Chapter 11
Increase of Grain Yields by Manipulating Starch Biosynthesis

Bilal Cakir, Aytug Tuncel, Seon-Kap Hwang, and Thomas W. Okita

Abstract Starch is the main carbohydrate storage reserve in many plants. This carbohydrate not only serves as the major caloric source for much of the world's population and as a key feedstock for renewable energy but is also utilized in many food and industrial applications. As the demand for starch as a source of food and energy feedstock will continue to increase in the coming years, increased production of starchy cereals and tuber crops will be needed, a goal that will be moderated by the loss of arable land and unpredictable environmental conditions due to global warming. In this chapter, we provide an update on starch biosynthesis occurring in photoautotrophic and heterotrophic organs, the role of ADPglucose pyrophosphorylase and other enzymes in this process, and the various molecular strategies for increasing starch in cereals. Although extensive efforts have been made, relatively small improvements have been accomplished in enhancing starch content and grain yield, necessitating the employment of new strategies to meet this goal.

Keywords Starch • AGPase • Biotechnological approaches • Crop yields

11.1 Introduction

Starch is the second most abundant biopolymer in the world. It is the main component of the major food crops and, therefore, constitutes the bulk of the daily diet for much of the world's population. The long-term storage properties of major food crops such as tubers and cereal grains enable them to provide a steady supply of calories as well as a significant proportion of other nutrients throughout the nongrowing season. As such, 2,500 million tons of starch-containing crops (Food and Agriculture Organization of the United Nations, http://faostat.fao.org) are harvested annually worldwide and used directly as food. In addition to being the major dietary source of worldwide caloric intake, more than 700 million tons of starch produced annually from roots and tubers are used for non-dietary purposes in industrial and manufacturer applications. For instance, more than 85 billion liters

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of automobile fuel, in the form of bio-ethanol, were produced from starch in 2011, and this production continues to increase (Amarasekara 2013). Starch is also used in many manufacturer applications such as an adhesive, thickener, polymer, and an inert material in many household and commercial products.

There have been significant efforts in recent years to generate plants with higher starch content due to the rising demand as a food by the world's growing population and as a feedstock for renewable energy. Such efforts, however, will be constrained by the limited acreage of arable lands and supply of water. Therefore, a better understanding of starch metabolism is essential to improve crop yields under less than ideal agricultural conditions for dietary, bioenergy, and industrial uses. Although starch metabolism has been a subject of intensive research for many years, there are still many questions left to be resolved that will provide new insights and strategies to enhance starch levels in plants.

Starch is synthesized in both photosynthetic and non-photosynthetic cells and plays essential roles during the life cycle of the plant. In leaves, a fraction of photoassimilate is retained in the chloroplasts and stored as transitory starch, a reserve which facilitates normal plant growth and development by providing a continuous supply of carbon and energy during the night. The bulk of fixed carbon, however, is converted into sucrose and exported to heterotrophic tissues and organs to support their growth and development. In storage organs such as cereal grains and tubers, starch is used as long-term storage reserve to support the next plant life cycle. Similar to transient starch in leaves, efficient metabolism of starch in storage organs has a profound effect on plant yields by maximizing the products of CO_2 assimilation.

11.2 The Pathway of Carbon into Starch in Photosynthetic and Heterotrophic Sink Tissues

The pathways leading to starch synthesis in photosynthetic and heterotrophic sink tissues differ. In photoautotrophic leaves, starch biosynthesis is restricted to the chloroplasts, while in heterotrophic sink tissues, the events leading to starch synthesis occur in both the cytoplasm and plastid. In cereals, the majority of the reactions, including synthesis of ADPglucose (ADPglc), take place in the cytosol, whereas production of ADPglc occurs in the amyloplast in non-cereal crops.

11.2.1 Biochemical Events in Leaves

In leaves, the fixed carbon is converted to sugars and amino acids during the day and transported to non-photosynthetic organs. A portion of this carbon is stored within the chloroplasts as transient starch which is broken down at night to provide

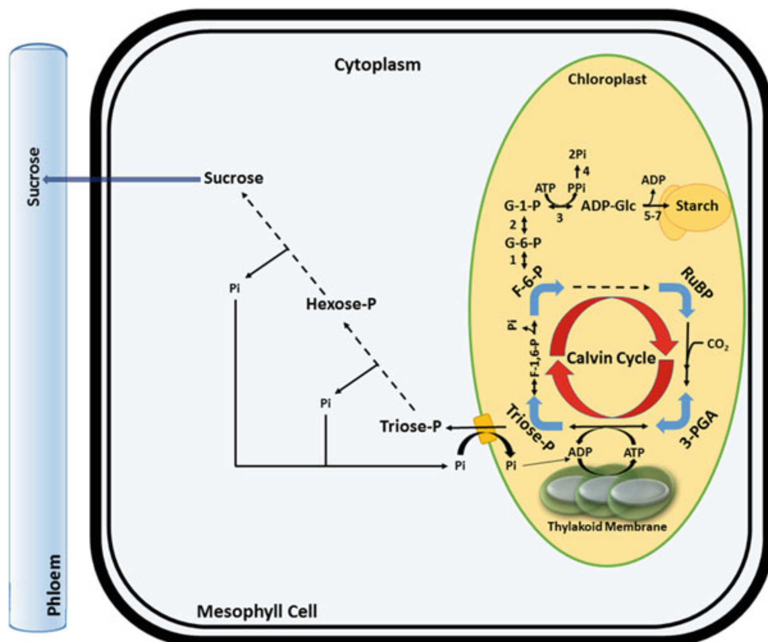

Fig. 11.1 Synthesis of starch in leaves. The fixed carbon in the form of 3-PGA is converted to triose Ps which are used for both the synthesis of starch and sucrose. Sucrose is transported to sink tissues to support plant growth. Starch synthesis is catalyzed by the following enzymes: *1* phosphoglucose isomerase, *2* phosphoglucomutase, *3* AGPase, *4* inorganic pyrophosphorylase, *5* SS, *6* SBE, *7* starch-debranching enzyme

reducing sugars for carbon precursors and energy (Fig. 11.1) (Stitt and Zeeman 2012). Although much of the carbon flux is through the Calvin-Benson-Bassham cycle, some of it is diverted into starch via the action of phosphoglucose isomerase (PGI) which converts fructose 6-phosphate (Fru6P) to glucose 6-phosphate (Glc6P). This hexose-P is then converted by phosphoglucomutase (PGM) into glucose 1-phosphate Glc1P, which together with ATP is used by ADPglucose pyrophosphorylase (AGPase) to generate ADPglc, the sugar nucleotide utilized by starch synthases. Since AGPase catalyzes the first committed step in the starch biosynthetic pathway, it is subjected to several regulatory mechanisms, which are discussed later in this chapter.

Although it is widely accepted that the flow of carbon into starch is dependent on the AGPase reaction and other plastid-localized events, an alternative cytoplasmic-based pathway has been suggested. Sucrose synthase (SuSy) also generates ADPglc by the hydrolysis of sucrose in the presence of ADP. This idea is corroborated by the presence of high amounts of ADPglc in leaves of *Arabidopsis* mutant lines which lack AGPase and PGM and the suggested existence of an ADPglc translocator on the plastid envelope that would support this alternate route to starch synthesis (Shannon et al. 1998). This alternative pathway in leaves involving cytoplasmic and plastidial

events has generated considerable debate in the literature (Baroja-Fernández et al. 2005; Ekkehard Neuhaus et al. 2005) that remains ongoing (Baroja-Fernandez et al. 2012). Suffice to say, irrespective of whether such an alternative pathway to starch synthesis exists, it only, at best, plays a minor role in starch synthesis as evident by the marked reduction in leaf starch in plants harboring a mutation in the AGPase small subunit APS1 gene (Ventriglia et al. 2008).

11.2.2 Biochemical Events in Seeds

Heterotrophic tissues such as developing seeds rely on sucrose produced in source leaves as the major resource to support growth and development. Sucrose is hydrolyzed by SuSy to generate UDPglc and fructose, two products which are eventually converted into Glc6P (Fig. 11.2). The plastid envelope has active Glc6P/Pi and ATP/ADP transporters (Jonik et al. 2012) which provide the carbon and energy for AGPase, respectively, and, in turn, starch biosynthesis.

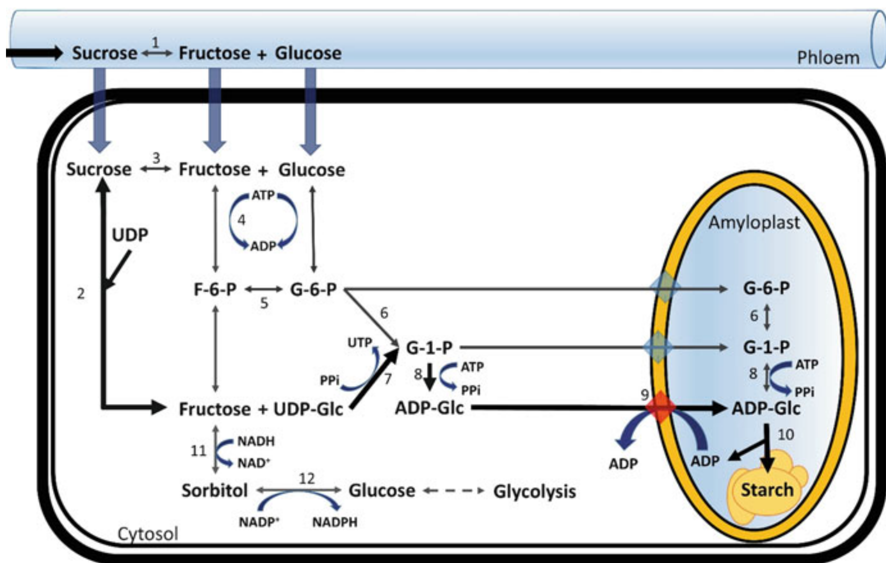

Fig. 11.2 Conversion of sucrose into starch in developing cereal endosperm (Adopted from Tuncel and Okita 2013). Formation of ADPglc and subsequent production of starch in seeds are depicted. Sucrose from leaves is converted into ADPglc in the cytoplasm and then transported into amyloplast. Major reactions of starch synthesis are shown by *bold arrows*. Enzymes shown are (*1*, *3*) invertase, (*2*) sucrose synthase, (*4*) hexokinase, (*5*) phosphoglucose isomerase, (*6*) phosphoglucomutase, (*7*) UGPase, (*8*) AGPase, (*9*) ADPglc transporter, (*10*) starch synthases, (*11*) sorbitol dehydrogenase, (*12*) aldose reductase

Cereal endosperms are unique in containing two different AGPase activities, a major cytoplasmic form and a minor plastidial form (Denyer et al. 1996; Thorbjørnsen et al. 1996; Sikka et al. 2001). Genetic studies (Tsai and Nelson 1966; Morrison et al. 1993; Lee et al. 2007) showed that mutations in the structural genes that code for the cytoplasmic enzyme result in a severe depression in starch accumulation, suggesting that the major site of ADPglc synthesis is cytoplasmic. Expression of an up-regulated bacterial AGPase gene (*glgC*-TM) in the cytoplasm but not in the amyloplast compartment resulted in an enhancement of starch synthesis and accumulation (seed weight) (Sakulsingharoj et al. 2004). Lastly, mutation in the BT1 (*Brittle-1*) sugar nucleotide transporter resulted in a severe reduction in starch levels but exceedingly high increases in ADPglc levels (Sullivan and Kaneko 1995; Shannon et al. 1998). These latter studies provide concrete evidence that the major route of carbon into starch in developing cereal endosperm requires the cytoplasmic AGPase.

Although the exact reason why cereals evolved new AGPase genes to be expressed in the cytoplasm is not exactly known, this subcellular localization provides a more efficient utilization of energy based on its status in maize endosperm (Rolletschek et al. 2005). As the maize endosperm develops, it becomes increasingly micro-aerobic where oxygen levels drop to about 1.4 % of that present in the atmosphere (Rolletschek et al. 2005), and hence, ATP is likely synthesized via glycolysis rather than by respiration. By generating ADPglc in the cytoplasm, high-energy pyrophosphate (PPi) and nucleotide triphosphate products (ATP and UTP) are conserved in this compartment. PPi consumed in the UGPase reaction is regenerated by the AGPase reaction. Conversely, ATP consumed in the AGPase reaction generates an equivalent UTP in the UGPase reaction. Hence, starch synthesis in maize and likely in other cereals recycles considerable energy, especially those contained in PPi. By contrast, in non-endosperm heterotrophic tissues where ADPglc is synthesized in the plastid, the hydrolysis of PPi by inorganic pyrophosphatase drives ADPglc synthesis and recycles Pi in vivo*,* at the expense of a loss of energy.

Despite extensive biochemical studies, the metabolic fate of fructose and UTP produced by SuSy and UGPase, respectively, has not been completely resolved. The generated UTP could be used by UTP-dependent fructokinase to synthesize Fru6P. However, UTP is more likely to support not only RNA synthesis but also DNA synthesis as there is extensive endoduplication of DNA that occurs during cereal endosperm development. An alternative model of fructose metabolism would be via sorbitol dehydrogenase (SDH) which converts fructose to sorbitol using NADH as a reductant (Fig. 11.2) (Oura et al. 2000), which would recycle NAD^+ to support glycolysis. In turn, sorbitol could then be converted into glucose by an aldose reductase where NADPH is also formed, which can be used for various biosynthetic reactions. The role of SDH in metabolizing fructose is supported by the inverse correlation between enzyme activity and fructose levels (Archbold 1999).

11.3 Structural and Functional Role of AGPase

Both bacteria and plants utilize the action of AGPase for synthesis of α-glucan reserves. Although carrying out the same reaction and subjected to allosteric regulation, the prokaryotic and higher plant AGPases have distinct structures. The prokaryotic enzymes are composed of four identical subunits (α4), while the higher plant enzymes contain a pair of two identical large (L) and two identical small (S) subunits ($\alpha_2\beta_2$) (Preiss 1972; Lin et al. 1988; Okita et al. 1990; Hwang and Okita 2012). Moreover, the genes that code for the higher plant enzymes are more complex and diverse than the prokaryotic genes as they code for multiple L and S subunit forms (Krishnan et al. 1986; Smith-White and Preiss 1992). For instance, *Arabidopsis* and rice have four L subunit genes (*APL1* to *APL4* in *Arabidopsis*, *OsAPL1* to *OsAPL4* in rice) and two S subunit genes (*APS1* and *APS2* in *Arabidopsis*, *OsAPS1* and *OsAPS2* in rice) (Crevillén et al. 2003, 2005; Akihiro et al. 2005; Lee et al. 2007). The *Arabidopsis* APS2 may not be a bona fide AGPase subunit as it does not display any catalytic activity by itself or when co-expressed with any of the four *Arabidopsis* L subunits (Crevillén et al. 2003). The rice AGPase S subunits display a further complexity as the *OsAPS2* gene is subject to alternative mRNA splicing that generates two S subunit isoforms, the leaf-specific OsAPS2a and the endosperm-specific OsAPS2b (Lee et al. 2007). Furthermore, the primary sequences of S subunits are highly conserved between species, whereas L subunits show a lower degree of conservation even within a species (Smith-White and Preiss 1992). Phylogenetic analysis shows that the primary sequences of the S subunits are more conserved than those of the L subunit indicating that amino acid substitutions in the S subunits are more evolutionarily constrained than the L subunits. This phenomenon was explained by the fact that S subunits have fewer gene copies, are less tissue specific, and, hence, have to interact with multiple L subunit isoforms (Georgelis et al. 2008). The L subunit may display more evolutionary flexibility as a means to fine-tune the catalytic and allosteric regulatory properties of the enzyme expressed in different tissues of the plant (Georgelis et al. 2008). The advantages of expressing tissue-specific AGPases with different regulatory and catalytic properties, however, are not readily apparent and would be a fruitful study in the future.

Earlier studies have suggested that the two subunits have different roles in enzyme function. When expressed alone in *Escherichia coli*, the recombinant potato tuber S subunit forms a catalytically active homotetramer with defective allosteric properties (Ballicora et al. 1995; Hwang et al. 2008). The L subunit, on the other hand, does not form an active enzyme without the S subunit. The capacity of the S subunit to form a fully catalytically active, albeit allosterically defective, enzyme suggests that the S subunit is the catalytic subunit and that the L subunit simply modulates the allosteric regulatory properties of the enzyme (Iglesias et al. 1993). This secondary role for the L subunit, however, was discounted by the study of mosaic enzymes composed of primary sequences from maize endosperm and potato

tuber AGPase where both subunits were found to equally contribute to the catalytic activity and allosteric regulatory properties of the enzyme (Cross et al. 2004, 2005). Likewise, detailed kinetic analysis of potato AGPase variants containing different combinations of mutant L and S subunits showed that both subunits contribute equally to the allosteric regulation as the properties are a product of synergy between the two subunit forms (Hwang et al. 2005).

The relative contribution of the L subunit in catalysis of the heterotetrameric enzyme varies considerably depending on the enzyme activity examined. The potato L subunit plays a minor role in catalysis (Ballicora et al. 2005; Hwang et al. 2007). Mutation of catalytic residue D145 of the S subunit resulted in a mutant enzyme ($L^{WT}S^{D145N}$) which displayed more than four orders of magnitude less catalytic activity than wild-type enzyme. By contrast, the comparable mutation (D160N) in the L subunit resulted in a minor reduction (~40 %) in catalytic activity of the mutant enzyme. Moreover, kinetic studies of the L subunit homotetrameric enzyme showed that it had only 4 % of the catalytic activity of the S subunit homotetramer. Interestingly, the L subunit was not activated by 3-PGA as a homotetramer but activated 30-fold when assembled with the catalytically silenced S subunit (D145N) (Ballicora et al. 2005). This observation indicates that the catalytic activity of the L subunit requires the presence of the S subunit and again supports the contribution of both subunit types in allosteric regulation.

A role for both subunits contributing to catalysis is also evident by mutations in the L subunit that have drastic consequences on catalytic activity and apparent substrate affinities (Hwang et al. 2007). The low catalytic efficiency of the potato L subunit is likely due to mutations at and/or near the catalytic site that occurred during evolution. The potato L subunit is capable of binding ATP (Hwang et al. 2006) and Glc-1-P (Fu et al. 1998b) but is apparently unable to undergo the catalytic cycle efficiently. Ballicora et al. (2005) showed that the L subunit with two amino acid substitutions (K41R and T51K) near the catalytic site, when assembled with catalytically silenced S subunit mutant, resurrected the catalytic activity of the $L^{WT}S^{D145N}$ enzyme nearly 250-fold such that it attained ~10 % of wild-type enzyme activity (Ballicora et al. 2005).

The low catalytic activity of the potato L subunit is not a conserved property among L subunits from other plant species. For instance, *Arabidopsis* L subunits (APL1 and APL2) exhibited significant catalytic activity when expressed with inactive S subunit (APS1) mutant, whereas heterotetramers containing mutant APS1 and APL3/APL4 were catalytically inactive. Both APL1 and APL2 have Lys residues (K271 and K267) at the corresponding positions of the potato L subunit, which at least partially account for catalytic activity (Hwang et al. 2008; Ventriglia et al. 2008). In addition, the tomato L3 subunit has been demonstrated to have catalytic activity as a monomer (Petreikov et al. 2010). Collectively, both subunits take part in catalysis and allosteric regulation of AGPase although the contribution of the L subunit to catalysis varies depending on the plant species and the isoform examined.

11.4 Allosteric Regulation of AGPase

11.4.1 Leaf Enzymes

In bacteria, cyanobacteria, and higher plants, α-glucans are synthesized during periods of excess sugars and consumed when carbon and energy become limited. Hence, the synthesis of α-glucans needs to be tightly regulated, which is accomplished mainly by controlling the synthesis of ADPglc via AGPase activity. The bacterial AGPase is allosterically activated by metabolic effector molecules that are intermediates of major metabolic pathways, glycolysis or the Entner-Doudoroff, and reflect the "high-energy state" of the cells (Ballicora et al. 2003). The glycolytic intermediate fructose-1,6-bisphosphate (FBP) is an activator of AGPase from *E. coli*, while the enzymes from *Agrobacterium tumefaciens* and *Rhodospirillum rubrum* are activated by Fru6P and pyruvate, respectively (Björn and Govindjee 2009; Ballicora et al. 2003). AGPases from cyanobacteria, green algae, and higher plants are activated by glycerate-3-phosphate (3-PGA) (Heldt et al. 1977; Preiss 1988; Preiss et al. 1989), which is the immediate product of CO_2 fixation. In addition to 3-PGA, AGPases from spinach leaves are also activated by Fru6P, FBP, phosphoenolpyruvate (PEP), and ribose 5-phosphate (Ghosh and Preiss 1966). These other metabolites, however, activate AGPases to a lesser extent, and higher concentrations are required to obtain maximum activation. For example, the most effective activator after 3-PGA is Fru6P, which enhances AGPase activity up to 40 % of the activation level observed for 3-PGA (Ghosh and Preiss 1966). Likewise, AGPase activity is suppressed by specific inhibitors including adenosine 5′-monophosphate (5′AMP) for many of the bacterial AGPases, while inorganic phosphate (Pi) is an inhibitor of the cyanobacterial and plant enzymes.

The regulation of AGPase activities and, in turn, starch synthesis in higher plants is under strict control. In leaves, AGPase is located in chloroplasts, the site of CO_2 fixation (Okita et al. 1979; Morell et al. 1988). During active photosynthesis, 3-PGA is produced and converted into triose phosphates which can reenter the Calvin cycle or be exported to the cytoplasm where it serves as the precursor for sucrose synthesis. An important by-product of the metabolic pathway leading to sucrose synthesis is Pi, which can then be recycled back into the plastid via the Pi translocator to support ATP synthesis. The 3-PGA/Pi ratio in chloroplast allows coordination of starch synthesis in this organelle and sucrose synthesis in cytoplasm according to the energetic state and needs of the cell (Stitt et al. 1987). When sucrose synthesis is not saturated, triose phosphates are exported to the cytoplasm where they are converted into sucrose enabling the by-product Pi to be imported back to the plastid where it can be recycled to generate ATP. Recycling of Pi into chloroplast results in relatively low 3-PGA/Pi ratio, a condition which assures less AGPase activity and, in turn, more carbon flow directed into sucrose synthesis. Hence, the allosteric regulation of AGPase operates in a way to suppress activity of the enzyme so that much of the fixed carbon is diverted for the biosynthesis of sucrose which is exported to support sink organs. However,

when sink tissues are not able to efficiently assimilate the transported carbon, the levels of sucrose and phosphorylated intermediates increase in the cytoplasm while Pi levels correspondingly decrease in the chloroplasts of leaf cells resulting in an increase in the 3-PGA/Pi ratio in the stroma. Therefore, AGPase is activated as a result of the high 3-PGA/Pi ratio enabling triose phosphates to be incorporated into transient starch while generating Pi to support photophosphorylation (Ballicora et al. 2004; Tuncel and Okita 2013). Thus, the allosteric regulation of AGPases in photosynthetic tissues is an essential mechanism enabling starch to serve not only as a transient reserve to support heterotrophic plant growth during the night but also as an important alternative metabolic process to recycle Pi and, thereby, minimize feedback inhibition of photosynthesis. This alternative mechanism to recycle Pi to support photophosphorylation is supported by analysis of transgenic *Arabidopsis* and rice plants which accumulated elevated leaf starch levels by expression of up-regulated AGPases (Gibson et al. 2011). Such plants exhibited increased photosynthetic capacity and, in turn, enhanced growth rates.

11.4.2 Sink Enzymes

One of the most extensively studied AGPases is the potato tuber enzyme. The potato AGPase is activated more than 30-fold by 3-PGA and inhibited by Pi. Fructose 2,6-bisphosphate (Fru2,6BP) and PEP are only partially effective (<30 % of levels obtained with 3-PGA) in activating the enzyme, while Fru6P and Glc6P are very weak activators (Iglesias et al. 1993; Wakuta et al. 2013). Unlike the leaf and stem AGPases, which are activated and inhibited by 3-PGA and Pi, respectively, the enzymes from developing seeds display considerable variation in their dependence on 3-PGA activation. The chickpea enzyme is highly responsive to 3-PGA activation (>30-fold activation) and Pi inhibition (Singh et al. 2003), whereas those from pea embryo (Hylton and Smith 1992) and bean cotyledon (Weber et al. 1995) are only slightly dependent (<30 % activation) on 3-PGA levels for attaining maximum catalytic activity. This variation in 3-PGA response observed for AGPases from developing seeds of dicots is also evident for those from cereal grains. The AGPase from barley endosperm (Doan et al. 1999) exhibits high catalytic activity when assayed in the absence of potential activators and was found to be insensitive to effector molecules. Likewise, the wheat AGPases (Tetlow et al. 2003) are not dependent on 3-PGA for maximum catalytic activity but are allosterically inhibited by Pi, ADP, and FBP. Although they have no impact on the enzyme activity alone, 3-PGA and Fru6P are able to reverse the inhibitory effect of Pi (Gómez-Casati and Iglesias 2002). In contrast, the rice endosperm native AGPase requires 3-PGA for maximal catalytic activity. The major AGPase activity in rice endosperm is stimulated more than 30-fold by 3-PGA, an activation which is reversed by the inhibitor Pi (Tuncel et al. 2014b).

The maize endosperm enzyme, extensively studied in detail by the Hannah laboratory, displays unique properties not seen in other AGPases. Although the enzyme

is activated by 3-PGA, the basal catalytic activity in the absence of 3-PGA is quite substantial resulting in a lower activation (ten-fold or less) than that seen for the leaf and potato tuber enzymes (Boehlein et al. 2005, 2009). It, therefore, shows an intermediate response to 3-PGA activation between that exhibited by the wheat (no 3-PGA response) and rice (high 3-PGA response) endosperm enzymes. Moreover, the enzyme is also appreciably activated by Fru6P and Glc6P, hexose-Ps that serve as poor activators for the leaf and tuber enzymes. In addition to its unique responses to the activators, the maize enzyme also exhibits unusual responses to Pi. In the absence of 3-PGA, the maize enzyme is not inhibited by Pi at low concentrations. In contrast, Pi surprisingly acts as an activator at low concentrations by significantly lowering the K_M values for the substrates ATP and Glc-1-P (Boehlein et al. 2010). Detailed kinetic studies have suggested that 3-PGA and Pi bind to the same or overlapping sites (Boehlein et al. 2013). By contrast, the potato enzyme has separate binding sites for these two effectors. Although the maize enzyme is inhibited by Pi at high concentrations, the K_i is one to two orders of magnitude higher than that of the leaf and tuber enzymes and, moreover, is only inhibited about 50 % compared to the nearly 100 % inhibition seen for the leaf and potato enzymes. Overall, the maize endosperm enzyme is not as sensitive to 3-PGA activation and Pi inhibition as the leaf and tuber enzymes. These unique allosteric regulatory properties suggest that the maize endosperm enzyme is not subject to the same stringent controls required by the leaf and tuber enzymes (Boehlein et al. 2009).

11.4.3 Redox Regulation

The leaf and tuber AGPases are also subject to redox regulation mediated by dimerization of the S subunits. This regulation was first observed in the potato tuber AGPase, where the extent of oxidation of the S subunits to form a 100 kDa dimer correlated with AGPase activity and affinity to ATP (Tiessen et al. 2002; Geigenberger et al. 2005). Incubation of the tuber disks in the presence of the reducing agent, dithiothreitol, or sucrose prevented S subunit dimerization and significant decrease in AGPase activity. Chemical labeling studies showed that activation was due to the reduction of an interchain disulfide bridge formed between Cys12 of the two S subunits (Fu et al. 1998a; Ballicora et al. 2000). Subsequently, Ballicora et al. showed that thioredoxin (Trx) was able to effectively reduce the S subunits (Ballicora et al. 2000). Interestingly, activation is very pronounced (three-fold or more) when the enzyme is reduced with DTT in the absence of 3-PGA. In the presence of 3-PGA, activation (four-fold) is only readily evident at lower activator concentrations (<1 mM), whereas at higher concentrations the difference in catalytic activities between the reduced and oxidized enzyme forms is minimal (Ballicora et al. 2000). Reduction of the S subunits increases the affinity of the enzyme to 3-PGA about 2-fold and affinity to ATP about 1.6-fold.

These studies were extended to the leaf AGPases from pea, potato, and *Arabidopsis* (Hendriks et al. 2003). Light and sugars were shown to modulate the

redox activation of AGPase in leaves. Redox activation of AGPase is similar to the light-dependent activation of enzymes involved in the Calvin-Benson cycle, ATP synthesis, and NADPH export from chloroplast by changing the redox status of the electron transport chain (Scheibe 1991). Photosynthetic electron transport results in the reduction of ferredoxin (Fdx) that can light-activate AGPase activation by two different processes. In one pathway, Fdx can reduce plastidial Trx *f* or Trx *m*, which can then reduce and activate AGPase and, in turn, starch synthesis in response to light. For instance, AGPase from pea leaf was shown to be activated three-fold by either 20 μM Trx *f* or Trx *m*. The second pathway involves the light-dependent activation of NADP-thioredoxin reductase C (NTRC) (Michalska et al. 2009). In the light, photoreduced Fdx activates NTRC by reducing Fdx-NADPH reductase. In turn, NTRC stimulates reductive activation of AGPase and starch synthesis. The knockout *ntrc* mutants of *Arabidopsis* displayed 5-fold decrease in AGPase monomerization in leaves and 2.7-fold decrease in starch levels compared to wild-type plants at the end of the day (Michalska et al. 2009).

In addition to light, sucrose also induces redox activation of AGPase in leaves (Hendriks et al. 2003). Increased sucrose levels elevate trehalose 6-phosphate (Tre6P) by regulating Tre6P synthase (TPS) or Tre6P phosphatase (TPP) (Kolbe et al. 2005), key enzymes that balance Tre6P levels in the cytosol. Overexpression of TPS, which converts Glc6P and UDPglc into Tre6P, in *Arabidopsis* leaves resulted in higher AGPase activation and starch levels compared to wild-type plants. On the other hand, plants overexpressing TPP, which dephosphorylates Tre6P to produce trehalose, had lower AGPase activation and starch levels. Further, intact chloroplasts were isolated from *Arabidopsis* leaves and fed with either 100 μM Tre6P or sucrose. The redox activation of AGPase was observed in chloroplasts fed with Tre6P, but not those fed with sucrose. Thus, it is clear that cytoplasmic synthesized Tre6P regulates starch metabolism by stimulating NTRC- or Trx-dependent redox regulation of AGPase although specific details on the signaling cascade between these players still need to be resolved (Kolbe et al. 2005; Lunn et al. 2006).

The physiological significance of redox regulation of AGPase activity was directly assessed in *Arabidopsis* (Hädrich et al. 2012). The APS1 subunit gene was mutagenized where Cys81 (equivalent to Cys12 of potato tuber enzyme) was replaced with Ser ($APS1_{C81S}$) and then transformed into the *adg1* mutant line lacking the functional APS1 subunit. The expressed mutant $APS1_{C81S}$ was unable to dimerize in the dark indicating that Cys81 was responsible for interchain disulfide linkage between the two S subunits. Despite having significantly lower levels (five- to 10-fold) of AGPase activity, the transgenic *adg1*/$APS1_{C81S}$ lines had higher levels of ADPglc and maltose as well as elevated starch synthesis or elevated starch levels at the end of the dark period than control plants. Hence, redox regulation of AGPase is essential for the diurnal regulation of starch turnover with the loss of regulation impacting starch breakdown. This latter condition would be consistent with the differences in the kinetic properties of the oxidized and reduced AGPase. In the presence of low 3-PGA or low 3-PGA/Pi ratio, the residual catalytic activity of the oxidized enzyme would be significantly smaller than the reduced enzyme. Hence, in the transgenic *adg1*/$APS1_{C81S}$ line, the reduced AGPase would have

higher catalytic activity in the dark allowing more starch synthesis to occur than that for the oxidized enzyme. Hence, the inability to form an oxidized enzyme impedes net starch turnover in the dark resulting in a starch-excess phenotype.

The contribution of redox regulation in controlling AGPase activity was not supported in another study (Li et al. 2012). No significant differences in the levels of reduced APS1 subunits and starch accumulation were evident between the wild-type and *ntrc* mutant lines grown at different light intensities. Therefore, Li and his colleagues (2012) suggested that redox regulation of AGPase via the NTRC pathway does not contribute to the fine regulation of transitory starch synthesis in *Arabidopsis* leaves. However, it should be noted that there were significant differences in the growth conditions (light intensity) of the *Arabidopsis* plants and preparation of protein samples (quenching of samples to prevent oxidation), which likely account for the different conclusions made in these two studies (Hädrich et al. 2012; Li et al. 2012). Likewise, the contribution of thioredoxin *f*/*m* was not discounted. Moreover, the fine down-regulation of AGPase activity in the dark, as suggested by Hädrich and his colleagues, was not evaluated in this study (Li et al. 2012).

11.4.4 Redox Regulation in Sink Organs

The carbon status, *i.e.*, the availability of glucose and sucrose in the cell, leads to redox activation of potato tuber AGPase by two different mechanisms (Tiessen et al. 2002, 2003). Firstly, the sucrose-dependent pathway involves SNF-1-like protein kinase (SnRK1), which stimulates the reductive activation of AGPase (Tiessen et al. 2003; McKibbin et al. 2006). In detached potato tubers, AGPase becomes less active and exhibits lower affinity to substrates and less sensitivity to 3-PGA activation, thus decreasing rates of starch synthesis (Tiessen et al. 2002). The differences in kinetic properties between the AGPases of detached and undetached tubers were quite remarkable in that AGPase activity was 10-fold lower in the former case. The AGPase from the detached tubers also exhibited significantly reduced 3-PGA sensitivity at physiological levels, a condition which was reversed by incubation of the detached tubers with sucrose. The components of sucrose signaling that stimulate reductive activation of tuber AGPase have yet to be identified. Secondly, a glucose-dependent pathway for redox activation of AGPase includes hexose phosphorylation by an endogenous hexokinase (Tiessen et al. 2003). When potato disks are incubated in the absence of sugars for 2 h, redox activation of AGPase is decreased due to limited sugar levels. When disks are fed with glucose, reductive activation of AGPase is recovered. Increased glucose supply elevates the $NADPH/NADP^+$ ratio in the cell which, in turn, will alter the plastidial redox modulation. Details on signal transduction between cytosol and the plastid are not fully resolved, but the results suggest that the redox transfer between Trx and its target may be influenced by additional factors.

In contrast to the potato tuber or leaf AGPases, the Cys12 residue is not conserved in S subunits of cereal endosperm AGPases, suggesting that endosperm AGPase

is not regulated by redox status of the cell. Nevertheless, the maize endosperm L subunits and not the S subunits are capable of forming a disulfide bond in the absence of DTT indicating a possible redox regulation of AGPase activity through the L subunit (Linebarger et al. 2005). Direct evidence in support of redox control of the cereal enzymes is seen for the rice endosperm AGPase, which is also subject to redox regulation through Cys residues at the N terminal region of the L subunit (AGPL2) (Tuncel et al. 2014a). Interestingly, the changes in kinetic properties of the native enzyme are more dramatic than the recombinant AGPase when they are reduced. The rice native reduced AGPase exhibits 6-fold higher affinity to 3-PGA and enhanced activity overall 3-PGA levels, whereas the recombinant AGPase only shows 3.4-fold more affinity to 3-PGA in the reduced state and higher activity only at low 3-PGA with no significant differences between the reduced and nonreduced forms at higher 3-PGA levels. Further studies are required to identify the basis for the differences in redox behavior between the native and recombinant enzymes in the presence of 3-PGA.

Lastly, in addition to finely controlling the catalytic activity, redox regulation may also play a role in controlling the enzyme's expression at the protein level. The activities of AGPases from cereal endosperms are significantly affected by heat exposure, an effect which also limits crop productivity. The maize AGPase activity is sensitive to heat and loses much of its activity ($\sim$95 %) when incubated at 57–60 °C for 5 min (Hannah et al. 1980; Greene and Hannah 1998). By contrast, the potato tuber AGPase is much more heat stable, and its activity is still maintained under these conditions (Okita et al. 1990; Hwang et al. 2008). To determine the basis for the differences in heat stability, the conserved QTCL motif which contains the Cys residue responsible for the interchain bond between the pair of potato S subunits was inserted in the maize endosperm S subunit (Linebarger et al. 2005). The mutagenized recombinant maize endosperm enzyme showed a 300-fold increase in the heat stability (Linebarger et al. 2005). A role for general stability is also evident in transgenic *Arabidopsis adg1* plants expressing the $APS1_{C81S}$. Although RNA levels were comparable to that seen in wild-type, net enzyme and $APS1_{C81S}$ subunit levels were significantly reduced. Hence, these results suggest that the AGPase capable of oxidoreduction cycle is more stable than the reduced fixed form.

11.5 Biotechnological Methods to Improve Grain Yields

Multiple approaches have been used to improve grain yields via manipulation of starch metabolism, most of which focused on the manipulation of single genes. Although some progress has been made with these approaches, relatively small improvements have been achieved in terms of starch content and grain yields (Van Camp 2005). In the following sections, the molecular strategies to increase starch levels in cereals and future prospects for enhancing starch yield are discussed.

11.5.1 Manipulation of AGPase Activity

Extensive attempts to increase starch content in cereal seeds have centered on the manipulation of AGPase activity since it catalyzes the first committed and "rate-limiting step" in starch biosynthesis. Indeed, this was first demonstrated in russet Burbank potatoes where expression of an allosteric-insensitive *E. coli glgC* gene elevated starch synthesis and the density of the tubers. Interestingly, a similar study using the potato cultivar Desiree failed to elevate net starch accumulation (Sweetlove et al. 1996). Although a four- to five-fold increase in AGPase activity and increase of carbon flux into starch were observed, elevated starch turnover was also apparent resulting in no net gain in starch (Sweetlove et al. 1996). The basis for the differences seen between these two studies is not clear although the use of different potato cultivars and their corresponding differences in starch content is a likely factor.

In view of the success obtained in elevating starch content in russet Burbank potatoes, considerable effort has exploited AGPase expression as a means to increase starch synthesis in developing cereal endosperm. As discussed in a recent review (Tuncel and Okita 2013), elevated AGPase expression increases the starch content of individual maize and rice grains (Giroux et al. 1996; Sakulsingharoj et al. 2004; Wang et al. 2007). Unexpectedly, expression of AGPase genes under the control of putative endosperm-specific promoters also increased the total number of maize kernels on individual cobs (Hannah et al. 2012) and number of developing grains per rice panicle or wheat head (Smidansky et al. 2002, 2003; Meyer et al. 2004). The increase in grain/seed number indicates that these transgenes are expressed at developmental stages prior to endosperm development. A more recent study also supports this view. AGPase cytosolic large subunit gene (TaLSUI) from wheat was overexpressed under the control of an endosperm-specific promoter in a wheat cultivar (Kang et al. 2013). The transgenic lines exhibited remarkably increased AGPase activity, starch levels, single grain weight, and grain number per spikelet.

11.5.2 Enhancement of Sucrose Synthase (SuSy) Activity

An alternative model for sucrose-starch conversion in cereal species has been proposed for starch biosynthesis where SuSy directly produces ADPglc from sucrose in the cytosol of seeds (Li et al. 2013). The newly synthesized ADPglc is then imported into the amyloplast for starch synthesis. This model also predicts that AGPase has a main role in scavenging glucose units generated by starch breakdown. Therefore, modulation of SuSy activity has been suggested to be a major determinant of the sink strength and crucial for increasing seed yields (Li et al. 2013).

The focus on SuSy originated from the belief that it is the major determinant of starch accumulation in tubers (Baroja-Fernández et al. 2009). Transgenic lines overexpressing SuSy under the CaMV 35S promoter had increased ADPglc and starch levels compared to the wild-type. The SuSy-overexpressing tubers contained 55–85 % higher starch levels per plant than wild-type plants. This approach was extended to maize endosperm where the potato SuSy was expressed under the control of ubiquitin promoter (Li et al. 2013). Transgenic maize lines exhibited increased SuSy activity and ADPglc levels and also had 10–15 % elevated starch levels. Authors argued that sucrose and starch pathways are connected by the action of SuSy, the main provider of ADPglc in the cytoplasm. However, this proposed model fails to account for the dramatic decrease in starch levels in *sh2* and *bt2* maize endosperms (Tsai and Nelson 1966) or significant increases in starch content by overexpression of the Sh2rev6 variant (Hannah et al. 2012). Similar results were also obtained in other cereal species when mutations in AGPase structural genes reduced cytosolic AGPase activity and, in turn, starch accumulation (Lee et al. 2007; Howard et al. 2012). Therefore, SuSy is unlikely to be the major determinant of ADPglc production, in turn, starch accumulation in cereals. A more plausible explanation of elevated starch levels with enhanced SuSy activity is the resulting increases in UDPglc levels and downstream metabolites including ADPglc. SuSy, UGPase, and AGPase catalyze readily reversible reactions, and increase in SuSy would result in elevated UDPglc and downstream metabolites, Glc-1-P, and ADPglc and, in turn, increase starch levels.

11.5.3 Overexpression of ADPglc Transporter (BT1)

Irrespective of the source of ADPglc, this sugar nucleotide must be imported from the cytoplasm into the amyloplast in cereal endosperm. This activity is carried out by BT1, whose gene sequences were first isolated by Sullivan et al. (Sullivan et al. 1991; Sullivan and Kaneko 1995) and found to encode a 39–44 kDa integral membrane protein. BT1 and its orthologs in other cereals belong to the mitochondrial carrier family (MCF) of proteins, a carrier class that has a broad range of substrates (Millar and Heazlewood 2003; Tjaden et al. 2004). Recent studies have demonstrated that BT1 from cereal storage tissues mediates the transport of ADPglc from the cytoplasm into the amyloplast in exchange with AMP or ADP (Emes and Neuhaus 1997; Bowsher et al. 2007; Kirchberger et al. 2007). The role of BT1 in the transport of ADPglc is supported by the analysis of genetic mutants. Maize kernels harboring the *bt1* mutation accumulate 80 % less starch than wild-type kernels (Tobias et al. 1992) but contain 11.5-fold higher levels of ADPglc (Shannon et al. 1996). Similar phenotypic properties were also observed for the barley *lys5* mutants that lack a functional form of the ADPglc transporter (Patron et al. 2004).

The study of transgenic rice plant, CS8, overexpressing the *E. coli glgC*-TM AGPase provides evidence that BT1 transport activity may be a limiting step in

starch biosynthesis (Sakulsingharoj et al. 2004). Transgenic CS8 lines show up to a 15 % increase in seed weight compared to WT plants. However, the extent of the increase in seed weight observed in CS8 lines grown in enriched CO_2 conditions was no different from plants grown under ambient conditions. The photosynthetic rates were significantly increased under elevated CO_2, leading to higher rates of sucrose transport into seeds (Rowland-Bamford et al. 1990; Chen et al. 1994). The inability of developing seeds of CS8 transgenic rice plants to further convert the increases in photoassimilate into starch under elevated CO_2 conditions indicates that starch synthesis is limited by some process other than AGPase. In support of this view, measurement of the carbon metabolites from seeds of CS8 and wild-type lines showed elevated levels of ADPglc in CS8 lines, which were three- to four-fold higher than the increases in seed weight (Nagai et al. 2009). These results suggest that AGPase is no longer a rate-limiting step in the transgenic plant and that maximum carbon flow into starch is constrained by one or more downstream steps, which would include the BT1 transporter and/or major starch synthases (SSs). SSs are present, however, in multiple forms and are unlikely to be limiting starch synthesis during the starch granule maturation phase based on available evidence. Genetic mutants of SSI (Fujita et al. 2006) or SSIII (Fujita et al. 2007) have starch levels comparable to wild-type plants. On the other hand, overexpression of SSIV increases starch content in both *A. thaliana* leaves and potato tubers, suggesting that initiation of starch synthesis is limiting (Gámez-Arjona et al. 2011). Therefore, BT1 and SSIV are likely candidates limiting carbon flow and are prime targets for manipulation to increase starch production in cereals.

11.5.4 Alternative Approaches to Increase Grain Yields

11.5.4.1 Changing the Expression of Starch Biosynthesis Regulators

An alternative strategy to increase starch synthesis is to globally increase the expression of the starch biosynthetic genes. An advantage of this approach is that, in addition to elevating the expression of some known starch biosynthetic genes, other genes yet to be identified that play a role in starch synthesis are also likely to be activated. The rice starch regulator 1 (*RSR1*) gene, which codes for an APETALA2/ethylene-responsive element-binding protein family transcription factor, was identified by genome-wide co-expression analysis. *RSR1* was found to down-regulate the expression of many starch-related genes, including AGPase, SS, granule-bound starch synthase (GBSS), starch-branching enzyme, and starch-debranching enzyme (Fu and Xue 2010). Suppression of *RSR1* gene expression in seeds elevated the expression of starch biosynthetic genes. *Rsr1*-deficient transgenic plants had altered amylose contents, altered formation of starch granule, and increased seed weight. On the other hand, overexpression of *RSR1* resulted in repression of starch synthesis genes, while starch structure and levels in the transgenic plants were comparable to wild-type (Fu and Xue 2010). Therefore, silencing *RSR1* gene seems to be a promising strategy to enhance rice grain yield.

In a recent study, mutation in the *FLO2* gene, which encodes a protein containing a protein-to-protein interaction tetratricopeptide motif, led to reduced grain size and altered starch quality in rice endosperm (She et al. 2010). The *flo2* mutation also decreased the expression of many genes that are involved in starch synthesis such as AGPase, SuSy, SSs, branching enzymes, and α-amylase. As expected, overexpression of *FLO2* resulted in enlarged, heavier grains and increased starch quality. This study proposes that rice grain size and starch quality are regulated by the *FLO2* gene. Further studies are needed to show the importance of *FLO2* orthologs in other grain species.

11.5.4.2 Regulation of Starch Degradation

A novel mechanism for increasing grain yields was described by blocking starch degradation since evidence showed that starch synthesis and breakdown occur concurrently in starch-accumulating organs (Baroja-Fernández et al. 2003). Phosphorylation of starch is critical for degradation of starch granule (Blennow et al. 2002; Smith et al. 2005). Glucan water-dikinase (GWD) and phosphoglucan water-dikinase (PWD) activities phosphorylate starch with the former activity transferring the β-phosphate of ATP to either the C6- or C3-position of amylopectin glucosyl residues (Kötting et al. 2005; Mikkelsen et al. 2006). The negative charges disrupt the ordered structure of amylopectin, thereby facilitating access of degrading enzymes such as α-amylase in cereals (Ritte et al. 2002). A recent study (Ral et al. 2012) showed that down-regulation of GWD by RNAi using a wheat endosperm-specific promoter resulted in significant increases in both wheat grain yields and plant biomass. Similar to the transgenic AGPase studies (Giroux et al. 1996; Wang et al. 2007), down-regulation of GWD under the control of a putative endosperm-specific promoter resulted in unanticipated changes in tissues that are formed well before endosperm development. This study opens up a new perspective on the significance of starch metabolism during the formation of reproductive organs as well as the balance between starch synthesis and breakdown. Collectively, they provide a novel mechanism for increasing grain yields by manipulation of starch metabolism in tissues that support overall plant growth and reproduction.

Sugar sensing and signaling in plants are far from being completely understood. The SnRK1 (for sucrose non-fermenting-1-related kinase-1) was proposed to be involved in regulating expression of many genes that are repressed by glucose and modulating the phosphorylation states of several enzymes (Gancedo 1998). SnRK1 also regulates enzymes that are responsible for starch breakdown in wheat and rice seeds during sugar deprivation (Laurie et al. 2003; Lu et al. 2007). It reduces the expression of α-amylase, and, thus, overexpression of SnRK1 under controlled spatial and temporal patterns could provide an alternative and complementary approach to enhance grain yields.

11.5.4.3 Enhancement of Hexose Levels Within Heterotrophic Cells

Starch production is ultimately dependent on the transport and uptake of sucrose by sink organs from source leaves. In cereals, grain filling is dependent on cell wall invertase. The loss of function of cell wall invertase in developing maize grains resulted in the miniature1 (*mn1*) seed phenotype (Kang et al. 2009), while a similar activity (GIF1) in rice rendered smaller seeds due to slower grain filling (Wang et al. 2008). In maize, expression of the cell wall invertase (*lncw*) occurs at the basal part of the endosperm, while in rice GIF1/OsCIN1 invertase resides on the ovular vascular and lateral stylar vascular traces of the developing grain. The spatial location of the cell wall invertase and pronounced reduction in grain size due to loss of function indicate that the cell wall invertase is required for the efficient unloading of sucrose from vascular tissues. Consistent with this view is the observation that transgenic rice plants overexpressing GIF1 had larger and heavier grains (Wang et al. 2008). Overall, these studies support the manipulation of cell wall invertase and other cellular processes involved in the efficient unloading of sucrose from vascular tissue and subsequent loading into endosperm tissue as potential targets to enhance grain yields.

11.6 Conclusions and Future Prospects

Starch metabolism is an essential process in plants and has a major impact on overall plant productivity and crop yields. The synthesis of starch is a complex and highly regulated process and involves the contribution of enzymes and metabolites in both the cytosol and plastid compartments. Despite several decades of study, the impact of starch metabolism on plant development is far from being understood as illustrated by the unanticipated results in studies attempting to manipulate starch genes during cereal endosperm development. Rather than the expected increase in grain weight due to increase in starch synthesis, unexpected increases in grain number per reproductive organ (wheat head, rice panicle, or maize cob) and number of reproductive organs per plant were evident. These unexpected increases in grain number suggest that the putative endosperm-specific promoters of AGPase are also active in non-endosperm tissue (e.g., the meristematic tissues that give rise to reproductive organs) and/or in ovule during the early stages of fertilization preventing seed abortion by increasing the sink strength of these tissues. Further studies are needed to identify the tissues where these promoters are active and whether enhancement of starch biosynthesis in these tissues stimulates flower development and, in turn, grain yields (Hannah et al. 2012). With such information in hand, a more rational approach can be used to enhance crop yields by the overexpression of single genes such as AGPase or by controlling global regulators that enhance overall expression of starch biosynthetic genes.

Likewise, many gaps in our understanding of starch synthesis still remain to be elucidated. For example, a CBM48 domain-containing protein has been

demonstrated to be essential for starch formation by its interaction with isoamylase and presumably aiding its debranching activity as a chaperone (Peng et al. 2014). As many other starch biosynthetic enzymes lack the ability to bind starch directly, similar carbohydrate-binding domain-containing proteins may be required to facilitate their action.

Moreover, recent studies showing the control of rice endosperm cytosolic AGPase activity by redox regulation (Tuncel et al. 2014a) open new avenues of investigation to identify the sugar signaling pathways which take part in carbon assimilation in developing rice endosperm. Redox activation of endosperm AGPase allows storage organs to adjust carbon storage according to levels of sucrose supplied from leaves. These signaling pathways will be perturbed by the up- or down-regulation of specific genes such as AGPase resulting in significant changes in overall gene expression at the RNA and protein level. Advances in next-generation sequencing, proteomics, and metabolite profiling now enable a more systematic and analytical means to assess these changes, heretofore not possible before. Such an integrated approach will provide a more complete view of starch metabolism, which will likely result in formulating novel approaches to engineer more efficient starch synthesis and, thereby, enhance plant productivity and yields.

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