Evidence for Distinct Mechanisms of Starch Granule Breakdown in Plants

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The aim of this work was to understand the initial steps of starch breakdown inside chloroplasts. In the non-living endosperm of germinating cereal grains, starch breakdown is initiated by α-amylase secreted from surrounding cells. However, loss of α-amylase from Arabidopsis does not prevent chloroplastic starch breakdown (Yu, T.-S., Zeeman, S. C., Thorneycroft, D., Fulton, D. C., Dunstan, H., Lue, W.-L., Hegemann, B., Tung, S.-Y., Umemoto, T., Chapple, A., Tsai, D.-L., Wang, S.-M, Smith, A. M., Chen, J., and Smith, S. M. (2005) J. Biol. Chem. 280, 9773–9779), implying that other enzymes must attack the starch granule. Here, we present evidence that the debranching enzyme isoamylase 3 (ISA3) acts at the surface of the starch granule. Atisa3 mutants have more leaf starch and a slower rate of starch breakdown than wild-type plants. The amylopectin of Atisa3 contains many very short branches and ISA3-GFP localizes to granule-like structures inside chloroplasts. We suggest that ISA3 removes short branches from the granule surface. To understand how some starch is still degraded in Atisa3 mutants we eliminated a second debranching enzyme, limit dextrinase (pullulanase-type). Atlida mutants are indistinguishable from the wild-type. However, the Atisa3/Atlida double mutant has a more severe starch-excess phenotype and a slower rate of starch breakdown than Atisa3 single mutants. The double mutant accumulates soluble branched oligosaccharides (limit dextrins) that are undetectable in the wild-type and the single mutants. Together these results suggest that glucan debranching occurs primarily at the granule surface via ISA3, but in its absence soluble branched glucans are debranched in the stroma via limit dextrinase. Consistent with this model, chloroplastic α-amylase AtAMY3, which could release soluble branched glucans, is induced in Atisa3 and in the Atisa3/Atlida double mutant.

Starch is the most abundant storage carbohydrate in plants. It is composed primarily of amylopectin, a branched polymer of glucose in which α-1,4-linked glucan chains are connected by α-1,6-bonds (branch points). In this way, amylopectin resembles glycogen, the soluble storage carbohydrate synthesized in prokaryotes, fungi, and animals. However, unlike glycogen, amylopectin molecules can form a semicrystalline granule. This capacity stems from the branching pattern of amylopectin, which produces clusters of linear chains that pack together in regular semicrystalline arrays. This gives rise to a macromolecular granule structure in which semicrystalline lamellae alternate with amorphous lamellae, where branch points are located (1, 2).

The way in which the insoluble starch granule is degraded to release stored glucose is understood only in the endosperm of germinating cereals. In this tissue, the process of starch breakdown is initiated by the action of α-amylase, secreted into the non-living starchy endosperm (3). This enzyme has an endoamylolytic action and releases a mixture of branched and linear oligosaccharides that serve as substrates for other hydrolytic enzymes including debranching enzymes (which hydrolyze the α-1,6-bonds) and exoamylases (β-amylases). There is mounting evidence that a different mechanism operates in living cells of other plant tissues, where the starch is degraded inside the plastid compartment. First, Arabidopsis mutants lacking all three α-amylases encoded in the genome have normal rates of starch breakdown under standard growth-room conditions, showing that the activity is not essential to initiate starch breakdown (4). Second, normal rates of starch breakdown in several plant organs require one or more enzymes belonging to the glucan water dikinase class (5–7), which phosphorylate glucosyl residues within amylopectin molecules (8). There is no evidence for a requirement for these enzymes in cereal endosperm starch degradation.

Evidence that chloroplastic enzymes other than α-amylase can attack starch granules is limited. Scheidig et al. (9) reported that a recombinant chloroplastic β-amylase from potato can liberate small amounts of maltose from isolated starch granules in vitro. Similarly, Hussain et al. (10) reported that a recombinant debranching enzyme from potato (StISA3) has a limited activity on isolated granules in vitro. In both cases, antisense repression of these enzymes resulted in starch accumulation in potato leaves (9, 11). Similarly mutation of the orthologous genes in Arabidopsis leaves causes starch accumulation (12, 13).

A model can be envisaged in which β-amylase degrades long linear chains at the granule surface to within a few glucosyl residues of the branch points. Debranching enzyme may then remove the short branches to reveal the next layer of linear chains for further exoamylolytic attack (14). To test this hypothesis we investigated in detail the phenotypes of mutant plants lacking debranching enzyme activity. If debranching enzymes attack the granule surface, we would expect to see distinctive and predictable changes in the structure of starch polymers in their absence.

Debranching enzymes fall into two classes in plants; isoamylase (ISA)3 and limit dextrinase (LDA; also known as pullulanase or PUL). The Arabidopsis genome encodes three proteins of the isoamylase-type (ISA1, ISA2, and ISA3) and one of the limit dextrinase-type. Although related, the two types are readily distinguishable at the level of their structure.

3 The abbreviations used are: ISA3, isoamylase 3; LDA, limit dextrinase; GFF, green fluorescent protein; dp, degrees of polymerization; MOPS, 4-morpholinepropanesulfonic acid; HPAGE-PAD, high performance anion-exchange chromatography-pulsed amperometric detection.
Mechanisms of Starch Degradation

Enzyme Assays—A method was developed for the quantitative assays of debranching enzyme using β-limit dextrin substrate. The β-limit dextrin was prepared as described previously (20), except that amylopectin (Sigma) was initially dissolved by boiling in water for 15 min. After digestion, the β-limit dextrin was precipitated by the addtion of 75% (v/v) methanol, 1% (w/v) KCl. Precipitate was collected by centrifugation, washed with 75% (v/v) methanol, dissolved by boiling in water for 15 min (final concentration of 4 mg ml⁻¹), and stored at −20 °C until used. Before use in the assay, the β-limit dextrin was re-treated with 50 units ml⁻¹ of β-amylase (Megazyme, Bray, Ireland) in 10 mM sodium acetate, pH 6.0. Residual maltose was removed by passage through a NAP-25 column pre-equilibrated with assay medium (50 mM MOPS, pH 7.2, 1 mM EDTA, 1 mM dithiothreitol, 1 mM CaCl₂). The β-limit dextrin and β-amylase were eluted in assay medium and residual maltose was retained in the column. Crude extracts were prepared by homogenizing leaf samples (200–300 mg), each from an individual plant, in 6 volumes of ice-cold extraction medium (50 mM MOPS, pH 7.2, 1 mM EDTA, 1 mM dithiothreitol) using an all-glass homogenizer. After centrifugation, the supernatants were desalted by passage through NAP-10 columns pre-equilibrated with assay medium. Duplicate assays (100 μl total volume) were started by the addition of the desalted extracts, incubated at 30 °C for 1 h, and stopped by boiling for 5 min. The assay contents were passed through sequential Dowex 50 and Dowex 1 mini-columns (24) to remove contaminating proteins and charged compounds, and neutral compounds were eluted with water. Maltose and maltotriose contents were determined by HPAEC-PAD, as described previously (25). No maltose was detected when the plant extract was omitted. Control reactions (in which the β-limit dextrin was omitted) were used for each extract and gave very low values, which were subtracted from the assay values.

For the assay of α-amylase the Crealpha™ kit (Megazyme) was used (18). This employs a linear substrate and is not liable to interference from debranching enzymes.

Carbohydrate Measurements and Structural Analyses—Samples were harvested into liquid N₂ and extracted using perchloric acid (17). Total glucans in soluble and insoluble fractions were determined by measuring the amount of glucose released by treatment with α-amylase and amyloglucosidase, as described previously (26). Chain length profiles of amylopectin were determined by HPAEC-PAD (17).

Molecular Methods—The full-length ISA3 cDNA was cloned into the binary vector pMDC83 (27) by recombinant cloning. This construct produced a full-length ISA3 protein with GFA fused to its carboxyl terminus, under the transcriptional control of a double 35S-cauliflower mosaic virus promoter. Transient expression was carried out in protoplasts from the wild-type and the starchless mutant pgm (28). Protoplasts were prepared as described by Fitzpatrick and Keegstra (29). Polyclonal glycol-mediated transformation was performed as described by Jin et al. (30). Localization was carried out using a Leica DM IRBE and a Leica TCS SP laser confocal scanning laser microscope (Leica, Unter- entfelden, Switzerland). Fluorophores were excited using a laser with 488 nm wavelength. The GFP emission signal was collected in a wavelength window between 495 and 525 nm. Chlorophyll autofluorescence was collected in a window of 625–690 nm.

RESULTS

Detection of ISA3 and LDA Mutants.—We identified mutant alleles of LDA and ISA3 from publicly available populations. Mutants lack-
ing LDA were obtained from the SALK collection (line SALK_060765) (31) and from the Cold Spring Harbor Gene Trap Collection (line GT7150) and designated \( \text{Atlda-2} \) and \( \text{Atlda-3} \), respectively. The positions of the T-DNA insertion in \( \text{Atlda} \) and the transposon insertion in \( \text{Atlda-3} \) occurred in exons 7 and 12, respectively. In \( \text{Atisa3-2} \), a T-DNA insertion occurred in exon 21 and in \( \text{Atisa3-3} \), a point mutation is present at the splice junction between intron 2 and exon 3.

A single band of activity was detected in the two wild-type lines. In both \( \text{Atlda-2} \) and \( \text{Atlda-3} \) no activity was detectable (Fig. 1B). Mutants lacking ISA3 were identified from the GABI-KAT collection (32) and via the Arabidopsis Tilling Program (33) and designated \( \text{Atisa3-2} \) and \( \text{Atisa3-3} \), respectively. The T-DNA insertion site in GABI_KAT_280G10 and the point mutation in CS88929 (TAIR polymorphism At4g09_120E3) were confirmed by PCR and DNA sequencing (Fig. 1A). In CS88929 the ethane methyl sulfonate-induced point mutation lies on a splice junction. Plants homozygous for each of the mutations were isolated from segregating populations obtained from the source laboratories. To confirm that these mutations abolished expression of the \( \text{ISA3} \) gene we acquired an antiserum raised to an AtISA3-specific peptide and used it to probe protein gel blots of crude extracts from the wild-type and the \( \text{Atisa3} \) mutants. A single 80-kDa protein was detected in the wild-type, which was missing in both mutants (Fig. 1C).

Both alleles of ISA3 had similar phenotypes. Likewise, both LDA mutants were alike. Unless specified, further work was carried out with \( \text{Atisa3-2} \) and \( \text{Atlda-2} \), both of which are mutants of the Columbia ecotype. We generated a double mutant by crossing \( \text{Atlda-2} \) and \( \text{Atisa3-2} \). The double mutant was selected from the segregating F2 population by screening for the T-DNA insertions in the respective genes using PCR.

**FIGURE 1. Identification and analysis of mutants at the \( \text{AtLDA} \) and \( \text{AtISA3} \) loci.**

A, structure of the \( \text{AtLDA} \) and \( \text{AtISA3} \) genes; exons are depicted as black boxes. Gray boxes depict 5’ and 3’ untranslated regions. T-DNA left border (LB) and transposon sequences are shown on a hatched background. Sequence lacking similarity to either the target gene or the T-DNA insert is underlined. T-DNA and transposon insertions in \( \text{Atlda-2} \) and \( \text{Atlda-3} \) occurred in exons 7 and 12, respectively. In \( \text{Atisa3-2} \), a T-DNA insertion occurred in exon 21 and in \( \text{Atisa3-3} \), a point mutation is present at the splice junction between intron 2 and exon 3.

B, proteins in crude extracts of leaves from the Columbia wild type (WT Col), \( \text{Atlda-2} \), Landsberg erecta wild type (WT L.er), and \( \text{Atlda-3} \) were separated by native PAGE in gels containing red pullulan. LDA activity was detected as a clear band against the background.

C, proteins in crude extracts of leaves of the wild-type (WT), \( \text{Atlda-2} \), and \( \text{Atlda-3} \) were separated by SDS-PAGE, blotted onto polyvinylidene difluoride membranes, and probed with anti-AtISA3 antibodies. Arrow indicates the migration position of a 75-kDa molecular mass marker. D, proteins in crude extracts of leaves from the wild-type (WT), \( \text{Atlda-2} \), \( \text{Atisa3-2} \), and the \( \text{Atlda/Atisa3} \) double mutant were separated by native PAGE in gels containing \( \beta \)-limit dextrin. After incubation of the gel, debranching enzyme activities were detected as blue-stained bands using iodine staining. D1 is the ISA1/ISA2 isoamylase. D2 is absent in \( \text{Atlda-2} \) and D3 is absent in \( \text{Atisa3-2} \). The colorless band A2 is the chloroplastic \( \alpha \)-amylase AMY3.

D, proteins in crude extracts of leaves from the wild-type (WT), \( \text{Atlda-2} \), \( \text{Atisa3-2} \), and the \( \text{Atlda/Atisa3} \) double mutant were separated by native PAGE in gels containing \( \beta \)-limit dextrin. After incubation of the gel, debranching enzyme activities were detected as blue-stained bands using iodine staining.
Determination of Debranching Enzyme Activity—Debranching enzyme activity is frequently determined using semiquantitative native PAGE (zymogram) methods that rely on the debranching of a glucan substrate (e.g. amyllopectin) incorporated into the gel. Activity is visualized by staining the gel with an iodine solution and the ISA1/ISA2 isoamylase and LDA activity are readily detected in Arabidopsis leaf extracts as clear or pale blue bands (12, 17, 18). No band of debranching enzyme activity has previously been attributed to ISA3 activity, leading to the suggestion that the protein is unstable (12). We tested a range of native gel conditions and substrates and observed that in gels containing the ISA3 orthologue from potato has high activity on \( \beta \)-limit dextrin (amylopectin treated with \( \beta \)-amylase to digest the external chains to within 2 or 3 glucosyl residues of a branch point), a dark blue band of activity was visible in wild-type extracts (Fig. 1D). This is consistent with the observation that the ISA3 orthologue from potato has high activity on \( \beta \)-limit dextrin in vitro (10).

We developed an assay to quantify \( \beta \)-limit dextrin debranching activity in extracts of Arabidopsis leaves (see “Experimental Procedures”). The assay uses \( \beta \)-limit dextrin in a saturating background of \( \beta \)-amylase. The external branches of \( \beta \)-limit dextrin are a mixture of “stubs” with degrees of polymerization (dp) of 2 and 3. Branches removed by debranching enzyme activity expose linear chains that are immediately degraded to maltose by \( \beta \)-amylase, thereby regenerating the \( \beta \)-limit dextrin substrate. The product of the assay is predominantly maltose (the dp2 stubs and the maltose produced by \( \beta \)-amylolysis of the newly exposed linear chains), together with smaller amounts of maltotriose (the dp3 stubs). We quantified both using HPAEC-PAD (Fig. 2A). Using wild-type extracts, we confirmed that this assay yields linear activity rates for both maltose and maltotriose production with respect to time and the volume of extract added in the ranges used in our experiments (data not shown).

Application of the debranching enzyme assay to extracts of wild-type and mutant plants showed that Atisa3 and Atilda both have reduced \( \beta \)-limit dextrin debranching activity. The production of maltose was reduced by 35 and 23% relative to the wild type in Atilda and Atisa3, respectively, and by 72% in extracts of the double mutant (Fig. 2B). Interestingly, the changes in maltotriose production did not follow the changes in maltose production. Atilda extracts released slightly less maltotriose than wild-type extracts (22% reduction), whereas a large reduction (62%) was seen with Atisa3 extracts. In the double mutant extracts, almost no maltotriose was produced (96% reduction). A second experiment with a separate batch of plants yielded very similar data. Thus, both LDA and ISA3 have appreciable activity on \( \beta \)-limit dextrin, but ISA3 may have a greater affinity than LDA for dp3 stubs.

We also used this assay to measure the activity in the Atisa1/Atasa2 double mutant (17). No reduction in activity was seen in terms of maltose or maltotriose production (Fig. 2B). This is intriguing, as the activity is visible on a native gel containing the same substrate. One explanation is that the ISA1/ISA2 does not remove the dp2 and dp3 stubs, but rather the longer branches that carry stubs (i.e. B-chains). This would result in very little dp2 and dp3 release.

It should be noted that \( \alpha \)-amylase may interfere with this assay as endoamylolysis will also expose chains for \( \beta \)-amylase. This may account for the residual maltose production in the Atilda/Atisa3 double mutant (39% of the wild-type activity). The absence of maltotriose produced in this line is also consistent with this explanation as \( \beta \)-amylase will release maltose from a branched substrate, but not maltotriose.

Phenotypes of Atisa3, Atilda, and the Double Mutant—In a controlled environment chamber with a 12-h photoperiod, wild-type and Atilda mutant plants grew at the same rate, whereas Atisa3 mutants grew more slowly. The growth of the double mutant was even slower (Fig. 3A and data not shown).

Based on measurements at a single time point it has been reported that an ISA3 mutant had a higher starch content than the wild-type but that an LDA mutant (Atpul) did not (12). We confirmed these phenotypes and extended the analysis to examine the diurnal turnover of starch. Wild-type and Atilda plants had similar starch contents through-
Mechanisms of Starch Degradation

out the diurnal cycle (Fig. 3B). This result was reproducible in three separate experiments and true for both Atlda mutant lines (data not shown). This result contrasts with the situation in maize leaves, in which the starch content was elevated in a mutant that lacks LDA (zpu1) (19).

The starch content of Atisa3 leaves was higher than that of wild-type leaves throughout the diurnal cycle (Fig. 3) supporting earlier suggestions of a role in starch degradation (10, 12, 34). Old leaves contained more starch than young leaves (data not shown). However, appreciable amounts of starch were still degraded during the night (67.5% of that degraded by the wild type; the mean of three separate experiments including the one shown in Fig. 3B), and extending the night for a further 48 h led to almost all the starch being remobilized (data not shown). These data suggest that the loss of ISA3 impairs but does not prevent the process of starch breakdown and that, in a day/night growth regime, starch accumulates in small increments due to an imbalance between daytime synthesis and nighttime degradation.

To investigate how the starch is still degraded in the absence of ISA3 we analyzed the Atisa3/Atlda double mutant. The phenotype of the double mutant was much more severe. Starch accumulated to higher levels than in Atisa3, and the extent of starch breakdown was further reduced (to 48.3% of the amount degraded by the wild type; the mean of two separate experiments including the one shown in Fig. 3B) and, even after an extended night of 260 h, all but the youngest leaves still stained darkly for starch with iodine solution (data not shown). This suggests that in the absence of ISA3, LDA plays an important role in debranching glucans.

Glucans present in the soluble fraction of the leaf extracts were also determined. The soluble glucan content in the wild-type and the two single mutant plants was very low, whereas significant amounts were detectable in the double mutant throughout the diurnal cycle. Soluble glucans in the double mutant increased during the night and decreased during the day (Fig. 3B). These glucans could not be precipitated using Pseudomonas isoamylase, Klebsiella pullulanase, or both. The chromatograms of the digested wild-type samples did not differ from the undigested sample and are not shown. The dotted lines indicate the retention times of linear malto-oligosaccharide standards with degrees of polymerization (dp) indicated.

To gain more information about the nature of these oligosaccharides, we isolated the neutral compounds from the soluble extracts and analyzed them by HPAEC-PAD. The major oligosaccharide peaks in the chromatogram indicated a size range between dp6 and dp20, but the peaks did not co-elute with linear malto-oligosaccharide standards (Fig. 4). This is typical of branched oligosaccharides that elute slightly earlier than linear molecules of the same size. To evaluate this further, we treated the neutral fraction with a bacterial isoamylase, a bacterial pullulanase, and representative chromatograms are shown in each case. Samples were digested with Pseudomonas isoamylase, Klebsiella pullulanase, or both. The chromatograms of the digested wild-type samples did not differ from the undigested sample and are not shown. The dotted lines indicate the retention times of linear malto-oligosaccharide standards with degrees of polymerization (dp) indicated.
molecules (note that the bacterial isoamylase and pullulanase enzymes may not have exactly the same substrate specificity as the Arabidopsis proteins ISA3 and LDA, respectively). These results indicate that, in the absence of both ISA3 and LDA, branched oligosaccharides are released from the granule surface and metabolized to limit dextrins that are then inefficiently debranched.

**Increased Chloroplastic α-Amylase Activity in Atisa3 and the Double Mutant—** Branched oligosaccharides could be released from the granule by the action of α-amylase. Although elimination of the chloroplastic α-amylase does not result in elevated starch content under our growth conditions, it may still contribute to breakdown (4). Our native gels suggested an increased activity of an endoamylase, migrating in the same location as the chloroplastic α-amylase AMY3 (Fig. 1D). Therefore, we performed quantitative α-amylase measurements on crude extracts of leaves (Fig. 5A). This revealed a significant increase in α-amylase activity in Atisa3 and an even greater increase in the Atisa3/Atlda double mutant. Protein gel blots probed with an antibody raised to the AMY3 protein confirmed that the amount of AMY3 protein was increased in both lines (Fig. 5B). This suggests that the loss of ISA3 results in a pleiotropic increase in AMY3, which is increased further in the double mutant line. We performed further native gels to search for additional pleiotropic effects on starch biosynthetic enzymes (starch synthase and starch branching enzymes) and degradative enzymes (α-glucan phosphorylase and starch hydrolyzing enzymes), but no other differences were observed (data not shown).

**Changes in Amylopectin Structure in Atisa3 and the Atisa3/Atlda Double Mutant—** Starch was extracted from leaves harvested at the end of the day and the chain length distribution of the amylopectin was determined by HPAEC-PAD. The distributions of wild-type and Atlda mutant amylopectins were indistinguishable (Fig. 6). In contrast, the chain length profile of amylopectin from Atisa3 differed markedly from that of the wild type, with an increase in the relative frequency of chains of dp3 and, to a lesser degree, dp4 and dp5 (Fig. 6). Chains between dp9 and dp16 were decreased in relative abundance. The chain length profile of the amylopectin from the Atlda/Atisa3 double mutant was very similar to that of Atisa3. In each case, the amylopectin chain length profiles were consistent between at least three different batches of plants.

Chains of amylpectin shorter than dp6 are not thought to be produced during normal starch biosynthesis in Arabidopsis leaves (35). Consequently, the short chains in Atisa3 and the Atisa3/Atlda double mutant most likely result from the action of degradative enzymes. Ritte et al. (36) showed that starch granules isolated from potato leaves at night have more short chains on their surface (notably dp3–dp5) compared with granules isolated from illuminated leaves. The inefficient removal of short chains from the granule surface, coupled with the repetitive diurnal cycles of synthesis and (partial) degradation could result in the build up of short chains within the granule to the levels that we observe.

**ISA3 Localizes to the Surface of the Starch Granule—** The change in amylopectin structure in Atisa3 suggests that ISA3 could directly attack the starch granule in vivo as well as in vitro (10). To investigate this further we examined the subcellular localization of ISA3 by expressing an ISA3-GFP fusion protein in protoplasts. Confocal microscopy of transformed wild-type cells revealed GFP fluorescence from within the chloroplasts (Fig. 7, A–G). However, the signal was not uniformly distributed, but was strongest from oval structures within the chloroplasts that were similar in size, number, and distribution to starch granules.

This pattern of fluorescence was observed in several independent transformation experiments. In most cases the GFP fluorescence surrounded the oval structures, from the middle of which there was no chlorophyll fluorescence (Fig. 7, B–D). This is consistent with the presence of a starch granule, which would place the chlorophyll-containing thylakoid membranes. We expressed the same construct in protoplasts isolated from the starchless mutant pgm (Fig. 7, H–N). In each case the GFP fluorescence signal was also exclusively from the chloroplast but the signal was uniform. These results provide evidence that ISA3 is present at the surface of the starch granule.

**DISCUSSION**

The work described here reveals the important role that two debranching enzymes play in starch breakdown inside chloroplasts and allows us to propose that there are distinct mechanisms of hydrolytic starch breakdown.

**ISA3 and LDA Are Both Active on β-Limit Dextrins—** Our results show that both Atisa3 and Atlda have lost single isoforms of debranching enzyme activity, detectable using β-limit dextrin-containing native gels. Both mutants also have a reduction in total β-limit dextrin debranching activity and the double mutant has a greater reduction. Furthermore, the results from the assay imply that ISA3 and LDA may have different specificities. Maltotriose production is reduced only slightly in Atllda, but markedly reduced in Atisa3, whereas the reduction in maltose production is similar in both lines. This could indicate that ISA3 preferentially removes dp3 stubs from the β-limit dextrin. However, an alternative explanation is that the increase in α-amylase detected in Atisa3 could interfere with the assay by enhancing maltose production resulting in an overestimated activity in Atisa3. Further experiments are required to confirm a preference of ISA3 for dp3 stubs. However, it is worth noting that the stubs that are overrepresented in Atisa3 amylopectin are primarily dp3. Interestingly, the stubs in the soluble limit dextrins that accumulate when both Atisa3 and Atllda are missing are predominantly dp2. Therefore, we speculate that the results of dp3 and, to a lesser degree, dp4 and dp5 (Fig. 6). Chains between dp9 and dp16 were decreased in relative abundance. The chain length profile of the amylopectin from the Atlda/Atisa3 double mutant was very similar to that of Atisa3. In each case, the amylopectin chain length profiles were consistent between at least three different batches of plants.

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of our assay indicate a preference of ISA3 and LDA for stubs of dp3 and dp2, respectively.

If the rate of maltotriose production is assumed to represent half the rate of $\beta$-limit dextrin debranching activity, it is possible to calculate an activity of 7.3 nmol of $\alpha$-1,6-linkages min$^{-1}$ g$^{-1}$ fresh weight for the wild-type (the average wild-type rate from Fig. 2B and a replicate experiment not shown). This is more than sufficient to catalyze the observed rate of branch point hydrolysis during starch breakdown (4 nmol of $\alpha$-1,6-linkages min$^{-1}$ g$^{-1}$ FW, assuming that 4% of the linkages in starch are branch points). We emphasize that this value should be treated with caution as the assays have not been optimized and the activities of the enzymes may be different when acting on substrates in vivo.

The Role of ISA3—Earlier results have implied that ISA3 is involved in starch degradation. A starch-excess phenotype is caused by antisense repression or mutation in potato and Arabidopsis, respectively (11, 12). In addition, ISA3 expression correlates with periods of starch breakdown in ripening banana fruit and Arabidopsis leaves (34, 37). Our results are consistent with these earlier reports and provide additional insight into the role that the enzyme plays in starch breakdown.

First, the starch structure of the amylopectin in Atisa3 is altered with respect to wild-type amylopectin with increases in very short chains (especially dp3). The variability of our data is low and the finding was consistent in numerous batches of plants grown in a range of different conditions. This result contrasts to the conclusions of Wattebled et al. (12) where no changes in amylopectin structure were found. However, chains shorter than dp5 were not determined in this study, explaining this discrepancy. The increase in short chains implies that ISA3 would normally remove them and is consistent with the observation that ISA3...
from potato has a particularly high activity on β-limit dextrin relative to other substrates (10). Second, our results show that the ISA3-GFP fusion protein localizes to the periphery of structures that are similar in size, shape, and number to starch granules within wild-type chloroplasts. The uniform GFP signal inside starchless chloroplasts is consistent with the idea that these structures are indeed starch granules. If so, these results suggest that ISA3 acts on the surface of the granule, most likely during starch breakdown. If exoamylases (i.e., β-amylase) attack the surface of the granules, as has been suggested (9), short branches would be produced and further β-amylolysis would not be possible until these branches had been removed. Thus, ISA3 could act in conjunction with β-amylase to progressively degrade the starch granule. According to this hypothesis, some of the short chains that are exposed during breakdown are not removed in Atisa3, limiting the rate of degradation. Many of these short chains could be elongated again upon subsequent starch biosynthesis in the light, but some may become buried in the granule leading to the changes in chain length distribution that we observe. Although such short branches are not thought to be produced during normal amylopectin synthesis (35), we cannot exclude the possibility that ISA3 is also active during starch biosynthesis.

Third, although the starch content of the Atisa3 leaves is significantly increased, there is still appreciable nighttime degradation. It is possible that this occurs via the activity of the chloroplastic α-amylase AMY3. α-Amylase could release branched glucans from the granule that would serve as substrates for other stromal enzymes. This would simultaneously expose linear chains on the granule surface for β-amylolysis.

The increase in chloroplastic α-amylase activity in Atisa3 provides some support for this hypothesis.

The Role of LDA—Arabidopsis mutants lacking LDA do not display a high starch phenotype (this study) (12), although in maize, the equivalent mutant does (19). However, our results show that when the LDA and ISA3 are both missing, the double mutant line has a much higher starch content and a slower rate of starch breakdown. This suggests that LDA is involved in starch breakdown, at least in the Atisa3 mutant background. Importantly, this double mutant accumulates small, soluble branched glucans during the dark. These have the characteristics of limit dextrins and are not observed in the single mutants or in the wild-type. Furthermore, the chloroplastic α-amylase is increased appreciably in this line. Together, these data suggest that soluble branched glucans produced by α-amylase give rise to limit dextrins in the stroma, which can then be metabolized by limit dextrinase into linear glucans. Low levels of limit dextrins were also observed in the LDA mutant of maize (19).

It remains possible that the ISA1/ISA2 isoamylase also contributes to debranching during starch breakdown. However, the slow rate of degradation observed, the accumulation of branched oligosaccharides in the Atisa3/Atlda double mutant, and the unaltered debranching enzyme activity on β-limit dextrin in the Atisa1/Atisa2 double mutant all suggest that its activity is either not sufficient or not suited to this role.

Two Pathways for Starch Granule Attack in Chloroplasts—Our data are consistent with the idea that there are several enzymes that can attack starch granules in vivo. We propose that in Arabidopsis, the major pathway involves β-amylase and ISA3, which progressively

Mechanisms of Starch Degradation

FIGURE 7. AtISA3 localizes to granule-like structures inside chloroplasts. Protoplasts from wild-type (A–G) and the starchless mutant pgm (H–N) were transformed with a construct encoding ISA3 with GFP fused to its COOH terminus. A and H, bright-field images of protoplasts. B and I, native chlorophyll fluorescence (the red objects are individual chloroplasts). C and J, GFP fluorescence. D–G, merged GFP- and chlorophyll-fluorescence images of wild-type protoplasts. G is an enlargement of D. E and F show different protoplasts. Note the non-uniform distribution of the GFP signal obtained in starch-containing wild-type chloroplasts. The uniform GFP signal is distributed around structures that do not show chlorophyll fluorescence (marked with arrows). K–N, merged GFP and chlorophyll fluorescence images of pgm protoplasts. N is an enlargement of K. L and M show different protoplasts. Note the uniform distribution of the GFP signal in the starchless pgm chloroplasts. Several independent transformation experiments were performed with each line, yielding the same results. Scale bars — 8 μm.
Mechanisms of Starch Degradation

A: Wild Type

B: Atisa3

C: Atisa3/Atlda

degrate the starch granule surface, releasing maltose for export from the chloroplast (38, 39) and short linear oligosaccharides for further metabolism (25) (Fig. 8A). The predominance of this pathway explains the absence of a starch excess phenotype in Arabidopsis mutants lacking either AMY3 or LDA (4, 12).

We suggest that α-amylase can also attack the starch granule to release branched oligosaccharides that are degraded in the stroma by a combination of β-amylase and LDA, but that this pathway is, under normal circumstances not required in Arabidopsis leaves. In the absence of ISA3, however, the amount of α-amylase is increased and the flux through this pathway accounts for the observed rate of degradation (Fig. 8B). The simultaneous removal of ISA3 and LDA would affect both of the proposed pathways, but would still allow some starch to be broken down. α-Amylase, further up-regulated in this line, can attack the granule exposing linear chains and the soluble glucans released can be degraded as far as branched dextrins (Fig. 8C). Therefore, a slow rate of degradation is still achieved.

This model requires further testing to confirm the distinct roles of each enzyme and to establish whether the data for Arabidopsis are generally applicable to the leaves of other plants. Under some conditions, or in other species, the relative importance of these pathways may be different. For instance, in maize leaves a greater fraction of the flux might proceed via α-amylase and LDA, explaining the reduced starch breakdown in the maize LDA mutant (19). Similarly, in rice, repression of α-amylase results in elevated levels of leaf starch (40). On the other hand, the importance of ISA3 and β-amylase are not yet known for these species. It seems likely that enzymes attacking the granule are dependent on its prior phosphorylation by enzymes of the glucan, water dikinase family. However, the nature of this dependence is not understood and also needs further investigation.

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