Bioregulation of starch accumulation in developing seeds

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Starch is a major constituent of cereal and most legume seeds and, thus, an important determinant of yield of these seeds. This paper reviews the processes controlling starch deposition in the developing seeds. The capacity of the seed (i.e. cell and amyloplast numbers) to accommodate starch is determined during the first one-third phase of seed development and the availability of carbohydrates to the seed during this phase influences this capacity. Sucrose is the primary source of reduced carbon for starch synthesis in the seeds. In the cytosol of starch-storing cells, sucrose is catabolized to generate hexose phosphates (Glc-1-P or Glc-6-P), which are transported into the amyloplast. These are used to generate ADPGlc by ADPGlc–PPase, which is then used by starch synthase(s) and branching enzymes to produce starch. Although the regulatory role of ADPGlc–PPase (i.e. its activation by 3-PGA and inhibition by Pi) in starch metabolism in photosynthetic tissues is well documented, based on kinetic characteristics and molecular biology studies, its similar role in the developing seeds has been questioned.

Both cereal and legume seeds and their by-products make up a major part of the human diet. Cereals contribute 50% of the per capita energy intake worldwide and 65% in the developing countries and Asian centrally planned economies[1]. Although the worldwide production of grain legumes is small relative to cereals, its relative contribution to human nutrition is greater than its relative production. Approximately 700 million people use legumes as an essential part of their diet[2]. Since starch is the main storage component of these seeds, an understanding of the mechanisms controlling the deposition of this component will allow the design of new genetic engineering strategies for improvement of quantity and quality of these seeds.

Several reviews over the past decade on the different aspects of starch synthesis have appeared[3–13]. Since there is ample evidence[14,15] that starch deposition in the seeds is most likely limited by the factors operating close to or within the grain, an attempt has been made to review our understanding of the processes that operate within the seed to control the synthesis and deposition of starch.

Ontogenetic pattern of starch accumulation

Early growth in all the seeds is primarily due to cell multiplication and cell expansion of the prospective storage tissue(s) and the surrounding structures like the seed coat, testa or pericarp without much reserve formation. In wheat, little starch is detectable during the coenocytic phase, but is present shortly after the cell wall formation begins[16]. Also, no starch is detectable in the cotyledons of certain leguminoseae seeds immediately after fertilization[17]. Only after the radical begins to differentiate are the starch granules detectable in the cotyledons. Considerable variation is observed among species and cultivars within the genus *Phaseolus* in the time lag between flowering and first appearance of starch[18]. Environmental conditions prevailing, especially the temperature, at the time of seed formation strongly affect the appearance of the first granules. For example, in wheat the starch granules first appear between day 4 and 14 after anthesis depending upon the temperature[19]: the higher the temperature, the earlier is the appearance.

After the initial lag phase, seeds show a striking transition from a very slow build-up of starch to the rates typical of actively accumulating seeds. Such a transition, in majority of the seeds, often accompanies the last phase of cell division and the onset of more rapid cell expansion. About four-fifths of the starch is deposited in the endosperm of wheat after cell division has ended, the mean weight of starch per cell rises by fourfold to fivefold while the volume of the cells themselves increases by about twofold[20]. Similarly, in pea, almost all the starch is laid down in the cotyledons without much change in the water content of the seed and with only a small increase (20%) in the mean volume per cell[21].

The rates of deposition of starch during active grain-filling stage varies greatly among species. Values as high as 10 mg per kernel per day are common for maize, in contrast to the much lower rates, less than 1–2 mg per grain per day, for small-grained cereals (wheat, rice, barley, rye, etc.) and millets (sorghum and pennisetum). Values of about 2 mg (*P. arvense*) to 4 mg (*P. sativum*) per day have been recorded[21,22]. Substantial differences between cultivars have been found too: maize

hybrids have higher rates than their inbred parents[23] and there are differences among the cultivars of wheat[24, 25] and barley[26]. Mutations strongly affect the rate of starch deposition. A 20% reduction in the starch content of mutant 1508 compared to normal Bomi barley is entirely due to the lower rate of starch accumulation[27].

Starch storage organelles

Starch in the seeds and other starch-storing tissues is deposited in the specialized organelles, the amyloplasts, which differentiate from proplastids[28]. This conversion is accompanied by changes in the transcriptional activities of plastid genes[29]. In most seeds, proplastids are recognizable at very early stages of cellular development[30]. They most likely arise from the division of other proplastids and are transmitted from the mother to the daughter cells[31].

At maturity, each amyloplast may contain only one starch granule (known as simple amyloplast) as in wheat[32], maize[33] and barley[34], or there may be several of them (known as compound amyloplast; numbers around 100 are not uncommon) as in rice[35] and oats[36]. Most of the seeds have only uniformly sized amyloplasts, but barley (some barley species do have unimodal size distribution[37]), wheat[36] and rye[38] have a bimodal size distribution: large (> 10μm in diameter) A-type and small (< 10 μm in diameter) B-type. A few days after anthesis, a single cell in the developing wheat endosperm may contain about 500 A-type amyloplasts[20]. However, with the passage of time, this number first declines and then rises swiftly as the more numerous B-type amyloplasts appear. This reduction in number is mainly ascribed to cell division continuing longer than the divisions in the A-type plastids (on an average, an A-type amyloplast undergoes about 3.5 divisions[20]) so that the younger cells (sub-aleurone) contain fewer amyloplasts than the older ones. Small B-type granules appear to be initiated in the stroma of A-type amyloplasts[32] and then separate by evagination or constriction of the A-type amyloplasts. B-type starch granules are also found within the narrow protrusions extending from the surface of A-type amyloplasts; but it is not certain whether they are initiated in the protrusions or passed into them from the stroma.

Changes in chemical composition

Amylose and amylopectin, the two major components of starch, are often deposited together during seed development, though not in a fixed proportion. The relative proportion of amylose increases with development in most of the seeds[3, 34, 35] and, at maturity, amylose in the normal cereal endosperm constitutes about 25% of the total starch[10]. In *waxy* (*wx*) genotypes of maize, rice, barley and sorghum, the percentage of amylose is very low, and changes little with time, but in double mutant *amylose extender* and *waxy* (*aewx*) of maize, the amylose percentage decreases during development[39]. The *r* (rugosus) mutation in pea seeds (resulting in wrinkled seeds) dramatically reduces the amylopectin content, whereas *rb* mutation increases it[40].

Sources of reduced carbon for starch synthesis

Sucrose is generally believed to be the primary carbon source for starch biosynthesis in the developing seeds[11, 14, 41]. It is translocated via the phloem from the photosynthetic tissues to the developing seeds. In wheat and barley, most of the photosynthate produced during the post-anthesis period and probably some produced prior to anthesis is used for grain filling[42]. The flag leaf, the stem immediately below the ear and the ear itself, are the main sources of carbon for the developing grain. Some of the reduced carbon may come from the stem reserves[42]. In pea and other legumes, the leaf and the stipule immediately subtending the pod serve the same function[43]. In legumes, the pod wall also plays an important role in the carbon economy of the developing seed[44]. It is photosynthetically active, and in pea it may contribute 30–40% of the carbon of the mature seed[43].

Seed coat in most of the legumes is also capable of reassimilating a proportion of the carbon dioxide lost through the respiration of the developing embryo. Although the contribution of seed coat to the carbon economy of the seed varies through development, it may reduce carbon dioxide loss by about 20% (ref. 45). Similarly, the pericarp in the case of cereal grains is photosynthetically active and may contribute some reduced carbon to the developing seed[46]. But how much contribution of the reduced carbon comes from this tissue is not known.

Biochemical pathway of starch synthesis

Before the mid-eighties, several workers[3, 47–50] proposed pathways for conversion of sucrose to starch; however, most of these pathways were based on the studies with isolated enzymes, where compartmentalization of enzymes and the metabolites was not considered, and hence, as such, are less likely to operate *in vivo*. Within the last decade, several investigators[51, 52] have developed methods for successful isolation of amyloplasts, which has provided a better understanding of the localization of enzymes and metabolites in different compartments of the cell.

Metabolism of sucrose in the cytosol

In the cytosol of the starch-storing cells, sucrose may be cleaved by sucrose synthase (SS) and/or by invertase (Figure 1). The presence of both the enzymes in the

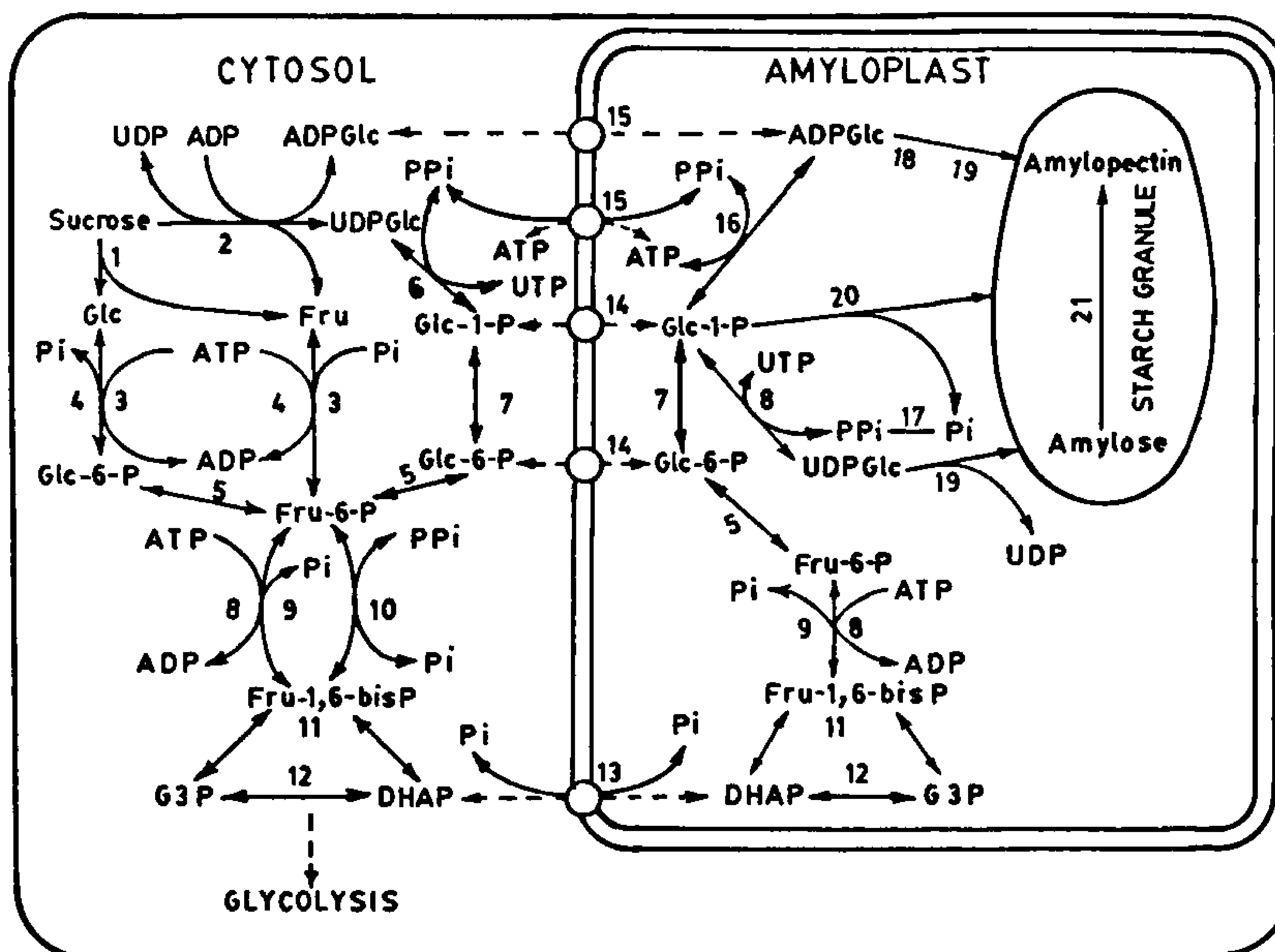

Figure 1. Proposed scheme of starch synthesis in the developing seeds[68]. Enzymes designated by numbers are. 1, invertase, 2, sucrose synthase; 3, hexokinase; 4, hexose-6-phosphatase; 5, glucose phosphate isomerase; 6, UDPGlc-PPase; 7, phosphoglucomutase; 8, FBPase; 9, PFKase; 10, PPi-PFKase; 11, aldolase; 12, triose phosphate isomerase; 13, triose-P/Pi translocator; 14, hexose phosphate translocator; 15, adenyltranslocator; 16, ADPGlc-PPase; 17, pyrophosphatase; 18, soluble starch synthase; 19, granule-bound starch synthase; 20, phosphorylase; 21, branching enzyme.

starch-storing tissues is well documented[15,53]. But there is overwhelming evidence that in seeds[4,13,54,55], SS-mediated cleavage of sucrose is a predominant pathway. The specificity of SS for nucleoside diphosphate and nucleoside diphospho-sugar is quite broad. In majority of the cases, UDP and UDPGlc are the preferred nucleoside diphosphate and nucleoside diphospho-sugar[15]. Recently, however, in sycamore suspension culture cells, ADP-specific SS has been reported[56]. But the relative contribution of this enzyme to sucrose metabolism or to starch biosynthesis is still not clear.

Fructose released by SS, and any hexose formed by invertase, is converted to hexose phosphates (Figure 1). The enzymes involved in the phosphorylation of hexoses and their interconversion are reported in the developing seeds of maize[57,58], rice[49,59,60], barley[61,62] and wheat[63]. Their activity sharply increases concomitant with active starch synthesis. From position-specific radiotracer and ^{13}C-NMR studies with wheat endosperm[64], cell suspension cultures of *Chenopodium rubrum*, tubers of potato, seeds of faba beans and maize[65,66], it is evident that rapid recycling between hexose phosphates and triose phosphates takes place in these tissues. The recycling may be brought about either by the reactions catalysed by ATP-dependent phosphofructokinase (PFK) and a fructose-1,6-bisphosphatase (FBPase) and/or by pyrophosphate-dependent phosphofructokinase (PPi-PFK). All the three enzymes have been reported in the developing seeds[57,67–69]. But, because of its wide distribution, higher activity than that of ATP-PFK or FBPase and its regulation by Fru-2,6-*bis*P, PPi-PFK is presumed to play a more dynamic role in the interconversion of hexose phosphates and triose phosphates. Nevertheless, the role of PPi-PFK is still controversial[69]. Since the reaction catalysed by PPi-PFK is readily reversible ($K_{eq} = 3.3$) and is close to equilibrium *in vivo*, it could be involved in glycolysis or gluconeogenesis. In fact, Hatzfeld *et al.*[70] have suggested that PPi-PFK could operate to generate PPi, which is required for sucrose mobilization via SS and UDPglucose pyrophosphorylase (UDPGlc-PPase)[71–73]. But, in contrast, many workers[57,74–76] have suggested that PPi-PFK may function in the glycolytic direction. The exact physiological role of this enzyme still remains unresolved.

UDPGlc formed by the action of SS is converted to UTP and Glc-1-P by the action of the enzyme UDPGlc-PPase[77]. The presence of UDPGlc-PPase is well documented in a number of seeds like wheat[47,78,79], maize[48], barley[61], rice[49] and sorghum[80]. The activity of this enzyme in majority of the cases is higher than that of ADPglucose pyrophosphorylase (ADPGlc-PPase) and shows a strong parallel with starch synthesis. In contrast, the developing potato tubers have lower UDPGlc-PPase activity than ADPGlc-PPase and it increases only 2- to 3-fold in

comparison with 16- to 24-fold increase in ADPGlc-PPase during the active period of starch synthesis[81]. But even in this case the enzyme activity is adequate to meet the demands of the observed rate of starch synthesis.

PPi required for the reaction(s) in the cytosol may come either from the reaction catalysed by PPi-PFK or from the synthesis of macromolecules (protein, RNA or DNA). Alternatively, PPi could come from the synthesis of starch in the amyloplast and may be exchanged with cytosol via adenylate/PPi exchanger (see Figure 1) on the amyloplast membrane[82]. Moreover, ample evidence exists that plant cells contain appreciable amounts of PPi[52,53,69] for the operation of UDPGlc-PPase.

Nature of the carbon compound(s) translocated to the amyloplast

The nature of the carbon compound(s) translocated from the cytosol into the amyloplast is currently highly controversial. At least three types of sucrose-derived products, viz. triose phosphate(s), hexose phosphate(s) and ADPGlc have been proposed to cross the amyloplast membrane (Figure 1).

Evidence in favour of the transport of triose-P

Several workers[3,50,58–60,73,83] have suggested that carbon is transported as triose phosphates into amyloplast via triose-P/Pi-translocator and, subsequently, these are used for starch synthesis via gluconeogenesis. In fact, a Pi-translocator has been identified by immunoblotting analysis in the envelope membrane of the amyloplasts from suspension-cultured cells of sycamore[82,84]. Also, the amyloplasts from developing seeds[58,59] and from other starch-storing tissues[83,85] contain all the enzymes necessary to convert triose-P into starch. Moreover, the isolated intact amyloplasts from 14-day-old maize endosperm[66] and from potato tubers[83] preferentially take up and convert [^{14}C] triose phosphates into an insoluble product, presumed to be starch. In the case of maize, however, it has subsequently been shown that the MeOH–KCl-insoluble product was not starch (J. C. Shannon, personal communication).

Evidence against the transport of triose-P

A plastidic-FBPase is the key feature of the above pathway. Without this enzyme, there is no known pathway for the conversion of triose-P to hexose-P in the amyloplast. Several workers[58,59,83,85,86] have indeed reported the presence of FBPase enzyme in the amyloplasts of heterotrophic starch-forming tissues. However, the presence of this enzyme has been questioned because the plastidic-FBPase activity reported to be present in non-photosynthetic tissues is difficult to distinguish from the activity of the cytosolic PPi-PFK[87]. Moreover, the recent efforts by Entwistle and ap Rees[88] have failed to detect this enzyme in a range of starch-storing tissues. Lloyd *et al.*[89] also reached the same conclusion when they analysed the expression of a chimeric β-glucuronidase gene driven by the promoter of wheat chloroplast-FBPase in transgenic tabacco. The expression of this gene was light-dependent and linked to the photosynthetically active tissues only. KoBmann *et al.*[90] cloned the cDNA encoding the plastidic-FBPase from potato and analysed the expression of this gene at RNA level in different photosynthetically active (leaf and stem) as well as in the non-photosynthetic tissues (tuber, root and stolon) of potato. The expression of this gene at RNA level was also restricted to photosynthetically active tissues and was regulated by light. These observations further support the view that the plastidic-FBPase is not active in heterotrophic tissues. Thus, even though the amyloplasts of these tissues may possess a triose-P/Pi-translocator, they do not have the conventional means of converting triose-P to hexose-P.

Evidence in favour of the transport of hexose-P

Evidence in favour of the transport of hexose-P(s) into amyloplast has been obtained by studying the degree of randomization which takes place with asymmetrically labelled sugar molecules as a result of their incorporation into starch[64–66], as well as by direct uptake studies with isolated intact amyloplasts[91–93]. When Tyson and ap Rees[91] incubated the isolated amyloplasts of wheat endosperm in the solutions of ^{14}C-labelled glucose, Glc-1-P, Glc-6-P, Fru-6-P, Fru-1,6-*bis*P, DHAP or G3P, they observed that Glc-1-P served as the most effective precursor for starch synthesis in the amyloplasts. The degree of labelling of starch was dependent on the intactness of the organelle. A somewhat different specificity of transport was exhibited by pea amyloplasts[93], which readily took up and incorporated Glc-6-P, but not Glc-1-P, into starch. These data are consistent with the view that in wheat and pea seeds it is a C-6 (Glc-1-P or Glc-6-P) compound that enters the amyloplast to provide carbon source for starch synthesis.

Keeling *et al.*[64] used specific position-labelled [^{13}C] sugars and ^{13}C-NMR to examine the extent of redistribution of ^{13}C between carbon 1 and 6 in starch glucosyl moiety during starch synthesis in wheat endosperms. When endosperms were fed [1-^{13}C] or [6-^{13}C] glucose or fructose, there was only 12–20% redistribution of ^{13}C into starch between the C1 and C6 atoms, again suggesting that hexose monophosphates, and not triose-P, are the most likely candidates for entry into amyloplasts of wheat. Similar observations of direct import of hexose units into amyloplasts of potato tubers, faba beans,

maize endosperm and suspension cells of *Chenopodium rubrum*[65,66] have been made.

Transport of ADPGlc into amyloplast

Recently, an alternative pathway for carbon transport into amyloplast has been proposed[94]. According to this pathway, ADPGlc is synthesized directly from sucrose via the action of cytosolic SS and then transported across (via ADP/ATP translocator) the amyloplast membranes, where it could be utilized by starch synthase (Figure 1).

An important distinction between this and hexose-P pathway is the subcellular localization of ADPGlc synthesis that is required for the synthesis of starch. Although SS in starch-synthesizing seeds functions with ADP[56], there is overwhelming evidence that the sugar nucleotide utilized by starch synthase is synthesized by ADPGlc-PPase in the plastid and not by SS in the cytosol. Lin *et al.*[95,96] isolated two *Arabidopsis thaliana* lines mutated at the *Adg1* and *Adg2* loci that were defective in starch synthesis and ADPGlc-PPase activity. Plants of the *adg1* line, which accumulated very little leaf starch, were devoid of both large and small subunits of the ADPGlc-PPase as viewed by immunoblot analysis[95], where plants of the line *adg2*, which accumulated 40% as much starch, were deficient in the large subunit of ADPGlc-PPase[96]. Therefore, the absence or depression in the levels of starch synthesis can be attributed to a direct causal relationship between defects in the structural genes for ADPGlc-PPase and the concomitant lower amounts of enzyme activity. Maize mutants, *sh-2* and *bt-2*, which contain about 25% of the starch in the normal endosperm, exhibit 10% ADPGlc-PPase activity as compared to the normal endosperm[97]. Further biochemical and molecular biology studies[98], using the native and subunit antibodies of spinach leaf enzyme, have shown that mutant *btu-2* lacks the 55 kDa subunit and the mutant *sh-2* lacks the 60 kDa subunit, and that both the subunits are required for maximal enzyme activity and starch synthesis[8]. Similarly, pea lines having recessive *rb* genes contained 38–72% of the starch found in the pea line having the *Rb* loci and about 3–5% of the normal ADPGlc-PPase activity. Other convincing evidence that the ADPGlc-PPase is the dominant enzyme, if not the sole enzyme, producing ADPGlc for starch synthesis in potato tubers has come from the use of 'reverse genetics'. Müller-Röber *et al.*[99] have shown that application of antisense RNA to the gene of the small subunit or the large subunit of potato tuber ADPGlc-PPase resulted in the production of minitubers containing only 3% of the starch observed in normal tubers. Recently, Stark *et al.*[100] have shown that only the expression of CTP-glgC16 (i.e. a plastid-directed ADPGlc-PPase), not glgC16, results in an increased starch content in transgenic potato tubers. These studies amply prove that the ADPGlc used for starch biosynthesis is formed via the activity of ADPGlc-PPase, and the SS-ADPGlc pathway plays little, if any, role in this process.

Metabolic reactions inside the amyloplast

Despite the controversy over the nature of the carbon compound(s) imported into the amyloplast, there is a consensus that Glc-1-P serves as a precursor for starch synthesis[5,8,9]. The activity of ADPGlc-PPase, the enzyme producing ADPGlc from Glc-1-P, has been monitored in a large number of developing seeds[4,5] and the evidence that this enzyme plays a cardinal role in starch synthesis has been discussed earlier. PPi produced during this reaction is inhibitory to ADPGlc-PPase and is either removed from the amyloplast via PPi/adenylate exchanger on the amyloplast membrane (Figure 1) or hydrolysed to Pi by alkaline pyrophosphatase. ADPGlc in the next step is added to the growing glucan chain by starch synthase. Starch synthase occurs both in soluble and starch-granule-bound forms[4,5]. There are two forms of soluble starch synthase, designated as starch synthase I and starch synthase II. These starch synthases are specific for the substrate ADPGlc. Though the two forms have the same affinity (K_m) for ADPGlc, type I enzyme in the presence of 0.5 M citrate is active without a primer whereas type II enzyme is inactive. From spinach leaf and maize endosperm starch synthase I has a molecular weight (MW) of about 70 kDa whereas starch synthase II has a MW of about 92 kDa. The granule-bound starch synthase (GBSS) can utilize both UDPGlc and ADPGlc as substrates. The rate of glucosyl transfer from ADPGlc, however, is 3 to 10 times higher and the K_m for ADPGlc is 15- to 30-fold lower than the K_m for UDPGlc. MacDonald and Preiss[101,102] solubilized (50–80% recovery) starch synthase activity from starch granules of *Zea mays* endosperm and resolved the enzyme extract into two fractions by fractionation of DEAE-sepharose CL-6B. GBSS I eluted off the column at lower salt concentrations than did starch synthase II. Both forms required a primer (amylopectin) for activity. GBSS II, however, had a higher affinity (lower K_m) for amylopectin than type I enzyme. GBSS I had a MW of 61 kDa and is different from soluble starch synthase I (MW 70 kDa). However, GBSS II and soluble starch synthase I have almost similar MWs (about 92 kDa). Antibodies against the soluble starch synthase I neutralized soluble starch synthase I effectively but had little or no effect on either GBSSI or II. Thus, kinetic data and the different MWs of starch synthases suggest that soluble starch synthase I and GBSS I are different proteins.

During the initial stages of maize endosperm development, the major form of starch synthase is the soluble one, becoming progressively bound as the tissue matures.

The relative contribution of the soluble and the granule-bound enzymes to starch synthesis in the developing seeds, however, is unclear. The studies with *wx* mutants of maize and *amylose-free* mutants in potato have revealed that these mutants had no amylose and had no granule-bound starch synthase activity, thus indicating that the soluble enzyme produces amylopectin and the bound one produces amylose. Further biochemical studies of GBSS in maize endosperm and genetic manipulation with potatoes is consistent with this view[103-106]. In pea embryo, the genes encoding the GBSSs are maximally expressed at different times during development. The levels of mRNA for GBSS II peak early in development, whereas those for GBSS I peak considerably later[107]. This ontogenetic pattern of appearance of GBSS I is consistent with the role of this enzyme in amylose synthesis, the amount of which increases with the maturity of the seed.

The last enzyme in the starch biosynthesis pathway is the starch branching enzyme. This enzyme catalyses the cleavage of a short chain of glucosyl units (20 or less) from the non-reducing end of an α1,4-linked chain, followed by reattachment of the cleavage product to the side of the same or an adjacent chain via α1,6 linkage to create a branch. Multiple forms of branching enzymes have been identified in developing grains of sorghum[108], maize[109], pea[110] and rice[111]. In immature maize endosperm, two forms (I and II) were distinguished on the basis of their behaviour in the assay system used. The form II could further be resolved into two fractions, IIa and IIb. The enzymes are monomers and have MWs in the range of 80 (fraction IIa and IIb) to 89 kDa (fraction I) and can be distinguished by their kinetic assays and immunological reactivity[112]. Similarly, the two forms of branching enzymes (type I and II) present in pea embryo also have different kinetic and physical properties, and different patterns of expression during seed development[13]. The two forms differ in their affinity for substrate (amylose), and in the solubility of their product[110]. One form is about 112 kDa and the other is about 100 kDa. A comparison of the deduced amino acid sequences reveals that they are about 50% identical. The levels of mRNA for branching enzyme I peaks early in development whereas that for branching enzyme II peaks much later. These differences suggest that the two forms must play different roles in the synthesis of starch.

Both forms of branching enzyme are present in the soluble fraction of the amyloplast and are bound on to the granule. Smith and Denyer[13] have suggested that branching occurs in the soluble phase at the periphery of the granule and that the branching enzymes, like the soluble starch synthases, become entrapped within the structure of the granules as their product crystallizes. But how the different isoforms of branching enzyme interact with the isoforms of starch synthase to determine the fine structure of the starch granule is not known.

Regulation of starch synthesis

Since the amount of starch deposited in the mature seed is dependent on the complex interplay of a large number of processes operating at different stages and at different levels of tissue organization during seed development, regulation of starch may take place at several sites.

Through the provision of carbon substrates

Most of the studies[113-115] with the developing cereal grains have indicated that the termination of starch accumulation in the grain is not due to the reduced supply of sugar precursors but due to a decline in the synthetic capacity of the endosperm. In fact, Cochrane[116] has shown that sucrose continues to enter the developing barley endosperm even after the end of grain filling. Similarly, there is much evidence[7,11,13,14] to suggest that sucrose supply under normal conditions does not limit starch accumulation, but the factors operating within, or close to the seeds control starch deposition. For example, artificially increasing CO_2 levels in barley[117] had little effect on grain filling, and decreasing the number of grains per ear[118-120] had variable effects, but seldom did the weight per grain increase by a large margin. Reducing photosynthesis by shading[118] or defoliation[121] does not result in very large decreases in weight per grain, and variation in irradiation during grain filling is not closely related to the rate of grain growth[122]. Further, under conditions which tend to reduce the production of assimilates, such as drought[123], leaf senescence[124], or chemical treatment[125], soluble carbohydrates from other parts of the plant are mobilized to sustain grain growth. Furthermore, Ugalde and Jenner[115] have estimated that sucrose present in the developing wheat endosperm and cavity is sufficient for about 4 h of starch synthesis. Thus, while there is a safety margin, starch synthesis clearly depends on a steady supply of assimilates from the rest of the plant, and this steady supply is ensured by the operation of temporary storage pools in the other parts of the plant[42]. Temporary storage pools serve to accommodate photosynthate during the period of surplus production, and to provide photosynthate when the current rate of photosynthate production is less than the needs of the grains.

More recent studies[126] with the heterotrophic suspension cultures of *Chenopodium rubrum* cells using [^{14}C] glucose suggest that sucrose concentration in the cells is regulated by rapid cycles of synthesis and degradation. These cycles allow the turnover of sucrose to respond very rapidly to changes in the levels of metabolic intermediates and sucrose, thus implying that sucrose turnover responds automatically to changes in the supply of sucrose and to the requirements for biosynthetic pathways.

More detailed information on the responses of starch deposition to variation in the concentration of sucrose in wheat has been obtained by Jenner *et al.*[7] by culturing detached ears/endosperms on solutions of sucrose. During grain filling, the rate of starch synthesis appears to be a function of the concentration of sucrose within the endosperm, but the concentration of endosperm sucrose seems to be tightly controlled. Rates of starch synthesis observed in ears cultured on solutions containing 40 gL^{-1} sucrose correspond to rates observed *in vivo*. At concentrations below 40 gL^{-1}, both the concentration of endosperm sucrose and the rate of starch synthesis are lower than the values observed *in vivo*; concentrations higher than 40 gL^{-1} have little promotive effect[24,127,128]. Trimming wheat ears after anthesis (by removing some spikelets) causes an increase in sucrose in the rachis, indicating that this treatment increases the amount of substrate available for distribution to the grain, but no corresponding increase in sucrose follows[129].

These apparent limitations to the accumulation of starch and intracellular concentration of sucrose are not the properties intrinsic to the endosperm, as they can be circumvented by supplying sucrose directly to the endosperm[130]. Instead, the limitations are explicable as a constraint imposing an upper limit to the transport of sucrose into and within the grain[131]. This is not imposed by the characteristics of transport of assimilates to the ear, as grain growth is unaffected by cutting through half the vascular bundles of the peduncle[132]; there appears to be a compensating increase in concentration and speed of movement through the vascular bundles that remain.

The simplest postulate explaining the upper limit to sucrose inflow and starch accumulation invokes a transport mechanism with saturable characteristics in the pathway of entry into the endosperm. Whether this operates in the stalk of the grain[133] or at the site(s) of unloading and transfer in the endosperm cavity[134] is not clear. According to Jenner *et al.*[7], the relationship between the supply of assimilates for grain filling and the rate of starch accumulation is the outcome of a concentration gradient in the pathway of entry into the grain and kinetic properties of the starch biosynthetic pathway in the endosperm.

Capacity to accommodate starch

Several studies in wheat[25,135,136] have suggested that the capacity of the grain to store starch is correlated with the number or size of endosperm cells or A-type starch granules. Since both the cell number and A-type starch granules are determined during the early phase of grain growth, the availability of assimilates during this phase may determine cell number and starch granule number, and the evidence that this appears to be the case has been reviewed[7].

Additional evidence that the number and size of starch granules determine the amount of starch deposited in the grains of wheat[137] and barley[138] has been obtained by growing plants at different temperatures. Bhullar and Jenner[137] exposed the plants of four cultivars of wheat (grown at 21/16°C) during grain filling (from 10 days after anthesis) to brief episodes of high temperatures of 33/25°C (day/night) and 27/22°C for 10 and 20 days, respectively. The final single-grain weight in all the cultivars was reduced by elevating the temperature, and to the same extent (14%). The number of B-type granules (but not A-type), were substantially reduced by warming, but this reduction did not account wholly for the smaller weight of starch per grain resulting from elevated temperatures. MacLeod and Duffus[138] also observed decreases in barley grain dry weight at elevated temperatures, which were due to reduction in both volume available for starch accumulation and numbers rather than sizes of starch granules.

Metabolic regulation

Several workers speculate that starch synthesis may be regulated by the *de novo* synthesis of enzymes of sucrose-to-starch conversion during seed development[4]. The above notion of coarse control of starch synthesis is supported by mutant studies in cereals like maize, barley, sorghum and rice. The maize mutants, *bt-2* and *sh-2*, which contain less starch than normal genotypes, are deficient in ADPGc-PPase. The *wx* maize mutant, containing little or no amylose, was shown to be limiting in GBSS I activity. Similarly, according to Boyer and Preiss[139], the deficiency of branching enzyme IIa and soluble starch synthase II is responsible for decrease in starch content and increase in amylose in maize mutant *dull*. Batra and Mehta[61,62] suggested that in barley, lower levels of PGM and starch synthase might be responsible for reduced accumulation of starch in mutant *notch-2* grain as compared to parent NP 113 during grain development. The *wx* rice grain, which has a high degree of branching, resulting in glutinous starch, contains much lower levels of bound starch synthase than non-waxy ones[140]. In non-waxy grains, it is postulated that the formation of a complex between newly synthesized amylose and bound starch synthase may reduce access to the branching enzyme and thus lower the proportion of amylopectin formed as the development proceeds.

Extensive studies by Preiss and his associates[5,8] suggest that ADPGlc synthesis is regulated by the activation of ADPGlc-PPase by 3-PGA and inhibition by inorganic phosphate (Pi). This view is almost certainly correct for photosynthetic tissues, where its flux control coefficient for carbon is reported to be much higher than that of the other enzymes of starch biosynthetic pathway[141]. But whether this enzyme plays a similar predominant

regulatory role in the seeds is not clear. Early work by Dickinson and Preiss[97] showed that maize endosperm ADPGlc-PPase was relatively insensitive to activation by 3-PGA and inhibition by Pi. However, Plaxton and Preiss[142] showed that the insensitivity of maize endosperm ADPGlc-PPase was due to the proteolytic loss of 1 kDa peptide during purification. They further demonstrated that the intact enzyme purified under non-proteolytic conditions was closely related to the enzyme from photosynthetic tissues. Like spinach leaf enzyme, it was activated by 3-PGA and inhibited by Pi. Similar characteristics have been observed for potato enzyme also[143]. These regulatory properties are consistent with an important role of this enzyme in the regulation of the flux of photosynthates into starch. However, the ADPGlc-PPase purified from wheat endosperm[144], barley[145] endosperms and pea embryo[13] appear to be relatively insensitive to regulation by 3-PGA/Pi. Like wheat endosperm enzyme, pea embryo enzyme is also insensitive to activation by 3-PGA and inhibition by Pi. Additional evidence that wheat endosperm enzyme differs from that of the leaf comes from molecular biology studies[146]. cDNA or genomic clones for ADPGlc-PPase gene for wheat endosperm and wheat leaf have been isolated. At the DNA level the isolated genes are quite different. For wheat leaf and wheat endosperm, nucleotide sequence analysis indicates that there is approximately 55% identity between leaf and wheat endosperm cDNAs and, on the basis of southern hybridization analysis and restriction mapping, there appear to be at least two distinct gene families. Similarly, for spinach leaf and rice endosperm, there is only about 50% identity[8]. In terms of amino acid sequences there was an overall low homology (40–44%) between wheat leaf, wheat endosperm and rice endosperm ADPGlc-PPases. There are, however, several well-conserved domains with the identity between the sequences of between 60 and 84% (ref. 146). Although it appears that the endosperm enzyme has a 3-PGA binding site, it was suggested that the differences between wheat leaf and endosperm ADPGlc-PPase amino acid sequences in the regions other than the putative 3-PGA binding site are responsible for the observed differences in allosteric properties of these enzymes. This would suggest that the wheat endosperm enzyme might not be able to undergo the necessary conformational changes required to convert it to a more active form in the presence of 3-PGA.

Summary and future prospects

Various aspects of starch synthesis and its regulation in the developing seeds have been reviewed. Capacity of the seed to store starch is determined during the early stages of seed growth (i.e. seed enlargement phase), and the processes during the subsequent stages (i.e. seed-filling phase) operate to fill this capacity. The number of cells and the amyloplasts, which are cardinal determinants of storage capacity, are determined during the earlier one-third stage of grain growth and both the parameters appear to be influenced by the availability of carbohydrates during this phase of seed growth.

The primary source of reduced carbon appears to be sucrose, the supply of which is most probably not limiting for starch storage in the developing seeds. In the cytosol of starch-storing cells, sucrose is first converted to UDPGlc by SS. Glc-1-P or Glc-6-P, derived either from UDPGlc or hexoses, may then be transported across amyloplast membranes, where it is converted to ADPGlc and thence into starch via ADPGlc-PPase, starch synthase and branching enzyme. Recent molecular biology and mutant studies suggest an important role of ADPGlc-PPase in the production of ADPGlc. The properties of ADPG-PPases from photosynthetic tissues and heterotrophic starch storage tissues appear to differ. In particular, the endosperm enzyme did not exhibit any of the activation characteristics reported for leaf enzyme. More detailed studies with different seeds are required before the regulation of ADPGlc-PPase by 3-PGA/Pi can be generally accepted. Both starch synthase and branching enzyme exist in multiple forms. But the precise role of these enzymes in starch synthesis or in determining the structure of starch granules is not known.

Recently, expression of an *E. coli* gene (glgC16) in potato from a patatin promoter and SSU leader sequence has been reported to lead to increased starch accumulation in potatoes[100]. Additionally, the technologies to transfer gene(s) to other starch-storing crops such as corn, wheat and rice are being developed. In the next 10 to 20 years, by using these techniques, it may be possible to enhance seed productivity and to produce starch of desirable quality.

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