Starch: Its Metabolism, Evolution, and Biotechnological Modification in Plants

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Abstract

Starch is the most widespread and abundant storage carbohydrate in plants. We depend upon starch for our nutrition, exploit its unique properties in industry, and use it as a feedstock for bioethanol production. Here, we review recent advances in research in three key areas. First, we assess progress in identifying the enzymatic machinery required for the synthesis of amylopectin, the glucose polymer responsible for the insoluble nature of starch. Second, we discuss the pathways of starch degradation, focusing on the emerging role of transient glucan phosphorylation in plastids as a mechanism for solubilizing the surface of the starch granule. We contrast this pathway in leaves with the degradation of starch in the endosperm of germinated cereal seeds. Third, we consider the evolution of starch biosynthesis in plants from the ancestral ability to make glycogen. Finally, we discuss how this basic knowledge has been utilized to improve and diversify starch crops.

Contents

INTRODUCTION

Starch is an insoluble glucan composed of two polymers of glucose, amylopectin and amylose. In higher plants, starch is synthesized in plastids in both photosynthetic and nonphotosynthetic cells. As the principal storage carbohydrate, starch plays important roles during the life cycle of the plant. In leaves, a fraction of the carbon assimilated through photosynthesis is retained in the chloroplasts as starch rather than being converted to sucrose for export to the sites of growth. This transitory starch is degraded at night to provide substrates for leaf respiration and for continued sucrose synthesis for export to the rest of the plant. The supply of carbon from starch can be vital for normal plant growth. Mutant *Arabidopsis* plants that cannot synthesize starch, or cannot degrade it at night, have reduced growth rates under most conditions (129). In nonphotosynthetic organs (e.g., stems, roots, tubers, and seeds), sucrose may be converted to starch for longer-term storage, often to high levels, in specialized plastids termed amyloplasts. This storage starch is remobilized to support phases of growth (e.g., seedling establishment after germination; 39) or to meet locally high demand for carbon for specific processes (e.g., nectar secretion; 109). Starch also accumulates transiently in certain cell types at specific developmental stages. For example, it is often present in tissues behind the division zones of meristems, possibly reflecting temporary imbalances between carbon import and utilization as cells move from division to expansion and differentiation (68).

The harvested parts of our staple crop plants are starch-storing organs. Cereal seeds (rice, maize, wheat, barley, sorghum, and others) are the most important group, followed by tubers (e.g., potato, sweet potato, yam) and storage roots (e.g., cassava, taro), and seeds of beans and peas. Much of our agricultural land is devoted to their growth. Most of the estimated 2500 million tonnes of starch crops harvested annually (Food and Agriculture Organization of the United Nations, values for 2007; **http://faostat.fao.org**) is consumed directly as food or used as animal feed, but there is increasing demand from nonfood industries for starch as a renewable raw material. In particular, starch is a major feedstock for first-generation biofuels due to the relative ease with which it can be converted to fermentable sugars (128). Understanding the pathways by which starch is synthesized and degraded in plants will facilitate the improvement of starch crops for both

food and nonfood uses. It is already clear that studies of model plants alone will not provide adequate information for this purpose. There are differences between species, and between leaves, roots, and seeds, in the factors controlling starch metabolism, the structure of starch itself, and the pathways by which it is degraded.

Starch Structure

Amylopectin and amylose together form semicrystalline, insoluble granules with an internal lamellar structure (**Figure 1**) (11). Amylopectin is the major component, typically making up 75% or more of the starch granule. A large, branched molecule, amylopectin has an estimated molecular weight of between 10^7 and 10^9 Daltons (11). The glucosyl residues of amylopectin are linked by α-1,4-bonds to form chains of between 6 and >100 glucosyl residues in length. The α-1,4-linked chains are connected by α-1,6-bonds (branch points). Amylopectin is responsible for the granular nature of starch. Although its exact molecular

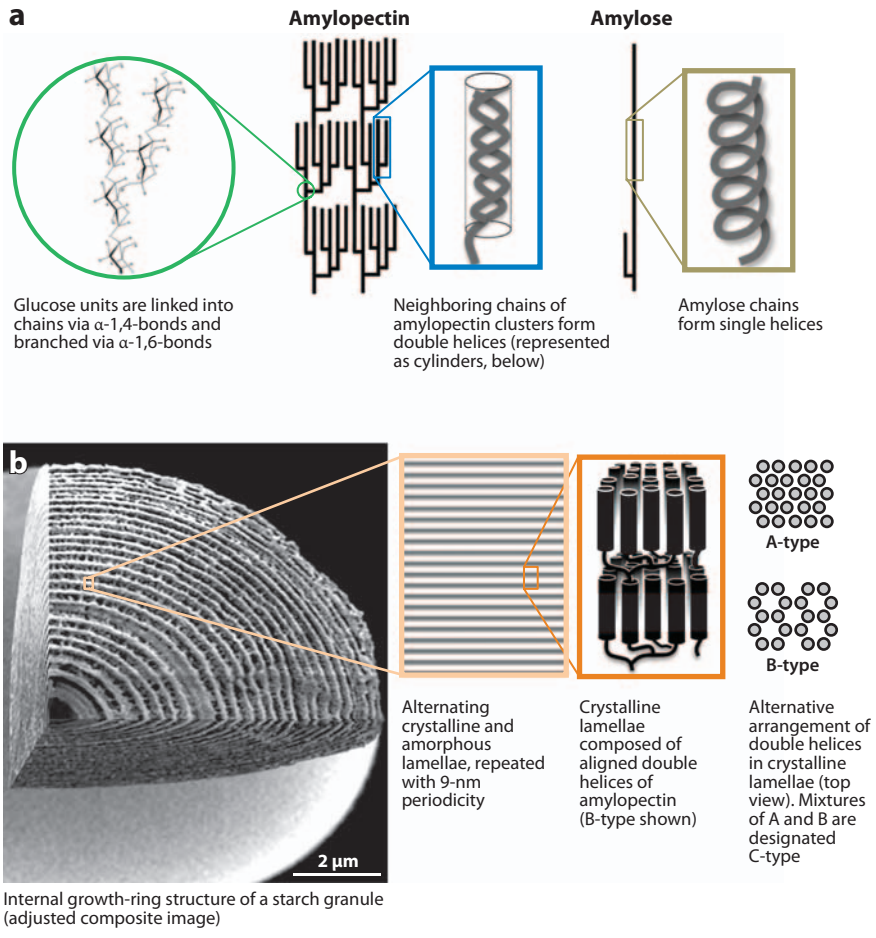

Figure 1

The composition and structure of starch granules. (*a*) A schematic representation of amylose and amylopectin, and the structures adopted by the constituent chains. (*b*) The relationship between the starch granule (composite image of potato granules, *left*) and amylopectin structure. Crystalline and amorphous lamellae arrange to form blocklets (*not shown; see text*) that make up the growth rings.

Crystalline lamellae: the alternating crystalline and amorphous lamellae of amylopectin have been likened to a side-chain, liquid-crystalline polymer (157)

architecture is not known, the combination of chain lengths, branching frequency, and branching pattern gives rise to a racemose, or treelike, structure in which clusters of chains occur at regular intervals along the axis of the molecule. Typically, chains within these clusters average between 12 and 15 glucosyl residues. The less abundant chains that span two clusters contain approximately 35–40 residues, while those that span three clusters contain approximately 70–80 residues (11, 59). Within the starch granule, amylopectin molecules are radially organized such that the free (nonreducing) ends of the chains point toward the periphery. Pairs of adjacent chains within clusters form double helices that pack together in organized arrays, giving rise to concentric, crystalline layers (lamellae) within the granule. These lamellae alternate with amorphous lamellae formed by the regions of the amylopectin molecule that contain the branch points. The lamellar organization is repeated with a 9- to 10-nm periodicity. This semicrystalline structure makes up the bulk of the matrix of the starch granule and is highly conserved in higher-plant starches (**Figure 1**) (65, 173). It seems possible that the organization of amylopectin to form the granule matrix is largely a physical process.

Higher-order structures also exist in starch granules (**Figure 1**). Most granules contain concentric "growth rings" that are visible by light microscopy and by scanning electron microscopy after etching the granule matrix with acids or hydrolytic enzymes (14, 104, 173). These rings have periodicities of several hundreds of nanometers. They are thought to reflect the organization of the alternating crystalline/amorphous lamellae into near-spherical blocklets, which vary periodically in diameter (between 20 and 500 nm) as the granule is laid down (49, 111). Based on electron optical tomography and cryo-electron diffraction experiments, it has also been proposed that 18-nm-wide, left-handed superhelices form from the double helices that comprise the crystalline lamellae (102). The relationship between these proposed structures and the blocklets has not been fully resolved.

Amylopectin is chemically similar to glycogen, the soluble polyglucan accumulated as a storage compound in animals, fungi, and bacteria. Glycogen is also a glucose polymer composed of α-1,4-linked, α-1,6-branched chains. However, glycogen has more branch points than amylopectin and these are arranged in a uniform rather than discontinuous pattern. Glycogen particles are limited in size (typically only 20–60 nm in diameter) (160) due to steric interference between branches as the diameter increases. Importantly, the branching pattern of glycogen does not allow the formation of the secondary and higher-order glucan structures that make up the matrix of the starch granule. The ability of plants to synthesize amylopectin has evolved from an ancestral capacity to make glycogen (see below and 5, 32, 103). Amylose, the second glucan component of starch, is smaller than amylopectin (relative molecular weight estimates vary between 10^5 and 10^6 Daltons) and only lightly branched (11). It is believed to exist primarily in an unorganized form within amorphous regions of the granule.

A significant number of enzymes involved in the metabolism of starch have been identified through studies of crop and model species. The proteins involved in starch metabolism in *Arabidopsis* have been tabulated previously (130). The subsequent sections describe the roles of these enzymes in the assembly and disassembly of the starch granule.

THE MECHANISM OF STARCH GRANULE BIOSYNTHESIS

Amylopectin Synthesis

The substrate for starch biosynthesis in higher plants is ADPglucose. The glucosyl moiety is transferred onto existing glucan chains by starch synthases (ADP-glucose: [1→4]-α-D-glucan 4-α-D-glucosyltransferase; EC: 2.4.1.21). Higher-plant starch synthases are encoded by five gene classes, designated GBSS (for granule-bound starch synthase), SSI, SSII, SSIII, and SSIV. Phylogenetic analyses separate the GBSS, SSI, and SSII classes from

the SSIII and SSIV classes (5, 81, 103). GBSS binds tightly to the starch granule and is responsible for amylose synthesis (see below). The other SS isoforms (often termed soluble SS) generate the chains in amylopectin and are either soluble in the plastid stroma, or part soluble and part associated with the granule. Genetic and biochemical data indicate that each SS isoform has different properties and a distinct role in amylopectin synthesis. Analysis of the distribution of chain lengths of amylopectin in mutant and transgenic plants lacking specific isoforms has led to the idea that the SSI, SSII, and SSIII classes preferentially elongate short, medium, and long chains, respectively (see 150 and references therein).

The branching of amylopectin proceeds concurrently with chain elongation (99). Branching is catalyzed by branching enzymes (BE; α-1,4-glucan: α-1,4-glucan-6-glycosyltransferase; EC: 2.4.1.18), which cut existing α-1,4-glucan chains and transfer the cut segment of six or more glucose units to the C6 position of a glucosyl residue of another (or the same) glucan chain. Higher-plant BEs fall into two classes, designated class I and class II (sometimes defined as B and A, respectively). Class I enzymes preferentially transfer longer chains than class II enzymes (see 150 and references therein). Genetic analysis shows that the two classes of BE make distinct contributions to the synthesis of amylopectin (150).

The evolution of multiple, specialized isoforms of SS and BE can be seen as a crucial factor in determining the architecture of amylopectin and therefore the capacity to synthesize starch rather than glycogen. However, the relative levels of SS and BE isoforms differ between starch-synthesizing organs. For example, in potato tubers SSIII accounts for about 80% of soluble starch synthase activity (90), in pea embryos about 60% of the activity is accounted for by SSII (30), and in maize endosperm about 60% is accounted for by SSI (16). These sorts of differences probably contribute to the observed variation in the structure of starches from different organs and species.

Debranching Enzyme Function in Amylopectin Synthesis

Besides SS and BEs, other glucan-modifying enzymes participate in the starch biosynthetic process. Debranching enzymes (DBEs; α-1,6-glucanohydrolase), which cleave branch points, are important determinants of the structure of amylopectin. This is evident from the dramatic changes in glucan structure and the form in which glucan accumulates in plants that lack specific DBE isoforms.

β-limit dextrin: a branched glucan digested with exo-acting β-amylase. External chains become stubs of two or three glucose residues. Internal chains are not degraded

Plants contain two types of DBEs—isoamylase (ISA; EC: 3.2.1.68) and limit-dextrinase (LDA, also called pullulanase; EC: 3.2.1.142)—distinguishable by their amino acid sequences and substrate specificities. The ISA type has three classes, designated as ISA1, ISA2, and ISA3 (61). ISA1 and ISA2 are strongly implicated in amylopectin synthesis. In potato tubers and *Arabidopsis* leaves, ISA1 forms a complex with ISA2 resulting in a heteromultimeric enzyme (26, 61, 162). In the endosperm of rice, and probably other cereals, ISA1 exists both as a homomultimer and as a heteromultimer with ISA2 (152). The primary role of LDA and ISA3 appears to be in starch degradation (see below). ISA1 is most active on glucan substrates with relatively long external chains, such as solubilized amylopectin, whereas LDA and ISA3 have high activities on glucans with short external chains (such as β-limit dextrins). ISA2 appears to be catalytically inactive (61). It probably modulates the action or stability of ISA1, rather than contributing directly to debranching (26, 162).

In mutant or transgenic plants deficient in ISA1, granular starch is reduced, or even absent, and is partly replaced by water-soluble glucans. This has been observed in developing cereal endosperms (12, 63, 97), green algae (24, 94), *Arabidopsis* leaves (26, 162), and potato tubers (13). In *Arabidopsis* and potato, loss of ISA2 has the same effect as loss of ISA1 (13, 26, 162, 178). The major soluble glucan is a glycogen-like polymer termed phytoglycogen. Phytoglycogen has a higher proportion of short chains (fewer than 10 glucosyl

residues) and more branch points than amylopectin. The average distance between branch points is less than for amylopectin. As for glycogen, it is presumed that the branching structure of phytoglycogen prevents the formation of the higher-order structures seen in starch granules. Phytoglycogen accumulation is interpreted to mean that "wrongly positioned" branch points introduced by BEs are normally hydrolyzed through selective debranching. This would liberate the misplaced branches as free oligosaccharides, leaving mature amylopectin capable of self-organizing to form the matrix of the starch granule (for earlier reviews, see 4, 95, 96, 176). If the misplaced branches remain, the glucan cannot self-organize to form a granule and instead accumulates as phytoglycogen.

α-amylase: an endoamylase that hydrolyzes α-1,4-bonds. Higher plants contain three classes: AMY1, AMY2, and AMY3 (134). AMY3 has a chloroplast transit peptide; AMY1 and AMY2 function in *Arabidopsis* is unknown

Other enzymes strongly influence whether glucans accumulate as insoluble granules or as soluble phytoglycogen in *isa1* mutants. Most *isa1* mutants still synthesize some starch granules, either alongside phytoglycogen accumulation in the same plastids (178), in different plastids in the same cell (12), or in different cell types of the same plant organ (26, 97). One explanation may be redundancy between DBEs, whereby debranching via ISA3 and/or LDA partially substitutes for the absence of ISA1. Maize and rice mutants with mild *isa1* alleles accumulate more phytoglycogen when LDA is also missing (35, 46), providing some support for this idea. However, the complement of other starch-synthesizing enzymes in the cell can also have a profound influence on the phenotype of *isa1* mutants. In maize, mutants that lack either GBSS or SSIII in addition to ISA1 (*wx su1* and *du su1* mutants, respectively) make 25–30% less phytoglycogen in developing endosperm than do mutants that lack only ISA1, and mutants that lack one branching enzyme isoform of the BEII class as well as ISA1 (*ae su1* mutants) make little or no phytoglycogen (3, 8).

Recent studies of *Arabidopsis* have provided new insight into the complexity of phytoglycogen accumulation. Mutants lacking all DBE activity (i.e., *isa1isa2isa3lda* quadruple mutants) do not have recognizable starch granules (136, 163). This result is superficially consistent with the idea that DBE activity is absolutely required for starch biosynthesis. However, further biochemical and genetic analyses reveal that the complete loss of starch in this mutant is the result of modification and turnover of branched glucans by starch-degrading enzymes rather than loss of DBE activity per se. Evidence for this is as follows. First, phytoglycogen extracted from the DBE-free mutant is rich in extremely short chains (fewer than six glucosyl residues) (136, 163). Such short chains are highly unlikely to be produced simply by the actions of SSs and BEs: Their presence is consistent with the action on a branched glucan of α-amylase (α-1,4-glucan glucanohydrolase; EC: 3.2.1.1) and β-amylases, enzymes that hydrolyze linear α-1,4-linked chains during normal starch degradation. Second, the DBE-free mutant accumulates small branched malto-oligosaccharides and maltose in addition to phytoglycogen. These are products of the actions of α- and β-amylases on branched glucans (136). Third, loss of the chloroplastic α-amylase, AMY3, from the DBE-free mutant partially restores starch granule biosynthesis. This implies that the branched glucan produced by SSs and BEs in the DBE-free mutant is actually capable of crystallizing to produce granules, but its partial hydrolysis by AMY3 destroys this property (136). These studies confirm that although DBEs are important in starch biosynthesis, they are not strictly necessary for starch granule formation to occur. Thus, ISA1 activity facilitates crystallization by removing wrongly positioned branches. In its absence crystallization is delayed, allowing the aberrantly branched glucans to be modified by enzymes that do not normally participate in starch biosynthesis (e.g., AMY3), such that they can no longer crystallize (136).

Protein Complex Formation Between Starch-Synthesizing Enzymes

There is increasing evidence for the occurrence in starch-storing organs of multienzyme

complexes comprising SSs, BEs, and other enzymes. The activities of the amylopectin biosynthetic enzymes must be interdependent because they act on a common glucan substrate. Physical association could serve to orchestrate the activities and thus allow the formation of specific glucan structures. Complexes containing both classes of BE (148) and complexes containing SSI, SSII, and class II BEs (147) have been found in wheat. Complexes containing combinations of SSII, SSIII, and class II BEs have been found in maize (57, 58). No complexes containing DBEs have been reported.

The stability of SS/BE enzyme complexes is dependent on the phosphorylation of some or all of the constituent proteins (147). Dephosphorylation in vitro by a nonspecific phosphatase resulted in the dissociation of the complexes (57, 147, 148) and also in the inactivation of a wheat BEII isoform (148). The protein kinases and phosphatases responsible for phosphorylation and dephosphorylation of the starch-synthesizing enzymes in vivo, the extent to which the enzymes are present as complexes, and the number of distinct types of complex remain to be established. The functional significance of complex formation is currently unclear. There is indirect in vitro evidence that enzymes in complexes have a higher affinity for their glucan substrates than the same enzymes as monomers (147), but there is no direct evidence that complex formation influences either the rate of starch accumulation or the structure of glucans synthesized in vivo. It is also not known whether protein complex formation is a general feature in starch biosynthesis. Complexes similar to those in the developing wheat endosperm were not found in starch-synthesizing wheat leaves (148).

Additional enzymes are present in protein complexes containing SS and BE in cereal endosperm (57, 148). These include subunits of ADP-glucose pyrophosphorylase, sucrose synthase, pyruvate, phosphate dikinase, and α-glucan phosphorylase. The discovery of α-glucan phosphorylase in a complex is interesting given new information about a possible role for this enzyme in starch biosynthesis (see below).

Despite the progress in identifying the enzymes of amylopectin synthesis, it is frustrating that there is still no detailed understanding of exactly what glucan structure SSs and BEs produce, how DBEs selectively debranch this nascent glucan, and how the actions of these three enzymes are coordinated to give rise to an amylopectin molecule that self-organizes to form a starch granule. In part, this problem arises from the technical difficulties associated with the determination of amylopectin structure and the isolation of nascent glucans from starch-synthesizing plastids (see 176 and the discussion therein). Its solution will require concerted efforts in the development of new analytical methods and molecular modeling techniques.

α-glucan phosphorylase: catalyzes reversible phosphorolysis at the nonreducing end of glucan chains, consuming orthophosphate and releasing glucose-1-phosphate (Glc-1-P). Higher plants contain plastidial and cytosolic classes (PHS1, PHS2 in *Arabidopsis*, respectively; 177). Cytosolic phosphorylase is probably involved in heteroglycan metabolism (38)

Amylose Synthesis

The amylose component of starch is synthesized by GBSS. Mutants and transgenic plants lacking this enzyme are essentially amylose free (see 29 and references therein). Amylose-free starch granules are normal in appearance, illustrating that only amylopectin is necessary for granule formation. GBSS differs from the other SS isoforms in its exclusive localization to the granule and in its mode of action. Unlike other starch synthase isoforms, GBSS transfers glucosyl residues from ADP-glucose to its glucan substrate processively, generating long chains (31). This occurs within the semicrystalline matrix formed by amylopectin (146), explaining why the newly formed amylose chains are not acted upon by stromal BEs. GBSS can use soluble malto-oligosaccharides as substrates for amylose production (28, 174). It can also act on the existing side chains of amylopectin (154) and contribute to the formation of long chains in amylopectin (47, 53, 161). It has been proposed that, in evolutionary terms, this may have been its original function (107). Amylose synthesis may render starch denser and improve the efficiency of carbon storage, explaining the conservation of GBSS in higher plants.

The Initiation and Control of Granule Formation

The initiation of starch granules and the mechanisms that determine granule size, number, and morphology are still not well understood. These factors are under genetic control and are highly species and organ dependent (64). In higher plants, there are usually multiple starch granules per plastid. Amyloplasts in heterotrophic tissues can contain either a few large granules (e.g., in potato tuber) or many small granules (e.g., in rice endosperm) that may fuse to form compound granules (e.g., in oat endosperm). In wheat endosperm, two successive rounds of granule initiation result in large lenticular A granules, initiated early in development, and small spherical B granules initiated late in development (124).

In mammals and in yeast, glycogen molecules are primed by the protein glycogenin, which glucosylates one of its own tyrosine residues (self-glucosylation) to create a chain of a few glucose residues (85). The chain is extended and elaborated by glycogen synthases (GSs) and glycogen branching enzymes. Glycogenin-like proteins are found in plants (19), as are other reversibly glucosylated proteins (RGPs; 127). Only one report has provided evidence of a role for these proteins in starch granule initiation; *Arabidopsis* plants in which expression of a glycogenin-like protein was repressed by RNAi had reduced starch, as judged by iodine staining (19). This observation was not substantiated with quantitative measurements or microscopic investigations. Studies of RGPs have concluded that they are cell-wall proteins preferentially localized to plasmodesmata, rather than being involved in starch metabolism (118).

Recent genetic evidence suggests that the SSIV class of starch synthase may have a role in granule initiation. *Arabidopsis* leaf chloroplasts contain about five starch granules. However, *Arabidopsis ssiv* mutants have just one large granule in each chloroplast (114). SSIV proteins differ from the other SS isoforms in possessing an N-terminal extension with a pair of coiled-coil domains and a putative 14–3-3 protein binding site (81). It is possible that features of the N-terminal extension enable SSIV to interact with other proteins and thus contribute to granule initiation. In the absence of SSIV, SSIII (the glucosyl transferase domain of which is most closely related to SSIV) seems to be responsible for the initiation of the single granule per chloroplast: Plants lacking both SSIV and SSIII lack starch in their leaves despite having 60% of the wild-type soluble SS activity (accounted for by the remaining SS isoforms; 140). SSIII also possesses a conserved 14–3-3 binding site (at its C-terminus; 123), and it can interact with other starch synthase and branching enzymes in a phosphorylation-dependent manner (see above). In *Agrobacterium tumefaciens*, GS itself has been proposed to prime glycogen synthesis through the formation of a glucan-GS intermediate (151). No SSs have been shown to have such a self-priming activity, although Szydlowski et al. (140) report that SSIII is able to synthesize glucans from ADP-glucose in a primer-independent way. Further work will be required to determine the precise roles of SSIV and SSIII in granule initiation.

Recently there has been renewed interest in the potential biosynthetic role of the plastidial α-glucan phosphorylase (E.C. 2.4.1.1), which can extend glucan chains using glucose-1-phosphate (Glc-1-P) as a substrate (releasing orthophosphate). This reaction is reversible and it has been assumed that the enzyme probably acts in the phosphorolytic direction (releasing Glc-1-P from glucan chains) due to the relatively high ratio of orthophosphate to Glc-1-P in plant cells (77). However, genetic studies in different species have yielded mixed results. Gene silencing in potato and mutation in *Arabidopsis* does not alter starch levels. In contrast, mutants of *Chlamydomonas* and of rice have conditional altered starch phenotypes (23, 119). *Chlamydomonas* has two plastidial phosphorylases (PhoA and PhoB), which have different properties. The *sta4* mutant lacks the PhoB isoform and has decreased starch contents under nitrogen-limited growth conditions (in which

starch normally accumulates to high levels; 23). In the rice mutant (lacking the single plastidial isoform, PHO1), starch content is normal when the seeds develop at 30°C but is decreased relative to controls at 20°C (119). The explanations offered for these phenotypes differ. Dauvillée et al. (23) observed that PhoB is very strongly inhibited by ADP-glucose so could well be inactive during starch biosynthesis. Accordingly, they proposed that the effect of loss of PhoB on starch accumulation was indirect, perhaps relating to potential interactions of the protein with other starch biosynthetic enzymes (e.g., with BEs, as in wheat endosperm; 148). In contrast, Satoh et al. (119) proposed that the rice endosperm PHO1 may synthesize linear glucans that serve as primers for new amylopectin and amylose molecules. To explain the temperature dependency of the mutant phenotype, they suggested that other factors must also be involved (potentially SS isoforms; see above). It is plausible that the role of phosphorylase differs between species and tissues, and further investigations of phosphorylase function are necessary.

Granule numbers are increased in ISA1-deficient plants of several species (12, 13, 178) and different explanations have been proposed for this phenomenon (12, 13). In *Arabidopsis*, a likely explanation is that small, soluble glucans resulting from the amylolytic turnover of aberrant glucans formed in the absence of ISA1 provide primers for granule initiation: Thus the effect of loss of ISA1 on granule number is probably indirect (136). In general, there is little reason to suppose that ISA1 normally controls granule number.

An intriguing study of *Ostreococcus tauri*, a single-celled green alga, resulted in the proposal that starch granules multiply by division in this species (108). *O. tauri* cells have a single chloroplast that contains one starch granule. Electron micrograph images suggest that during cell division the granule divides, with half retained in the chloroplast of each daughter cell. If this is indeed what happens, our current understanding of starch structure and metabolism is clearly insufficient to explain how a starch granule can be split in this way.

STARCH DEGRADATION

There has been rapid progress recently in understanding starch degradation (reviewed in 38, 82, 130, 171, 175, 176). It is clear that pathways of degradation differ between plant organs, and that distinct pathways may operate within the same organ. Starch degradation is best understood in leaves, where transitory starch is degraded at night, and in cereal endosperms where storage starch is broken down over several days after seed germination. The pathways in these two organs share some components but also have major differences. Our understanding of the pathway in other starch-containing tissues such as tubers, roots, and noncereal seeds is more fragmentary (130).

The Pathways of Starch Degradation in Chloroplasts

Transitory starch in leaves is degraded primarily by hydrolysis of the constituent glucans to maltose and glucose, both of which can be exported from the chloroplast and metabolized in the cytosol (**Figure 2**) (101, 164, 165). Mutations affecting key degradative enzymes decrease starch breakdown, resulting in the accumulation of starch over repeated diurnal cycles (so-called starch-excess or *sex* phenotypes; 15). There is good evidence that starch degradation is dependent on the reversible phosphorylation of glucans at the surface of the starch granule, which serves to solubilize the granule surface, thus allowing hydrolases access to the glucan chains.

Hydrolysis of the linear chains is catalyzed primarily by β-amylases (α-1,4-glucan maltohydrolase; EC: 3.2.1.2), exo-acting enzymes that release maltose from exposed nonreducing ends of chains. β-Amylases cannot hydrolyze α-1,6-branch points or act immediately adjacent to them. Thus, the complete degradation of amylopectin also requires hydrolysis of

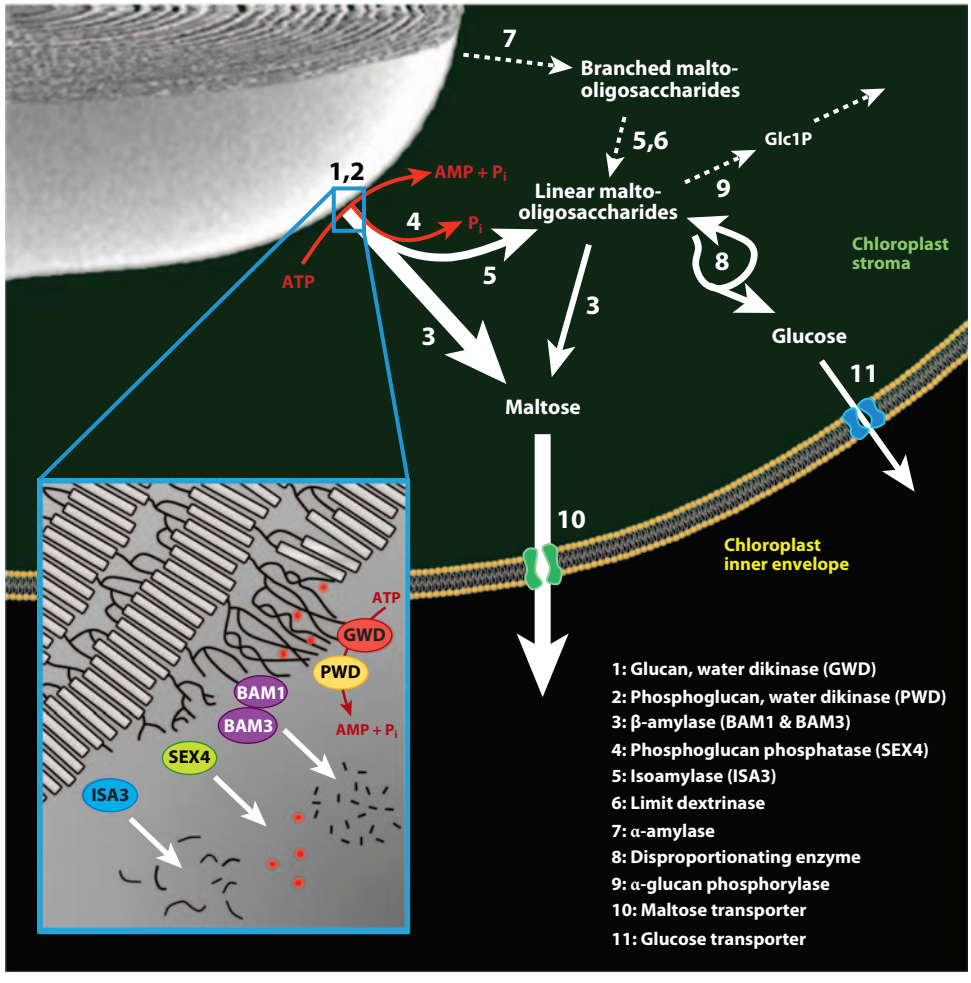

Figure 2

The pathway of starch degradation in chloroplasts and the role of transient glucan phosphorylation. Maltose and malto-oligosaccharides are released from the surface of the starch granule during degradation. Malto-oligosaccharides are metabolized in the stroma. Maltose and glucose are exported to the cytosol. Estimated fluxes are indicated by relative arrow size. Dashed arrows represent the minor steps in *Arabidopsis* (*see text*). Inset is a model depicting the role of phosphorylation by GWD and PWD in disrupting the packing of amylopectin double helices (*gray boxes*). This allows the release of maltose and malto-oligosaccharides (*black lines*) by β-amylases (BAMs) and DBE (ISA3). Phosphate (*red dots*) is concomitantly released by SEX4 to allow complete degradation.

branch points by DBE activity. Higher-plant genomes encode large numbers of β-amylase-like proteins. *Arabidopsis*, for example, has nine β-amylase genes (*BAM1–9*). The β-amylase family can be divided into four distinct classes and higher plants contain one or more genes of each class (48). The importance of chloroplastic β-amylase activity for transitory starch degradation was established by experiments with transgenic potatoes, in which repression of a chloroplastic isoform led to a *sex* phenotype (120). This finding has been confirmed and extended in *Arabidopsis* (48, 70). Four of the nine *Arabidopsis* BAM proteins (BAMs 1–4) have been shown to be chloroplastic (48). BAM1 and BAM3 are active enzymes and partially

redundant: *bam1* and *bam3* mutants have a wild-type and a mild *sex* phenotype, respectively, but the *bam1bam3* double mutant has a strong *sex* phenotype (48, 70). No function has yet been ascribed to BAM2. The recombinant BAM2 protein has a very low specific activity compared with BAM1 and BAM3, and *bam2* mutants are indistinguishable from wild-type plants (48).

BAM4 is unusual because its putative active site has multiple substitutions of amino acid residues that are strictly conserved in other, active β-amylases, and the recombinant protein does not have β-amylase activity (48). Other members of this class of BAMs, including *Arabidopsis* BAM9 and proteins from other species have similar substitutions in their putative active sites (48), and recombinant BAM9 has no enzymatic activity (S. K. Lee and S. C. Zeeman, unpublished data). Although BAM4 is apparently noncatalytic, the *bam4* mutant has a *sex* phenotype, indicating that it is required for starch degradation. Genetic analysis shows that BAM4 does not act simply by modulating the activity of the active β-amylases, as its loss in the *bam1bam3* double mutant background enhances the *sex* phenotype (48). One possible explanation for the requirement for BAM4 is that it senses maltose levels and transduces this information to modulate the starch degradation rate by interaction with other starch-degrading enzymes. Further work is needed to test this hypothesis.

Several isoforms of BAM lack discernable plastid transit peptides. If they are indeed outside the plastid, they are unlikely to be involved in starch degradation. A high proportion of the β-amylase activity in *Arabidopsis* leaves is contributed by one such isoform, BAM5 (also called RAM1), which has been shown to be present within phloem sieve elements (158). Studies of the *bam5* mutant failed to discern a starch-related phenotype (78) and its function remains unclear.

DBEs release short, linear malto-oligosaccharides into the plastid stroma (27, 171). Two DBEs (ISA3 and LDA) function in starch degradation in *Arabidopsis* leaves. Both enzymes preferentially remove short branches and have their highest activity on β-limit dextrins. They differ in the range of branch point configurations that they are able to degrade, as illustrated by the ability of LDA-type, but not ISA-type, to degrade the fungal glucan pullulan (27, 61, 143, 168). There is functional overlap between ISA3 and LDA. Loss of LDA alone has no effect on starch metabolism in *Arabidopsis* leaves, whereas loss of ISA3 leads to a *sex* phenotype (27, 162). Loss of both enzymes together (the *isa3lda* double mutant), however, results in a phenotype more severe than that of *isa3* (27). The relative importance of ISA3 and LDA in leaf starch degradation may differ between species. Unlike *Arabidopsis*, maize mutants lacking LDA alone (*Zmpu1*mutants) have reduced rates of starch degradation in leaves at night (35).

The shortest malto-oligosaccharide on which β-amylase can act is maltotetraose. Maltotriose formed during the degradation of starch is metabolized by disproportionating enzyme (D-enzyme; α-1,4-glucan 4-α-glucanotransferase; EC: 2.4.1.25), which transfers a maltosyl group from maltotriose to another glucan, generating glucose and a longer glucan that can be further degraded by β-amylase. This activity is necessary for normal starch degradation in *Arabidopsis* leaves: In mutants lacking D-enzyme (*dpe1* mutants: 22), the rate of starch degradation is reduced and maltotriose accumulates in leaves at night. Two additional enzymes, plastidial α-amylase and plastidial α-glucan phosphorylase (see above), are widely conserved in plants and may participate in starch degradation. In *Arabidopsis*, mutations resulting in loss of activity (the *amy3* and *phs1* mutants, respectively) do not result in *sex* phenotypes under normal laboratory conditions (170, 177). However, AMY3 does participate in starch degradation in *Arabidopsis* plants in which other starch-degrading enzymes are missing. For example, in mutants lacking the two DBEs that participate in starch degradation (the *isa3lda* double mutant), AMY3 is induced and is responsible for releasing branched malto-oligosaccharides from the starch granule surface (27; S. Streb and S. C. Zeeman, unpublished data). The importance of chloroplastic

Linear malto-oligosaccharides: named using the number of glucosyl residues; maltotriose, maltotetraose, and maltopentaose contain three, four, and five glucosyl residues, respectively

Pullulan: a glucan polymer produced by the fungus *Aureobasidium pullulans* and consisting of maltotriose units linked end to end by α-1,6-bonds. It is not present in plants

α-amylase in leaf starch degradation appears to differ between species. Rice plants with reduced activity of chloroplastic α-amylase have a *sex* phenotype (2).

Reversible Glucan Phosphorylation

Two classes of enzyme bind to starch and modulate the phosphate content of amylopectin. These are glucan, water dikinases, which add phosphate groups using the β-phosphate of ATP, and phosphoglucan phosphatases, which remove these phosphate groups. Glucan, water dikinase (GWD; ATP: α-1,4-glucan, water phosphotransferase; EC: 2.7.9.4, initially designated R1) was discovered as a starch granule–binding protein in potato tubers (88). In transgenic potatoes deficient in GWD, fewer than 1 in 10,000 glucosyl residues of leaf starch amylopectin have a phosphate group, compared with ~1 in 2000 in control plants. Furthermore, starch accumulates to very high levels in leaves and cold-induced starch degradation in tubers (leading to cold-sweetening; see below) is prevented (88). Similarly, loss of GWD in *Arabidopsis* (the *sex1* mutant) leads to a very severe *sex* phenotype (169). Subsequent work established that GWD phosphorylates the C6 position of glucosyl residues in amylopectin (112, 113). A second glucan, water dikinase, PWD (for *p*hosphoglucan, *w*ater *d*ikinase, ATP: phospho-α-1,4-glucan, water phosphotransferase; EC: 2.7.9.5), phosphorylates the C3 position of glucosyl residues (7, 74) and is also required for normal starch breakdown: *Arabidopsis pwd* mutants have a mild *sex* phenotype (7, 74). The action of PWD on amylopectin requires the prior action of GWD (hence its name). This suggests that PWD activity is dependent on the presence of the C6 phosphate group added by GWD, or the change in glucan structure caused by the C6 phosphate (56). *Arabidopsis* also contains an extraplastidial glucan, water dikinase (GWD2), expressed primarily in the vasculature. Mutant analysis shows that this is not required for leaf starch metabolism and its function is unknown (52).

The impact of phosphorylation on starch degradation has been studied using stable suspensions of insoluble, crystalline malto-oligosaccharides as models for starch granules (54, 56). GWD has a high affinity for crystalline malto-oligosaccharides, and phosphorylation results in extensive solubilization of the constituent oligosaccharide chains. These experiments support the idea that phosphorylation in vivo serves to disrupt the crystalline structure of amylopectin (**Figure 2**) (113, 169). The importance of this disruption for starch degradation was shown by studies of the activities of recombinant β-amylases and DBEs (BAM1 and ISA3; see above) on starch granules isolated from leaves. The rate of degradation of phosphate-free starch granules (isolated from *sex1* mutants) by these enzymes was significantly increased by the simultaneous phosphorylation of the granule surface, brought about by the addition of recombinant GWD and ATP (36).

Recent research shows that removal of the phosphate groups added by GWD and PWD is necessary for complete starch degradation. The loss from *Arabidopsis* leaves of a glucan-binding phosphatase encoded at the *SEX4* locus (also designated as PTPKIS1 and DSP4; 42, 71, 100, 132) decreases the rate of starch degradation (100, 172) and leads to the accumulation during the night of soluble phospho-oligosaccharides (75). Recombinant SEX4 can dephosphorylate glucans, including semicrystalline amylopectin (51, 75), acting on phosphate groups at either the C6- or the C3-positions (55). The need for dephosphorylation during starch degradation probably relates to the substrate requirements of β-amylases. These enzymes cannot act on a glucan chain carrying a phosphate group close to the nonreducing end (75, 144). Thus, having served to disrupt the granule surface, phosphate groups must be removed to allow full degradation of the glucan chains. In the absence of SEX4, some β-amylolysis still occurs and the phosphorylated starch granule can also be attacked by DBEs and α-amylase—giving rise to the observed build-up of phospho-oligosaccharides in the *sex4* mutant—but

complete degradation to maltose and glucose is not possible. Further in vitro analyses support these ideas: The rate of starch degradation by β-amylase and DBE is enhanced by the addition of GWD and ATP (see above) and is further enhanced when SEX4 is also added (75).

Arabidopsis chloroplasts contain a second glucan phosphatase–like protein, LSF1 (*l*ike *S*EX *F*OUR1), which is required for normal starch degradation. *lsf1* mutants have a *sex* phenotype and reduced rates of starch degradation, although the accumulation of starch is not as pronounced as in *sex4* mutants. Genetic analysis suggests that SEX4 and LSF1 have distinct rather than mutually redundant roles, but the substrate for LSF1 in vivo has yet to be established (21).

Based on the research discussed above, the current model for starch degradation in leaves involves transient amylopectin phosphorylation proceeding concurrently with glucan hydrolysis (**Figure 2**) (36, 75). Each crystalline lamella of amylopectin is structurally disrupted via phosphorylation, allowing progressive degradation and dephosphorylation by the hydrolytic enzymes and phosphoglucan phosphatases, respectively. As the next crystalline lamella is exposed, the process of disruption begins again (**Figure 2**). The development of this model has coincided with a renewed interest in the phosphorylation of glycogen in mammals. Glycogen, like amylopectin, contains covalently linked phosphate (41, 86). The mechanism of glycogen phosphorylation is not known, but recent research shows that dephosphorylation is carried out by a glycogen phosphatase (laforin) related in sequence to SEX4 (142, 166). Mutations abolishing laforin function result in the accumulation of aberrant glycogen that aggregates to form insoluble polyglucosan bodies (Lafora bodies). Lafora bodies have been described as starchlike due to their dark staining when treated with iodine solution, suggestive of a branching architecture different from that of glycogen (18). The accumulation of Lafora bodies in neurons is proposed to be the underlying cause of the neurodegenerative condition Lafora disease (18). As in plants, the function of glucan phosphorylation may be to promote or maintain glucan accessibility for hydrolysis, with a requirement for the removal of phosphate groups for complete degradation. If this is the case, loss of laforin could affect both turnover and structure of glucans, as in the *sex4* mutant of *Arabidopsis* (51, 141). However, laforin also appears to function in the turnover of glycogen-metabolizing enzymes through interaction with a ubiquitin ligase called malin (155, 167). Much remains to be elucidated about laforin function and the significance of glycogen phosphorylation.

Differences in Starch Degradation Between Leaves and Cereal Endosperm

Starch degradation in cereal endosperm occurs in a radically different physical setting from the process in the leaf mesophyll cell. At the time of germination, the starchy endosperm is dead: Cellular integrity is lost during the drying-out phase of seed development. The tissue consists largely of cell walls, starch granules, and storage proteins. The product of starch degradation is primarily glucose, which is taken up by living cells in the embryo adjacent to the endosperm (the scutellum). The enzymes that degrade starch are either released into the endosperm from the scutellum or the aleurone (a layer of living cells that surrounds the starchy endosperm) or in the case of β-amylase, laid down within the endosperm during seed development and converted to active forms during germination by the action of proteases released from the aleurone.

Biochemical studies established the presence in the endosperm of four starch-degrading enzymes, α-amylase, β-amylase, DBE (specifically LDA), and α-glucosidase (maltase; EC: 3.2.1.3), and their properties and structure-function relationships have been studied in detail (e.g., 10, 40, 76, 91). However, relatively little is known about the importance of each of the enzymes in the process of starch degradation, in part because of the difficulty of manipulating

their levels independently and in a controlled manner.

It is generally accepted that α-amylase plays a central role in endosperm starch degradation. Upon germination, there is massive synthesis of α-amylase in the aleurone and scutellum, followed by secretion into the endosperm (39). There it initiates the attack on the starch granule, probably at specific sites (pores) on the granule surface, resulting in a visible pitting effect. Its endoamylolytic action releases a mixture of soluble linear and branched oligosaccharides. In barley, members of two classes of α-amylase genes (*Hv*AMY1 and *Hv*AMY2) are expressed during germination. The proteins encoded by both families are more closely related to the extraplastidial AMY1 of *Arabidopsis* than to the chloroplastic AMY3 (134, 170). The properties of *Hv*AMY1 and *Hv*AMY2 differ, and it seems likely that they play distinct roles in starch degradation (6). *Hv*AMY2 has a strong affinity for starch as a substrate, while *Hv*AMY1 has a higher affinity for linear malto-oligosaccharides (131). Repression of an AMY1-type α-amylase in the rice endosperm delays seedling establishment (2), confirming its importance in starch mobilization.

The genes encoding the enzymes of transient glucan phosphorylation (i.e., GWD, PWD, and the glucan phosphatases) are conserved in cereal species and are probably involved in the degradation of starch inside plastids. However, there is no evidence that transient glucan phosphorylation occurs in the endosperm of germinated seeds. In fact, cereal seed starches have very low levels of covalently linked phosphate (9), and it is unlikely that there would be sufficient ATP in the endosperm of germinated seeds to sustain the repeated cycles of phosphorylation proposed to occur during degradation of leaf starch. The initial stage of starch degradation in cereal endosperm thus differs markedly from that in chloroplasts.

Linear and branched glucans released from starch by α-amylase can be degraded further by α-amylase, DBE and β-amylase to yield maltose and short malto-oligosaccharides. Unlike the situation in chloroplasts, β-amylase seems to be at least partly redundant in this process. Some cultivars of barley originating from Tibet almost completely lack endosperm β-amylase, yet seedlings grow normally (69). Maltose and short malto-oligosaccharides produced in the endosperm are degraded to glucose by α-glucosidase (maltase). There is evidence that α-glucosidase may also act synergistically with α-amylase to remove glucose directly from the granule surface (137). This contrasts markedly with the fate of starch degradation products in leaves. In *Arabidopsis*, maltose produced by β-amylase in the chloroplast is exported to the cytosol (101, 165), where it is metabolized by a transglucosidase (DPE2; 20, 89). A similar transglucosidase is necessary for cytosolic maltose metabolism in potato leaves (82). The metabolism of maltose in the leaf cytosol has been reviewed recently (38). There is currently no evidence for the involvement of an α-glucosidase in maltose or malto-oligosaccharide metabolism in *Arabidopsis* leaves.

Archaeplastida (kingdom Plantae): the three lineages are Chloroplastida (green plants and algae), Rhodophyceae (red algae), and Glaucophyta (92)

EVOLUTION OF STARCH BIOSYNTHESIS

Starch biosynthesis is unique to plants (defined as the Archaeplastida) and evolved from the ancestral ability to make glycogen. The recent expansion of genome sequence information has allowed comparative analysis of different starch- and glycogen-synthesizing organisms from the archaeplastidal lineages. Together with cytological and biochemical studies, this information has shed new light on the possible evolutionary steps leading to the starch biosynthetic machinery that exists in higher plants today.

The Archaeplastida are generally considered to be monophyletic; that is, all members are descended from a single ancestor in which a primary endosymbiotic event occurred [entailing the uptake of a cyanobacterial cell (the symbiont) by a nonphotosynthetic eukaryotic cell (the host); 92]. Most modern cyanobacteria synthesize glycogen, as do nonplant eukaryotes. However, the recent identification

of modern cyanobacterial species that make polymers more like starch (designated as either semiamylopectin or cyanobacterial starch) means that the primary endosymbiont itself may have been able to synthesize a starchlike polymer (32, 98, 125, 126). Differences in the starch biosynthetic pathways between the archaeplastidal lineages have arisen during subsequent evolution. Most notably, in green plants starch is synthesized in the plastid compartment, whereas in red algae and in glaucophytes it is synthesized in the cytosol. Interestingly, some rhodophyte species appear to have reverted to synthesizing glycogen (126).

Analyses of the phylogeny of the protein sequences of starch metabolic enzymes have revealed a mixture of host- and symbiont-derived genes in each branch of the Archaeplastida (32–34, 103). In green plants, the ADP-glucose-utilizing soluble and granule-bound starch synthases are derived from the symbiont. Red algae and glaucophytes contain both UDP-glucose-utilizing starch synthases derived from the host and GBSS-like proteins derived from the symbiont. The ancestry of other starch metabolic enzymes is also a mosaic; in all cases, branching enzymes, phosphorylase, and β-amylases are derived from the ancestral host, whereas DPE1 and isoamylase are proposed to be symbiont derived (32, 103). The sequence of events that resulted in cytosolic starch biosynthesis in some Archaeplastida and plastidial starch biosynthesis in others remains the subject of speculation (32, 103).

Components of the starch biosynthetic machinery that are found in all starch-synthesizing organisms are likely to have made an important contribution at some stage in the evolution of glucan polymers able to form starch granules. For example, GBSS-like proteins are present in all starch-synthesizing lineages examined thus far. Even though GBSS is not essential for amylopectin synthesis in higher plants today, its capacity to produce long glucan chains may have been an important factor in the evolutionary transition to the synthesis of amylopectin-like rather than glycogen-like polymers (107). The subsequent acquisition of other starch synthase isoforms in the green plant lineage may have rendered this original function of GBSS redundant, and amylose synthesis may be a secondary function. Isoamylases are also present in all starch-synthesizing organisms. Their original function was probably in glucan degradation (as is the case in glycogen-synthesizing bacteria; 25, 138). Their recruitment to glucan synthesis is likely to have been an important step toward the synthesis of glucan polymers able to form starch granules. This step may have been facilitated by gene duplication events that allowed the evolution of multiple isoforms with distinct substrate specificities. Further insight into the evolution of starch metabolism from ancestral glycogen metabolism will be facilitated by the recent development of model organisms from the different branches of the Archaeplastida (33, 105).

BIOTECHNOLOGICAL MODIFICATION OF STARCH METABOLISM

Starch is the major carbohydrate of nutritional importance in the human diet and is also an important industrial material. Starch for industrial purposes is extracted predominantly from corn, but significant amounts are also extracted from a range of other species, including rice, wheat, cassava, potato, arrowroot (*Maranta arundinacea*), and sago palm (*Metroxylon sagu*). Starches from different botanical sources have different polymer compositions and structures and, hence, different physicochemical properties (gelatinization temperature, viscosity of cooked pastes and gels, etc.). These properties—referred to as the functionality of the starch—determine the range of applications for which a given starch is used. Starch content is also an important consideration in crops used as forage and during the storage of harvested crop organs (e.g., fruits, in which ripening involves the conversion of starch to sugars, and potato tubers, in which conversion of starch to sugars leads to deterioration).

The modification of starch metabolism in crops could be beneficial to increase starch

accumulation in harvested organs, to prevent or increase starch degradation (depending on the crop and/or use), or to modify starch structure to enhance or diversify its functionality in food and as an industrial material. The latter is of particular interest, as specialty applications of starch often require it to be chemically or physically modified after extraction to achieve optimum functionality. Given the large number of enzymes that are involved in determining starch structure in a specific plant organ, the potential number of different starches that could be bioengineered by up- or downregulation of the respective genes is enormous. In diploid species that are sexually propagated, breeding, potentially combined with TILLING approaches, is the best method to introduce combinations of mutations affecting the genes that encode enzymes of starch metabolism. Biotechnological methods may be preferable or required if the target crop is polyploid and/or asexually propagated, if the target gene needs to be upregulated or only partially downregulated, or if the desired trait results from expression of a gene from another species.

TILLING: targetted induced local lesions in genomes; a method for the identification of chemically induced mutations in specific genes of interest

AGPase: in plants, a heterotetramer comprising two large and two small subunits, evolved from a common ancestor protein. In *Escherichia coli*, AGPase is a homotetramer of the GlgC protein

Biotechnological Approaches to Increase Starch Biosynthesis

Most attempts to enhance starch accumulation have focused on engineering ADP-glucose pyrophosphorylase (AGPase) activity in plants. This enzyme provides the substrate for SSs and is subject to tight allosteric regulation. Therefore a mutant variant of the AGPase gene from *Escherichia coli* (*glgC16*), encoding a differently regulated form of the enzyme, has been widely used for overexpression in plants. When *glgC16* was targeted to plastids in transgenic potatoes, some of the lines had up to 60% more starch in tubers on a fresh-weight basis than control plants (135). However, in experiments with a different variety of potato (139), as well as in cassava (62), maize (159), and other species, increases of this magnitude have not been seen. Therefore, manipulating AGPase alone may not be the most promising strategy for increasing starch contents in plant storage organs (128).

There is good evidence that increasing the supply of ATP to the plastid can stimulate the production of ADP-glucose and hence the rate of starch biosynthesis. First, overexpression of a plastidial envelope adenylate translocator from *Arabidopsis* in potato increased ADP-glucose levels twofold and increased starch content by 16–36% compared with control tubers (50, 149). Second, the downregulation of a plastidial adenylate kinase, an enzyme that interconverts two molecules of ADP into ATP and AMP, resulted in a tenfold increase in ADP-glucose levels and a doubling of the starch content in potato tubers, in both greenhouse and field trials (110). It is possible that the downregulation of adenylate kinase leads to enhanced transport of ATP into the plastids in exchange for ADP, which is released from ADP-glucose upon glucan polymerization.

Manipulation of Starch Breakdown to Enhance Crop Quality

An alternative approach to enhancing starch content in crops in which starch turnover occurs is to reduce the rate of starch degradation. As discussed above, the addition of phosphate groups to amylopectin by GWD is necessary for starch degradation. To enhance the proportion of starch—an easily digestible carbohydrate—in fodder crops and products, the corresponding GWD has been downregulated in transgenic clover (*Trifolium repens*), alfalfa (*Medicago sativa*), and ryegrass (*Lolium perenne*; 45), as well as in maize varieties used for silage production (44). In all of these cases, the downregulation of GWD led to substantial increases of the starch content in leaves, which ultimately should lead to enhanced digestibility of the fodder crops. Downregulation of GWD to prevent starch breakdown has also been exploited to enhance the quality of potato tubers. Potato tubers degrade their starch during cold storage and accumulate the reducing sugars fructose and glucose (cold sweetening). The presence of reducing sugars allows the Maillard reaction

to occur during frying, leading to an undesirable dark coloring of the fried products and to the production of toxic acrylamide (93). Downregulating GWD in potato tubers inhibits cold sweetening (88) and reduces acrylamide production during frying (115). Surprisingly, the enzymes responsible for starch degradation in potato tubers during cold storage remain unknown. Their identification will be assisted by the recent progress in *Arabidopsis* and will provide additional options for the inhibition of cold sweetening.

An increased capacity for starch degradation is of potential value in starch crops grown for bioethanol production. Expression in corn (*Zea mays*) of a thermostable α-amylase (80) has been used to create so-called self-processing plants that improve the economic viability of conversion of starch to ethanol. The amylase is expressed in subcellular compartments that do not participate in starch biosynthesis or storage, so its presence does not affect starch accumulation during growth of the crop. When milled kernels are heated in water to gelatinize the starch, the thermostable amylase initiates the conversion of the starch to fermentable sugars.

Manipulation of Starch Structure to Diversify Its Uses

The major targets for the manipulation of starch structure in plants are to modify the relative proportions of amylopectin and amylose, to change the chain length distribution in the amylopectin, or to increase the phosphate content of the starch. Due to the contrasting physicochemical properties of amylopectin and amylose, it is generally advantageous if starches for industrial applications are composed mainly of one or the other. Many mutants have been described in various cereal and legume species that contain starches composed of only amylopectin or that have a very high proportion of amylose (73). Efforts have been made to introduce these traits into potato and cassava, which are generally difficult to breed, using transgenic approaches. This is partly because the mutants in other species frequently show yield penalties, which might not occur in cassava and potato, and partly because starches from these sources already have distinctive properties with specific industrial value (e.g., in the case of potato, a relatively high phosphate content). The first transgenic potato and cassava plants with modified starch were those in which amylose was eliminated via the downregulation of GBSS (106, 145, 156). No penalties on the starch content were observed and plants seemingly suited for commercialization were produced almost 20 years ago. Problems with gaining approval for commercialization of transgenic crops in Europe mean that this has still not been achieved. Instead, a mutant GBSS from a nontransgenic amylose-free mutant of potato, originally identified in a diploid variety (60), has been bred into a commercial cultivar and commercially produced amylose-free potato starch is derived from this. A nontransgenic amylose-free mutant has now also been described for cassava (17), opening the possibility of commercially produced amylose-free cassava starch without facing the regulatory hurdles surrounding transgenic lines.

It has proved more difficult to produce high-amylose potato starch. The main approach has been through downregulation of isoforms of branching enzyme. When the major isoform (BEI) was downregulated, only minor changes in starch structure and no increases in the amylose content were observed, despite the fact that more than 95% of the measurable activity was lost (72, 117). Subsequently, the minor tuber isoform (BEII) was downregulated, giving an apparent amylose content of the starch of 38% compared with 30% in the starch from control plants (66). Only when both isoforms were simultaneously downregulated were substantial increases in the amylose content achieved (reaching 75% of the granule; 122). Interestingly, there seems to be an interaction between the branching of starch and its phosphorylation during starch biosynthesis in potato tubers. Simultaneous downregulation of GWD and the BEs resulted in higher amylose contents (up to

90%) than downregulation of BEs alone (87, 153). Downregulation of BEs led to marked increases of the phosphate content of the starch (117).

There are numerous reports in the literature on diverse transgenic manipulations that affect amylopectin structure (e.g., 84). Those that were done in an amylose-free background are arguably most suitable for further exploitation. Based on the observation that the simultaneous reduction of SSII and SSIII in potato led to the synthesis of an amylopectin with an elevated ratio of short to long chains (37, 83), Jobling et al. (67) downregulated SSII and SSIII in combination with GBSS, resulting in the production of short-chain amylopectin almost free of amylose. When heated in water this starch produces a gel that is stable through repeated cycles of freezing and thawing—a highly desirable trait. A similar approach was used to produce high-phosphate amylopectin. Based on the observation that downregulation of BEI (117) or SSIII (1) leads to an increased phosphate content, each has been simultaneously downregulated with GBSS (133).

In contrast to potato and cassava starches, cereal endosperm starches are generally low in phosphate (9). With the discovery of GWD and PWD, it is now possible to produce cereal starches that contain higher amounts of phosphate through overexpression of those proteins during seed development. This is especially interesting because the superior quality of potato starch for some applications relies partly on its high phosphate content. The overexpression of GWD has now been achieved in wheat (121), maize (43, 79), and rice (43). This reportedly leads to the production of starches with unprecedented swelling power when heated in water (43).

Outlook for the Development of Crops with Altered Starch Metabolism

The biotechnological development of crops with altered starch metabolism is hampered by two main problems. First, as mentioned above, there are enormous numbers of enzyme variations—and thus starch structures—that could be generated. However, the relationship between a given starch structure and its resultant physicochemical properties is not well understood. Thus, it is difficult to predict what uses might be found for a starch with a novel structure. Large amounts of starch are generally needed for testing in different applications; these are difficult to produce for large numbers of genotypes. Second, the high costs associated with the regulatory procedures surrounding transgenic plants make it commercially unattractive to develop such crops, especially when a nontransgenic alternative exists. Following the cisgenic (or intragenic) route in which plants are transformed only with their native DNA presents an exciting opportunity. This approach involves the introduction of native promoter elements and coding sequences, flanked by species-specific P-DNA elements rather than by *Agrobacterium*-derived T-DNA (115). One example of such an event where, among other traits, cold-induced sweetening has been suppressed in cisgenic potatoes is currently in the process of deregulation (116). The outcome of this attempted commercialization may have a large impact on the future of starch biotechnology.

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Errata

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