of malto-oligosaccharides. Trimming would predict that these would be transiently labeled. Work is under way to perform these studies.

We also propose that branching occurs principally as soon as chains are sufficiently long to be accepted as donors for the branching reaction, resulting primarily in short chains (DP6–11). This proposition is based on the observations of: 1) efficient formation of DP6 chains, 2) excess of chains DP6–11 during pulse labeling, 3) and the deficiency in chase-labeled chains peaking around DP15–16. Thus, most of the branch transfers appear to take place from medium length chains forming short chains. This is a plausible model; a very efficient branching, as observed in our experiments, would promote branching of chains as soon as they are long enough. This will result in preferential branching of medium length chains, which are also more abundant than longer chains, and will simultaneously ensure preferential transfer of short chains (DP6–11). Possibly longer chains also serve as donor chains, but because of the organization of crystalline layers restricting accessibility, only the outer glucose units are likely to participate in the branching reactions. This situation appears to be entirely changed in the *dbe* mutant. Phytoglycogen is less organized, and presumably our data reflect that the glucan chains remain accessible to branching enzymes over a longer period. Long chains may therefore remain to serve as substrates for the branching reaction, and efficient branching (relative to elongation) will result in increased branching with time, which would explain the considerably lower amount of very long chains in phytoglycogen compared with starch.

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