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REVIEW ARTICLE

Recent developments in understanding the regulation of starch metabolism in higher plants

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Abstract

This article reviews current knowledge of starch metabolism in higher plants, and focuses on the control and regulation of the biosynthetic and degradative pathways. The major elements comprising the synthetic and degradative pathways in plastids are discussed, and show that, despite present knowledge of the core reactions within each pathway, understanding of how these individual reactions are co-ordinated within different plastid types and under different environmental conditions, is far from complete. In particular, recently discovered aspects of the fine control of starch metabolism are discussed, which indicate that a number of key reactions are controlled by post-translational modifications of enzymes, including redox modulation and protein phosphorylation. In some cases, enzymes of the pathway may form protein complexes with specific functional significance. It is suggested that some of the newly discovered aspects of fine control of the biosynthetic pathway may well apply to many other proteins which are directly and indirectly involved in polymer synthesis and degradation.

Key words: ADPglucose pyrophosphorylase, amylopectin, amyloplasts, amylose, protein phosphorylation, protein–protein interactions, starch branching enzyme, starch degradation, starch phosphorylase, starch synthase, starch synthesis.

Introduction

Starch is an insoluble polymer of glucose residues produced by the majority of higher plant species, and is a major storage product of many of the seeds and storage organs produced agriculturally and used for human consumption. All higher plant starches are synthesized inside plastids, but their function therein will depend upon the particular type of plastid, and the plant tissue from which they are derived. Transient starches synthesized in leaves during the day are 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, whilst storage starches in developing seeds are a long-term carbon store for the next generation. The starch granule is a complex structure with a hierarchical order composed of two distinct types of glucose polymer; amylose, comprising largely unbranched α-(1→4)-linked glucan chains, and amylopectin, a larger, highly 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. The polymodal distribution of glucan chain lengths, and branch point clustering, within amylopectin allows the chains to form double helices which can pack together in organized arrays, which are the basis of the semi-crystalline nature of much of the matrix of the starch granule (for reviews of starch structure, see Buléon *et al.*, 1998; Thompson, 2000). 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 (French, 1984; Hizukuri, 1986; Myers *et al.*, 2000). The crystalline structure of starch granules is highly conserved in plants at the molecular level (Jenkins *et al.*, 1993), as well as at the microscopic level, where alternating regions of

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Abbreviations: ADPGlc, ADPglucose; AGPase, adenosine 5′ diphosphate glucose pyrophosphorylase; AGP-L, large subunit AGPase; AGP-S, small subunit AGPase; DAP, days after pollination; DBE, debranching enzyme; D-enzyme, disproportionating enzyme; DP, degree of polymerization; GBSS, granule-bound starch synthase; Glc1P, α-D-glucose 1-phosphate; MOS, malto-oligosaccharides; MS, mass spectrometry; 3-PGA, 3-phosphoglyceric acid; Pi, inorganic orthophosphate; PPi, inorganic pyrophosphate; SBE, starch branching enzyme; SDS, sodium dodecyl sulphate; SP, starch phosphorylase; SS, starch synthase; TAP, tandem affinity purification; UGPase, uridine 5′ diphosphate glucose pyrophosphorylase.

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semi-crystalline and amorphous material, commonly known as growth rings, are present in all the higher plant starches studied to date (Hall and Sayre, 1973; Pilling and Smith, 2003). A common feature of all starches, is that at some point in time they must be degraded (e.g. leaf starch at the end of the light period, or storage starches during germination), and hence the granule structure must have built-in entry points (either channels, cavities or zones of attack) for the enzymes involved in the degradation process.

The synthesis of this architecturally complex polymer assembly is achieved through the co-ordinated interactions of a suite of starch biosynthetic enzymes, including some which had traditionally been associated with starch degradation. The complement of these starch metabolic enzymes, which is a reflection of the starch biosynthetic and degradative pathways, is well conserved between plastids/tissues which 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 various enzymes involved in starch metabolism are highly conserved (Jesperson *et al*., 1993; Smith *et al*., 1997; Ball and Morell, 2003). 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.

Details of the key components of the starch metabolic pathways have been adequately reviewed elsewhere, dealing with starch synthesis in higher plants and green algae (Ball and Morell, 2003), and also more specifically with starch biosynthesis in cereal endosperms (James *et al*., 2003), as well as starch degradation in leaves (Smith *et al*., 2003*b*). The application of this knowledge of key reactions of starch synthesis, and their impact on granule structure has been reviewed recently in relation to attempts at creating designer starches possessing novel functionalities (Jobling, 2004). This review is primarily concerned with aspects of the control and regulation of starch metabolic pathways in higher plants, firstly dealing briefly with gene expression/localization of the enzymes in the core pathway (coarse control) and then with more recent research showing that the fine control of starch metabolic pathways is achieved through post-translational modifications of key reactions, some of which are co-ordinated via the assembly of functional multiprotein complexes.

The core pathway of starch biosynthesis

The formation of ADPglucose by ADPglucose pyrophosphorylase

In all plant tissues capable of starch biosynthesis, adenosine 5′ diphosphate glucose (ADPGlc) pyrophosphorylase (AGPase, EC 2.7.7.27) is the enzyme responsible for the production of ADPGlc, the soluble precursor and substrate for starch synthases. The AGPase reaction is the first committed step in the biosynthesis of both transient starch in chloroplasts/chromoplasts, and storage starch in amyloplasts. 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 endosperms of maize (Denyer *et al*., 1996*b*), barley (Thorbjørnsen *et al*., 1996*a*), rice (Sikka *et al*., 2001), and wheat (Tetlow *et al*., 2003) which have been shown to correspond to plastidial and cytosolic isoforms of AGPase. In the developing endosperms of wheat, maize, barley, and rice the cytosolic isoform accounts for 65–95% of the total AGPase activity, implying that most of the storage starch biosynthesis in these tissues occurs through import of ADPGlc into amyloplasts.

Plants possess multiple genes encoding either the AGP-L or AGP-S subunits, or both, 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 tissues such as potato (La Cognata *et al*., 1995), rice (Nakamura and Kawaguchi, 1992), and barley (Villand *et al*., 1992*a*). 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 both barley and wheat (Olive *et al*., 1989; Villand *et al*., 1992*a*, *b*) or induced under specific conditions, such as increased sucrose or glucose levels in potato (Müller-Rober *et al*., 1990; Duwenig *et al*., 1997). 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 cDNAs 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 the section on allosteric regulation), which are suited to the particular metabolic demands of a given plant tissue/organ. In cereal endosperms, the subcellular localization of AGPase isoforms is thought to be regulated 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 alternate first exons (Thorbjørnsen *et al*., 1996*b*).

Elongation of the glucan chain by starch synthases

The starch synthases (SS, EC 2.4.1.21) catalyse the transfer of the glucosyl moiety of the soluble precursor ADPGlc to the reducing end of a pre-existing α-(1→4)-linked glucan

primer to synthesize the insoluble glucan polymers amylose and amylopectin. Plants possess multiple isoforms of SSs, containing up to five isoforms that are categorized according to conserved sequence relationships. The isoforms within each of the major classes of SS genes are highly conserved in higher plants through the dicots and monocots (Ball and Morell, 2003). The major classes of SS genes can be broadly split into two groups, the first group primarily involved in amylose synthesis, and the second group principally confined to amylopectin biosynthesis.

Amylose biosynthesis: The first group of SS genes contains 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 found, essentially, completely within the granule matrix (one of the so-called granule-associated proteins[†]). In addition to its role in amylose biosynthesis, GBSSI was also found to be responsible for the extension of long glucans within the amylopectin fraction in both *in vitro* and *in vivo* experiments (Delrue *et al*., 1992; Maddelein *et al*., 1994; van de Wal *et al*., 1998). 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 which accumulate transient starch (Nakamura *et al*., 1998; Fujita and Taira, 1998; Vrinten and Nakamura, 2000). An interesting aspect of the control of polymer (amylose) elongation has been observed in the leaves of sweet potato (*Pomoea batatas*) where GBSSI transcript abundance and protein levels were shown to be under circadian control, in addition to being modulated by sucrose levels (Wang *et al*., 2001).

One of the unique properties of GBSSI is its stimulation by malto-oligosaccharides (MOS) when synthesizing amylose (Denyer *et al*., 1996*a*). 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). Other *in vitro* experiments have shown that glucan chain extension from amylopectin to yield amylose is possible, given extended incubation times, suggesting that two different mechanisms may explain the synthesis of distinct categories 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 (Denyer *et al*., 1999; Dauvillée *et al*., 1999).

Amylopectin biosynthesis: The second group of SS genes (designated SSI, SSII, SSIII, and SSIV) are exclusively involved in amylopectin biosynthesis, and their distribution within the plastid between the stroma and starch granules varies between the species, tissue, and developmental stages (Table 1). The individual SS isoforms from this group probably play unique roles in amylopectin biosynthesis. The study of SS mutants in a number of systems has been helpful in the assignment of *in vivo* functions/roles for the soluble and granule-associated SS isoforms in amylopectin synthesis. Valuable information about the roles of the SS isoforms *in vivo* is being derived from mutants lacking specific isoforms, and analysis of plants appears to show that each isoform performs a specific role in amylopectin synthesis. However, such data should be treated with caution, as in some cases there are pleiotropic effects of mutations on other enzymes of starch synthesis.

While no mutants for SSI have been reported, biochemical evidence suggests that SSI is primarily responsible for the synthesis of the shortest glucan chains, i.e. those with a DP of 10 glucosyl units or less (Commuri and Keeling, 2001), and further extension of longer chains is achieved by the activities of SSII and SSIII isoforms, each of which act on progressively longer glucan chains. Two classes of SSII genes are found in monocots: SSIIa and SSIIb. The role of the latter in starch biosynthesis is unknown as no mutants have been identified to date. *In vitro* studies of the two SSII forms from maize reveal different substrate specificities and kinetic properties (Imparl-Radosevich *et al*., 2003). SSIIa predominates in cereal endosperms, whilst SSIIb is mostly confined to photosynthetic tissues. Loss of SSIIa (in monocots) and SSII (in dicots) results in reduced starch content, reduced amylopectin chain-length distribution, altered granule morphology, and reduced crystallinity, suggesting that the SSII forms have similar roles in starch biosynthesis across different species boundaries. In monocots, SSIIa plays a specific role in the synthesis of the intermediate-size glucan chains of DP 12–24 by elongating short chains of DP $\leqslant 10$, and its loss/down-regulation has a dramatic impact on both the amount and composition of starch, despite the fact that SSIIa is a minor contributor to the total SS activities in cereal endosperms, as opposed to SSI and SSIII. 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. Antisense suppression of SSIII in potato has a major impact on the synthesis of amylopectin, resulting in amylopectin with modified chain-length distribution and decreased starch synthesis (Edwards *et al*., 1999). However, mutations in maize eliminating

[†] The designation of granule-associated protein is arbitrary, and is generally based on the ability of a protein to be retained within the starch fraction following vigorous aqueous (buffers, SDS- and proteinase treatments) and non-aqueous (acetone or ethanol treatments) extraction and washing techniques described in a number of research articles (Denyer *et al*., 1995; Rahman *et al*., 1995; Mu-Forster *et al*., 1996). Many other proteins may have a functional role at or inside the granule matrix, but are far more loosely associated with the granules than those normally designated as ‘granule-associated’.

Table 1. *Localization of enzymes constituting the known core pathway of starch biosynthesis in higher plants*

Enzyme	Monocotyledon			Dicotyledon		
	Endosperm	Leaf	Granule[a]	Embryo/tuber	Leaf	Granule[a]
AGPase (cytosolic)	+	–	S	–	–	–
AGPase (plastidial)	+	+	S	+	+	S
GBSSI	+	–	G	+	–	G
GBSSII	–	+	G	–	+	G
SSI	+	+	G/S	+	+	G/S
SSIIa	+	–	G/S			
SSIIb	–	+	?			
SSII				+	+	G/S
SSIII	+	+	S	+	+	S
SSIV	?	?	?	?	?	?
SBEI[b]	+	+	S	+	+	S
SBEIc[c]	+	–	G	–	–	–
SBEIIa	+	+	G/S			
SBEIIb	+	–	G/S			
SBEII				+	+	G/S
Isoamylase-type DBE (Iso-1)	+	+	S	+	+	S
Iso-2	?	?	?	?	?	?
Iso-3	?	?	?	?	?	?
Pullulanase-type DBE	+	+	S	+	+	S
SP[d]	+	+	S	+	+	S
D-enzyme[d]	+	+	S	+	+	S
R1 Protein	?	+	G/S	+	+	G/S
α-amylase[d]	+	+	S	+	+	S
β-amylase[d]	+	+	S	+	+	S

[a] G, granule-associated; S, soluble. Granule-associated proteins are defined as those proteins remaining attached/associated with the starch matrix following extensive washing treatments with buffers/SDS and acetone.
[b] Not all plant tissues appear to possess an SBEI gene; for example, there is no SBEI homologue in the *Arabidopsis* genome.
[c] SBEIc appears to be restricted to the A-type granules of storage starches from the endosperms of *Triticum* sp., *Hordeum* sp., and *Secale* sp.(Båga *et al*., 2000; Peng *et al*., 2000); these species (Festucoideae) show a bimodal granule size distribution.
[d] Enzymes possessing both plastidial and extra-plastidial forms; the latter are not presumed to have any role in starch metabolism within the plastid and are not shown in the table.

SSIII (*du1*) lead to a subtle phenotype which is only conspicuous in *Waxy* backgrounds (Gao *et al*., 1998). Sequences for SSIV appear in a wide range of higher plants in EST databases although, to date, no mutants have been isolated with lesions in this gene and no role has been assigned for this class of SS in the process of starch biosynthesis.

Branching of the glucan chain by starch branching enzymes

Starch branching enzymes (SBEs, EC 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. 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 (Table 1). Analysis of the primary amino acid sequences of higher plant SBEs reveals two major classes; SBEI (also known as SBE B) and SBEII (also known as SBE A). The two classes of SBE 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 (Takeda *et al*., 1993; Guan and Preiss, 1993). The construction of chimeric forms of maize SBEI and SBEII and analysis of their catalytic properties by Kuriki *et al*. (1997) indicated that the N- and C-termini of these proteins play important roles in determining substrate preference, catalytic capacity, and chain length transfer. In monocots, the SBEII class is made up of two closely related but discrete gene products, SBEIIa and SBEIIb (Rahman *et al*., 2001).

To date, only mutations in SBEII isoforms give clear phenotypes, and in monocots this is confined to SBEIIb mutants. 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 (Blauth *et al*., 2002; Satoh *et al*., 2003*b*; Flipse *et al*., 1996). Recent analysis of a maize SBEIIa mutant showed a clear phenotype in the leaf starch, but showed no apparent alterations in the storage starch of the endosperm (Blauth *et al*., 2001). This observation suggests a primary role for SBEIIa in leaf (transient) starch synthesis, and either no critical role for SBEIIa in amylopectin biosynthesis in the endosperm, or else a role that can easily be compensated for by other SBEs in its absence. SBEII isoforms are also partitioned between the plastid stroma and the starch granules (Table 1). As with

the granule-associated SSs (above), 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, although it has recently been suggested that alternative splicing of a 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 by Seo *et al*. (2002) has shed further light on the roles of different SBE isoforms in the construction of the starch granule, which would not have been possible by analysing mutations in single SBE genes. Expression of three functional maize SBE genes in a yeast strain lacking the endogenous yeast glucan branching enzyme showed that SBEI was unable to act in the absence of SBEIIa or SBEIIb, and that SBEII may act before SBEI on precursor polymers. Both of the maize SBEII isoforms heterologously expressed by Seo *et al*. (2002) could complement the lack of yeast glucan branching enzyme, and produce glucans with unique chain distributions and branch frequencies. These data suggest that SBEI does not play a central role in this *in vitro* system, leaving the role of SBEI in the starch biosynthetic pathway still an open question.

There is also evidence for proteins regulating the co-expression of starch metabolic enzymes. The *floury-2* (*flo2*) mutation in rice produced reductions in gene expression and protein content of SBEI (down to 10% of wild-type levels). In addition, significant reductions in the expression of SBEIIb, SSI, GBSS, pullulanase-type DBE, and SP were also observed, suggesting that the wild-type *Flo2* gene encodes a regulatory protein responsible for simultaneously modulating the expression of a number of starch biosynthetic genes (Satoh *et al*., 2003*a*).

The role of debranching enzymes in polymer synthesis

The analysis of low-starch mutants that accumulate a water-soluble polysaccharide termed phytoglycogen have been described in a wide range of higher plants, including *Arabidopsis* and maize, as well as the unicellular alga *Chlamydomonas* (Zeeman *et al*., 1998*b*; James *et al*., 1995; Mouille *et al*., 1996) and indicate that starch synthesis involves isoamylases, also termed debranching enzymes (DBEs, EC 3.2.1.41, and EC 3.2.1.68) in addition to SSs and SBEs. Two groups of DBEs exist in plants; isoamylase-type, and pullulanase-type (also known as limit-dextrinases) which efficiently hydrolyse (debranch) α-(1$\rightarrow$6)-linkages in amylopectin and pullulan (a fungal polymer of maltotriose residues), respectively, and are part of the α-amylase 'super-family' of enzymes. The *Arabidopsis* genome contains three isoamylase-type DBEs (isa-1, isa-2, and isa-3) and one pullulanase-type DBE. Both groups of DBEs in higher plants share a common N-terminal domain whose function is yet to be elucidated. The decrease/loss of either isa-1 or isa-2 isoamylase-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 it is thought that, in rice endosperm, residual pullulanase-type DBE activity modulates these phenotypic effects (Nakamura *et al*., 1998). In maize endosperm the pullulanase-type DBE activity is thought to have a bi-functional role, assisting in both starch synthesis and degradation (Dinges *et al*., 2003). In wheat, the expression of a cDNA for the isoform of an isoamylase-type DBE (Iso-1) is highest in developing endosperm and undetectable in mature grains, which suggests a biosynthetic role for isoamylase in this tissue.

The precise roles for the isoamylase-type and pullulanase-type DBEs in starch biosynthesis are not yet known. 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 would be 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. Recent observations, which show that the surface of the immature granules contains numerous short chains, are consistent with this model (Nielsen *et al*., 2002). An alternative to the glucan-trimming model proposes that the DBEs function in starch synthesis indirectly in a 'clearing' role, removing soluble glucan that is not attached to the granule from the stroma. This removes a pool of substrates for the amylopectin synthesizing enzymes (SSs and SBEs), and thereby prevents the random/futile synthesis of glucan polymers by these enzymes which could cause the accumulation of phytoglycogen, ultimately leading to a reduction in the rate of starch synthesis. This model could also explain the accumulation of phytoglycogen at the expense of amylopectin observed in DBE mutants (Zeeman *et al*., 1998*b*).

Starch phosphorylase: Starch phosphorylase (SP, EC 2.4.1.1) catalyses the reversible transfer of glucosyl units from glucose 1-phosphate (Glc1P) 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 higher affinity for amylopectin than glycogen. Kinetic analysis of the maize endosperm Pho1 showed that the phosphorolytic reaction is favoured over the synthetic reaction in the presence of MOS (Mu *et al*.,

2001). Although the precise role of Pho1 in starch metabolism is unclear, available experimental evidence indicates that Pho1 probably contributes to starch synthesis, as a number of studies have found that SP/Pho1 gene expression and activity measurements both correlate with starch biosynthesis (Duwenig *et al*., 1997; van Berkel *et al*., 1991; Y Yu *et al*., 2001). Studies with sweet potato roots have shown that the activity of the plastidial isozyme (L-form) of SP may be regulated by proteolysis of a 78-amino acid peptide (L78). Removal of L78 by an endogenous protease increased the catalytic activity of SP in the phosphorolytic direction (Chen *et al*., 2002). One possible role for Pho1 would 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 (above). It has been suggested that disproportionating enzymes (D-enzymes, see section on starch degradation) may 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 Glc1P used to synthesize ADPGlc by plastidial AGPase. Indeed, the phosphorolytic SP reaction has been shown to be stimulated by the presence of D-enzyme (Colleoni *et al*., 1999*b*).

Starch degradation in plastids: Starch degradation is part of the overall process of starch turnover which occurs in all starch-containing plastids to varying degrees. Much of the research on starch degradation has focused on understanding the diurnal fluctuations of starch in leaves, whereby the starch synthesized in leaves during the day is degraded at night, and the carbon exported from the chloroplasts used to meet various metabolic demands of the plant. In common with the starch biosynthetic pathway (above), most, if not all, of the enzymes involved in the pathway of starch degradation are known, but the details of its operation and regulation are poorly understood. Little (Fondy *et al*., 1989), or no (Zeeman *et al*., 2002) starch turnover has been reported in leaves during the day, suggesting that the process of starch degradation is switched on, or strongly up-regulated, during the night, and switched off/ down-regulated in the light by as yet unknown/undetermined mechanisms. Interestingly, studies with isolated chloroplasts showed diurnal oscillation of amylolytic (α- and β-amylase and maltase), but not phosphorolytic, activities (Pongratz and Beck, 1978; Ghiena *et al*., 1993) indicating that some type of regulation of the former enzyme activities occurs during the diurnal cycle.

At the simplest level, the process of starch degradation requires an initial hydrolytic attack on the intact starch granule, followed by debranching (hydrolysis) of α-(1→6)-linkages to produce linear glucan chains, and finally, the degradation of the linear chains to glucosyl monomers. There is a range of plastidial enzymes with starch-degrading capabilities which may participate in the process of starch degradation and turnover. α-Amylase (EC 3.2.1.1) and other endoamylases hydrolytically cleave α-(1→4)-glucosyl bonds resulting in the production of a mixture of linear and branched MOS and, ultimately, glucose, maltose, malto-triose, and a range of branched α-limit dextrins. In addition, β-amylase (EC 3.2.1.2) catalyses the hydrolysis and removal of successive maltose units from the non-reducing end of the α-glucan chain. Alternatively, α-(1→4)-glucosyl bonds may be cleaved phosphorolytically by SP to produce Glc1P from successive glucosyl residues at the non-reducing end of the α-glucan chain.

The initial hydrolytic attack on the intact, semi-crystalline starch granule is thought to be via endoamylases (Steup *et al*., 1983; Kakefuda and Preiss, 1997). This idea was tested recently by Smith *et al*. (2003*a*) using *Arabidopsis*, whose genome contains three α-amylase genes, one of which is predicted to be plastidial due to the presence of a putative transit peptide. Analysis of a knock-out mutant for the putative plastidial α-amylase showed that mutant plants had normal rates of starch degradation, indicating that the initial hydrolysis must be catalysed by another endoamylase, or as yet unidentified protein/proteins. The discovery of the R1 protein in potato by Lorberth *et al*. (1998), and studies of the effects of down-regulating the gene, indicate that this protein may play an important role in starch degradation. Antisense plants showing reduced R1 protein levels showed a reduction in the phosphate content of the starch and a reduced ability to degrade starch (Lorberth *et al*., 1998). Mutations at the *sex1* locus in *Arabidopsis* result in leaf starch accumulation and an inability to degrade starch at night. The mutation has subsequently been mapped to a gene encoding a homologue of the potato R1 protein (TS Yu *et al*., 2001), a starch-water dikinase (EC 2.7.9.4) associated with the starch granules which phosphorylates glucose residues on amylopectin (Ritte *et al*., 2002). Interestingly, there are few or no phosphate groups in the amylopectin from the *sex1* mutants. It was recently hypothesized by Smith *et al*. (2003*b*) that either the R1 protein, or the presence of the phosphate groups on amylopectin, are necessary for the action of an enzyme (or enzymes) which catalyse the initial attack on the starch granule.

Both β-amylase and SP activities are present in plastids (Zeeman *et al*., 1998*a*; Lao *et al*., 1999), and each could be responsible for the degradation of linear glucan chains to glucosyl monomers *in vivo*. Analysis of the *Arabidopsis* genome sequence predicts four plastidial β-amylases and one plastidial SP. A plastidial SP knock-out mutant in *Arabidopsis* showed normal rates of leaf starch synthesis and degradation during the diurnal cycle (Smith *et al*., 2003*a*), suggesting that SP is not necessary for starch degradation, and that one or more of the isoforms of β-amylase probably plays an important role in the process.

Evidence in support of this idea comes from studies of a down-regulated form of plastidial β-amylase in potato leaves which showed reduced rates of starch degradation at night (Scheidig *et al*., 2002), and is the first clear evidence for β-amylase having a key role in transitory starch degradation in leaves. The chloroplast envelope is permeable to maltose (Rost *et al*., 1996), and the recent identification and analysis of a maltose transporter (MEX1) indicates that MEX1 is the major route by which the products of starch degradation are exported from the chloroplast at night in higher plants (Niittylä *et al*., 2004). Malto-triose released from the glucan chain by the action of β-amylases, and which is unavailable for further degradation by these enzymes, may be utilized by a plastidial 1,4-α-D-glucan:1,4-α-D-glucan, 4-α-D-glucanotransferase (disproportionating enzyme, D-enzyme, EC 2.4.1.25) found 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 onto a longer glucan chain, making them available to the β-amylases, and the resulting glucosyl monomer available for export from the plastid via a glucose transporter at the inner envelope membrane (Weber *et al*., 2000; Niittylä *et al*., 2004). In addition to plastidial isoforms of D-enzyme, a cytosolic form exists, termed DPE2. Analysis of an *Arabidopsis* mutant in the DPE2 gene suggests that DPE2 utilizes the maltose released from the chloroplast by MEX1, and is therefore likely to be an important component of the pathway of utilization of starch for sucrose synthesis in leaves (Chia *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 reaction plays a part in the pathway of (chloroplast) starch degradation. Available evidence also indicates that MOS levels can modulate starch degradation in leaves, and since D-enzyme probably does not catalyse the initial hydrolytic attack on the granule, the effect of the D-enzyme knock-out mutant could be to cause the accumulation of MOS levels, which in turn may inhibit the initial step of starch degradation (Smith *et al*., 2003*b*). However, the role of D-enzyme in starch metabolism in the unicellular alga *Chlamydomonas reinhardtii* appears to be quite different from that of the *Arabidopsis* enzyme. Analysis of mutants of *C. reinhardtii* specifically lacking D-enzyme (in the STA11 locus) showed a severe decrease in starch content and clearly indicated a role for the enzyme in starch (amylopectin) synthesis (Colleoni *et al*., 1999*a*, *b*; Ball *et al*., 2003). The precise role of D-enzyme in plant storage tissues is unclear, as no mutants have yet been described in any of the major starch storing crops.

Fine control of starch metabolic pathways

The above sections briefly describe the key components of the likely pathway of starch synthesis and degradation in the plastids of higher plants, and their inter- and intracellular tissue localization. However, description and appreciation of the main reactions in each pathway does not in itself explain how the starch granule is synthesized or degraded, nor account for the varied patterns of starch turnover in different plant tissues with essentially the same complement of starch metabolic enzymes. The mechanisms underlying the distinct structure of amylopectin are still unknown, and attempts to synthesize the molecule *in vitro* or to reconstitute the system have not been successful. It is the co-ordination of these expressed proteins inside different types of plastids which allows the controlled synthesis and degradation of this architecturally complex polymer, and the current rudimentary knowledge of these regulatory processes is discussed below.

Allosteric modulation by metabolites

The activities of a number of starch metabolic enzymes have been shown to be modulated by effector molecules, which are often metabolic intermediates. Allosteric regulation of AGPase is the most highly characterized example of this method of control; the catalytic activities of higher plant AGPases generally being stimulated by 3-phosphoglycerate (3-PGA), and inhibited by inorganic orthophosphate (Pi). The relative sensitivity of 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. The chloroplast AGPase, which synthesizes ADPGlc from the Glc1P 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 believed 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*., 2003) and barley (Kleczkowski *et al*., 1993) endosperms suggests that measurable activity (the majority of which is cytosolic, see above) is much less sensitive to 3-PGA activation and Pi-inhibition than other forms of AGPase. Heterologously co-expressed AGP-L and AGP-S subunits of the barley cytosolic AGPase showed insensitivity to allosteric effectors (Doan *et al*.,1999), as did the plastidial AGPase from wheat endosperm amyloplasts (Tetlow *et al*., 2003). However, the plastidial AGPase from the storage tissues of dicots (e.g. potato tuber) appears to be as sensitive to the allosteric effectors as their counterparts in the chloroplast (Ballicora *et al*., 1995; Hylton and Smith, 1992). The sensitivity of plastidial AGPase to allosteric regulation in other plastid types, such as leucoplasts and chromoplasts is unknown.

Information on the allosteric properties of other starch metabolic proteins is sparse. Early work on SP indicated that phosphorolytic activity of Pho1 is strongly inhibited

by physiological concentrations of ADPGlc. Studies with SBEs in wheat showed that the catalytic activity of SBE forms was modulated by a number of metabolites, but in particular, SBEI was stimulated by Pi (Morell *et al*., 1997). Recently, it has been shown that the activity of the maize endosperm pullulanase-type DBE is inhibited in mutants with elevated levels of soluble sugars, such as *shrunken2* (with a disrupted AGPase AGP-L subunit), indicating that sucrose may be a negative regulator of pullulanase-type DBE activity (Wu *et al*., 2002).

Post-translational modifications of starch metabolic enzymes

Redox modulation

Post-translational modification of AGPase involving thioredoxin was suggested 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). In potato, post-translational redox modulation of AGPase occurs in a time frame of 30–60 min, and activation was shown to occur in response to factors directly or indirectly related to increased sucrose availability (Tiessen *et al*., 2002). Working with a recombinant potato tuber AGPase, Fu *et al*. (1998) determined the regulatory site to be a cysteine at position 82 (Cys^{82}) on the AGP-S. It is thought that under oxidizing conditions the Cys^{82} residues from AGP-Ss form intramolecular disulphide bonds, resulting in an inactive dimer (Fig. 1). The Cys^{82} is highly conserved amongst other forms of AGP-S, with the notable exception of the cytosolic isoform of AGP-S from monocots, implying this form of regulation is restricted to plastidial AGPases. However, preliminary studies indicate that cytosolic AGPases in wheat and barley can dimerize under oxidizing conditions *in vitro*, suggesting that different cysteine residues may be involved in this case. The subunit composition of the cytosolic AGPase dimer in these tissues has also not been established (IJ Tetlow, K Denyer, MJ Emes, P Geigenberger, unpublished observations). Recent work has demonstrated that this phenomenon is relatively widespread, and includes photosynthetic, as well as non-photosynthetic tissues from a number of species (Hendriks *et al*., 2003). Starch synthesis in leaves from a broad range of plants is controlled by redox modulation of AGPase activity in response to light and sugar levels. At night, leaf AGPase was converted to an inactive dimer (Hendriks *et al*., 2003). The signalling components leading to redox modulation of AGPase are beginning to be understood, and are thought to involve sucrose and glucose acting via a SNF1-related protein kinase and hexokinase, respectively (Tiessen *et al*., 2003).

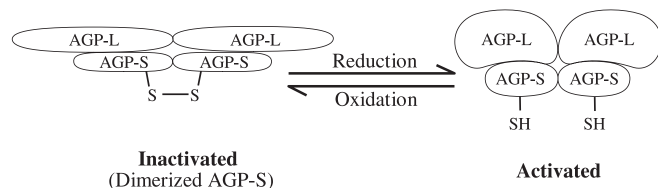

Fig. 1. Current model for redox modulation of AGPase activity in higher plants. Activation of higher plant AGPases is thought to occur via a reduction of an intermolecular disulphide bridge between the AGP-S subunits at a conserved cysteine residue, in addition to a conformational change induced by the substrates. Reductive activation operates in response to cellular sucrose levels through an, as yet, uncharacterized signalling system.

There is some evidence to suggest that the activities of certain members of the α-amylase 'super-family' may be redox modulated. Redox regulation of pullulanase-type DBEs has been proposed in a number of tissues, including spinach leaves and the endosperms of barley and maize (Schindler *et al*., 2001; Beatty *et al*., 1999). *In vitro* studies with β-amylase also showed reversible inactivation via disulphide interchanges (Spradlin and Thoma, 1970), and the amylolytic activity in spinach chloroplasts doubled upon the addition of the sulphydryl reagent dithiothreitol (Pongratz and Beck, 1978), suggesting redox modulation may play a role in the diurnal regulation of amylolytic activities in leaves.

Protein phosphorylation

Until recently, virtually nothing was known about the role that protein phosphorylation plays in the processes of starch synthesis and degradation. Earlier protein phosphorylation studies with heterotrophic plastids suggested a likely role for this regulatory mechanism in non-photosynthetic plastid metabolism (Macherel *et al*., 1986; Vescovi and Viale, 1993; Lukaszewski *et al*., 2001), but few phosphoproteins have been identified from the limited number of such studies, and none connected with starch metabolism. A recent study with isolated amyloplasts from wheat endosperm identified a number of phosphoproteins, including some involved in starch metabolism, indicating that some aspects of starch (amylopectin) biosynthesis may be controlled by protein phosphorylation (Tetlow *et al*., 2004). In this study, all the stromal isoforms of SBE and SP (Pho1) were shown to be phosphorylated at one or more serine residues by plastidial protein kinase(s) in amyloplasts following short incubations of intact organelles with [γ-^{32}P]-ATP. *In vitro* dephosphorylation of the phosphoproteins reduced the activity of SBEIIa and SBEIIb in amyloplasts, and SBEIIa in chloroplasts, whilst having no measurable effect on the activity of SBEI. Phosphoproteins were also detected in the starch granules from isolated wheat endosperm amyloplasts, and among those identified were the

granule-associated isoforms of SBEII, SSIIa, and an unidentified form of SS.

Formation of multiprotein complexes

A potentially important mechanism for co-ordinating the multiple actions of different proteins involved in starch polymer synthesis (and degradation) has recently been proposed involving protein–protein interaction. The sequencing of complete genomes in rice and *Arabidopsis*, and the availability of partial sequences for the major crops such as maize and wheat, provides a list that includes proteins responsible for general cellular regulation, and also includes the proteins predicted to participate in starch metabolic pathways. However, this information does not immediately reveal what all these proteins do *in vivo*, nor how they are assembled into the functional networks that control cellular and subcellular behaviour. It is becoming apparent that the regulation of many different cellular processes is achieved by the use of protein interaction domains which direct the association of polypeptides with one another and with subcellular structures, small molecules or nucleic acids. The possibility that enzymes involved in starch metabolism may operate as part of functional multiprotein complexes arises from the analysis of the pleiotropic effects of various mutations in genes of starch biosynthesis, and then more directly by *in vitro* experiments with isolated plastids. For example, the *amylose extender* (*ae*) mutant of maize lacks SBEIIb, but also shows loss of activity of SBEI and altered properties of an isoamylase-type DBE (Colleoni *et al*., 2003). Mutations in maize that affect both a pullulanase-type DBE (*zpu1-204*) and an isoamylase-type DBE (*su1-st*) both cause a loss in SBEIIa activity, although the amount of SBEIIa protein is apparently unchanged (James *et al*., 1995; Dinges *et al*., 2001, 2003). In addition to the pleiotropic loss of SBEIIa activity in the *zpu1-204* mutation, a reduction in β-amylase activity and a shift in β-amylase migration on native gels has also been observed (Colleoni *et al*., 2003). In both the *zpu1-204* and *su1-st* mutants, the inactive SBEIIa polypeptide accumulated to seemingly normal levels, suggesting the possibility of post-translational modifications and altered interactions with the DBEs. The *ae* mutation in rice endosperm (lacking SBEIIb) caused a dramatic reduction in the activity of soluble SSI (Nishi *et al*., 2001). A recent study of the barley *sex6* mutant suggests that starch granule-associated proteins form protein complexes (or that existing protein complexes in the stroma become granule-associated), as loss of SSIIa activity in the developing endosperm was shown to abolish binding of SSI, SBEIIa, and SBEIIb within the granule matrix, with no apparent loss in the affinity of these enzymes for amylopectin/starch (Morell *et al*., 2003). It has been speculated that the co-ordination of debranching, branching, and SS activities required for starch synthesis might be accomplished by physical association of the enzymes in a complex/complexes within the amyloplast (Ball and Morell, 2003). Thus, the various mutations in different components of a putative protein complex would disrupt or alter the complex and cause a loss or reduction in biosynthetic capacity; this would at least partially explain some of the observed pleiotropic effects associated with a number of well-characterized mutants in cereal endosperms.

Recent experiments with isolated amyloplasts from wheat endosperm have shown that some of the key enzymes of the starch (amylopectin) biosynthetic pathway form protein complexes that are dependent upon their phosphorylation status (Tetlow *et al*., 2004). Phosphorylation of SBEI, SBEIIb, and SP by plastidial protein kinase(s) resulted in the formation of a protein complex between these enzymes which was lost following *in vitro* dephosphorylation. The role of protein complex formation between these starch biosynthetic enzymes in the process of starch synthesis is not fully understood, but it is thought that functional assemblies of this kind improve the efficiency of polymer construction as the product of one reaction becomes a substrate for another within the complex (substrate channelling), and may be part of a wider multiprotein signalling network. The association of SP with SBEI and SBEIIb in a putative complex in amyloplasts, as proposed by Tetlow *et al*. (2004), suggests SP is involved in starch synthesis. There are two alternative means by which SP can perform such a biosynthetic role. Firstly, SBEs could utilize the products of SP activity, i.e. adding branch points to the glucan chains elongated by SP (operating in a glucan-forming direction). A second, more likely scenario, sees SP operating after SBE activity by modifying the structure of amylopectin via phosphorolysis. Many biological multiprotein complexes contain one or more large proteins that are believed to be essential components, both functionally and structurally (Nakayama *et al*., 2002). SP normally functions as a large multimeric protein, and may also act as a scaffold in addition to other functions within the protein complex. The model shown in Fig. 2 represents a working hypothesis based on the available evidence. Other interacting proteins are probably present, forming a larger complex than the one identified so far, and these components await identification. Such schemes have recently been hypothesized following analysis of heterologously expressed starch biosynthetic proteins in yeast cells (Seo *et al*., 2002). The findings of Tetlow *et al*. (2004) may point to a wider role for protein phosphorylation and protein complex formation in the regulation of starch synthesis and degradation in plastids. 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 such a multiprotein complex could be the shielding of the growing polymer from degradative enzymes. At a higher level of organization, the formation of protein complexes during starch biosynthesis

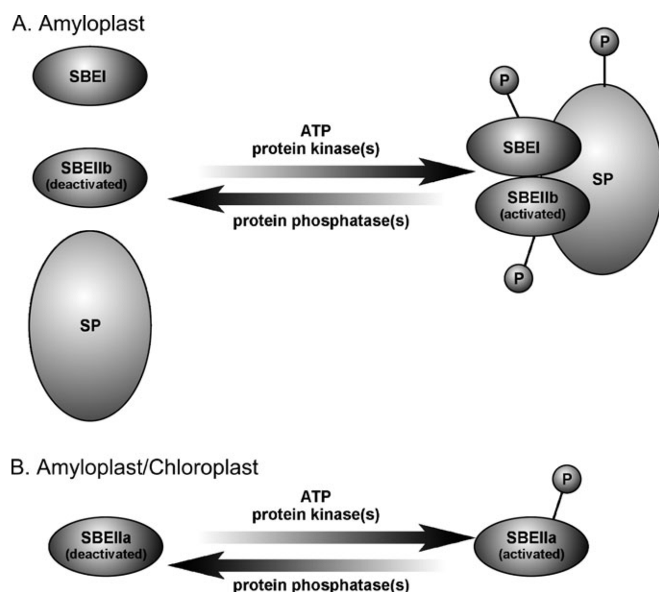

Fig. 2. Model of phosphorylation-dependent protein complex formation involved in storage starch biosynthesis. Activation of SBEIIa (in chloroplasts and amyloplasts, A and B), and activation and complex formation involving SBEI, SBEIIb, and SP by protein phosphorylation in the amyloplast stroma (A) stimulates amylopectin biosynthesis. The functional relationships between the different components of the putative protein complex are unclear. It is notable that in mutants lacking SSIIa, that starch granules are also observed to be devoid of SSI, SBEIIa, and SBEIIb, suggesting that these components may also be capable of forming a complex under *in vivo* conditions.

may promote a certain favoured, necessary three-dimensional structure within the growing polymer, for example, in the case of amylopectin this could be a structure necessary for crystallinity (clustered branch points, side chains of defined length, particular side chain packing). In this hypothetical context, such multiprotein complexes may act as a form of 'carbohydrate chaperonin'. It is expected that the study of protein complexes between starch metabolic enzymes in plastids will reveal a dynamic system, whereby single and multiple complexes operate which are comprised of different permutations of starch metabolic/regulatory proteins, depending on plastid type and environmental circumstances. Data from model systems suggests that the specific complex observed in wheat endosperm is also likely to be formed in the storage tissues of other higher plants, since comparisons of yeast and human protein complexes showed that conservation across species not only applied to single proteins at the amino acid level, but also extended to their immediate molecular environment (Gavin *et al.*, 2002).

14-3-3 proteins

Direct regulation of enzyme activity and/or the regulation of whole series of reactions by protein complex formation, in some cases involve the phosphorylation of target proteins followed by the formation of a complex with 14-3-3 proteins, and 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 Finnie *et al.*, 1999; Chung *et al.*, 1999; Sehnke and Ferl, 2002). The 14-3-3 proteins are a structurally highly conserved group of proteins of approximately 30–35 kDa, and 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 transient starch metabolism has recently been proposed by Sehnke *et al.* (2001) who showed that a form of 14-3-3 protein (from the ε sub-group) was present in *Arabidopsis* leaf starch. Suppression of the granule-associated 14-3-3 proteins resulted in leaf starch accumulation (Sehnke *et al.*, 2001). These workers speculated that one possible target for the 14-3-3 proteins in *Arabidopsis* was SSIII, implying phosphorylation of SSIII at a (putative) conserved 14-3-3 (mode 1) binding motif, causing inactivation of the enzyme, although there is no evidence that this form of SS is granule-associated (Table 1). In addition, 14-3-3 proteins have also been detected inside starch granules from immature maize pollen (Datta *et al.*, 2002), further implicating this family of proteins in starch metabolic pathways. The presence of both 14-3-3 proteins and phosphoproteins within starch granules places these putative signalling components in an ideal situation to regulate starch synthesis and degradation, as well as to perform structural modifications within the growing granule. However, few of the major starch metabolizing enzymes have well conserved 14-3-3 binding motifs (one notable exception is β-amylase), which suggests that if 14-3-3 proteins are involved in facilitating protein–protein interactions between starch metabolizing enzymes in plastids, then binding to the target phosphoproteins may be at, as yet, uncharacterized binding sites. Despite the circumstantial evidence cited above, a direct role for 14-3-3 proteins in starch metabolism has yet to be established.

Summary and future prospects

In addition to the important contribution being made by plant breeding, future increases in starch yield and improvements/modifications of agriculturally produced starches will be driven by the isolation of natural mutants or through the use of genetic engineering approaches. Underpinning this latter technology is the cloning and characterization of genes directly and indirectly associated with starch metabolism for any given crop.

Analysis of the complete *Arabidopsis* and rice genomes now offers a complete overview of the potential genes involved in starch metabolism, and points to key differences between the two 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 represent a note of caution when attempting to make generalized conclusions about the roles of isoforms from one model system to another.

The isolation and analysis of natural and insertion mutants has made, and will continue to make, an invaluable contribution towards understanding of starch metabolism in higher plants. In many cases, the tendency has been to try to explain the functions of genes based solely on their mutant phenotype. The many pleiotropic effects observed arising from single mutations, coupled with preliminary research which indicates that some of the starch metabolic enzymes may operate within complexes, suggests this approach may, in some cases, be 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. Despite the advances made in understanding of starch metabolism by the analysis of mutants, many more mutants are required at key points in the biosynthetic and degradative pathways in order to understand starch metabolism fully in the economically important crops, for example, cereals. To date, no mutants have been characterized in cereals which lack SSI, SSIIb, SSIIc, Iso-2, Iso-3, D-enzyme, SP (Pho1), and plastidial AGPase.

The varied pleiotropic effects observed with various mutations in genes of the starch metabolic pathways (see above) indicates that there are probably many more interactions between 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 which govern this aspect of metabolic regulation, as well as to identify the components controlling protein complex formation in the plastid. This research effort will require a multidisciplinary approach, combining biochemical analyses of putative complexes (e.g. protein purification, immunoprecipitation, yeast two-hybrid studies) and a bioinformatics-based strategy, utilizing the sequence information derived from both complete (rice, *Arabidopsis*) and partial plant genomes, in order to identify common interaction domains, for example, predicting recognition/binding motifs for 14-3-3 protein interactions (see the section on 14-3-3 proteins). However, such predictive data must be validated by direct analysis of protein complexes from cells/plastids. Rapid progress has been made in the understanding of a variety of protein complexes in yeast cells using tandem affinity purification (TAP) of tagged recombinant proteins in conjunction with mass spectrometry (MS) (Rigaut *et al*., 1999; Puig *et al*., 2001). TAP-MS-based approaches facilitate the identification of the various components of protein complexes under *in vivo* conditions, and are better suited to the characterization of protein complexes than yeast two-hybrid screening, which produces binary interactions (see Gavin *et al*., 2002, for analysis of the two systems). Protein chemistry techniques will also be valuable tools in the identification of the various post-translational modifications (phosphorylation, hydroxylation, acetylation, methylation, ubiquitination) which are often necessary prerequisites for binding and assembly of protein complexes in other eukaryotic systems (Pawson and Nash, 2003). Recent advances in the use of MS to identify phosphorylated sites raise the possibility of following post-translational modifications (Ficarro *et al*., 2002; Salomon *et al*., 2003), which can then be linked to the dynamic assembly of protein complexes in the plastid under different environmental conditions, and under varied (artificial) genetic backgrounds.

Metabolic (flux) control analysis has been shown to be a valuable tool in studying the regulation of the conversion of sucrose to starch in the developing potato tuber, particularly in response to environmental changes such as water deficit, rising temperature, or altered oxygen supply (Geigenberger *et al*., 2004). All the major genes of the potato tuber sucrose-to-starch pathway have been cloned in recent years, making this a tractable system for such analysis. As the full complement of genes in this pathway is cloned in the major cereal crops, metabolic control analysis will become an important tool for plant breeders and agronomists to assess crop performance. However, there are limitations in applying metabolic control analysis to the enzymes of starch metabolism, which do not necessarily apply to the soluble and membrane-bound proteins involved in other metabolic pathways, and which will add considerable complexity to such analyses. The partitioning of some of the key starch metabolic enzymes within the plastid changes during development from soluble to granule-associated forms, possibly altering the kinetic characteristics of these enzymes. The relative importance of each enzyme in controlling flux is therefore likely to change during the growth of the starch granule, as may its contribution to controlling not only the amount of starch made, but the structure of the polymer. An unusual feature of the pathway is the fact that the substrate for the starch metabolizing enzymes (the starch granule) is continually changing/growing and altering its three-dimensional structure. In addition, the characteristics of some of the enzymes of starch metabolism may be continually altering as they act upon the granule both individually, and as components of different multi-enzyme complexes.

It is becoming clear that a new level of complexity is emerging in understanding of the starch metabolic pathway. Proteins within the core pathway (and probably other auxiliary proteins) are regulated at the post-translational level and, in addition, may form functional protein complexes, contributing towards the short- to mid-term control of the biosynthetic and degradative pathways. Knowledge has been gained about starch metabolism from recent advances in the understanding of the core synthetic (and degradative) enzymes in different tissues derived from

molecular biology and genomics-based technologies. The next challenge will be to use these tools to identify the regulatory systems controlling the core pathway (e.g. protein kinases and protein phosphatases). These novel regulatory mechanisms need to be fully characterized, and the knowledge of these processes integrated into current understanding of the synthetic and degradative pathways in order to provide a rational basis for attempts at future improvements of starch-storing crops.

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