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REVIEW ARTICLE

The diurnal metabolism of leaf starch

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Starch is a primary product of photosynthesis in leaves. In most plants, a large fraction of the carbon assimilated during the day is stored transiently in the chloroplast as starch for use during the subsequent night. Photosynthetic partitioning into starch is finely regulated, and the amount of carbohydrate stored is dependent on the environmental conditions, particularly day length. This regulation is applied at several levels to control the flux of carbon from the Calvin cycle into starch biosynthesis. Starch is composed primarily of branched glucans with an architecture that allows the formation of a semi-crystalline insoluble granule. Biosynthesis has been most intensively studied in non-photosynthetic starch-storing organs, such as developing seeds and tubers. Biosynthesis in leaves has received less attention, but recent reverse-genetic studies of *Arabidopsis* (thale cress) have produced data generally consistent with what is known for storage tissues. The pathway involves starch synthases, which elongate the glucan chains, and branching enzymes. Remarkably, enzymes that partially debranch glucans are also required for normal amylopectin synthesis. In the last decade, our understanding of starch breakdown in leaves has advanced considerably. Starch is hydrolysed to maltose and glucose at night via a pathway that requires recently discovered proteins in addition to well-known enzymes. These sugars are exported from the plastid to support sucrose synthesis, respiration and growth. In the present review we provide an overview of starch biosynthesis, starch structure and starch degradation in the leaves of plants. We focus on recent advances in each area and highlight outstanding questions.

Key words: *Arabidopsis thaliana* (thale cress), carbohydrate metabolism, amylopectin, starch, photosynthesis, Poaceae (Gramineae, grasses), *Solanum tuberosum* (potato).

PHYSIOLOGICAL ROLE OF LEAF STARCH METABOLISM

Starch is arguably the most important storage carbohydrate in plants. It is typically associated with perennating organs, such as seeds, roots and tubers, as these are the major sources of carbohydrate in the human diet. Starch is also a primary product of photosynthesis, along with sucrose, in the leaves of most plants. It is a simple substance composed of glucose polymers which are packed to form dense osmotically inert granules. The starch made in chloroplasts during daytime photosynthesis is degraded during the following night, providing a continued supply of sugars to sustain metabolism in the leaf and for export to sink organs throughout the night. On a global scale, the daily fluxes into and out of starch amount to tens of millions of tons of carbon.

There is variation in the extent to which plants use chloroplastic starch to store photo-assimilates. In some species, such as *Glycine max* (soybean), *Beta vulgaris* (sugar beet) and *Arabidopsis* (thale cress), starch is the major storage form [1–3], whereas in others, such as *Phaseolus vulgaris* (French bean) and *Spinacia oleracea* (spinach), sucrose as well as starch accumulates in leaves during the day [2,4]. Raffinose-family oligosaccharides and fructans are also synthesized in the leaves of some plants [5,6]. In some species, including *Pisum sativum* (garden pea) and spinach, starch might best be viewed as an overflow for photo-assimilates, synthesized when the supply of sucrose exceeds both the demand from sink tissues and the storage capacity of the leaf. Thus assimilate partitioning into starch increases as sucrose synthesis declines through the day [4]. This is probably not the case in *Arabidopsis* and other plants in which half or more of the photo-assimilates are partitioned into starch [3]. In such cases, the division of assimilates between starch and sucrose is constant throughout the day and controlled to suit the environmental conditions (especially day length) [7–9]. Plants grown in short days partition relatively more of their photo-assimilates into starch than those grown in long days. Thus the longer the night, the higher the proportion of photo-assimilates stored as starch to provide a supply of sugars during this period.

Mutations affecting starch metabolism have been instrumental in defining the biosynthetic [10–14] and degradative [15–26] pathways, and are now giving us the first indications of the mechanisms that control them [27–29]. Such mutations result in decreased plant growth, illustrating the importance of the daily starch turnover [9,10,20,21,30–32]. Enormous progress has been made in this field in the last 6 years, using new and enhanced genetic approaches stemming from the publication of the *Arabidopsis* genome sequence. Below, we provide an overview of current understanding of the pathways leading to and from starch in leaves. The first section discusses the biosynthesis of ADP-Glc (ADP-glucose), the activated glucose donor for starch synthesis. Subsequent sections describe the biosynthesis of the starch granule and discuss the limitations in our understanding and methodologies. The last sections describe the emerging pathways of starch degradation and discuss how they may be regulated.

Abbreviations used: ADP-Glc, ADP-glucose; AGPase, ADP-glucose pyrophophorylase; AMY, α-amylase; BAM/BMY, β-amylase; BE, branching enzyme; DBE; debranching enzyme; d.p., degree of polymerization; DPE1, disproportionating enzyme 1 (D-enzyme); GBSS, granule-bound starch synthase; GWD, α-glucan,water dikinase; ISA, isoamylase; LDA, limit dextrinase; 3-PGA, 3-phosphoglycerate; PGI, phosphoglucose isomerase; PGM, phosphoglucomutase; PHS1, chloroplastic α-glucan phosphorylase; PWD, phosphoglucan,water dikinase; SBE, starch branching enzyme; SEX, starch excess; SnRK1, sucrose-non-fermenting-1-related protein kinase; SS, starch synthase; Tre6*P*, trehalose 6-phosphate.

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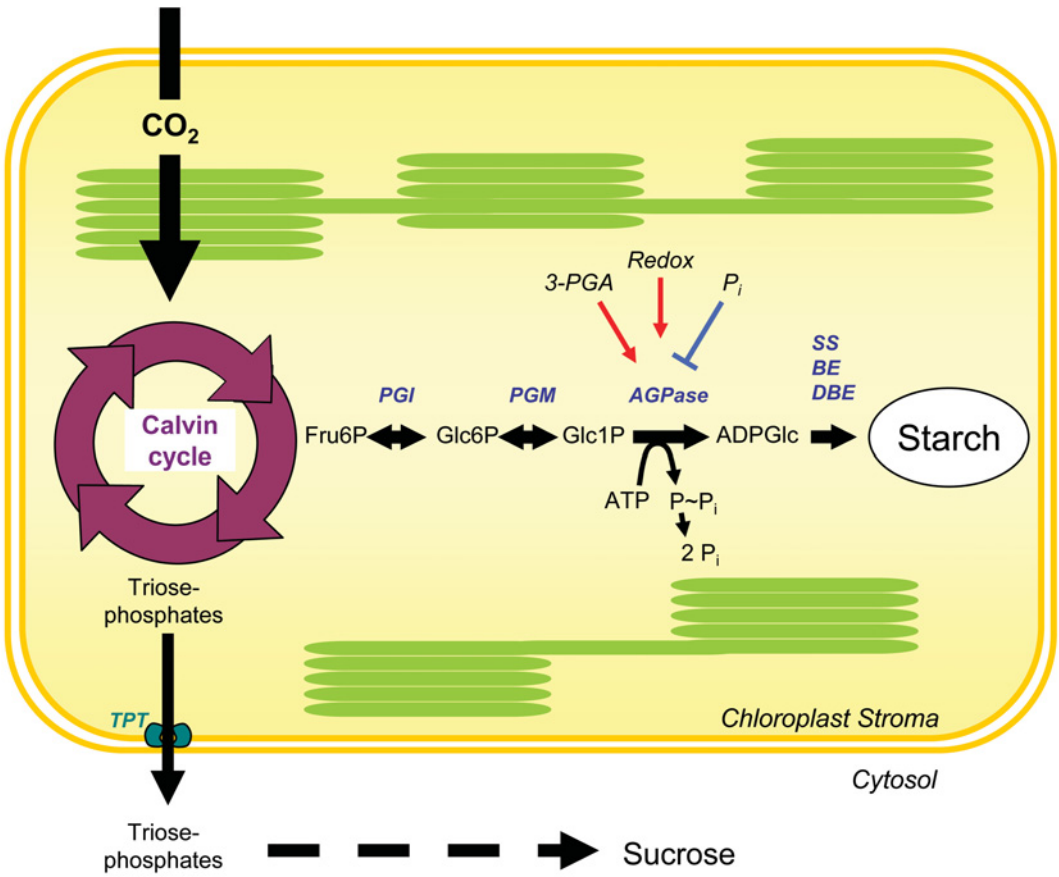


Scheme 1 Pathway of starch synthesis in chloroplasts

Carbon assimilated via the Calvin cycle is partitioned with a fraction exported to the cytosol for sucrose synthesis and a fraction retained in the chloroplast for starch synthesis. Redox activation and allosteric regulation of AGPase controls the flux of carbon into starch. Abbreviations: Fru6P, fructose 6-phosphate; Glc1P, glucose 1-phosphate; Glc6P, glucose 6-phosphate; TPT, triose-phosphate/phosphate translocator.

PROVISION OF SUBSTRATES FOR STARCH BIOSYNTHESIS

The widely accepted pathway for the conversion of Calvin cycle intermediates into ADP-Glc, the substrate for starch synthases, is shown in Scheme 1. Chloroplastic isoforms of phosphoglucose isomerase and phosphoglucomutase catalyse the conversion of fructose 6-phosphate into glucose 1-phosphate. AGPase (ADP-Glc pyrophosphorylase) uses glucose 1-phosphate and ATP to generate ADP-Glc and PP_i. Genetic and biochemical evidence shows that all of these steps occur within the chloroplast in *Arabidopsis* and in other species. Mutations affecting these enzymes decrease or abolish starch synthesis in leaves [10–12,14,33–35]. In non-photosynthetic tissues, a similar pathway from the hexose-phosphate pool operates. In most cases, sucrose entering the non-photosynthetic cell is converted into glucose 6-phosphate in the cytosol; this then enters the plastid as the substrate for the starch-biosynthetic pathway [33,36]. Until relatively recently, ADP-Glc production was thought to be confined to the plastid in all higher plants. While this is probably true in most plant organs [37,38], there is compelling genetic and biochemical evidence that, in the developing endosperm of cereal seeds, there are both plastidial and cytosolic forms of the enzyme and ADP-Glc is synthesized in both compartments [39–42]. Mutations affecting the cytosolic form of AGPase markedly reduce starch accumulation, suggesting that most of the ADP-Glc is synthesized there. Furthermore, mutations affecting a transporter in the plastid envelope capable of translocating ADP-Glc (brittle1) also decrease starch biosynthesis and result in a high concentration of ADP-Glc in the cytosol [43,44].

Despite the strong genetic evidence in support of the plastid-localized pathway of ADP-Glc production in leaves, it has been suggested recently that this model is wrong and that an alternative pathway exists, whereby ADP-Glc is predominantly synthesized in the cytosol via the action of sucrose synthase [45]. Evidence for this view comes first from the overexpression of a bacterial enzyme capable of ADP-Glc hydrolysis in the cytosol of *Solanum tuberosum* (potato) leaves – the reported consequence was a reduction in ADP-Glc levels and a reduction of starch synthesis [46]. Secondly, Muñoz et al. [45] reported that ADP-Glc levels are unaffected in *Arabidopsis* mutants lacking plastidial PGM (phosphoglucomutase) and AGPase. On the one hand, these results, if substantiated, cannot be explained by the current model for ADP-Glc biosynthesis. On the other hand, they offer only circumstantial evidence for the newly proposed model. Several lines of evidence argue against the newly proposed pathway. First, it is difficult to explain why mutants deficient in plastidial enzymes required to convert Calvin-cycle intermediates into ADP-Glc [PGI (phosphoglucose iosomerase), PGM and AGPase] are starchless if ADP-Glc is made in the cytosol from sucrose and imported into the chloroplast. Baroja-Fernández et al. [46] explained these phenotypes by proposing that starch is continuously turned over in the light, and that PGM, PGI and AGPase are involved in

scavenging the products of this turnover. However, double mutants resulting from crosses between *pgm* and mutant plants deficient in starch breakdown are still starchless ([20]; S. C. Zeeman, S. M. Smith and A. M. Smith, unpublished work), suggesting that PGM is needed for primary synthesis rather than scavenging. Secondly, restriction of triose-phosphate export [47–50] or of the gluconeogenic flux towards sucrose in the cytosol [51,52] causes a shift in partitioning in favour of starch and away from sucrose, arguing against sucrose-derived substrates for starch biosynthesis. Thirdly, the potato homologue of the brittle1 transporter (which imports ADP-Glc into plastids in cereal endosperm) appears not to transport ADP-Glc [53], and no other ADP-Glc transporter in the chloroplast envelope has been identified. Thus this newly proposed model has been met with some scepticism [54] and needs to be independently evaluated. The current weight of evidence favours the classical model (Scheme 1).

REGULATION OF AGPase ACTIVITY

The flux of carbon into the starch-biosynthetic pathway in leaves is thought to be controlled by modulation of AGPase activity. The reaction catalysed by AGPase is reversible under physiological conditions (Scheme 1), but the high activity of plastidial alkaline pyrophosphatase (hydrolysing pyrophosphate to P_i) is assumed to drive the reaction towards ADP-Glc production [55]. It has long been established that AGPase activity is allosterically regulated by the levels of P_i, an inhibitor, and 3-PGA (3-phosphoglycerate), an activator [56]. The ratio of these two metabolites changes according to supply of photo-assimilates and the demand for them. Under conditions where supply exceeds demand, high levels of photo-assimilates translate into a high 3-PGA/P_i ratio in the chloroplast stroma, the activation of AGPase and the synthesis of starch [57].

It is now apparent that AGPase activity is also redox-regulated [58,59] in both non-photosynthetic [60] and photosynthetic tissues [61]. Inactivation of AGPase occurs when the when sulfur-containing groups of cysteine residues in the C-terminal domains of the small subunits become oxidized, forming a disulfide bridge between the two subunits. Reduction of the enzyme breaks the bridge and results in activation [58]. In chloroplasts, redox activation of AGPase is probably mediated by the chloroplast ferredoxin/thioredoxin system, and the activation state changes between day and night, being (partially) activated during the day and mostly inactivated during the night [61]. This mechanism may prevent drainage of metabolic intermediates into the starch-biosynthetic pathway at night.

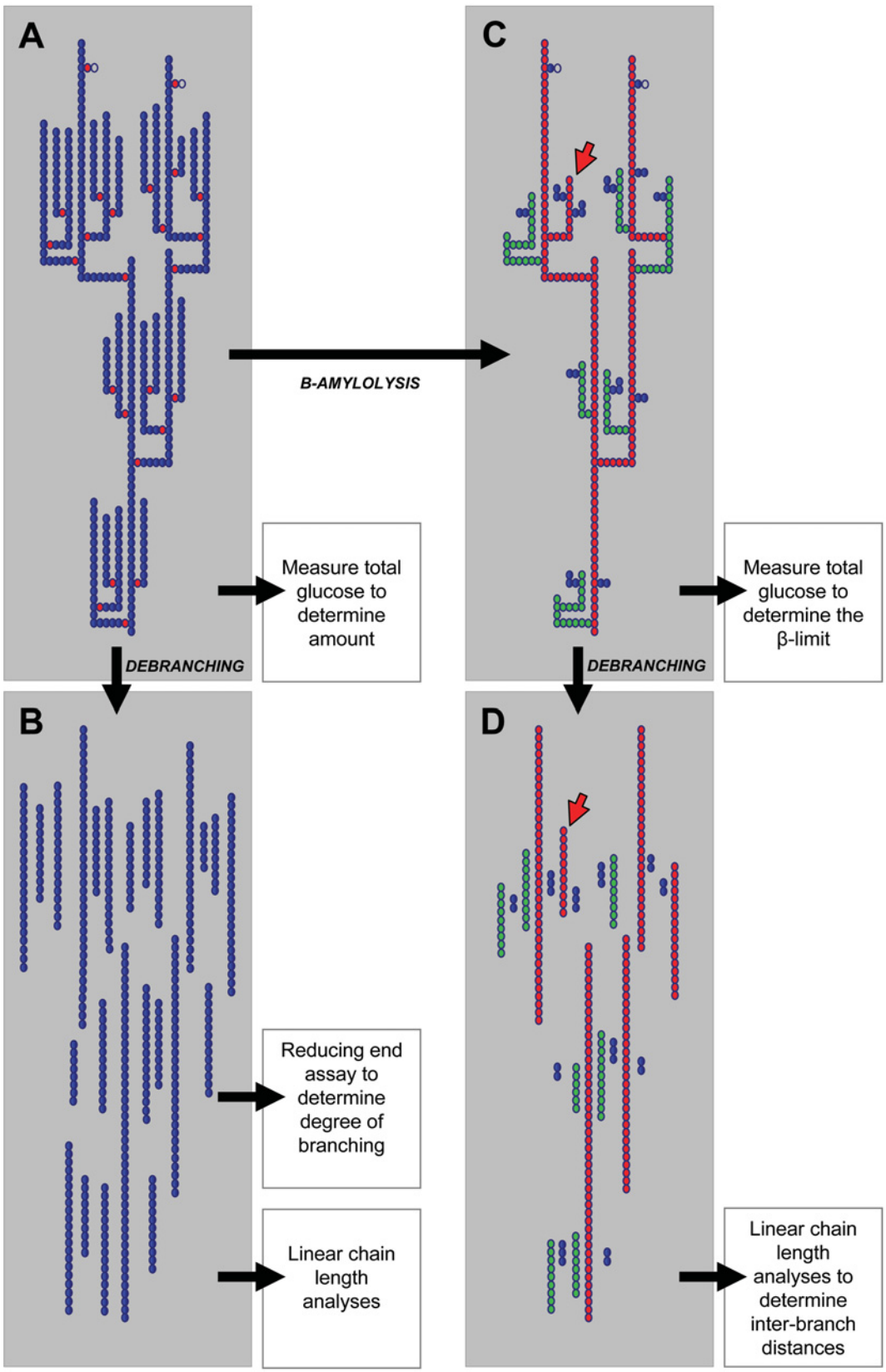
The degree of reductive activation of AGPase appears to be sensitive to metabolic changes. Activation and hence increased starch biosynthesis can be triggered by high levels of sucrose or glucose, involving SnRK1 (sucrose-non-fermenting-1-related protein kinase)- and hexokinase-mediated sugar signalling pathways respectively [62]. Kolbe et al. [63] provided evidence that sucrose-dependent activation also occurs in leaves and that Tre6*P* (trehalose 6-phosphate), a newly identified signalling compound in plants [64–66], may act as a signalling intermediate. The treatment of isolated chloroplasts with Tre6*P* results in almost complete reductive activation of AGPase [63].

The factors controlling Tre6*P* levels have not been elucidated, but the expression of genes predicted to encode enzymes of Tre6*P* metabolism is sensitive to changes in metabolic status, especially carbohydrate depletion [67]. Thus carbohydrate depletion may lead to increased capacity for Tre6*P* synthesis, which, in turn, could activate AGPase and promote starch synthesis. Evidence consistent with this was recently provided by Lunn et al. [68], who developed a sensitive method for the determination of Tre6*P* levels in *Arabidopsis* leaves. They demonstrated that Tre6*P* amounts increase at the onset of the light period when AGPase becomes activated. After an extended night, the increase in Tre6*P* is considerably enhanced upon re-illumination. In an extended night, starch reserves are exhausted, resulting in carbohydrate depletion. This causes an arrest of growth [9]. The elevation of the Tre6*P* level at the start of the following day is preceded by a rapid accumulation of sucrose and is also accompanied by (and presumed to be the cause of) an increased level of AGPase activation. This results in increased partitioning of photo-assimilates into starch relative to that in the previous light period. Thus a single night during which starch supply was not adequate to meet demand could trigger a mechanism ensuring that a greater reserve of starch is built up during the subsequent light period, sufficient to allow for an extension of the subsequent night [68].

It seems plausible that there might be a dual role for Tre6*P* in signalling, namely in carbohydrate depletion, as described above, and when carbohydrate is abundant. Both are situations in which future or immediate starch accumulation might be beneficial. The *Arabidopsis* genome encodes 22 genes putatively involved in the metabolism of trehalose and Tre6*P*. Many of the encoded proteins are putative targets for post-translational modification [69,70]. This probably reflects a complex system for the regulation of the levels of these metabolites in different cell types and in response to internal and external cues. It seems likely that these metabolites will prove to be central signals for the appropriate utilization of carbohydrates or their storage as starch.

STARCH BIOSYNTHESIS – THE PRIMING OF STARCH GRANULES

Starch is composed of branched and linear polyglucans. It is generally accepted that the ability of plants to synthesize semi-crystalline starch granules in plastids has evolved from an ancestral capacity to make glycogen – a simpler, more highly branched polymer, which is soluble [71]. SSs (starch synthases) catalyse the formation of new glucosidic linkages by transferring the glucose residue of ADP-Glc to the non-reducing end of an existing α-1,4-linked glucan chain, thereby elongating it. Branch points are introduced by BEs (branching enzymes), and DBEs (debranching enzymes) may be involved in tailoring the branched glucans into a form capable of crystallization (see below). The origins of these enzymes can be traced back to proteins involved in glycogen synthesis in both the eukaryotic host and the cyanobacterial endosymbiont ancestor of the chloroplast [72]. For glucan elongation and branching to occur, there must be primers on which the SSs can act. There is no robust evidence that SSs can produce glucan chains from ADP-Glc alone: a glucan acceptor (primer) is required (see, for example, [73]). There is good reason to believe that granule priming is under genetic control. Granule number is highly species- and organ-dependent [74]. Most *Arabidopsis* leaf chloroplasts contain about five starch granules. Starch-storing amyloplasts in heterotrophic tissues can contain either very few large granules (e.g. in potato tubers) or very many small granules [e.g. in rice (*Oryza sativa*) endosperm]. Depending on the species and organ, granules may be simple (derived from one initiation event) or compound (from multiple initiation events after which material subsequently merges to form a single granule). During the development of some cereal endosperms, there are two rounds of granule initiation, resulting in two distinct populations of starch granules, namely large lenticular ‘A’ granules, initiated early in development, and small spherical ‘B’ granules, initiated late in development [75]. It is not known what factors vary between species to generate this diversity in starch granule number and morphology.



Scheme 2 Determining the structural characteristics of amylopectin

Several characteristics of amylopectin can be reliably measured and give insight into its architecture (**A**). First, it is possible to measure the absolute frequency of the branch points (red circles). This can be achieved in a number of ways, such as comparing the quantity of glucose residues after complete hydrolysis with the number of linear chains obtained by debranching (**B**). The latter is measurable by quantifying the reducing ends of linear glucan chains after debranching. Secondly, it is possible to determine the chain-length distribution (i.e. the relative numbers of linear chains

In mammals and in yeast, glycogen molecules are primed by glycogenin, a self-glucosylating protein [76,77]. A chain of a few glucose residues attached to a tyrosine residue of glycogenin is extended and elaborated by glycogen synthases and BEs. Proteins similar in amino acid sequence to glycogenins are found in plants, as are other, distantly related self-glucosylating proteins, but evidence that they are involved in the priming of granule initiation is sparse [78,79]. One recent report suggests that reductions in the level of chloroplast-targeted glycogenin-like proteins in *Arabidopsis* results in alterations in starch metabolism [80], but further studies will be required to substantiate this claim. It is also possible that granules are initiated from small, soluble glucans. If so, there must be a mechanism that controls the *de novo* synthesis, turnover and limitation of their levels and thus governs the frequency with which they give rise to granules.

Recent studies have highlighted the fact that granule numbers are increased in mutants that lack DBEs of the ISA (isoamylase) class [81,82]. Certain isoforms of ISAs are required for normal starch synthesis (see below). When these are decreased in abundance, or absent, a proportion of the synthesized glucans does not form granules and remains soluble. This phenomenon is often accompanied by increases in the numbers of granule initiation events per plastid, leading to an abundance of small granules with irregular morphologies. Such observations have led to two models that seek to explain how DBEs might directly control granule number [81]. First, ISA might cleave glucan chains from glycogenin-like proteins that act as primers for granule initiation, thereby limiting the number of initiations. In the absence of ISA, more glycogenins with glucan chains would be present, hence more granules would be initiated. Secondly, ISAs might limit the number of small soluble branched glucans that act as primers for granule initiation – again in the absence of ISA; levels of these glucans would be increased, hence more granules would be initiated. It is difficult to devise robust and objective means of testing these hypotheses, and there is no direct evidence that ISA exerts a significant level of control over granule number in normal plants.

STARCH BIOSYNTHESIS – PRODUCTION OF AMYLOSE AND AMYLOPECTIN

After initiation, starch-granule formation proceeds by the production of amylopectin, a branched glucan containing 70 % or more of starch. Amylopectin forms an insoluble, semi-crystalline matrix in which the second component of starch, amylose, is synthesized. Although the enzymes that synthesize amylopectin are known, the way in which their actions are orchestrated to produce a crystallization-competent polymer is not fully understood. This is arguably because of experimental limitations in understanding the structure of the polymer.

Amylopectin is composed of chains of α-1,4-linked glucose residues between six and several hundred units in length, with an average length of 20–25 units. The chains are linked by α-1,6-bonds (branch points) to generate a tree-like structure (Scheme 2). Approx. 5 % of the total linkages in amylopectin are branch points. Each amylopectin molecule contains 10 000–100 000 glucose residues. The key feature of amylopectin is the existence of clusters of neighbouring unbranched chains 10–15 glucose residues in length (Scheme 2). Pairs of unbranched chains form double helices which pack into crystalline lamellae that alternate with amorphous lamellae containing the branch points. The resultant semi-crystalline structure has been likened to a side-chain liquid-crystalline polymer [83]. Biophysical, microscopic and chemical studies have provided the important data underlying this understanding of the nature of starch. The evidence for double-helix formation and packing is strong and widely accepted (for a review, see [84]). However, the exact molecular architecture of amylopectin is not known. First, the positioning of branch points with respect to one another is not known. Secondly, structural analyses generally give an average measure for amylopectin as a whole, which may mask structural heterogeneity in different parts of the molecule. Thirdly, the way in which branch frequency, chain-length distribution and branching pattern interrelate to produce a molecule capable of crystallization is not fully appreciated. Part of the reason for this lack of understanding is the size and complexity of amylopectin and its relative uniformity (simply glucose joined with two glucosidic linkages) compared with other biological macromolecules. This combination presents challenges when attempting to analyse amylopectin fine structure (see Scheme 2).

In spite of analytical limitations, genetic and molecular-biological studies – thus far conducted mainly on non-photosynthetic organs – have yielded an insight into how individual enzymes contribute to amylose and amylopectin biosynthesis. Chains of α-1,4-linked glucose residues are elongated from the non-reducing end by isoforms of SS. These isoforms can be classified into five families [GBSS (granule-bound SS), SSI, SSII, SSIII and SSIV], based on amino acid sequence [72]. GBSS is found exclusively bound to, and buried within, the starch granule. It synthesizes the linear amylose fraction of starch, using ADP-Glc, which diffuses into the granule matrix [85–89]. It may also contribute to the synthesis of long amylopectin chains [90,91]. GBSS is thought to elongate either soluble malto-oligosaccharides [92,93] or the side chains of amylopectin [89]. Its processive mode of action allows the generation of unbranched chains hundreds or even thousands of glucose residues in length [94]. The starch of storage organs generally contains about 20–30 % amylose. The amylose content of leaf starch is variable, but generally lower than that of storage organs [95–97]. This might be because the diurnal pattern of synthesis and breakdown of starch affords little time for the

of particular lengths). This is typically achieved using two approaches; either the separation/quantification of each chain length {e.g. by HPAEC-PAD (high-performance anion-exchange chromatography with pulsed amperometric detection) [133] or FACE/FA-PAGE (fluorophore-assisted capillary electrophoresis/fluorophore-assisted PAGE) [102]} or the overall analysis of the linear glucan size distribution by GPC (get-permeation chromatography) (see, e.g., [90]). The former technique has the advantage of giving clear resolution of individual chain lengths, but is insensitive to changes in the frequency of long chains, which are too similar in size and few in number to be reliably separated and detected. GPC is the preferable technique in this respect. Thirdly, it is possible to determine what fraction of the molecule is composed of 'external' and 'internal' chains. This is achieved by analysing the extent to which the molecule can be degraded by an exo-acting enzyme (typically BAM), which will degrade chains from the non-reducing ends until a branch point is reached (**C**). Fourthly, it is possible to combine the techniques described above to gain a measure of the inter-branch distances (see, e.g., [195]). This technique relies on first digesting the external chains (A-chains) with an exo-acting enzyme, then analysing the linear chain-length distribution of the resultant limit dextrin (**D**). Using this approach, external chains are reduced to stubs of two or three glucose units in length, whereas chains that carry other chains via branch points (B-chains) are protected from complete digestion. Subsequent debranching allows the lengths of these residual B-chain segments to be measured. For singly branched chains, this gives a measure for the inter-branch distance (green chains). However, for doubly or multiply branched chains, it only gives the distance to the outermost branch point (e.g. red chains). Such chains are likely to be long, inter-cluster B-chains, but if short B-chains carry two or more branches, closely spaced branch points may not be detected (e.g. on the chain marked with an arrow in **C** and **D**). Few, if any, studies compare all measurable aspects of amylopectin, even when mutant or transgenic plants are created specifically to understand starch biosynthesis. Furthermore, there is as yet no rigorous way to incorporate and interpret such data, limiting our ability to gain a full understanding of amylopectin structure.

accumulation of amylose. Higher amylose contents are seen in starch synthesized in leaves in continuous light, or in the leaves of mutants in which starch builds up to high levels [97].

The SS families SSI, SSII and SSIII are all involved in the elongation of amylopectin chains. They are localized in the plastid stroma and, in some cases, a fraction of the protein is bound to the granule. The function of the SSIV class has yet to be established. Most of the SS activity in *Arabidopsis* leaves is contributed by SSI and SSIII. [98,99]. There is good evidence that each SS family preferentially elongates amylopectin chains of particular lengths [90,98–106]. Although most of these studies have been conducted on non-photosynthetic tissues, the situation is likely to be similar in leaves. Loss of a particular SS class leads to specific characteristic changes in amylopectin structure. For example, two recent reports described changes in amylopectin chain-length distribution in *Arabidopsis* leaves [98] and developing rice endosperms [104] upon loss of SSI. In both cases, chains with a degree of polymerization (d.p.) of nine and ten glucose residues were reduced in relative abundance, and longer chains (d.p. 16–18) were increased. Such observations, in some cases supported by work on the isolated SS proteins (e.g. [100]), have led to the idea that SSI preferentially elongates the shortest chains (generated by the action of BEs; see below), SSII preferentially elongates medium-length chains and SSIII preferentially elongates longer chains [107]. Thus SSI may generate the preferred substrate of SSII and so on.

BEs introduce the α-1,6 branch points via a glucanotransferase reaction in which an existing α-1,4-linked chain is cut and a glucan segment of six or more glucose residues is transferred to the same, or an adjacent, chain [108]. Multiple isoforms of BE contribute to amylopectin synthesis. These fall into two classes [referred to as I (or B) and II (or A)] [109,110]. Class I enzymes preferentially transfer longer chains than class II enzymes [111, 112]. Most plants studied to date have representatives of both BE classes. Numerous enzymatic studies and analyses of mutant and transgenic plants reveal that the actions of the BEs are to an extent interdependent, and it has been suggested that they act sequentially during the formation of amylopectin clusters [113]. Studies of purified and recombinant BE isoforms have suggested interactive effects. For example, when expressed in yeast, *Zea mays* (maize) BEI was only able to contribute to branched glucan synthesis in the presence of a class II enzyme [114]. This was interpreted to mean that BEII produces substrates for BEI. Removal of the class I enzyme has minimal effects on starch quantity and composition in potato tubers [115] and also in cereal endosperm [116,117]. Removal of class II enzymes reduces starch synthesis and results in amylopectin with fewer branches and longer chains, in organs as diverse as potato tubers, pea and maize leaves, and cereal grains [96,118–122], suggesting that the class I enzyme alone is unable to mediate efficient branching. Repression of both of classes simultaneously in potato tuber has a much more dramatic effect than removal of one or the other, replacing essentially all of the amylopectin with long-chain glucans and giving rise to highly abnormal granules [123]. This indicates that the actions of the two isoforms are interdependent *in vivo*. Interestingly, the *Arabidopsis* genome appears to encode two members of the class II enzyme, but no enzyme conforming closely to the consensus for class I. Mutation of both genes encoding class II enzymes abolishes starch synthesis and results in the accumulation of short linear malto-oligosaccharides [124].

Similar studies to those described above, in which two SS of different classes, or BE and SS, are simultaneously removed from starch-storing organs, have produced evidence for interactions between isoforms of SS [90,102,125,126], and between isoforms of SS and BE (A. M. Smith, unpublished work). The possibility that the functional interactions suggested by these studies stems from the formation of starch-synthesizing 'complexes' containing multiple enzymes is discussed below.

THE ROLE OF DBEs IN STARCH SYNTHESIS

In all cases where changes in the complement of SS or BE isoforms cause changes in amylopectin structure, the resulting glucan still forms insoluble semi-crystalline granules, albeit with altered morphology and properties. Mutations affecting particular classes of DBEs, on the other hand, result in the partial or complete replacement of granules with soluble glucan (known as phytoglycogen), which has twice the level of branching of amylopectin. Different models to explain the accumulation of phytoglycogen in the absence of DBE have been proposed [127–129]. The prevailing view is that DBEs directly modify the structure of amylopectin during its biosynthesis, although there is no unambiguous evidence that this model is correct. According to this model, the actions of SS and SBE (starch branching enzyme) produce a glucan that cannot crystallize to form a granule. This may be because the branches are too numerous, the transferred chains too short or the branch points incorrectly positioned relative to one another. DBE activity is proposed to tailor these branched glucans selectively by removing branches, facilitating crystallization. There is as yet no evidence as to which branch-point configurations are removed, how selectivity is achieved or how this contributes to amylopectin synthesis.

Just as the SSs and BEs can be classified into subgroups, plants contain several genes encoding DBEs, which can be classified into two groups; limit-dextrinases (LDAs or pullulanase-type) and ISAs. The ISA group can be subdivided into three: ISA1, ISA2 and ISA3 [130,131]. The mutations that result in phytoglycogen synthesis in cereal endosperm are all in genes encoding ISA1 [81,132,133]. Phytoglycogen synthesis in *Arabidopsis* and potato is brought about by reductions in either ISA1 or ISA2 {[82,129,130], [134] (but see [134a]), [135]}. Mutations in genes encoding LDA and ISA3 do not by themselves result in phytoglycogen synthesis [26,135,136]. Thus genetic evidence indicates that the DBE activity required for normal starch synthesis is a function of ISA1, or ISA1 and ISA2. It seems likely that the main role of LDA and ISA3 is in starch degradation rather than in synthesis (see below).

Biochemical studies show that the ISA activity involved in starch synthesis has native molecular mass of between 350 and 500 kDa, indicative of a multimeric enzyme [134,137–139]. The subunit composition of the enzyme appears to vary between species. The enzyme in *Arabidopsis* leaves and potato tuber contains both ISA1 and ISA2 subunits [130,134]. In potato, the enzyme probably contains two ISA1 and two ISA2 subunits. By contrast, most of the activity in cereal endosperm appears to be attributable to a homomultimeric protein containing only ISA1 subunits [138]. However, recent work indicates that a heteromultimeric enzyme containing both ISA1 and ISA2 subunits is also present in rice endosperm, but the ratio of ISA2 to ISA1 subunits is low [140]. Interestingly, sequence analysis of *ISA2* genes and assays of recombinant ISA2 protein indicate that, although this class of proteins may be able to bind glucans, it may lack DBE activity due to substitutions of key active-site residues [130]. This has led to the suggestion that ISA2 may have a regulatory function [130] or may help to confer substrate specificity to the catalytically active ISA1 subunit [134]. The variation in subunit composition between species may thus be reflected in a variation in the properties of the enzyme. The significance of such predicted variation will only become apparent when the precise role of ISA in starch synthesis is elucidated.

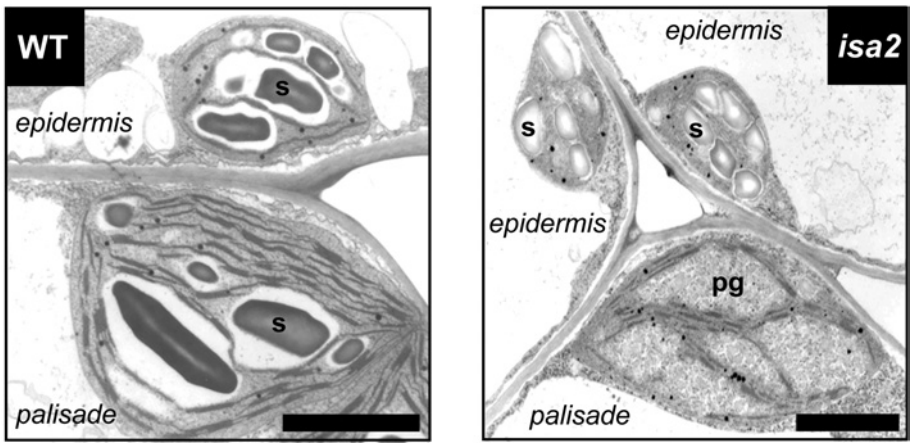


Figure 1 Starch and phytoglycogen accumulation in the ISA-deficient mutant *atisa2-1* (= *dbe1-1*)

Loss of the multimeric ISA caused by mutation of either the *isa1* or *isa2* genes results in the accumulation of phytoglycogen ('pg') in the spongy mesophyll and palisade cells. However, some cell types, such as epidermal cells, still synthesize starch granules ('s') which are normal in appearance, but contain altered amylopectin. This indicates that other factors are involved in determining whether the glucan synthesized crystallizes into granules or remains soluble (for details, see [134]).

Comparisons of phytoglycogen and amylopectin have revealed several differences in structural characteristics in addition to the greater branch-point frequency in phytoglycogen. For example, phytoglycogen has a greater number of very short chains than amylopectin [134,141–143]. It also seems likely that the distance between branch points is different in the two polymers. Delatte et al. [134] analysed the chain-length distributions of phytoglycogen and amylopectin from *Arabidopsis* after BAM (β-amylase) digestion of the external chains. The results indicated that branch points in amylopectin are relatively well spaced (approximately eight glucose residues between branch points), whereas those of phytoglycogen appear to be much closer together. However, it should be noted that the technique used in that study had limitations and the spacing of branch point is difficult to measure accurately (see Scheme 2). Delatte et al. [134] suggested that, during amylopectin synthesis, the removal by ISA of branches that are very close to other branches may be important in the production of a structure capable of crystallization. This view is generally consistent with models of amylopectin secondary structures which conclude that interbranch distance is a critical factor, with branch point spacing of four to six glucose units optimal for double-helix formation and local packing [144].

A complicating factor in understanding the role of ISA is that the phenotypes of ISA-deficient plants are very variable: some species accumulate phytoglycogen almost exclusively (e.g. in the unicellular green alga *Chlamydomonas* [142]), whereas others accumulate mostly starch {e.g. barley (*Hordeum vulgare*) [81]}. Furthermore, there are several examples of localized accumulation of phytoglycogen and/or starch in different cell types. The *isa1* (sugary1) mutants of rice [145], maize [141] and barley [81] all show heterogeneous distributions of starch and phytoglycogen within their endosperms. In the *isa1* and *isa2* mutants of *Arabidopsis*, the leaf mesophyll accumulates predominantly phytoglycogen, whereas the epidermal cells and vascular bundle-sheath cells accumulate starch granules which are normal in appearance but which contain slightly altered amylopectin (Figure 1) [134]. This suggests that the loss of the ISA activity results in the formation of an altered branched glucan which may, or may not, form a granule. The fate of the glucan may depend on the complement of other starch-synthetic and degradative enzymes present in that cell type [134].

In general terms, interpretation of the phenotypes of mutant and transgenic plants lacking isoforms of SS, BE and DBE is complicated by three factors: the fact that all of these enzymes act on the same glucan substrates, the large pleiotropic effects seen in some starch-storing organs, and the possibility that some of these enzymes physically interact in starch-synthesizing 'complexes'. Here, we briefly consider each of these factors in turn.

The glucan product of any given enzyme of starch synthesis serves as a substrate for several other enzymes. For example, a branch created by a class II BE may then be acted on by three or four different SSs and then by a class I BE, possibly followed by ISA. The alteration in structure upon the loss of one enzyme may thus have profound consequences for subsequent modifications of the polymer. Alternatively, other enzymes might partially or wholly compensate for the loss of a particular activity. Either possibility makes it difficult to assign a specific function to a particular enzyme of starch synthesis based on examination of the alterations in the structure of starch polymers in a plant in which it is absent or reduced.

Mutations affecting one enzyme of starch synthesis may result in changes in the amount or activities of other such enzymes. These effects are particularly prevalent in developing cereal endosperms [132,136,146–148]. As examples, mutation of the rice BEIIb results in a reduction in activity in SSI [147], whereas loss of the starch DBE LDA (pullulanase-type) from maize causes a reduction in BEIIa [136]. Such pleiotropic effects may lead to changes in amylopectin structure that are not due to the original mutation: this is clearly shown in the study performed by Dinges et al. [136].

There is growing evidence that starch-biosynthetic enzymes form multienzyme complexes. Complex formation could influence both enzyme properties and the product of their combined actions. The existence of complexes has been inferred from the pleiotropic effects caused by particular mutations [71]. Further indirect evidence arose from the effects of reductions in the level of chloroplast-targeted 14-3-3-binding proteins in *Arabidopsis*: starch levels in the leaves were increased [149]. 14-3-3 proteins can mediate protein–protein interactions [150], and there is theoretical and *in vitro* evidence that SSs and SBE may interact with these proteins [149,151]. The correlation between 14-3-3 protein levels and starch content may thus reflect a role for

14-3-3 proteins in regulating the formation of complexes of starch-synthesizing enzymes necessary for normal rates of starch metabolism. Direct evidence for complex formation between starch-biosynthetic enzymes was provided by Tetlow et al. [152]. They showed that, in plastids isolated from wheat (*Triticum aestivum*) leaves or developing endosperms, BEI and BEII were able to associate into a complex (also containing α-glucan phosphorylase). Complex formation was dependent on protein phosphorylation, which also specifically enhanced BEIIa activity. The consequences of complex formation for enzymatic activity and interactions, in particular the interdependence of the actions of BEs described above [114], and the impact of complex formation on amylopectin biosynthesis, have yet to be established. Future developments in this field are likely to enhance our understanding of the orchestration of starch-biosynthetic enzymes.

REMOBILIZATION OF STARCH

There have been major advances in our understanding of starch breakdown in leaves in the last few years (reviewed in [153–156]). At least for *Arabidopsis*, the major steps in the pathway are known and the key genes/proteins have been identified. The initial event, seemingly required for subsequent degradation, is the phosphorylation of amylopectin by enzymes of the GWD (α-glucan,water dikinase) class [157]. Subsequently, starch is hydrolysed to maltose and glucose inside the chloroplast. These metabolites are exported to the cytosol and metabolized further.

THE IMPORTANCE OF GLUCAN PHOSPHORYLATION

GWD is a newly identified enzyme, discovered on the basis of its importance in starch degradation. Elimination from potato plants of a starch-bound protein of unknown function (originally named the R1 protein) caused starch accumulation in leaves and reduced levels of sweetening in cold-stored tubers, phenomena both indicative of reduced rates of starch degradation [16]. This was associated with loss of the phosphate groups covalently linked to the amylopectin component of both leaf and tuber starch. The protein was found to be a GWD that transfers the β-phosphate group of ATP to a small proportion of the glucose residues of amylopectin chains, specifically at the C^6 position [158,159]. Subsequent work revealed that a mutant of *Arabidopsis* selected because of the accumulation of very high levels of starch in its leaves [the *sex1* (starch excess 1) mutant] carries a mutation in the gene encoding GWD [17]. This plant also accumulates elevated levels of starch in seeds, flowers and root tips [15] and lacks phosphate groups in the amylopectin component of starch [17]. These results suggest that GWD-mediated phosphorylation of amylopectin is required for starch breakdown in both photosynthetic and non-photosynthetic tissues.

Glucan phosphorylation occurs concurrently with starch biosynthesis [160], resulting in a low level of phosphorylation throughout the granule (e.g. 1 in 2000 of the glucose residues in *Arabidopsis* leaf starch carry phosphate groups [17]). However, GWD is reported to be more active during periods of starch breakdown. First, GWD in potato leaves has been shown to bind to the surface of starch granules at night, but not during the day [158]. Secondly, the outer surface of potato leaf starch granules contains higher levels of phosphate at night than during the day [161]. Thirdly, pulse–chase radiolabelling experiments conducted in photoautotrophic *Chlamydomonas* cells suggest that the rate of phosphorylation at the surface of the granule is higher during the night [161]. The subsequent removal of the outer glucans during degradation means that such night-time phosphorylation would not be readily detected when analysing the phosphate content of whole starch granules. Although these experiments suggest that GWD is most active at night, Mikkelsen et al. [28] recently presented evidence that the enzyme is strongly regulated via redox activation and that the granule-bound fraction of GWD is in its oxidized (inactive) form. Further work is required to resolve these apparently inconsistent results. This is important, because GWD is placed at the start of the starch catabolic pathway. Regulation of its activity could potentially control flux through the pathway.

By searching for proteins that bind to phosphorylated starch granules, Kötting et al. [23] identified a second enzyme that phosphorylates amylopectin, exclusively on the C^3 position of the glucose residues. Although related in amino acid sequence to GWD, this enzyme requires that its glucan substrate is already phosphorylated, hence it has been named PWD (phosphoglucan, water dikinase). The function of this protein was discovered independently by Baunsgaard et al. [24]. This requirement for a phosphorylated glucan substrate, coupled with the fact that starch in plants lacking GWD contains no detectable phosphate, implies that PWD acts downstream of GWD and is dependent on the prior action of GWD. It seems likely that the combined actions of GWD and PWD result in phosphorylated glucose residues in close proximity to each other.

It is important to emphasize that the way in which glucan phosphorylation allows or facilitates starch breakdown is not yet known. A plausible explanation is that single or adjacent phosphate groups disrupt the packing of double helices within the semicrystalline layers formed by amylopectin, allowing access by glucan-hydrolysing enzymes into the hydrated cleft [17]. Adjacent phosphate groups are likely to have a considerably greater effect in this respect than single groups.

ENDO-AMYLOLYSIS OR EXO-AMYLOLYSIS OF THE GRANULE SURFACE?

There is uncertainty over which enzymes actually attack the starch granule surface in leaves. The three most likely candidates are AMY (α-amylase), BAM and DBE (ISA3). In the endosperm of germinating cereal seeds, where starch breakdown is relatively well understood, AMY (an endoamylase) is thought to initiate the process. AMY is capable of attacking the insoluble granule, cutting internal α-1,4 linkages to release both linear and branched glucans. This action may firstly facilitate the attack of the granule by other enzymes [162,163] and secondly release soluble glucan fragments to serve as substrates for other enzymes. AMY action causes a characteristic visible pitting of the granule surface. It seems likely that this process is independent of GWD and PWD; cereal endosperm starch contains little or no phosphate, and the endosperm – a non-living tissue at the stage of germination – has no means of generating ATP during starch degradation. The available evidence suggests that, in leaves, AMY has a less important role in starch breakdown. Three distinct AMYs (AMY1, 2 and 3) are encoded in the genome of *Arabidopsis* [164]. AMY3 is localized within chloroplast [32,165], but mutations that knockout the *AMY3* gene do not prevent normal rates of starch breakdown [165]. AMY1 and AMY2 do not have predicted transit peptides, suggesting that they are probably not chloroplastic, and starch metabolism appears normal in *amy1*/*amy2*/*amy3* triple mutants [165]. These results indicate that 'classical' AMY is not required for starch degradation in *Arabidopsis*, at least when grown under normal growth-room conditions. It remains possible that there are other non-classical endoamylases (i.e. not recognizable because they have distinct amino acid sequences) that compensate for the

loss of AMY3. It is also possible that AMY3-mediated starch breakdown is important in tissues or in conditions that have not been evaluated to date. Indeed, our recent data [26] suggest that, at least in some circumstances, AMY3 protein levels are elevated and it does participate in starch breakdown (see below).

Although endo-amylolysis has long been accepted as the first step in the degradation of starch granules, the lack of requirement for AMY in *Arabidopsis* leaves raises the possibility that an altogether different mechanism may operate. Recent data from studies of *Arabidopsis* and potato are consistent with the idea that there may be a progressive degradation of the granule surface by exo-amylolysis and debranching. The loss of a specific isoform of the exoamylase BAM [BAM3 in *Arabidopsis*, also called BMY8 and ct (chloroplast-targeted)-BMY; PCT (potato chloroplast-targeted)-BMY1 in potato] and the DBE ISA (ISA3 in *Arabidopsis* and potato) results in reduced rates of starch breakdown and excess accumulation of starch, indicating that these two enzymes are involved in breakdown [19,25,26,135]. Recombinant forms of both chloroplastic BAM and ISA3 have some activity on intact starch granules *in vitro*, although their activities are much greater on soluble substrates [19,130]. The low activity against intact isolated granules is not surprising, as one or both enzymes might require the substrate to be phosphorylated by GWD during degradation.

From these data it is credible to propose a model whereby BAM and ISA3 attack the granule surface in an interdependent manner. BAM acts only on the outer chains of a branched glucan: it cannot act inside the branch points. Thus its action on the granule would produce a surface of short chains on which it could act no further. These short chains could then be removed by the debranching action of ISA3, uncovering longer chains on which BAM could continue to act. Experimental evidence supporting this model is as follows. First, Ritte et al. [161] showed that the abundance of short chains (d.p. 3–5) on the surface of granules extracted from potato leaves increased markedly in the first hours of the night, consistent with exo-amylolysis. Secondly, Delatte et al. [26] showed that such short chains accumulate in the starch of *Arabidopsis isa3* mutants, suggesting that they are no longer efficiently removed by debranching. Thirdly, expression of an ISA3–green fluorescent protein construct in *Arabidopsis* leaf protoplast showed that the fluorescent fusion protein was preferentially localized to the starch granule surface, consistent with its proposed role there [26].

The model outlined above would result in the release of maltose via β-amylolysis and short linear oligosaccharides via debranching from the starch granule surface, rather than a mixture of linear and branched glucans typical of α-amylolysis. However, it is noteworthy that the down-regulation/mutation of neither *BAM* nor *ISA3* genes completely abolishes starch breakdown [19,25,26,135], suggesting that other enzymes can compensate at least partly for those that are missing. Evidence for such compensation comes from studies of *Arabidopsis* plants lacking either ISA3 or the DBE LDA, or both of these enzymes. Whereas loss of ISA3 decreases the rate of starch breakdown, loss of LDA alone has no measurable effect [26,135]. However, simultaneous loss of ISA3 and LDA resulted in a further reduction in starch breakdown compared with loss of ISA3 alone, indicating that in the absence of ISA3, LDA is required for breakdown [26]. Furthermore, in the *isa3*/*lda* double mutant, soluble branched oligosaccharides appeared during starch breakdown and the activity of the AMY was increased, attributable to a higher amount of the chloroplastic isoform, AMY3. This suggests that, in the absence of ISA3, AMY3 may degrade the granule and thereby provide soluble substrates for LDA. Thus, even though AMY3 and LDA can each be mutated in *Arabidopsis* without affecting leaf starch breakdown, they can nonetheless contribute to breakdown [26]. Currently, it is not clear whether AMY3 and LDA participate in starch breakdown in wild-type plants, or whether they have specific functions under certain environmental or developmental conditions.

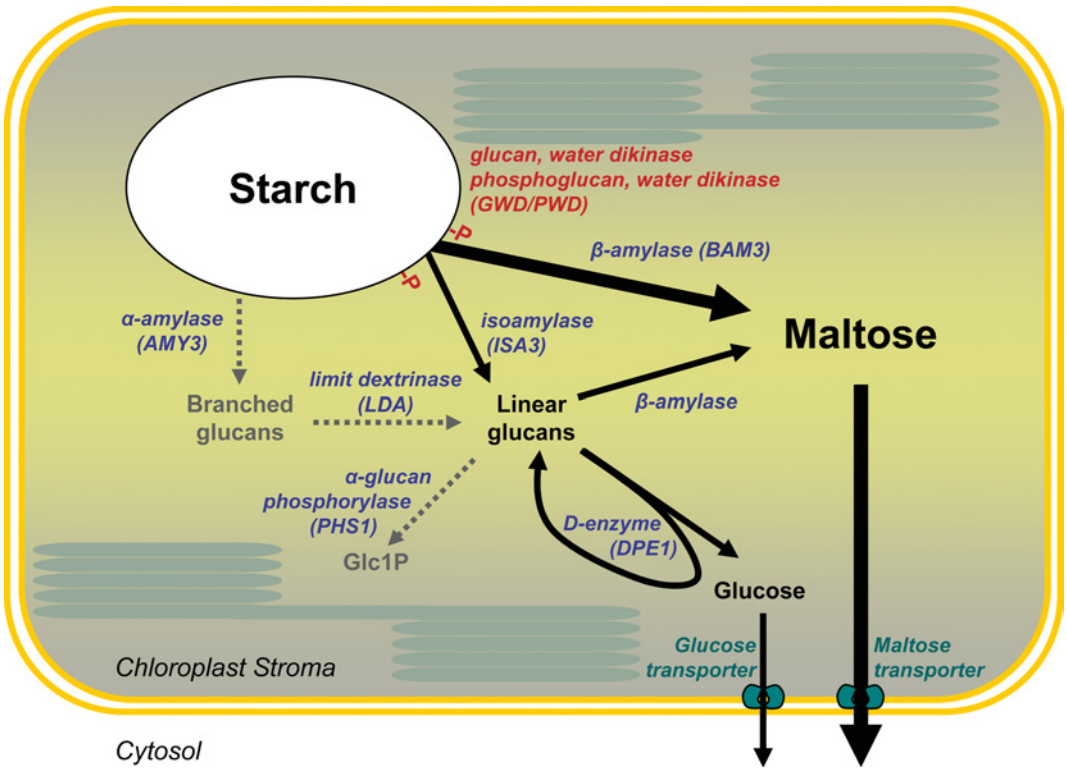
The likely pathways for the release of glucans from the starch granule in *Arabidopsis* leaves are shown in Scheme 3. As discussed above, a major gap in our understanding is the significance of GWD- and PWD-mediated glucan phosphorylation. It is tempting to propose that phosphorylation is important for the operation of exo-amylolysis and debranching, as these appear to represent the major pathway. Furthermore, there is no reason to suppose that endo-amylolytic attack by AMY requires phosphorylation. It will be important to determine experimentally which starch-degrading enzymes require prior glucan phosphorylation in order to act. It is noteworthy that AMY3 and GWD both contain similar carbohydrate-binding modules within their N-terminal (non-catalytic) domains [17,28,165,166]. Thus AMY3 and GWD might bind to the same glucan structures to mediate endo-amylolysis or to prime exo-amylolysis/debranching respectively.

The data obtained from studies of starch degradation in the leaves of *Arabidopsis* and potato are generally in agreement and point towards a common primary mechanism of starch breakdown. However, the situation may be different in other plants. In maize, unlike *Arabidopsis*, loss of LDA alone results in a reduced rate of starch breakdown in leaves [136]. In rice, it was reported recently that an AMY (related in sequence to *Arabidopsis* AMY1) possessing a signal peptide for targeting to the secretory pathway actually appears in the chloroplasts and that repression of its expression leads to starch accumulation in leaves [167]. Thus in the grasses (Poaceae, formerly Gramineae), starch breakdown in leaves may be initiated by AMY. Little is known about the importance of GWD and BAM in the leaves of these species.

METABOLISM OF SOLUBLE GLUCANS

The studies described above suggest that maltose, larger malto-oligosaccharides (predominantly linear, d.p. 3–5) and a few phosphorylated malto-oligosaccharides may all be released by the enzymes that attack the starch granule. Nothing is known about how the phosphorylated malto-oligosaccharides are metabolized. If branched malto-oligosaccharides are produced, they are most likely debranched by LDA and/or ISA3 [26]. Linear oligosaccharides can be metabolized from their non-reducing ends by BAM to yield maltose and maltotriose (the latter being three α-1,4-linked glucose residues, which is too short to act as a substrate for BAM [168]). There is good evidence that maltotriose is metabolized via a glucanotransferase reaction catalysed by DPE1 (disproportionating enzyme 1, D-enzyme). In the *dpe1* mutant of *Arabidopsis*, maltotriose accumulates during the dark, and starch breakdown is retarded [18]. The disproportionation of maltotriose produces glucose and maltopentaose (five α-1,4-linked glucose residues). The former can be exported to the cytosol through the chloroplast-envelope glucose transporter [169–171], while the latter is long enough to serve once more as a substrate for BAM, which will produce maltose and maltotriose. The net products of linear oligosaccharide metabolism through this pathway would be chiefly maltose, together with some glucose (Scheme 3).

Linear glucans can also potentially be metabolized by the chloroplast-localized α-glucan phosphorylase. The preferred substrates of this enzyme are linear oligosaccharides of five glucose residues or longer [172]. Phosphorylase liberates glucose 1-phosphate from the non-reducing ends of the chains. As with BAM, DPE1 could recycle short oligosaccharides that are poor substrates for phosphorylase. The precise role and importance



Scheme 3 A model for the pathways of starch degradation in *Arabidopsis* chloroplasts

Starch is hydrolysed to maltose and glucose during the dark. Phosphorylation of the granule surface by GWD and PWD may allow the direct action of BAM3 and ISA3. Linear glucans can be metabolized by DPE1, releasing glucose. Loss of any of these enzymes reduces starch breakdown and causes a SEX phenotype. AMY3 may also attack the starch granule to release branched glucans which can then be debranched in the stroma by LDA. In addition to further degradation by BAM, PHS1 may liberate glucose 1-phosphate (Glc1P) to support chloroplast metabolism. The reactions catalysed by AMY, LDA and PHS1 are shown in grey with broken lines, as loss of these proteins does not prevent a normal rate of starch breakdown. The thickness of the arrows reflects our estimates of the respective fluxes. The importance of the glucose transporter for starch breakdown has not yet been established.

of phosphorolysis in leaf starch breakdown is still not clear. Potato plants with reduced plastid phosphorylase activity have a wild-type phenotype [173]. *Arabidopsis phs1* (chloroplastic α-glucan phosphorylase) mutant plants lacking the enzyme also have normal starch levels [174], although mutant plants display sensitivity to abiotic stress, resulting in small necrotic lesions bordered by starch-accumulating tissues. These observations lead to the suggestion that phosphorylase might supply substrates for the plastidial oxidative pentose phosphate pathway, potentially important for the provision of reductant (NADPH) during stress tolerance [174]. Interestingly, mutants lacking chloroplastic NADPH-dependent thioredoxin reductase, proposed to be important in the tolerance to oxidative stress, also display necrotic lesions and sensitivity to abiotic stress [175]. More recently, Weise et al. [176] showed that starch breakdown is induced under photorespiratory conditions, yielding both maltose derived from amylolysis and hexose phosphates derived from phosphorolysis. This observation led to the suggestion that phosphorylase may play a role in degrading glucans to replenish Calvin-cycle intermediates lost during photorespiration.

On balance, the available evidence suggests that hydrolysis rather than phosphorolysis is the predominant pathway of starch breakdown in leaves under normal growth conditions. First, studies on several species have shown that maltose and glucose are exported from isolated chloroplasts that are breaking down their starch [177–180], and a recent study indicated that maltose is the major sugar exported [181]. Secondly, maltose accumulates to high levels at night inside the chloroplasts of *Arabidopsis* plants deficient in the chloroplast-envelope maltose transporter MEX1 (maltose excess 1), and starch breakdown is decreased [20,182]. Larger malto-oligosaccharides are not thought to cross the chloroplast envelope [183]. Thirdly, potato and *Arabidopsis* plants lacking a chloroplastic BAM exhibit starch-excess phenotypes and decreased rates of starch breakdown [19,25]. This contrasts with the lack of phenotype in plants lacking chloroplastic α-glucan phosphorylase [173,174]. Fourthly, loss of DPE1 results in the accumulation of maltotriose [18]. This is consistent with the operation of β-amylolysis rather than phosphorolysis: phosphorolysis would be expected to result in maltotetraose accumulation [172]. The apparent importance of β-amylolysis and of maltose production during starch breakdown is reflected in the *Arabidopsis* genome. Nine genes encode BAM-like proteins. Four of these proteins have N-terminal sequences which could be chloroplast transit peptides, and recently obtained findings (S. C. Zeeman, A. M Smith and S. M. Smith, unpublished work) suggest that at least three of these are involved in starch degradation.

REGULATION OF STARCH BREAKDOWN

The initiation and rate of starch breakdown are strongly regulated [156]. Upon an abrupt transition from the light to the dark, starch breakdown usually commences after a short delay. When *Arabidopsis* plants are entrained to a particular photoperiod, the rate of degradation is controlled so that the starch synthesized during the day is sufficient to provide a steady supply of carbon

throughout the night. Two recent studies have illustrated the flexibility of this regulation in *Arabidopsis*. Lu et al. [184] showed that plants entrained to a long photoperiod, exhibiting a high rate of starch breakdown during the short night, have a slower rate of degradation if the plants were placed in the dark prematurely, halfway through their normal photoperiod. Conversely, plants entrained to a short day, exhibiting a low rate of starch breakdown during the long night, have a higher rate of breakdown when placed in the dark if their normal photoperiod was doubled in length. Although the adjustment of the degradation rate was not sufficient to ensure that the starch supplies lasted out the night exactly in plants prematurely placed in the dark (which ran out of starch) or plants entrained to an extended day (which contained a surplus), these results suggest that the rate of starch mobilization at night is somehow influenced by the length of the preceding photoperiod. Similar experiments by Gibon et al. [9] showed that full adjustment of the rates of starch biosynthesis and degradation to an altered light regime was achieved in just 3 days. The mechanisms that control starch degradation are not yet understood [155], but several possible mechanisms, including transcriptional control, feedback inhibition, redox regulation and reversible protein phosphorylation, have emerged recently.

Several of the genes encoding starch-metabolizing enzymes (including *GWD*, *PWD*, *ISA3*, *AMY3*, *DPE1* and *PHS1*) appear to be co-expressed, and transcript levels fluctuate appreciably between day and night in *Arabidopsis*. The peak of expression is at the end of the day, just prior to the onset of starch breakdown [185]. For several genes this diurnal fluctuation has been shown to be under light-dependent circadian control [184,186,187]. However, where studied, protein levels do not show the same diurnal fluctuations, suggesting that post-translational mechanisms are probably more important in the immediate control of the rate of starch breakdown [17,184,185]. The significance of the diurnal patterns of transcript abundance for these enzymes is not known, but the 'integration' of mRNA levels over time may control protein levels over the medium-to-long term. The modulating effects of external and internal inputs (e.g. day length or metabolite levels) on endogenous expression patterns might allow gradual adjustment of protein levels necessary to maintain the plant's diurnal carbon balance [188].

There are several possible post-translational mechanisms that might regulate the enzymes of starch degradation. First, accumulation of the intermediates of starch breakdown might feed back to prevent further release of glucans from the granule [155,156]. This view is based on the reduced rates of starch breakdown observed in *Arabidopsis* mutants such as *dpe1*, *mex1* and *dpe2*, all of which accumulate malto-oligosaccharides [18,20–22]. However, the levels of malto-oligosaccharides accumulated in these mutants are greatly in excess of normal night-time levels, so the importance of such feedback in a normal leaf is questionable. Secondly, the enzymes of starch breakdown might be regulated by reversible protein phosphorylation [27]. Mutation of a dual-specificity protein phosphatase in *Arabidopsis* [SEX4, also called PTPKIS (protein-tyrosine phosphatase kinase interaction sequence) and DSP4 (dual-specificity phosphatase 4)] results in a reduction in starch breakdown and the accumulation of very large starch granules [3,27,32,189]. In addition to its phosphatase domain, the SEX4 protein has a carbohydrate-binding module, allowing it to bind soluble glucans and starch granules [27,189,190]. Remarkably, SEX4 has sequence similarity to a mammalian protein phosphatase, laforin, implicated in the control of glycogen metabolism. Laforin deficiency leads to the accumulation of aberrant glycogen particles (polyglucosan or Lafora bodies), resulting in neuronal dysfunction and death [191]. In both cases, excess glucan could result from increased synthesis or decreased degradation. In the *sex4* mutants, the rate of starch synthesis is decreased with respect to wild-type plants, but the rate of starch degradation is lower still [3,32], suggesting that SEX4 primarily regulates breakdown.

An exciting possibility is that SEX4 is actually a glucan phosphatase, responsible for the removal of phosphate groups added by GWD and PWD. A recent study suggests that, unlike other known protein phosphatases, human laforin is capable of dephosphorylating potato amylopectin [192]. These authors state that SEX4 is also capable of glucan dephosphorylation. Further work will be necessary to identify whether the downstream targets of SEX4 are glucans or proteins *in vivo*, and to understand the role of SEX4 in controlling starch metabolism.

A third mechanism implicated in the regulation of starch breakdown is redox activation of the enzymes. Potato GWD and an isoform of BAM from *Arabidopsis* (BAM1, also called BMY7) are activated by reduction [28,29]. The SEX4 protein may also be subject to reductive activation [189]. These observations are puzzling, given that the enzymes are expected to be active at night, when the chloroplast is in a more oxidized state than during the day. Other well-studied chloroplast proteins are activated directly or indirectly by reduction [for example, the Calvin-cycle enzymes fructose-1,6-bisphosphatase, sedoheptulose 1,7-bisphosphatase, phosphoribulokinase, NADP:glyceraldehyde-3-phosphate dehydrogenase and Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase)], using electrons from Photosystem I transferred via the ferrodoxin–thioredoxin system [193]. Their activation is thus light-dependent, and they are oxidized and inactive at night. This system clearly cannot be responsible for activation of proteins that are active at night. It is possible that such proteins might be activated by a light-independent thioredoxin, such as that recently described in the amyloplasts of non-photosynthetic tissues [194].

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