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INVITED REVIEW

Starch biosynthesis in developing seeds

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Abstract

Starch is globally important as a source of food and, in addition, has a wide range of industrial applications. Much of this agriculturally produced starch is synthesized in developing seeds, where its biological function is to provide energy for seedling establishment. Storage starch in developing seeds is synthesized in heterotrophic plastids called amyloplasts and is distinct from the transient synthesis of starch in chloroplasts. This article reviews our current understanding of storage starch biosynthesis occurring in these organelles and discusses recent advances in research in this field. The review discusses starch structure and granule initiation, emerging ideas on the evolution of the pathway, the enzymes of starch synthesis, and the post-translational modification and regulation of key enzymes of amylopectin biosynthesis.

Keywords: amylopectin, amyloplasts, amylose, debranching enzyme, protein phosphorylation, protein–protein interactions, seed development, starch, starch branching enzyme, starch-granule-associated proteins, starch phosphorylase, starch synthase, starch synthesis

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Abbreviations: ADP-glucose, adenosine 5′-diphosphate glucose; *ae*$^{-}$, *amylose extender*; AGPase, adenosine 5′-diphosphate glucose pyrophosphorylase; AGP-L, large subunit of AGPase; AGP-S, small subunit of AGPase; DAP, days after pollination; DBE, debranching enzyme; D-enzyme, disproportionating enzyme; DP, degree of polymerization; GBSS, granule-bound starch synthase; Glc 1-P, glucose 1-phosphate; Glc 6-P, glucose 6-phosphate; GS, glycogen synthase; GSK, glycogen synthase kinase; MOS, malto-oligosaccharides; 3-PGA, 3-phosphoglyceric acid; Pi, inorganic phosphate; PPi, inorganic pyrophosphate; SBE, starch branching enzyme; SDS, sodium dodecyl sulphate; SNF1, sucrose non-fermenting-1 protein kinase; SP, starch phosphorylase; SS, starch synthase; UDP-glucose, uridine 5′-diphosphate glucose; WSP, water-soluble polysaccharides.

Introduction

Starches produced by higher plants function as seed storage reserve carbohydrates and are probably the most important dietary source of energy for humans, representing a major proportion of the daily calorific intake. Plant starches are synthesized inside specialized subcellular organelles called plastids and provide the cell and tissue with the means to store high-density energy in the form of carbohydrate. Transient starches are synthesized in leaf chloroplasts during the day and degraded at night to provide carbon for non-photosynthetic metabolism. Starch produced in tuberous tissues also acts as a carbon store, and may need to be accessed as environmental conditions dictate, while storage starches in developing seeds, such as cereal endosperms, are a long-term carbon store for the next generation. All storage starches produced in heterotrophic tissues are made inside specialized plastids termed amyloplasts. The storage starches produced in cereal endosperm amyloplasts account for over 90% of the world market for starch (the major cereals in terms of tonnage of yield are maize and wheat). Starch is a cheap, natural renewable raw material, the varied structures of which are exploited in the agri-food sector, in many different industrial applications and as a source of energy after conversion to ethanol (Röper, 2002; Smith, 2008), making it a versatile and highly useful commodity (Burrell, 2003). Many of the desired physicochemical properties underpinning the different uses of starches can be produced and altered through chemical and enzyme modification, and physical treatment (Wurzburg, 1986; Jobling, 2004; Xie and Liu, 2004).

Characterization of natural mutants through plant breeding, and identification and isolation of the major genes encoding the core components of the starch biosynthetic pathway have provided a basic understanding of starch granule formation (see recent review by Keeling and Myers, 2010). Knowledge of the basic

pathway has led to the production of a number of functional starches based upon predictions of their structures resulting from various mutations/gene deletions (see below). However, there still remain significant gaps in our understanding of the pathway, in particular how the growth of starch granules is initiated and terminated, and important details regarding the regulation and coordination of the many enzyme activities involved in polymer synthesis. Such information is a prerequisite for the rational production of 'designer' starches *in planta*, which will reduce the need for costly and environmentally hazardous post-harvest chemical treatments.

The following is a review of recent developments in our understanding of starch biosynthesis in higher plants, with an emphasis on production of storage starches in the developing seeds of cereals. I will present a brief overview of the biological significance of starch synthesis in the context of the developing seed, discuss the origins of the starch biosynthetic pathway in higher plants, outlining some emerging ideas, and then present a current overview of starch structure, the core pathway involved in its synthesis and recent developments in our understanding of regulation of the pathway at the post-translational level.

Seeds as storage organs

Human civilization is dependent on the agricultural production of seeds which provide the majority of the world's staple foods, such as cereals and legumes, and in addition provide an important source of industrial biomaterial. The development of seeds has given the Angiosperms a great selective advantage, allowing fertilization in a non-aqueous habitat and providing an ideal dispersal unit with long-term viability during quiescent periods due to the evolution of desiccation-tolerant embryos. Seeds are produced following reproduction, and an important requirement for the survival of seeds is the development of long-term carbon stores to power eventual seedling establishment. These can be in the form of starch, lipid or hemicelluloses and are deposited in storage cells usually in the cotyledons or persistent endosperm. The relative partitioning of assimilated carbon (usually in the form of sucrose) into different storage compounds in different tissues is regulated by a complex interplay of gene expression and metabolic activity during seed development (Lin *et al.*, 1999; Wobus and Weber, 1999; Rolletschek *et al.*, 2005). Starch is present in most plant tissues as a carbon store, and in many agriculturally important crops, notably the cereals, it can represent as much as 70% of the dry weight of the seed (Dale and Housely, 1986). The structure and composition of starch make it an ideal storage material in seeds as it is chemically inert and water insoluble, allowing large amounts of sugar to be stored in cells without adverse effects on solute potential. Starch is deposited in endosperm cells shortly after a period of rapid cell division and expansion of cell volume following pollination (Briarty *et al.*, 1979; Olsen, 2001). The number of endosperm cells formed may be a strong indicator of final yield (Brocklehurst, 1977). This cell division phase is completed within a few days [2–4 days after pollination (DAP) in wheat, and 4–6 DAP in maize], and cell volume increases up to maturity (approximately 35 DAP). The deposition of storage material usually occurs between 10 DAP and maturation (Bewley and Black, 1994). The following reviews our current knowledge of starch biosynthesis in plant tissues, with an emphasis on the storage starches produced in amyloplasts of cereal endosperms.

Overview of the origins of the starch biosynthetic pathway in plants

Polyglucans are the most important and widespread form of reserve carbohydrate found in nature; glycogen and starch being the predominant forms. Both glycogen and starch are comprised of glucose chains (glucans) linked in an α-(1 → 4) configuration, and branched at α-(1 → 6). Glycogen is a homogeneous water-soluble polymer with relatively uniformly distributed branches (Roach, 2002) and is found within the archaea, bacteria and certain eukaryotes. Starch is made up of amylose (a largely unbranched, minor component) and amylopectin (an asymmetrically branched major component) and is present in the cytoplasm of Rhodophyceae (red algae) and Glaucophyta (Dauvillée *et al.*, 2009), but is confined to the plastid stroma of Chloroplastida (green algae and land plants). In fact, starch synthesis is restricted to the Archaeplastida, whose origins are thought to be via a single endosymbiotic event involving ancestors of cyanobacteria and a heterotrophic host (Cavalier-Smith, 2009), rendering the organelle known as the plastid, which is capable of oxygenic photosynthesis. Recent phylogenetic studies indicate that the plastidial starch pathway is complex, and made up of genes with both cyanobacterial and eukaryotic origins (Patron and Keeling, 2005; Deschamps *et al.*, 2008a), and is in sharp contrast to the lower-complexity pathway of cytosolic starch synthesis found in the Rhodophyceae and Glaucophyta (Deschamps *et al.*, 2008b). Phylogenetic analysis of the enzymes of the starch biosynthetic pathway strongly suggests that the pathway was originally cytosolic (in the common ancestor of the Archaeplastida), and then re-directed (reconstructed) to plastids via three discrete steps (see Deschamps *et al.*, 2008a for a detailed discussion), leaving some enzymes involved in the metabolism of malto-oligosaccharides

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(MOS) and amylopectin degradation in the cytoplasm. The three evolutionary steps involved: (1) plastidial synthesis of unbranched MOS; (2) glycogen synthesis (including priming steps and branching activities); and (3) plastidial starch synthesis, resulting in the eventual loss of cytosolic starch synthesis. Interestingly, the proposed timing of the relocation of the starch synthesis pathway to plastids coincides with the evolution of light-harvesting complexes, and it has been suggested that the presence of starch, and its degradation via starch phosphorylase, could generate the required ATP to overcome protoporphyrin IX-induced oxidative stress, thus imparting improved fitness (evolutionary benefit) to the organism (Deschamps *et al.*, 2008a).

Overview of starch structure

The starch reserves found in the tissues of higher plants are packaged as discrete granules inside plastids. The structure of the starch granule is complex and has a hierarchical order composed of two distinct types of glucose polymer: amylose, a polymer of 100–10,000 glucosyl units comprising sparsely branched α-(1 → 4)-O-linked glucan chains, and amylopectin, a larger, highly and regularly branched glucan polymer typically constituting about 75% of the granule mass, produced by the formation of α-(1 → 6)-linkages between adjoining straight glucan chains. There is approximately one branching point for every 20 glucose residues in amylopectin, which is approximately half the value normally found in glycogen. Over the years many models have been proposed for the structure of amylopectin and its organization into granules; however, the cluster model has emerged as the most likely structure (Fig. 1). The polymodal distribution of glucan chain lengths and branch point clustering within amylopectin allows the short linear chains to pack together efficiently as parallel left-handed double helices (with approximately six monomers per turn, with a pitch of 2.13 nm). These glucan chains cluster in organized arrays, forming the basis of the semi-crystalline nature of much of the matrix of the starch granule, and resulting in the water-insoluble nature of starch. Granule formation is driven by both the semi-crystalline properties of amylopectin, as determined by the length of the linear chains of amylopectin, and the clustering and frequency of α-(1 → 6)-linkages (Robin *et al.*, 1974; French, 1984; Hizukuri, 1986; Myers *et al.*, 2000). The products of partial α-amylolysis of various cereal starches, resulting from the non-random hydrolysis of glucosidic bonds between unit clusters (known as long B-chains, or B_2 and B_3 chains, depending on the number of clusters interconnected), are fully consistent with the

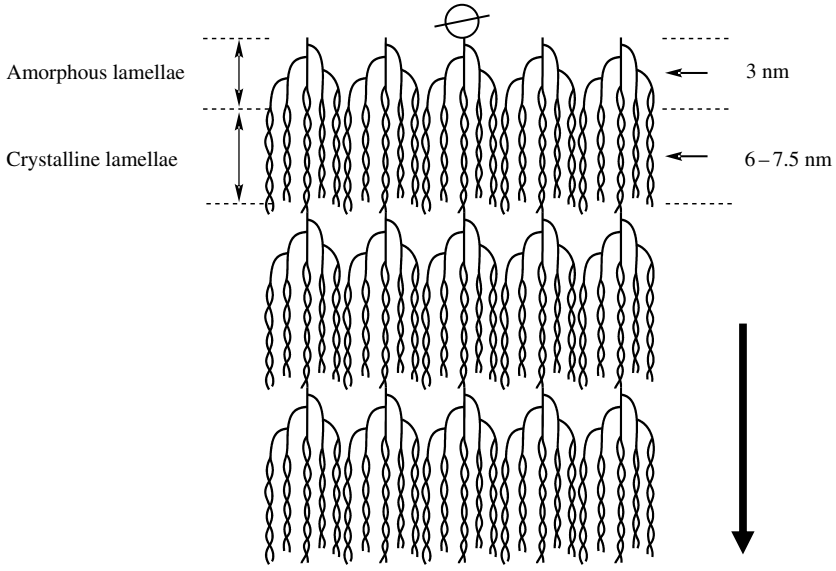

Figure 1. Schematic representation of the basic structural unit of amylopectin – the cluster model. The starch granule (which may be as much as 50 μm in length) is made up of many regions of alternating amorphous and semi-crystalline zones, several hundred nanometres in width; pairs of these constitute a single growth ring. The growth rings, and the more structured elements of the granule, are organized within an amorphous background containing amylopectin in a less ordered state, and amylose interspersed between these amorphous zones and the amylopectin clusters. In the tandem-linked glucan clusters shown, clusters of parallel α-(1 → 4)-O-linked glucan chains of short–intermediate length [degree of polymerization (DP) ≥ 10] glycosyl units pack together to form double helices, contributing to the water-insoluble properties of the polymer. The amorphous lamellae are characterized by clustered α-(1 → 6)-linked glucosidic linkages (branch-points) and longer glucan chains connecting the crystalline lamellae. The length of the crystalline and amorphous regions (approximately 9–10 nm) appears to be highly conserved, irrespective of the source of the starch. Growth of the polymer is from the non-reducing end in the direction of the arrow.

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cluster model for amylopectin (Bertoft, 1986). Pairs of adjacent chains within the clusters form double helices which help pack the glucan chains into crystalline layers (lamellae). At a higher order of organization, the lamellae form repeat units with a periodicity of approximately 9 nm. Groups of these semi-crystalline and amorphous lamellae associate into units termed blocklets (Gallant *et al.*, 1997), which in turn associate into large crystalline structures several hundred nanometres wide, termed growth rings. These are visible by light and scanning electron microscopy following treatment of broken granules with acid or amylolytic enzymes (Buttrose, 1960; Zeeman *et al.*, 2002; Pilling and Smith, 2003). The crystalline structure of starch granules is highly conserved in higher plants at the molecular level (Kainuma, 1988; Jenkins *et al.*, 1993; Zeeman *et al.*, 2002), as well as at the microscopic level, where growth rings have been observed in all the higher plant starches studied (Buttrose, 1960; Hall and Sayre, 1973; Pilling and Smith, 2003). By contrast, glycogen produced by animals and bacteria, including many cyanobacteria, has a more homogeneously and highly branched (approximately 10%) open structure which expands in a globular fashion (Roach, 2002) resulting in a water-soluble polymer. However, certain cyanobacteria synthesize a polyglucan, termed semi-amylopectin, which is intermediate between amylopectin and glycogen in terms of the α-(1 → 4)-chain-length distribution (Nakamura *et al.*, 2005). For more detailed reviews of starch structure models, see Manners (1989), Buléon *et al.* (1998), Thompson (2000) and Bertoft (2004).

The precise location of amylose within the granule is a matter of much debate, but it is thought to be predominantly interspersed in a single-helical or random-coil form in the amorphous, less crystalline regions (Jane *et al.*, 1992; Jenkins and Donald, 1995) and synthesized within a pre-existing amylopectin matrix. Amylose is readily leached from starch granules, suggesting weak associations with the amylopectin granule matrix (Cowie and Greenwood, 1957).

A common feature of all starches is that, at some point, they must be degraded (e.g. seed storage starches following germination, and leaf starch at the end of the light period), and hence the granule structure must have built-in entry points (either channels, cavities or zones of attack) for the enzymes involved in its degradation (Fannon *et al.*, 1992; Huber and BeMiller, 1997).

The synthesis of this architecturally complex polymer assembly is achieved through the coordinated interactions of a suite of starch biosynthetic enzymes, including some that have traditionally been associated with starch degradation. The complement of these starch metabolic enzymes is well conserved between plastids in tissues that make different types of starches, for example, transitory starch (made in chloroplasts) and storage starch (made in amyloplasts). With few exceptions, the various isoforms of the many starch metabolic enzymes can be found in both chloroplasts and amyloplasts, and the amino acid sequences of the enzymes involved in starch metabolism are highly conserved (Jespersen *et al.*, 1993; Smith *et al.*, 1997; Ball and Morell, 2003). This remarkable conservation in form and function of the starch biosynthetic enzymes is highlighted in a recent study by Sawada *et al.* (2009) which demonstrated complementation of function by expressing a starch branching enzyme from the alga *Chlorella* in transgenic rice. In addition, mutations in analogous starch biosynthetic and degradative genes in higher plants show consistent trends, which illustrates conservation of their biological roles, although their impact varies depending upon the genetic background. Models have been proposed which attempt to explain how the observed structure of starch is synthesized, based upon knowledge of the enzymes of the core pathway, essentially derived from *in vitro* experimental evidence and analysis of mutants (Ball *et al.*, 1996; Waigh *et al.*, 1998; Zeeman *et al.*, 1998; Nakamura, 2002). The following section summarizes our knowledge of the known components of the starch biosynthetic pathway in higher plants, beginning with the formation of ADP-glucose from common metabolic intermediates (hexose-phosphate and ATP), and ending with the architecturally complex starch granule. Figure 2 presents an overview of the pathway of storage starch biosynthesis in heterotrophic plastids from developing monocot and dicot seeds.

The pathway of starch biosynthesis

The formation of ADP-glucose by ADP-glucose pyrophosphorylase

Higher plant tissues capable of starch biosynthesis employ a prokaryote-like pathway for the formation of adenosine 5′-diphosphate glucose (ADP-glucose) (Greenberg and Preiss, 1964) which is the soluble precursor and substrate for starch synthases (Recondo and Leloir, 1961). ADP-glucose is synthesized by ADP-glucose pyrophosphorylase (AGPase, E.C. 2.7.7.27). The AGPase reaction is the first committed step in the biosynthesis of both transient starch in chloroplasts and chromoplasts, and storage starch in amyloplasts, and is subject to different mechanisms of post-translational regulation (see below). It also catalyses a rate-limiting step in glycogen synthesis in bacteria. The reaction scheme for AGPase is as follows:

$$\text{Glucose 1-phosphate} + \text{ATP} \leftrightarrow \text{ADP-glucose} + \text{PPi}$$

Thus, AGPase catalyses an equilibrium reaction. *In vivo*, the plastidial reaction is shifted away from

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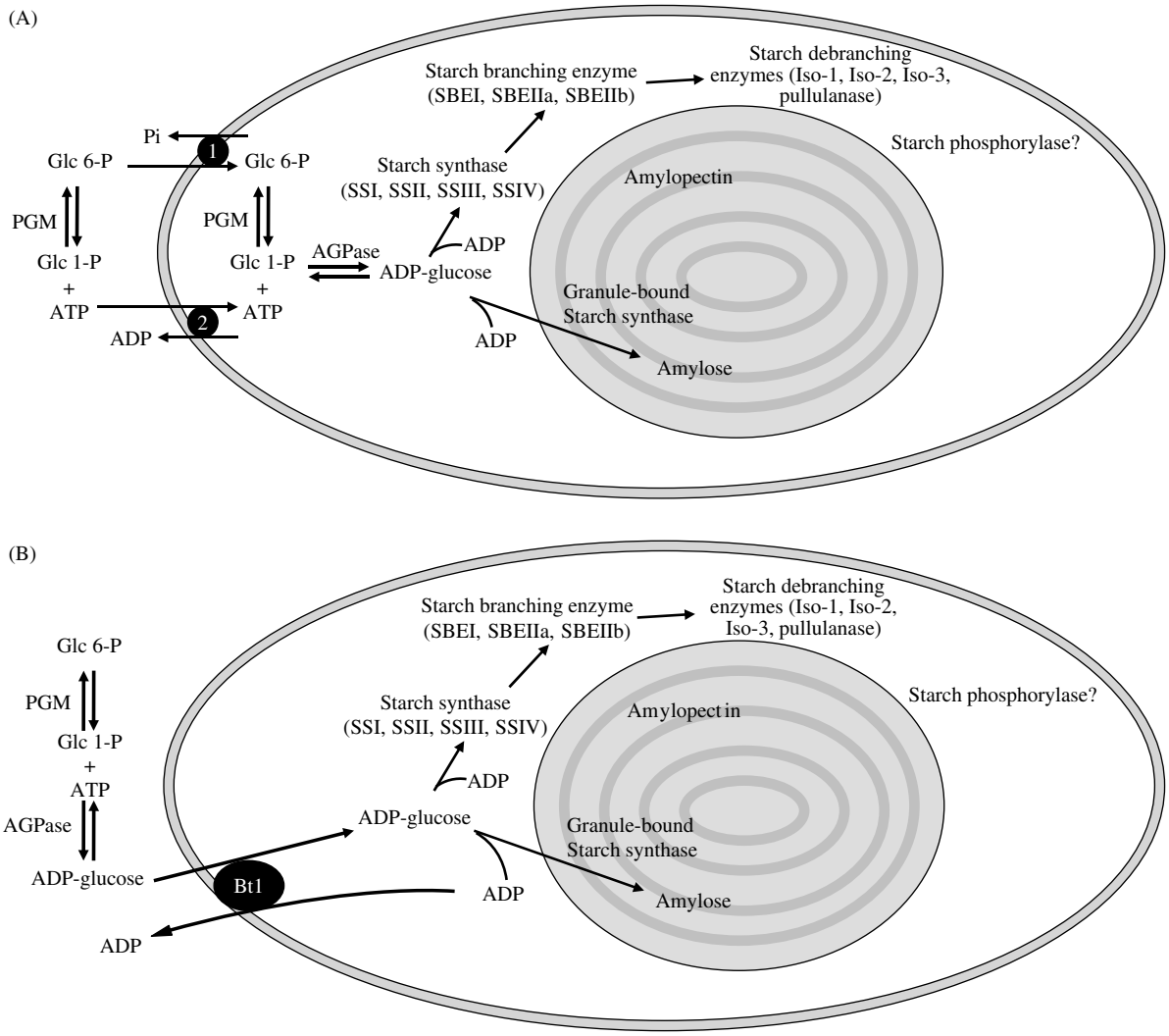

Figure 2. Overview of storage starch biosynthesis in the heterotrophic plastids (amyloplasts) of seed storage tissues. (A) The pathway of starch biosynthesis in storage tissues of dicots, where AGPase, responsible for the synthesis of ADP-glucose, is exclusively localized in the plastid stroma. Hexose-phosphates and ATP for this reaction are imported into the plastid from the cytosol via, respectively, the Glc 6-P/Pi antiporter (1) and the ATP/ADP transporter (2) located in the inner envelope membrane of the plastid. Cytosolic and plastidial isoforms of phosphoglucomutase (PGM) interconvert Glc 6-P and Glc 1-P. The antiport substrates for these transporters are generated from plastidial reactions involved in starch synthesis; Pi is generated from pyrophosphate produced by AGPase, and ADP is a by-product of the starch synthase (SS) reaction. ADP-glucose is utilized by the SSs, and the glucan chain branched and debranched by starch branching enzymes (SBE) and debranching enzymes, respectively, to form amylopectin. Granule-bound starch synthase also uses ADP-glucose, and is exclusively involved in amylose synthesis. (B) The pathway of starch synthesis operating in the endosperms of monocots such as cereals. These plants possess a cytosolic form of AGPase and import ADP-glucose from the cytosol via an ADP-glucose/ADP transporter termed Bt1. Monocots also possess a plastidial form of AGPase (as seen in A), but much of the AGPase in these tissues is extra-plastidial.

equilibrium in favour of ADP-glucose synthesis by the action of plastidial alkaline inorganic pyrophosphatase (E.C. 3.6.1.1) (Gross and ap Rees, 1986) that converts inorganic pyrophosphate (PPi) into inorganic phosphate (Pi), which can be transported across the plastid envelope membrane via different routes (for a review see Weber *et al.*, 2005). AGPase from higher plants is heterotetrameric, consisting of two large (AGP-L) subunits and two small (AGP-S) catalytic subunits encoded by at least two different genes (Preiss and Sivak, 1996). In addition to the plastidial AGPase present in all starch-synthesizing tissues, biochemical evidence indicates the presence of at least two distinct AGPase enzymes in the developing endosperms of maize (Denyer *et al.*, 1996b), barley (Thorbjørnsen *et al.*, 1996a), rice (Sikka *et al.*, 2001) and wheat (Tetlow *et al.*, 2003b) which correspond to plastidial and cytosolic isoforms of AGPase.

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Further analyses have shown that cytosolic AGPase is restricted to the endosperms of the agriculturally important *Graminaceae* (Beckles *et al.*, 2001). Starch synthesis, and therefore ADP-glucose formation, via a cytosolic AGPase is likely to be very dependent upon the activities of cytosolic PPi-consuming reactions which are able to shift the cytosolic AGPase reaction from equilibrium. In this regard, PPi-dependent fructose 6-phosphate 1-phosphotransferase (E.C. 2.7.1.90) and UDP-glucose pyrophosphorylase (E.C. 2.7.7.9) have been proposed to be important cytosolic PPi-consuming reactions enabling the provision of cytosolic ADP-glucose for plastidial starch biosynthesis (ap Rees and Morrell, 1990; Kleczkowski, 1994). In the developing endosperms of wheat, maize, barley and rice, the cytosolic isoform accounts for 65–95% of the total AGPase activity. Consequently, most of the storage starch biosynthesis in these tissues occurs through import of ADP-glucose into amyloplasts via a specialized nucleotide sugar transporter which imports ADP-glucose in exchange for ADP, the by-product of the plastidial starch synthase reaction (Fig. 2B) (Möhlmann *et al.*, 1997; Tetlow *et al.*, 2003a; Bowsher *et al.*, 2007). The amino acid sequence of the maize endosperm ADP-glucose transporter, termed Bt1 has been determined (Kirchberger *et al.*, 2007). Storage starch biosynthesis in non-graminaceous plant tissues and algae appears to be solely dependent on a plastidial AGPase and import of hexose phosphates and ATP from the cytosol (Fig. 2A) (see review by Comparot-Moss and Denyer, 2009).

Plants possess multiple genes encoding the AGP-L or AGP-S subunits and these are differentially expressed in different plant organs. This means that the AGPase subunit composition may vary in different parts of the same plant in species such as rice (Nakamura and Kawaguchi, 1992), barley (Villand *et al.*, 1992a) and potato (La Cognata *et al.*, 1995). The multiple genes encoding the AGP-L subunits show strong specificity in their expression, for example, being restricted to leaf, or root and endosperm in barley (Villand *et al.*, 1992a, b), wheat (Olive *et al.*, 1989) and rice (Lee *et al.*, 2007), or induced under specific conditions, such as increased sucrose or glucose levels in potato (Müller-Rober *et al.*, 1990; Duwenig *et al.*, 1997) and *Arabidopsis* (Crevillén *et al.*, 2003, 2005). Multiple isoforms of the AGP-S subunit in bean show organ-specific expression patterns; one form is expressed only in leaves, the other in both leaves and cotyledons (Weber *et al.*, 1995). Different mRNAs encoding the AGP-S subunit in maize also have distinct tissue expression patterns (Giroux and Hannah, 1994; Prioul *et al.*, 1994). The differential expression of subunits in different tissues may produce AGPases with varying degrees of sensitivity to allosteric effectors (see below), which are suited to the particular metabolic demands of a given tissue/organ. In developing cereal endosperms, the subcellular localization of some AGPase isoforms is thought to be determined by differential splicing of AGPase genes. Studies with barley indicate that the plastidial and cytosolic AGP-S subunit mRNAs are produced from a single gene by the use of two alternative first exons (Thorbjørnsen *et al.*, 1996b).

The catalytic activities of AGPases are, to varying degrees, subject to allosteric control. In higher plants AGPases are generally stimulated by 3-phosphoglyceric acid (3-PGA) and inhibited by inorganic orthophosphate (Pi) (Ghosh and Preiss, 1966). By contrast, the homotetrameric bacterial AGPase is stimulated by fructose 1,6-bisphosphate and inhibited by AMP (Haugen and Preiss, 1979). The relative sensitivity of plant AGPases to these allosteric effectors appears to depend on the tissue and plastid type, and, in the case of cereal endosperms, the subcellular location of the enzyme. A small polymorphic motif within the small subunit of AGPase may influence both catalytic and allosteric properties by modulating subunit interactions (Cross *et al.*, 2005). The chloroplast AGPase, which synthesizes ADP-glucose from the glucose 1-phosphate (Glc 1-P) produced from photosynthesis, is tightly regulated by metabolite concentrations, being activated by micromolar amounts of 3-PGA and inhibited by Pi (Ghosh and Preiss, 1966). The ratio of these two allosteric effectors is thought to play a key role in the control of starch synthesis in photosynthetic tissues (Preiss, 1991). However, evidence from wheat (Gómez-Casati and Iglesias, 2002; Tetlow *et al.*, 2003b) and barley (Kleczkowski *et al.*, 1993) endosperms suggests that measurable activity in monocot storage tissues (the majority of which is cytosolic, see above) is much less sensitive to 3-PGA activation and Pi-inhibition than other forms of AGPase. However, the plastidial AGPase from the storage tissues of dicots (e.g. potato tuber) appears to be as sensitive to the allosteric effectors as its counterparts in the chloroplast (Hylton and Smith, 1992; Ballicora *et al.*, 1995). Thus in tissues committed to the long-term storage of starch, such as developing seeds, there is reduced sensitivity of AGPase to allosteric effectors. The sensitivity of plastidial AGPase to allosteric regulation in other plastid types, such as leucoplasts and chromoplasts, is unknown.

Post-translational modification of AGPase involving thioredoxin was proposed by Fu *et al.* (1998) following the observation of partial inactivation of the recombinant potato enzyme by the formation of intramolecular disulphide bonds between the N-termini of the AGP-S subunits. Redox control of AGPase through sucrose supply in potato tubers has recently been proposed, whereby reductive activation causes channelling of carbon to starch and away from respiratory/glycolytic metabolism (Tiessen *et al.*, 2002), and is probably a general mechanism operating

in other storage tissues, such as developing seeds. The signalling components leading to redox modulation of AGPase are beginning to be understood, and are thought to involve sucrose and glucose acting via an SNF1-related protein kinase (SNF1 is sucrose non-fermenting-1 protein kinase, originally described in *Saccharomyces cerevisiae*) and hexokinase (E.C. 2.7.1.1), respectively (see Tiessen *et al.*, 2003). Studies with transgenic *Arabidopsis* indicate a role for trehalose 6-phosphate in promoting the SNF1-related protein kinase-dependent redox activation of AGPase (Kolbe *et al.*, 2005). Sugar-induced redox activation of AGPase is lost in transgenic plants expressing a trehalose 6-phosphate phosphatase (E.C. 3.1.3.12), which causes a reduction in trehalose 6-phosphate content (Kolbe *et al.*, 2005). Changes in allosteric effectors (3-PGA and Pi) and light-dependent changes in redox activation of AGPase are likely to be more important factors in controlling rates of transient starch synthesis over a diel cycle than for storage starch synthesis in developing seeds.

Much research attention has been focused on modulating the activity of AGPase in crop plants in order to increase starch yield. Plant productivity is regulated by both the rate and duration of carbon fixation in 'source' leaves, and the capacity of developing 'sink' tissues and organs to assimilate fixed carbon (Woodrow and Berry, 1988). Under environmental conditions when photosynthesis is not limiting, overall productivity appears to be regulated by sink strength (i.e. the efficiency of utilization of photosynthates for metabolism and biomass/yield production). Productivity can be enhanced if plants possess a greater capacity to store fixed carbon due to increased sink strength. This strategy has been successfully demonstrated in potato tubers (Stark *et al.*, 1992) and in maize endosperm (Giroux *et al.*, 1996) using allosterically unregulated forms of AGPase. In rice, yields are limited by sink strength, in particular the capacity of developing seeds to take up and convert photosynthates (sucrose) into starch (Rowland-Bamford *et al.*, 1990; Chen and Sung, 1994). The sink strength of developing rice seeds is increased by the expression of an allosterically insensitive bacterial mutant AGPase (the *Escherichia coli glgC* mutant) in the cytosol, resulting in increased starch production (Sakulsingharoj *et al.*, 2004). Overall, these studies show that modulation of AGPase activity in sink tissues is a promising target for increasing the carbon flow into starch in a number of important crop species. However, the flux control coefficient of AGPase is low in some tissues (as low as 0.08 in developing bean seeds) (Weber *et al.*, 2000; Rolletschek *et al.*, 2002), and in insertional mutant pea plants deficient in AGPase limited starch synthesis still occurs (Weigelt *et al.*, 2009). It has recently been suggested that alternative routes of starch biosynthesis are available using hexose phosphates via a starch phosphorylase (SP)-mediated pathway (Fettke *et al.*, 2010).

Glucan chain formation by starch synthases

Higher plant starch synthases (SS, E.C. 2.4.1.21) catalyse the transfer of the glucosyl moiety of the soluble precursor ADP-glucose to the non-reducing end of a pre-existing α-(1 → 4)-O-linked glucan primer to synthesize glucan polymers, which eventually, in conjunction with other enzymes (see below), form the insoluble polymers amylose and amylopectin. Plants possess multiple isoforms of SSs, containing at least five isoforms that are categorized according to conserved sequence relationships. Isoforms within each of the major classes of SS genes are highly conserved in higher plants (see Ball and Morell, 2003). In the SSs of land plants and green algae the conserved region is localized to a 'core' region in the C-terminus of approximately 60 kDa, but in prokaryotic glycogen synthases (GS, E.C. 2.4.1.11) these residues/domains are distributed across the protein sequence. The highly conserved K–X–G–G–L motif responsible for substrate binding in prokaryotic GSs and higher plant SSs (Furukawa *et al.*, 1990, 1993; Busi *et al.*, 2008) is found within the C-terminus in higher plant SSs (Nichols *et al.*, 2000). Detailed biochemical analysis of maize SSs indicates the importance of glutamate and aspartate residues for catalytic activity and substrate binding (Nichols *et al.*, 2000), and the involvement of lysine in the K–X–G–G–L domain in determining glucan primer preference (Gao *et al.*, 2004). Variation amongst the SS isoforms occurs within the N-terminus upstream of the catalytic core, which can vary greatly in length, from 2.2 kDa in granule-bound starch synthase I (GBSSI) to approximately 135 kDa in maize SSIII (Gao *et al.*, 1998) (Fig. 3). Research on the wheat endosperm suggests that soluble starch synthases have a high flux control coefficient, indicating their importance in carbon allocation to starch in this tissue (Jenner *et al.*, 1993; Keeling *et al.*, 1993; Tomlinson and Denyer, 2003). The major classes of SS genes can be broadly split into two groups, the first primarily involved in amylose synthesis, and the second principally confined to amylopectin biosynthesis.

Amylose biosynthesis

One group of SS genes encodes the granule-bound starch synthases (GBSS), and includes GBSSI and GBSSII. GBSSI is encoded by the *Waxy* locus in cereals, functioning specifically to elongate amylose (De Fekete *et al.*, 1960; Nelson and Rines, 1962) and is completely within the granule matrix (one of the so-called granule-associated proteins). Mutations in the *Waxy* locus leading to loss of GBSS activity result in amylose-free (*waxy*) starches, and in maize there is a clear effect of *Waxy* gene dosage on the production of GBSSI activity and resulting amylose content of the storage

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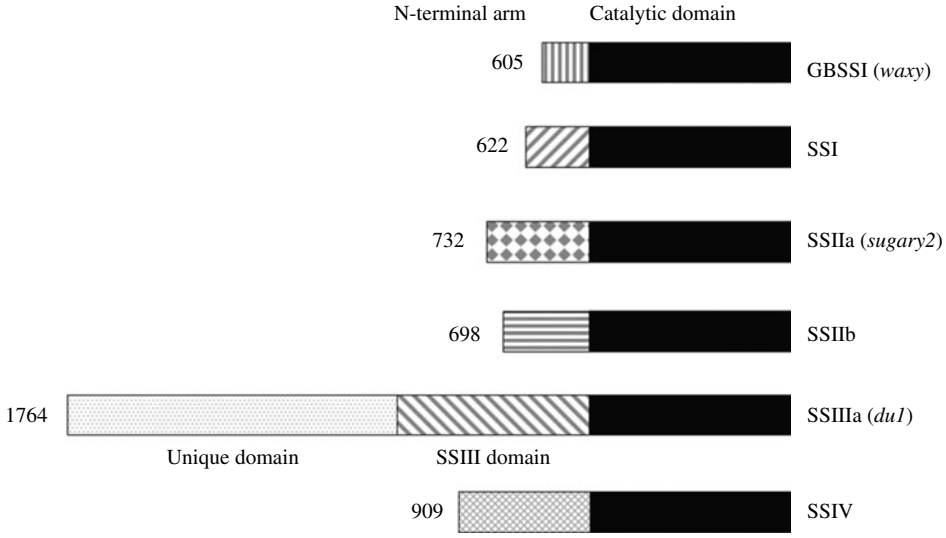

Figure 3. Domain comparison of cereal SS sequences showing C-terminal catalytic domains (including the ADP-glucose binding domain) in black (not drawn to scale). The five known SS isoforms are shown with the name of the corresponding maize mutant in parentheses. The different SSs have N-terminal domains of varying lengths (shown as hatched bars); SSIII in particular has a unique N-terminal extension thought to be involved in controlling protein–protein interactions. Numbers of amino acids constituting each isoform in maize are shown.

starch (Tsai, 1974; Shure *et al.*, 1983). In addition to its role in amylose biosynthesis, GBSSI is thought to be responsible for extension of long glucans (termed extra-long chains) of amylopectin in both *in vitro* and *in vivo* experiments in sweet potato (Baba *et al.*, 1987), *Chlamydomonas reinhardtii* (Delrue *et al.*, 1992; Maddelein *et al.*, 1994; van de Wal *et al.*, 1998), pea and potato (Denyer *et al.*, 1996a) and rice endosperm (Hanashiro *et al.*, 2008). Further support for this hypothesis is the observed reduction in extra-long chains in amylopectin in *Waxy* rice (Hizukuri *et al.*, 1989). Experiments with potato have shown that, in certain genetic backgrounds, GBSS can have a profound influence on the granule structure and morphology of tubers (Fulton *et al.*, 2002). Expression of GBSSI appears to be mostly confined to storage tissues, and a second form of GBSS (GBSSII), which is encoded by a separate gene, is thought to be responsible for amylose synthesis in leaves and other non-storage tissues that accumulate transient starch (Fujita and Taira, 1998; Nakamura *et al.*, 1998; Vrinten and Nakamura, 2000).

A unique property of GBSSI is the stimulation of its catalytic activity by MOS when synthesizing amylose (Denyer *et al.*, 1996a, 1999). One hypothesis is that MOS diffuse into the granule matrix, where GBSSI synthesizes amylose by elongating the MOS primers (for a review of amylose biosynthesis, see Denyer *et al.*, 2001). *In vitro* experiments have shown that glucan chain extension from amylopectin to yield amylose is possible with extended incubation times, suggesting that there are two different mechanisms for the synthesis of amylose (van de Wal *et al.*, 1998). The properties of *Waxy* starches demonstrate that amylose synthesis is not required for the formation of semi-crystalline granules, and its synthesis probably takes place within a pre-existing amylopectin matrix as the granule is formed (Dauvillée *et al.*, 1999; Denyer *et al.*, 1999) and the absence of amylose has no effect on the crystallinity of starch granules (Cooke and Gidley, 1992; Bogracheva *et al.*, 1999). There is also evidence that the amylose content of starch in storage tissues is affected by substrate (ADP-glucose) availability. GBSSI has a lower affinity for ADP-glucose than the soluble starch synthases involved in amylopectin synthesis [K_m of 1.4 mM for pea (Clarke *et al.*, 1999) is five- to ten-fold greater than other soluble isoforms of starch synthases], and estimates of the ADP-glucose concentration in plastids (approximately 0.9 mM), mean that the GBSSI reaction is not saturated and fluctuations in ADP-glucose concentration can reduce amylose synthesis more than amylopectin synthesis (Clarke *et al.*, 1999). This idea is supported by studies with plant mutants predicted to modify ADP-glucose content. The *rb* and *rug* mutants of pea (Clarke *et al.*, 1999), *STA1* and *STA5* mutants of *Chlamydomonas* (Van den Koornhuyse *et al.*, 1996), and potato expressing antisense AGPase constructs (Lloyd *et al.*, 1999a) are expected to have lowered amounts of ADP-glucose, and all contain less amylose in storage starch.

Amylopectin biosynthesis

A second group of SS genes (designated SSI, SSII, SSIII, and SSIV) are exclusively involved in amylopectin biosynthesis and individual SS isoforms from this

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group probably play unique roles. The distribution of the SSs within the plastid between the stroma and starch granules varies between species, tissue and developmental stage (Ball and Morell, 2003). The study of SS mutants in a number of plant species has been helpful in the assignment of *in vivo* functions/roles for the soluble and granule-associated SS isoforms. Valuable information about their roles in amylopectin synthesis *in vivo* is being derived from mutants lacking specific isoforms, and is summarized below. However, in some cases there may be pleiotropic effects of mutations on more than one starch synthesis enzyme.

SSI

SSI is primarily responsible for the synthesis of the shortest glucan chains, i.e. those with a degree of polymerization (DP) of 10 glucosyl units or less (Guan and Keeling, 1998; Commuri and Keeling, 2001), and extension of these glucan chains is achieved by the activities of SSII and SSIII isoforms, each of which acts on progressively longer glucan chains. SSI (and SSII) shows stimulated catalytic activity with glycogen in the presence of 0.5 M citrate (Imparl-Radosevich *et al.*, 1998, 1999). Analyses of mutants and transgenic plants lacking SSI activity tend to support the notion that SSI elongates short (DP 4–7) glucan chains. Studies of mutants of *Arabidopsis* (Delvallé *et al.*, 2005) and rice (Fujita *et al.*, 2006) lacking SSI show deficiencies in shorter (DP 6–12) glucan chain lengths of the starches, supporting its proposed role in the synthesis of short glucan chains. The fact that loss of SSI, a major SS isoform in cereal endosperms, has no effect on the size and shape of developing seeds and starch granules, nor on the crystallinity of endosperm starch in rice (Fujita *et al.*, 2006), suggests that other SS forms are capable of partly compensating for its function. In cereals SSI is expressed early in development (5–10 DAP in wheat endosperm), and considerable amounts of the protein become entrapped as 'granule-associated' proteins (see later) (Peng *et al.*, 2001).

SSII

Two classes of SSII genes are present in monocots: SSIIa and SSIIb. The role of the latter in starch biosynthesis is unknown as no mutants have been identified. *In vitro* studies of the two SSII forms from maize reveal different substrate specificities and kinetic properties (Imparl-Radosevich *et al.*, 1999, 2003). SSIIa predominates in cereal endosperms, while SSIIb is mostly confined to photosynthetic tissues. Both SSI and SSII are also localized within the starch granules in many cereals (Mu-Forster *et al.*, 1996; Morell *et al.*, 2003; Umemoto and Aoki, 2005). Loss of SSIIa from barley and wheat seeds results in their reduced starch content, reduced amylopectin chain-length distribution, altered granule morphology and reduced crystallinity (Morell *et al.*, 2003; Kosar-Hashemi *et al.*, 2007). In maize endosperm, SSIIa is the product of the *sugary2* gene, a mutation resulting in more short chains of DP 6–10, fewer chains of DP 12–30 and an elevated amylose content in the endosperm of up to 40% (Zhang *et al.*, 2004). In monocots and green algae, SSIIa appears to play a specific role in the synthesis of the intermediate-size glucan chains of DP 12–24 by elongating short chains of DP $\leq$ 10 (Fontaine *et al.*, 1993; Imparl-Radosevich *et al.*, 2003; Morell *et al.*, 2003). Despite SSIIa being a minor contributor to the total measurable SS activities in cereal endosperms, its loss/down-regulation has a major impact on both the amount and composition of starch. In cereals, important food-processing properties, such as starch gelatinization temperature, are controlled by the gene for SSII (Bao *et al.*, 2009). The simultaneous down-regulation of multiple SS reactions, such as in potato following reduction of SSII and SSIII activities, causes extensive alterations in starch structure (Edwards *et al.*, 1999; Lloyd *et al.*, 1999b). In *Arabidopsis* loss of SSII results in an increase in the amylose:amylopectin ratio, an increase in total amylose and a reduction in amylopectin glucan chains of DP 12–28, whereas a double mutant deficient in SSII and SSIII causes a severe phenotype with reduced growth rate and lowered starch content (Zhang *et al.*, 2008). The synergistic effects of the loss of SSII and SSIII are more severe than those with each individual mutation, suggesting only partial redundancy with respect to the functions of these SSs in amylopectin biosynthesis.

SSIII

After SSI, SSIII catalyses the second most abundant measurable activity in cereal endosperms such as maize and rice (Cao *et al.*, 1999; Fujita *et al.*, 2006). Two genes are responsible for expression of SSIII in the endosperm (*SSSIIIa*) and leaf (*SSIIIb*) of rice (Hirose and Terao, 2004; Dian *et al.*, 2005). Studies with plants lacking SSIII suggest that the primary role of this enzyme is amylopectin synthesis, although the impact of loss of SSIII appears to differ with the genetic background. Mutations in maize eliminating SSIII (*du1*) produce an endosperm with a glassy, dull appearance (Mangelsdorf, 1947), a phenotype which is only conspicuous in *Waxy* backgrounds (Gao *et al.*, 1998). Analysis of amylopectin from *du1* maize and rice show altered granule morphology and crystallinity and a reduction in long glucan chains (DP $\geq$ 30), suggesting a role for SSIII in their elongation (Inouchi *et al.*, 1983; Fujita *et al.*, 2007; Ryoo *et al.*, 2007). Partially purified SSIII from maize endosperm has a higher affinity for amylose (longer glucan chains) compared with amylopectin and glycogen (Cao *et al.*, 2000). It has been proposed, therefore, that SSIII functions in the provision of long chains which extend between clusters of amylopectin (James *et al.*, 2003). In addition to alterations in starch structure and physical

properties, loss of SSIII from maize endosperm is also associated with pleiotropic effects on other starch synthases, causing increased activities of SS (Cao *et al.*, 1999), and a reduction in starch branching enzyme (SBE) IIa activity (Boyer and Preiss, 1981). In rice endosperm increased transcripts of both SSI and GBSSI in SSIIIa-deficient plants are proposed to contribute to the observed starch phenotype (Fujita *et al.*, 2007), and in *Chlamydomonas* loss of SSIII from the *sta3* mutant is compensated by GBSSI (Ral *et al.*, 2006). These observations have led to the proposal that, in addition to its catalytic role, SSIII possesses regulatory properties with respect to control over the starch biosynthetic pathway. SSIII mutants of *Arabidopsis* show a starch excess phenotype in the leaves caused by an increased rate of starch biosynthesis (Zhang *et al.*, 2005), suggesting that this enzyme acts as a negative regulator of transient starch biosynthesis. Although the mechanism by which SSIII influences the rate of starch synthesis and/or other biosynthetic enzymes in the pathway is not clear, analysis of the structure suggests potential regulatory properties which may also control the pathway in developing seeds. SSIII is the largest of the SS isoforms [rice SSIII is a 230 kDa polypeptide (Dian *et al.*, 2005)] and has a long extension of amino acids at the N-terminus, termed the SSIII homology domain (SSIIIHD), which contains a putative 14-3-3 protein binding domain within the C-terminal (catalytic) domain. The SSIIIHD is involved in protein–protein interactions with other starch biosynthetic enzymes (see below) and glucan binding (Hennen-Bierwagen *et al.*, 2008, 2009; Valdez *et al.*, 2008; Wayllace *et al.*, 2010).

SSIV

SSIV is the most recently discovered form of higher plant SSs (Dian *et al.*, 2005), and has structural characteristics conserved amongst other SS forms, including two ADP-glucose binding domains (a K–X–G–G–L motif) and a unique (SSIV-specific) N-terminus, which includes two coiled-coil domains and a putative 14-3-3 protein binding domain (Leterrier *et al.*, 2008). SSIV is phylogenetically related to SSIII, and two isoforms exist in plants, SSIVa and SSIVb, which are differentially expressed in endosperm and leaf tissues, respectively (Leterrier *et al.*, 2008). Structural features of the ADP-glucose binding domains of SSIII and SSIV suggest they have different glucan specificities. The catalytic activity of SSIV is dependent on ADP-glucose (unlike other SSs, SSIV shows no activity with UDP-glucose) and the *Arabidopsis* enzyme has affinity for ADP-glucose in the same range as other SS forms (K_m for ADP-glucose of 0.47 mM). *In vitro* studies with SSIV indicate that it only elongates glucan chains in the presence of pre-existing glucan, and is particularly active with maltotriose (shows >90% of activity with amylopectin) (Szydlowski *et al.*, 2009). Reverse genetics studies indicate a role for SSIV in controlling the number of starch granules within a plastid. Loss of SSIV from *Arabidopsis* causes chloroplasts to accumulate a single, large starch granule, without observable effects on starch composition (Roldán *et al.*, 2007). Studies with a SSIV/SSII double mutant in *Arabidopsis* indicates that loss of both SSs is required to prevent granule initiation, leading to the proposal that both of these phylogenetically related SSs regulate starch granule initiation (Szydlowski *et al.*, 2009). It has not been determined whether SSIV in storage (seed) tissues functions in an analogous manner to the leaf enzyme.

Branching of the glucan chain

Starch branching enzymes (SBEs, E.C. 2.4.1.18) generate α-(1 → 6)-linkages by cleaving internal α-(1 → 4) bonds and transferring the released reducing ends to C6 hydroxyls to form the branched structure of the amylopectin molecule. SBEs are related to the α-amylase super-family of enzymes (Jespersen *et al.*, 1993) and are able to generate α-(1 → 6)-linkages on linear and branched glucan substrates. Following cleavage of a α-(1 → 4)-linkage, SBEs can transfer the cleaved glucan to an acceptor chain which is either part of the original glucan chain, or part of an adjacent glucan chain (known as inter-chain transfer). Studies with potato SBE (originally termed Q-enzyme) suggest inter-chain transfer predominates, and that close association of glucan chains, for example, in a double helical configuration, create a more favourable environment for SBE catalytic activity (Borovsky *et al.*, 1979). As with the elongation of glucan chains by SSs, SBE activity is also a function of multiple isoforms, some of which are tissue- and/or developmental-specific in their expression patterns (Yamanouchi and Nakamura, 1992; Gao *et al.*, 1997; Sun *et al.*, 1998; Regina *et al.*, 2005). Analysis of the primary amino acid sequences of higher plant SBEs reveals two major classes; SBEI (also known as SBE B) and SBEII (SBE A). These differ in terms of the length of the glucan chain transferred *in vitro* and their substrate specificities; SBEII proteins transfer shorter chains and show a higher affinity towards amylopectin than their SBEI counterparts, which show higher rates of branching with amylose (Guan and Preiss, 1993; Takeda *et al.*, 1993; Rydberg *et al.*, 2001). Phylogenetic analysis indicates that SBEI evolved prior to the monocot–dicot divergence, and its retention in higher plants suggests that it plays an important role in starch biosynthesis/metabolism (Gao *et al.*, 1996). It is not known whether SBEI branches amylose *in vivo*. The construction of chimeric forms of maize SBEI and SBEII and analysis of their catalytic properties by Kuriki *et al.* (1997) indicate that the N- and C-termini of these proteins

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play important roles in determining substrate preference, catalytic capacity and chain length transfer. SBEs and SSs utilize the same substrate, so it is perhaps unsurprising that structural similarities exist between the active sites of these different classes of enzymes; aspartate and glutamate residues are important for the catalytic function of SBEs and α-amylase (E.C. 3.2.1.1) (Boel *et al.*, 1990; Kuriki *et al.*, 1996) as with SSs (above). Studies with wheat endosperm indicate that the catalytic activity of SBEII forms is regulated by protein phosphorylation (Tetlow *et al.*, 2004). In monocots the SBEII class is made up of two closely related but discrete gene products, SBEIIa and SBEIIb (Rahman *et al.*, 2001). In developing wheat endosperm there are marked differences in the expression of these enzymes, with the SBEIIb form being at much lower levels than the IIa (Morell *et al.*, 1997; Regina *et al.*, 2005). This is in contrast to the maize endosperm, where SBEIIb is the predominant form, being expressed at approximately 50 times the level of the IIa form (Gao *et al.*, 1997); it is the most abundant protein in the maize endosperm amylopast stroma (Mu *et al.*, 2001).

Mutations in SBEII isoforms give the clearest phenotypes. In maize and rice, mutation of the gene encoding SBEIIb [also known as '*amylose extender*' (*ae*$^{-}$)] produces a high-amylose starch phenotype characterized by longer internal chain lengths of amylopectin than in normal starches and less frequently branched outer chains (Nishi *et al.*, 2001; Klucinec and Thompson, 2002; Evans and Thompson, 2004). In wheat such starches are only produced by suppression of both genes encoding the SBEIIa and SBEIIb forms, resulting in starches containing >70% amylose (Regina *et al.*, 2006). In potato, down-regulation of the equivalent SBE form produces a high-amylose starch (Schwall *et al.*, 2000). Down-regulation or elimination of SBEI activity in both monocots and dicots appears to have minimal effects on starch synthesis and composition in photosynthetic and non-photosynthetic tissues (Flipse *et al.*, 1996; Blauth *et al.*, 2002; Satoh *et al.*, 2003). In addition, loss of SBEI from a SBEIIb-deficient background causes increased branching, suggestive of a regulatory role for SBEI in influencing other SBEs (Yao *et al.*, 2004). Physical interactions between SBEI and SBEIIb occur in amyloplasts from wheat endosperm (Tetlow *et al.*, 2004).

The maize SBEIIa mutant shows a clear phenotype in leaf starch, but no apparent alterations in the storage starch in the endosperm (Blauth *et al.*, 2001). This suggests a primary role for SBEIIa in leaf (transient) starch synthesis, and either no critical role in amylopectin biosynthesis in the endosperm, or else one that can easily be compensated for by other SBEs. *Arabidopsis* has two SBE isoforms belonging to the SBEII class (Fisher *et al.*, 1996), plus a third putative form unrelated to the SBEII class, and with no assigned function. Loss of both SBEII forms from *Arabidopsis* results in a failure to synthesize starch, and accumulation of maltose in the cytosol (Dumez *et al.*, 2006). SBEII isoforms are also partitioned between the plastid stroma and the starch granules (Mu-Forster *et al.*, 1996). As with the granule-associated SSs, the factors/mechanisms involved in partitioning the SBE proteins to the starch granules remain undetermined. The ability of proteins to become granule-associated may be a function of the relative affinities of their active sites for the glucan polymer, as with some isoforms of SS (Commuri and Keeling, 2001), although it has been suggested that alternative splicing of an SBEII form in *Phaseolus vulgaris* causes an alteration in the properties of the enzyme, and partitioning within the starch granule (Hamada *et al.*, 2002).

In vitro analysis of heterologously expressed maize SBEs (Seo *et al.*, 2002) has shed further light on the roles of different SBE isoforms in the construction of the starch granule. Expression of three functional maize SBE genes in a yeast strain lacking the endogenous yeast glucan branching enzyme showed that SBEI is unable to act in the absence of SBEIIa or SBEIIb, and that SBEII may act before SBEI on precursor polymers. These data suggest that SBEI does not play a central role in this *in vitro* system, leaving its role in the starch biosynthetic pathway still an open question.

The role of debranching enzymes in amylopectin synthesis

In addition to SSs and SBEs, isoamylases, also termed debranching enzymes (DBEs, E.C. 3.2.1.41 and E.C. 3.2.1.68, originally termed R-enzyme) may be important in the formation of crystalline amylopectin. Analysis of low-starch mutants that accumulate a disordered water-soluble polysaccharide, termed phytoglycogen, have been described in a wide range of higher plants, including *Arabidopsis* and maize, the unicellular alga *Chlamydomonas* (Morris and Morris, 1939; James *et al.*, 1995; Mouille *et al.*, 1996; Zeeman *et al.*, 1998) and recently in rice (Fujita *et al.*, 2009), indicating that starch synthesis involves debranching enzymes working in conjunction with the SSs and SBEs. Two groups of DBEs exist in plants: the isoamylase-type (of which at least three forms exist in angiosperms: isoamylase-1, isoamylase-2 and isoamylase-3) and the pullulanase-type (also known as limit-dextrinases) which efficiently hydrolyse (debranch) α-(1 → 6)-linkages in amylopectin and pullulan (a fungal polymer of malto-triose residues), respectively, and are part of the α-amylase 'super-family' of enzymes. The *Arabidopsis* genome contains three isoamylase-type DBEs and one pullulanase-type DBE; these enzymes are present in most starch-synthesizing organisms (Deschamps *et al.*, 2008b). Both groups in higher plants share a common N-terminal domain, the function of which is yet to be elucidated. The decrease or loss of

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either isoamylase-1 or isoamylase-2 type DBE activities is thought to be responsible for the accumulation of phytoglycogen rather than starch in mutant/transgenic plants (Bustos *et al.*, 2004) and algae (Mouille *et al.*, 1996), and, in rice, endosperm residual pullulanase-type DBE activity may modulate these phenotypic effects (Nakamura *et al.*, 1998). Although isoamylase-2 lacks amino acid residues essential for catalytic activity, isoamylase-1 and isoamylase-2 polypeptides constitute a hetero-oligomeric complex to form a functional enzyme (Hussain *et al.*, 2003). In maize this complex is approximately 400 kDa, and is also found with a 300 kDa complex containing isoamylase-1, but not isoamylase-2. Studies with an isoamylase-1-null line show that isoamylase complexes are absent, indicating the importance of this isoform for complex assembly (Kubo *et al.*, 2010). The idea of isoamylase catalytic activity being a result of a hetero-enzyme complex was given support by the observation that targeted mutants of *Arabidopsis* lacking one or both of isoamylase-1 and isoamylase-2 exhibit identical phenotypes (Delatte *et al.*, 2005; Wattebled *et al.*, 2005). Complementation of *sugary-1* mutants with isoamylase-1 results in restoration of amylopectin synthesis, suggestive of a role for this DBE isoform (Kubo *et al.*, 2005). The isoamylase-1 and isoamylase-2 forms, therefore, are probably involved in developing amylopectin crystallinity by trimming inadequately spaced glucan branches, thus allowing tighter packing of glucan chains. A third isoform of isoamylase (isoamylase-3) appears to be compulsory for starch degradation at night in *Arabidopsis* leaves, and is catalytically more active on water-soluble polysaccharides that have been processed by β-amylase and starch phosphorylase (Wattebled *et al.*, 2005). Studies with barley mutants and transgenic rice suggest that isoamylases play a crucial role in starch granule initiation (Burton *et al.*, 2002; Kawagoe *et al.*, 2005). In maize endosperm the pullulanase-type DBE activity is thought to have a bifunctional role, assisting in both starch synthesis and degradation (Dinges *et al.*, 2003). Pullulanase tends to work on tightly branched glucans (including pullulan and amylopectin), and has weak affinity for loosely spaced glucan polymers such as glycogen, whereas the isoamylases are unable to hydrolyse pullulan, but can hydrolyse α-(1 → 6)-linkages in amylopectin and glycogen. In rice, reduction in pullulanase activity has no pleiotropic effects on other enzymes of starch synthesis; double mutants showing loss of pullulanase and isoamylase-1 show increased accumulation of phytoglycogen and water-soluble polysaccharides over those in the isoamylase-1 mutant alone, indicating partial overlap of function between pullulanase and isoamylase-1 (Fujita *et al.*, 2009).

The precise roles for the isoamylase-type and pullulanase-type DBEs in starch biosynthesis are not fully understood. Two models have been proposed which could define a role for the DBEs in starch synthesis and phytoglycogen accumulation. The 'glucan-trimming' (pre-amylopectin trimming) model proposes that glucan trimming is required for amylopectin aggregation into an insoluble granular structure (Ball *et al.*, 1996; Myers *et al.*, 2000). DBE activity is responsible for the removal of inappropriately positioned branches (pre-amylopectin) generated at the surface of the growing starch granules, which would otherwise prevent crystallization. As such, the debranched structure would favour the formation of parallel double helices, leading to polysaccharide aggregation. The surface of immature starch granules contains numerous short chains, consistent with this model (Nielsen *et al.*, 2002). An alternative to the glucan-trimming model proposes that DBEs function in starch synthesis indirectly in a 'clearing' role, removing soluble glucans from the stroma which are substrates for the amylopectin-synthesizing enzymes (SSs and SBEs), and thereby preventing the random/futile synthesis of glucan polymers by these enzymes which could cause accumulation of phytoglycogen (leading to a reduction in the rate of starch synthesis). This model could also explain the accumulation in phytoglycogen at the expense of amylopectin observed in DBE mutants in a number of species (Morris and Morris, 1939; James *et al.*, 1995; Mouille *et al.*, 1996; Zeeman *et al.*, 1998; Wattebled *et al.*, 2005, 2008; Fujita *et al.*, 2009).

Despite this experimental evidence, some recent studies have argued against a mandatory role for DBEs in starch granule formation. The loss of all four DBEs from *Arabidopsis* leads to the accumulation of maltose and soluble branched glucans which are degraded by α- and β-amylases; however, additional loss of a chloroplastic form of α-amylase (AMY3) restores starch synthesis in this quadruple mutant (Streb *et al.*, 2008). Furthermore, it has been suggested that the role played by DBEs in amylopectin crystallization is a sole feature of the Chloroplastida (see Deschamps *et al.*, 2008b). For example, apicomplexan parasites, such as the starch-accumulating *Toxoplasma gondii*, do not contain functional isoamylases (Coppin *et al.*, 2005), suggesting that, in these organisms at least, crystalline amylopectin formation is possible in the absence of DBEs.

Other enzymes implicated in the pathway

D-enzyme

Plastidial 1,4-α-D-glucan:1,4-α-D-glucan, 4-α-D-glucanotransferase (disproportionating enzyme, D-enzyme, E.C. 2.4.1.25) is present in many different starch-containing organs of plants (Lin *et al.*, 1988; Takaha *et al.*, 1993). D-enzyme transfers two of the glucosyl units from malto-triose on to a longer glucan chain, making them available to the β-amylases, and the resulting glucosyl monomer becomes available for

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export from the plastid via a glucose transporter in the inner envelope membrane (Weber *et al.*, 2000; Niittylä *et al.*, 2004). Knock-out mutants of D-enzyme in *Arabidopsis* show reduced rates of nocturnal starch degradation (Critchley *et al.*, 2001), indicating that this enzyme plays a part in the pathway of (chloroplast) starch degradation. Mutants of *C. reinhardtii* specifically lacking D-enzyme (in the STA11 locus) show a severe decrease in starch content and clearly indicate a role for the enzyme in starch (amylopectin) synthesis (Colleoni *et al.*, 1999a, b; Ball *et al.*, 2003). Analysis of D-enzyme levels in the developing endosperm of wheat is consistent with a role in starch biosynthesis (Bresolin *et al.*, 2005). Its precise role in plant storage tissues remains unclear.

Starch phosphorylase

Starch phosphorylase (SP, E.C. 2.4.1.1) catalyses the reversible transfer of glucosyl units from glucose 1-phosphate to the non-reducing end of α-1,4-linked glucan chains and may be driven in either a synthetic or a degradative direction by the relative concentrations of the soluble substrates. However, the role of SP in higher plant starch metabolism is unclear. Plastidial SP (referred to as Pho1 or the L-form) is characterized by a higher affinity for amylopectin than glycogen, and is inhibited by ADP-glucose (Dauvillée *et al.*, 2006). Kinetic analysis of maize endosperm Pho1 shows that the phosphorolytic reaction is favoured over the synthetic reaction in the presence of MOS (Mu *et al.*, 2001). Phosphorylases from various sources occur as homotetrameric or homodimeric assemblies (Nakano and Fukui, 1986; Albrecht *et al.*, 1998; Buchbinder *et al.*, 2001). Elution profiles of maize amyloplast SP from gel permeation chromatography are consistent with a homotetrameric form of SP, although evidence suggests SP may exist as monomers or lower complexity multimers when associating with other enzymes of starch synthesis (Liu *et al.*, 2009). Although the precise role of Pho1 in starch metabolism is unclear, it probably contributes to starch synthesis, as a number of studies have found that both SP/Pho1 gene expression and activity measurements correlate with starch biosynthesis (Van Berkel *et al.*, 1991; Duwenig *et al.*, 1997; Yu *et al.*, 2001), and SP transcripts in potato increase in the light (Albrecht *et al.*, 2001). Analysis of *STA4* mutants of *C. reinhardtii*, lacking the plastidial SP isoform, show reduced starch content and abnormally shaped granules with high amylose content, arguing for a role for SP in starch biosynthesis (Dauvillée *et al.*, 2006). In storage tissues the activity of the plastidial isozyme (L-form) of SP may be regulated by proteolysis of a 78-amino acid peptide (termed L78). Removal of L78 by an endogenous protease increased the catalytic activity of SP in the phosphorolytic direction (Chen *et al.*, 2002). Phosphorylation of L78 at a single serine residue increases the susceptibility of the plastidial L-form of SP to proteolytic degradation (Young *et al.*, 2006). This suggests a role for protein phosphorylation in the regulation of catalytic activity of SP, which is supported by other studies that show direct phosphorylation of the protein in storage tissues such as cereal endosperm (Grimaud *et al.*, 2008; Liu *et al.*, 2009). One role for Pho1 could be in controlling the availability of MOS, which are required for amylose synthesis (see above), and acting in a 'clearing' role similar and complementary to that proposed for the DBEs. It has been suggested that D-enzymes work in conjunction with SP, contributing to starch synthesis via the phosphorolytic SP reaction (Takaha *et al.*, 1998). According to this model, which is based on the 'glucan-trimming' model proposed by Ball *et al.* (1996), short-chain MOS liberated in the trimming reaction by DBEs are converted to longer-chain glucans by D-enzyme, which in turn are available for phosphorolysis by SP, liberating glucose 1-phosphate used to synthesize ADP-glucose by plastidial AGPase. Indeed, the phosphorolytic SP reaction is stimulated by the presence of D-enzyme (Colleoni *et al.*, 1999b).

Coordination of enzyme activities during starch granule synthesis

During starch deposition in the seed, all of the enzyme classes (and most of the isoforms within each class) involved in amylopectin synthesis (and degradation) are expressed simultaneously. This implies that some degree of coordination between these potentially counter-productive enzyme activities is required for granule assembly. Functional interactions and physical associations between enzymes involved in amylopectin biosynthesis have been suspected for some time, based on the analysis of enzyme kinetics and mutations in the pathway (Hawker *et al.*, 1974; Boyer and Preiss, 1979, 1981; Gao *et al.*, 1998; Beatty *et al.*, 1999; Dinges *et al.*, 2001, 2003; Nishi *et al.*, 2001; Seo *et al.*, 2002; Yao *et al.*, 2002, 2004; Colleoni *et al.*, 2003). More recently, biochemical analysis of plastid extracts has provided direct evidence for protein–protein interactions between enzymes of starch synthesis (Tetlow *et al.*, 2004, 2008; Hennen-Bierwagen *et al.*, 2008). Experiments with isolated amyloplasts from wheat endosperm have shown that some of the key enzymes of the starch (amylopectin) biosynthetic pathway form heteromeric protein complexes, the formation of which is dependent upon phosphorylation status. Phosphorylation of SBEI, SBEIIb and SP by plastidial protein kinase(s) results in the formation of a protein complex between these enzymes which is lost following *in vitro* dephosphorylation (Tetlow *et al.*, 2004). Our knowledge of the regulation of the enzymes of starch synthesis and degradation by protein

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phosphorylation and complex assembly has been summarized in a recent review by Kötting *et al.* (2010). Protein phosphorylation modifies the catalytic activities of starch branching enzymes in amyloplasts and chloroplasts of wheat (Tetlow *et al.*, 2004), and may also play a role in their association with starch granules (Grimaud *et al.*, 2008). Early studies with SSs and SBEs showed that the activity of the former can be stimulated in the presence of the latter and salts such as sodium citrate (Hawker *et al.*, 1974), and the potentially cyclic catalytic activities of SSs and SBEs led some researchers to propose amylopectin synthesis via a 'synthetase–branching enzyme complex' (Schiefer *et al.*, 1973). Recent work with wheat and maize amyloplasts demonstrated protein–protein interactions between SSI, SSII and SBEII isoforms, and the improved stability of these protein complexes under relatively high salt (NaCl) concentrations (Hennen-Bierwagen *et al.*, 2008; Tetlow *et al.*, 2008). Formation of a protein complex of approximately 260 kDa between SSI, SSII and SBEII in the endosperms of wheat and maize requires protein phosphorylation, and *in vitro* dephosphorylation causes its disassociation (Tetlow *et al.*, 2008; Liu *et al.*, 2009) (Fig. 4). Further analysis of this protein complex in maize endosperm showed that the SBEIIb in the complex is phosphorylated (Liu *et al.*, 2009). Other, larger protein complexes (of approximately 670 kDa), which include the SSIII isoform, are present in maize endosperm, and formation of these complexes is also phosphorylation dependent (Hennen-Bierwagen *et al.*, 2009). Biochemical analysis of mutants will likely benefit our understanding of the regulation of the pathway of starch biosynthesis. For example, the *ae*$^{-}$ mutant of maize lacks SBEIIb, which

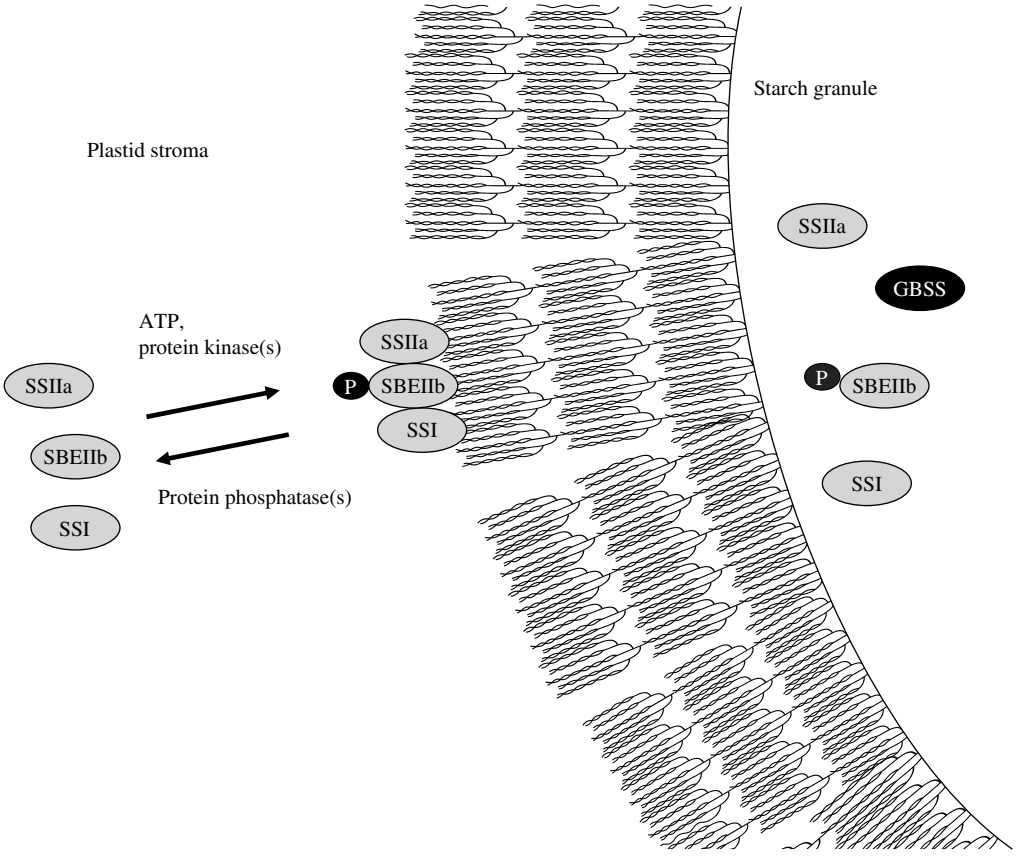

Figure 4. Protein–protein interactions between amylopectin-synthesizing enzymes during starch granule formation. Many of the enzymes of amylopectin biosynthesis are in the plastid stroma and also associated with a number of protein complexes (see text for details). One such protein complex (in wheat and maize) occurring between SBEII, SSI and SSIIa is shown, the assembly of which is dependent on protein phosphorylation (Hennen-Bierwagen *et al.*, 2008; Tetlow *et al.*, 2008). In maize SBEIIb is phosphorylated (denoted as 'P' symbols in the diagram) (Liu *et al.*, 2009). *In vitro* dephosphorylation with alkaline phosphatase causes disassociation of the protein complex assembly; *in vivo* this function is presumably performed by a plastidial protein phosphatase. The diagram also shows how the proteins, which are present as complexes in the stroma, are partitioned within the starch granule as 'granule-associated' proteins, and also shows the granule-associated GBSS. It is proposed that the SS/SBEII protein complexes work at the periphery of the nascent granule, being involved in assembly of the clusters of short- to intermediate-sized glucan chains which eventually form the semi-crystalline lamellae of starch granules within which the enzymes become entrapped. There is currently no evidence to suggest they remain as complexes when they become granule-associated.

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is the major form of SBEII in the endosperm, resulting in starches with reduced branch point frequency and longer glucan chains within clusters (termed a 'high-amylose' starch, but is in fact a starch with modified amylopectin) (Hilbert and McMasters, 1946; Banks *et al.*, 1974; Klucinec and Thompson, 2002). Analysis of protein complexes in the amyloplasts of the *ae*$^-$ mutant reveals a distinct pattern of protein–protein interactions which indicate functional complementation for the loss of SBEIIb by SBEI (which has a low branching frequency with amylopectin, and transfers longer glucan chains than SBEII). In *ae*$^-$ mutant endosperm amyloplasts the wild-type protein complex (SSI, SSIIa and SBEIIb) is replaced by a novel complex consisting of SSI, SSIIa, SBEI, SP and SBEIIa (Liu *et al.*, 2009). In the protein complex in *ae*$^-$ amyloplasts both SBEI and SP are phosphorylated (Liu *et al.*, 2009). Furthermore, differences in stromal protein complexes are mirrored in the complement of starch-synthesizing enzymes detected within the starch granules (granule-associated proteins, see below) of each genotype, supporting the idea that protein complexes play a functional role in amylopectin biosynthesis. The presence of phosphorylated forms of proteins in starch granules (granule-associated proteins), which are also components of phosphorylation-dependent protein complexes (Tetlow *et al.*, 2004; Grimaud *et al.*, 2008), reinforces the idea that complexes involved in amylopectin biosynthesis constitute the functional units which become entrapped within the granule (Liu *et al.*, 2009). The operation of SSI, SSIIa and SBEII isoforms (working as one or more protein complexes) in semi-crystalline cluster formation, and SSIII and SBEI (probably working with other SSs and SBEs) involved in the synthesis of the cluster-connecting glucan chains in the amorphous zones, fits well with the 'two-step branching and improper branch clearing' model proposed by Nakamura (2002). In this model, glucan extension and branching activities occur in both the cluster and amorphous regions of the granule. During the whole process DBEs play critical roles in trimming the cluster shape at the periphery of the growing granule, since they act on sparsely localized branch points more rapidly than those in the densely packed semi-crystalline lamellae (Ball *et al.*, 1996; Mouille *et al.*, 1996; Nakamura *et al.*, 1998). Consequently, those enzymes involved in synthesis of the amorphous region and in the trimming process, remain as soluble proteins in the amyloplast stroma, or are removed during the washing of starch granules.

The role of protein complex formation between these starch biosynthetic enzymes in starch synthesis is not fully understood, but it is thought that such functional assemblies improve the efficiency of polymer construction as the product of one reaction becomes a substrate for another within the complex (substrate channelling). The formation of complexes of starch metabolic enzymes via protein–protein interactions may directly alter the kinetic properties of individual components of the complex through conformational changes. Other, not mutually exclusive, functions of these multiprotein complexes could be the shielding of the growing polymer from degradative enzymes also present within the plastid. At a higher level of organization, the formation of protein complexes during starch biosynthesis may promote a certain favoured, necessary three-dimensional structure within the growing polymer, e.g. in the case of amylopectin this could be a structure necessary for, or promoting, crystallinity (clustered branch points, side chains of defined length, particular side-chain packing). In this context, such multiprotein complexes may act as a form of 'carbohydrate chaperonin'.

Direct regulation of enzyme activity, and the regulation of groups of reactions by protein complex formation, in some cases involves the phosphorylation of target proteins followed by the formation of a complex with 14-3-3 proteins; this appears to be a general mechanism for regulating enzymes and pathways in eukaryotic systems (for general reviews of plant 14-3-3 proteins, see Sehnke and Ferl, 2002; Comparot *et al.*, 2003; Roberts, 2003). These proteins are a structurally highly conserved group of approximately 30–35 kDa, and exist as dimers. Isoforms have been identified in chloroplasts from pea leaves (Sehnke *et al.*, 2000), although there is currently no evidence for the presence of 14-3-3 proteins in other plastid types. The involvement of 14-3-3 proteins in the regulation of starch metabolism was proposed by Sehnke *et al.* (2001), who showed that a form of 14-3-3 protein (from the ϵ sub-group) is present in *Arabidopsis* leaf starch as a granule-associated protein. 14-3-3 Proteins have also been detected inside starch granules from developing barley endosperm (Alexander and Morris, 2006) and immature maize pollen (Datta *et al.*, 2002), further implicating this family of proteins in starch metabolic pathways. Phosphorylation-dependent binding of key starch metabolic enzymes (SSI, SSII, SBEIIa and GBSS) to 14-3-3 proteins was demonstrated in the amyloplasts of developing barley endosperm (Alexander and Morris, 2006), suggesting a mechanism for protein complex formation between the enzymes of starch biosynthesis described above. Despite the above circumstantial evidence, a direct role for 14-3-3 proteins in starch metabolism remains to be established.

Starch granule proteins

Starch granule proteins are a group of proteins which are consistently found associated with these structures following extensive washing with detergents and acetone as well as protease treatments (Denyer *et al.*,

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1993, 1995; Rahman *et al.*, 1995; Mu-Forster *et al.*, 1996; Borén *et al.*, 2004). Granule-associated proteins are routinely found in higher plants and green algae, but it is not clear whether they play a functional role within the granule matrix, or are present as a result of entrapment during polymer biosynthesis. Some proteins appear to be exclusively associated with the starch granules, e.g. GBSS isoforms involved in amylose biosynthesis (Denyer *et al.*, 1996a). Other proteins that are detected exclusively in starch granules include: SBEIc, a large 152 kDa form of SBEI within the large A-type starch granules of storage starches from the endosperms of *Festucoideae* (e.g. *Triticum*, *Hordeum* and *Secale* species) (Båga *et al.*, 2000; Peng *et al.*, 2000), and α-glucan-water dikinase [ATP:α-1,4-glucan, water phosphotransferase (GWD), E.C. 2.7.9.4, previously designated R1], which phosphorylates glucose residues at the C6 position in amylopectin, probably preparing the polymer for degradation by hydrolytic enzymes (Ritte *et al.*, 2002, 2004; Kötting *et al.*, 2005; Hejazi *et al.*, 2008; Zeeman *et al.*, 2010). Another enzyme transiently associated with starch granules is phospho-α-glucan-water dikinase [ATP:phospho-α-1,4-glucan, water phosphotransferase (PWD) E.C. 2.7.9.5] involved in phosphorylating amylopectin at the C3 position of glucose residues pre-phosphorylated by GWD during starch degradation (Baunsgaard *et al.*, 2005; Kötting *et al.*, 2005). However, most other granule-associated enzymes appear to be partitioned to varying degrees between the soluble (stromal) phase and starch granules. Other proteins in the starch pathway probably play a functional role at, or inside, the growing granule, but are more loosely associated. The amylopectin-synthesizing enzymes routinely found as granule-associated proteins in cereal endosperms are all involved in the synthesis of short- to intermediate-length glucan chains, which are known to form clusters, resulting in semi-crystalline lamellae (see Ball and Morell, 2003). The gradual, periodic synthesis of amylopectin clusters joined via amorphous lamellae, as proposed by the French (1984) and Hizukuri (1986) models, has been suggested to be the cause of entrapment of proteins (specifically, amylopectin biosynthetic enzymes) within granules. However, detailed kinetic analysis of some SS isoforms suggests that granule association of these proteins, at least, may be a product of their increased affinity for longer glucan chains during catalysis (Commuri and Keeling, 2001). In higher plants, only a specific group of amylopectin-synthesizing proteins are consistently observed within granules (SSI, SSIIa and isoforms of SBEII), whereas other enzyme classes, which likely play an important role in amylopectin biosynthesis, are either absent from the granule (SSIV, SBEI, SP, isoforms of isoamylase, pullulanase-type DBE and D-enzyme) or are found in very small amounts (SSIII). SSIII and SBEI, respectively, elongate and branch relatively long glucan chains (Gao *et al.*, 1998; Blauth *et al.*, 2002), and it has been proposed these enzymes may function in glucan chain formation between clusters in the amorphous region of the starch granule (Nakamura, 2002; James *et al.*, 2003). Notably, all of the granule-associated proteins known to be involved in amylopectin synthesis are also components of identified soluble protein complexes in the endosperms of wheat and maize (Hennen-Bierwagen *et al.*, 2008; Tetlow *et al.*, 2008), and it has been suggested that they become entrapped as functional protein complexes during amylopectin cluster formation (Liu *et al.*, 2009) (see above). Of the starch granule-associated proteins, some are phosphorylated, e.g. SBEII forms (Tetlow *et al.*, 2004; Grimaud *et al.*, 2008). It is not yet clear whether granule-associated phosphoproteins are present as a consequence of protein complex assembly, or if protein phosphorylation causes granule-association.

Analysis of mutants of the starch pathway strengthens the notion that stromal protein complexes involved in α-polyglucan cluster formation become granule localized. A number of mutants in the pathway display loss of proteins from the starch granule proteome (Morell *et al.*, 2003; Borén *et al.*, 2004; Regina *et al.*, 2005; Umemoto and Aoki, 2005; Grimaud *et al.*, 2008), consistent with the idea that their loss (they are components of multi-enzyme complexes in amyloplasts) causes disruption of the complex, preventing the remaining proteins from becoming granule-associated. Some mutations (notably the *ae*$^{-}$ mutant of maize) cause association of novel proteins within the starch granule, which appear to correlate with the altered composition of protein complexes in the amyloplast stroma (see section on protein complexes).

Other proteins that are not directly involved in starch biosynthesis or degradation, but have no assigned function, have been identified in starch granules. These include a plastid-localized starch granule-associated glycogen synthase kinase (GSK-3)-like protein kinase in *Medicago* and *Arabidopsis*. Increased expression of the GSK-3-like kinase, termed MsK4, results in starch accumulation in leaves under saline conditions (Kempa *et al.*, 2007). It is hypothesized that this granule-associated protein kinase regulates starch synthesis or turnover during stress. In addition, starch granule-associated amylopectin phosphatases have been identified to play a key role in starch degradation by priming the polymer for degradation via phosphorylation of glucose residues (Fordham-Skelton *et al.*, 2002; Kerk *et al.*, 2006; Niittylä *et al.*, 2006; Sokolov *et al.*, 2006; Kötting *et al.*, 2009; Comparot-Moss *et al.*, 2010) and are related to the laforin protein which regulates mammalian glycogen metabolism (Fernandez-Sanchez *et al.*, 2003; Lohi *et al.*, 2005). Pores have been observed at the surface of starch granules from a number of species (Fannon *et al.*, 1992;

Glaring *et al.*, 2006), and are present in both A- and B-types in cereals (Kim and Huber, 2008). The pores are openings of channels connecting the external surface to the inside of the granule (Huber and BeMiller, 1997). Recent analysis of these channels in maize endosperm indicates the presence of actin-like and tubulin-like (FtsZ) proteins, along with proteins involved in starch biosynthesis such as AGPase and SS (Benmoussa *et al.*, 2010). Their presence implies physical connections between the cytosol, plastid stroma and starch granule. Analysis of developing endosperm cells in wheat by Briarty *et al.* (1979) suggested to these authors that enzymes of starch synthesis associate with plastid tubuli at the initial stages of granule deposition, and that there is a direct relationship between starch granule volume and amyloplast tubule volume.

Granule initiation and control of starch granule size

Despite the considerable advances in our knowledge of the pathway of starch biosynthesis, the factors controlling starch granule initiation and granule size remain unclear. For a detailed current overview of initiation of storage glucan synthesis the reader is referred to a recent review by D'Hulst and Mérida (2010). The locus of granule initiation is termed the hilum, located in the centre of the growing granule and thought to be essential for granule initiation/priming (Ziegler *et al.*, 2005). It has been suggested that the granule forms from the hilum, from which radially oriented microtubules emanate, and these become the channels that terminate as openings (pores) on the granule surface (Fannon *et al.*, 2004). A system of glucan polymer (starch) initiation similar to the proteinaceous priming mechanism of glycogen biosynthesis (Rodriguez and Whelan, 1985; Whelan, 1986) has been proposed in higher plants, with the discovery of a glycogenin-like starch initiation protein in *Arabidopsis* (PGSIP1) (Chatterjee *et al.*, 2005). Loss of isoamylase activity from barley, rice and potato leads to an increase in granule initiation (Burton *et al.*, 2002; Bustos *et al.*, 2004; Kawagoe *et al.*, 2005), consistent with the idea that isoamylases suppress the sites of new granule initiation, although DBEs are probably not directly involved in initiating granule synthesis (Delatte *et al.*, 2005; Wattebled *et al.*, 2005; Streb *et al.*, 2008). Pullulanase-type DBE (limit dextrinase) activity may also play a role in determining granule size. Down-regulation of a pullulanase-type DBE inhibitor activity in barley causes a reduction in the small (B-type) granules, reduces amylose content, alters amylopectin glucan chain-length distribution and reduces starch content (Stahl *et al.*, 2004). Specific classes of SS may initiate starch granule formation. Mutants of *Arabidopsis* lacking SSIV are incapable of synthesizing more than one starch granule per plastid (the single granule has a distinct glucan organization in the hilum), indicating its involvement in granule initiation (Roldán *et al.*, 2007). Recent data by Szydlowski *et al.* (2009) suggest that the role of SSIV in granule initiation can be replaced by the phylogenetically related SSIII, and that elimination of both SSIII and SSIV prevents starch synthesis altogether in *Arabidopsis*, thus indicating a dual role for these proteins in granule initiation. Glycogen-like structures have been associated with the priming of insoluble starch-like polyglucans (Puteaux *et al.*, 2006), leading Szydlowski *et al.* (2009) to speculate upon a role for SSIV in the interaction and formation of these polyglucans in the seeding of the starch granule. To date there is no evidence for autoglucosylation activity by SSIII or SSIV acting in a priming role analogous to glycogenin or prokaryotic glycogen synthase.

There is huge variation in the size and morphology of starch granules from different species and genotypes within a species, although in many species granule size is homogeneous for a given developmental stage (Jane *et al.*, 1994). Populations of different granule size show different physicochemical characteristics (reviewed by Lindeboom *et al.*, 2004). Granule size and morphology range from $<0.5\,\mu m$ diameter for the spherical granules in cells of the picophytoplanktonic green alga *Ostreococcus tauri* (Ral *et al.*, 2004), $\sim 5\,\mu m$ for the round, flattened granules of *Arabidopsis* (Delvallé *et al.*, 2005; Wattebled *et al.*, 2008), $2-30\,\mu m$ for polyhedral granules of maize (Buléon *et al.*, 1998) and $20-100\,\mu m$ for the large, oval granules in potatoes and pea (*Pisum sativum* L.) cotyledons (Buléon *et al.*, 1998). Cereals belonging to the Festucoid family of grasses, such as wheat, barley, oats and rye, are characterized by a bimodal (or trimodal) distribution of starch granules in storage tissues (Evers, 1973; Meredith, 1981; Bechtel *et al.*, 1990). Large lenticular-shaped A-type granules ($15-30\,\mu m$ diameter) are formed early in endosperm development, while the smaller B-type ones (averaging $5-9\,\mu m$ in diameter) are formed later (Parker, 1985). Recent observations suggest a third, smaller, class of starch granules ($<5\,\mu m$, termed C-type granules) in cereals such as wheat (Wilson *et al.*, 2006). The difficulty in isolating and quantifying C-type granules has led to their inclusion as B-type granules in many studies. Analysis of A- and B-type starch granules from developing wheat endosperm indicates that they have distinct physicochemical properties (Wei *et al.*, 2010). The selective advantage conferred upon plants displaying a bi/trimodal distribution of starch granules in storage tissues is at present unclear, but the formation of smaller-sized granules may offer a more efficient means of packing more stored carbon in a cell. It has been proposed that the different classes of starch granules in wheat endosperm (the large A-type,

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and smaller B- and C-type granules) are produced in different cell types at different stages of endosperm development (May and Buttrose, 1959; Briarty *et al.*, 1979; Parker, 1985). The smaller B-type granules present in wheat and barley form inside protrusions from the amyloplast termed stromules (stroma containing tubules) (Buttrose, 1963; Köhler *et al.*, 1997; Langeveld *et al.*, 2000; Bechtel and Wilson, 2003). Experiments with potato suggest that the presence of proteins with starch-binding domains may in some way limit glucan chain extension, and ultimately, granule size (Ji *et al.*, 2004). The specific factors controlling the size of starch granules in any species are unknown, however.

Summary

Starch is a raw material of fundamental significance to many human activities, and ultimately to civilization. It is therefore of crucial importance to understand the various processes involved in starch granule assembly, and the regulatory factors governing carbon allocation for this process inside plastids, so that increased food demands and the desire for specific starches in the food and non-food sectors can be met. In addition to the important contribution being made by plant breeders, future increases in starch yield and improvements in quality of agriculturally produced starches will be driven by the isolation of natural mutants or through the use of genetic engineering. Underpinning this latter technology is the cloning and characterization of genes directly and indirectly associated with starch metabolism for any given crop.

The availability of complete plant genome sequences now offers more detailed insight into the potential genes involved in starch metabolism, and points to key differences between different model systems in terms of the number of isoforms of particular enzymes (e.g. multiple SSII and SBEII genes in rice, which are not present in *Arabidopsis*). Different suites of enzymes are clearly involved in the synthesis of leaf and endosperm starches in rice, and such information should present a note of caution when attempting to make generalized conclusions from one species to another about the roles of particular isoforms.

The isolation and analysis of natural and insertion mutants has made, and will continue to make, an invaluable contribution towards our understanding of starch metabolism in higher plants. The tendency has been to try to explain the functions of genes based solely on their mutant phenotype. The many pleiotropic effects arising from single mutations, coupled with research indicating that some of the starch metabolic enzymes may operate within complexes, suggest that this approach is, in some cases, too simplistic. Some of the mutant phenotypes, therefore, are probably the result of the disruption of various protein complexes and associations, and not just the mutated gene.

The diverse pleiotropic effects observed with various mutations in genes of the starch metabolic pathways indicate that there are probably many more interactions between the enzymes of starch metabolism and other signalling pathways than have been identified to date. More work is required to discover new interactions between starch metabolic enzymes in starch-storing crops, and to elucidate the mechanisms and signalling cues that govern this aspect of metabolic regulation, as well as to identify the components controlling protein complex formation in the plastid. Signalling pathways involved in connecting photosynthate delivery in source leaves with plastidial carbon metabolism in sink tissues are still not clearly understood (Smith and Stitt, 2007). The exact role of the different protein complexes involved in starch biosynthesis needs to be elucidated. Given that assembly of most of these is dependent on protein phosphorylation, further research promises to uncover many new potential targets for manipulation of starch structure and yield.

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