Redesigning Starch Metabolism to Increase Rice Yields

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

Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340 USA. Email: okita@wsu.edu

Abstract

Rice provides much of the caloric dietary needs for a significant percentage of the world's population. Despite this dependence on this crop plant as a staple food, the annual net increases in rice yields have slowly regressed since the 1990s. These nominal net increases in yields have not kept abreast of the increases in population growth, resulting in a gradual decline in rice stockpiles and, in turn, increase commodity price. A continuation of this trend will result in a growing percentage of the world's poor population unable to afford this food staple to attain the required caloric needs for normal health and well-being. New advances in breeding higher yielding rice varieties and more efficient production and post-harvest technologies are needed to reverse this trend where rice can meet the growing dietary needs of population growth. The generation of higher yielding rice varieties is dependent on the introduction of new genetic traits. These include new genes that enhance plant growth and development under conditions of elevated CO_2 and higher temperature as well as those that counteract the deleterious effects of the multitude of biotic and abiotic stresses that impact yields. Here, we will describe our efforts to genetic engineer rice for higher yields by manipulating starch metabolism. We will first discuss the rationale behind these efforts, describe past and present efforts from our laboratory as well as those from others in engineering rice and related cereals, describe the general approaches to improve starch synthesis, and finally discuss prospects and pitfalls of achieving new rice types via manipulation of starch metabolism.

Keywords: Starch metabolism, rice, yield, source-sink relationship

Introduction: manipulation of source-sink relationship in rice and the role of starch

Under ideal growth conditions, the genetic yield potential of rice is dependent on source-sink relationships (Ho 1988; Turgeon 1989). Source leaves capture light energy and fix CO_2 to produce sugars, amino acids and other metabolites (Fig. 1). The bulk of these newly synthesized organic compounds are exported from the source leaves and transported to developing sink tissues, *e.g.* new tillers and root tissue, which utilize these basic precursors for growth and eventually the formation and maturation of reproductive organs. Source-sink relationship is a dynamic process which varies during the day as well as during plant development. In the morning hours the rates of CO_2 assimilation steadily increase with the increase in light intensity. Maximum photosynthetic rates are not maintained, however, and mid-day depression in photosynthetic rates is commonly observed due to photosynthetic feedback or decrease in stomatal conductance (Horton and Murchie 2000). Photosynthetic feedback occurs mainly due to the limitation of sinks to utilize photosynthate which, in turn, has long distance effects on leaf carbon metabolism and a depression in photosynthesis (Paul and Foyer 2001). Because of their importance as a food source, rice has been studied extensively with regard to source-sink relationships. Moreover, this short grain cereal is an excellent experimental system as the bulk of the total carbon (60-90%) accumulated in developing seeds is produced from photosynthesis that occurs during heading and seed development, and much of this photosynthate is produced by the flag leaf (Yoshida 1981). The role of photosynthesis in determining total biomass and the number of reproductive organs of rice is supported by genetic and CO_2 enrichment studies. Net photosynthesis and yields are significantly elevated in a hybrid rice compared to the parent lines (Black et al 1995). Under enriched CO_2 conditions, rice plants exhibit initially high photosynthetic rates which lead to an increase number of tillers and reproductive structures and, in turn, higher seed yields than plants grown under normal atmospheric conditions (Chen and Sung 1994; Hocking and Meyer 1991; Imai et al 1985; Rowland-Bamford et al 1990; Ziska and Teramura 1992).

Such enhancement in photosynthesis under higher levels of CO_2 does not, however, increase individual grain weight. As the levels of total non-structural carbohydrate in the vegetative parts of the plants (Rowland-Bamford et al 1990) or sucrose levels in peduncle exudates (Chen and Sung 1994) are elevated, developing rice seeds are unable to convert the excess photosynthate into dry matter (starch) (Chen and Sung 1994) and, hence show a strong sink-limited phenotype. The large linear decline in Rubisco content by high CO_2 grown rice plans indicate that there is strong acclimation and feedback effect on source activity due to inability of sinks to utilize additional photosynthate (Rowland-Bamford et al 1991).

Past studies from this laboratory readily support the view that rice yields are strongly affected by sink strength which, in turn, limits photosynthesis even under moderate conditions of temperature, light, and vapor pressure deficit. Photosynthesis of the flag leaf during the reproductive stage saturates at near ambient CO_2 levels especially under conditions that favor high stomatal conductance (Sun et al 1999; Winder et al 1998). This saturation at near ambient CO_2 levels is atypical for C_3 species which normally requires two to three times higher ambient levels before CO_2 assimilation rates become saturated. This apparent saturation is associated with limitations on the capacity to convert fixed carbon into sucrose and starch in rice leaves. Rice is a poor accumulator of leaf starch and enhanced carbon flow into this transient reserve would utilize excess photosynthate and recycle inorganic orthophosphate (Pi), the latter required to support ATP synthesis and the C_3 cycle (Sun et al. 1999; Winder et al 1998). A major goal of this laboratory is to increase the capacity of rice leaves to convert triose-P to storage carbohydrates and the sink strength of developing seeds to convert sucrose to starch through genetic manipulations.

Recent advances in engineering rice starch

Efforts to increase yield in rice, as well as in other plants, have mainly focused on ADPglucose pyrophosphorylase (AGPase) due to its pivotal role in catalyzing the first committed step in starch biosynthetic pathway in both leaves and storage organs. The enzyme, composed of a pair of large subunits (LSs) and a pair of small subunits (SSs) catalyzes a reversible reaction where glucose 1-phosphate (G1P) and ATP is utilized to produce ADPglc, the activated form of glucose utilized by starch synthases, as well as inorganic pyrophosphate (PP_i). The latter is hydrolyzed to Pi in the plastid by a potent inorganic pyrophosphatase. This reaction not only drives the reaction towards ADPglucose formation but also recycles Pi which is needed for photophosphorylation (Fig. 1). A different pathway for starch synthesis is utilized in cereal endosperm. Although a plastid-localized AGPase is present, the major enzyme activity is cytoplasmic. This intracellular location requires the newly synthesized product ADPglucose to be transported into the plastid where it can be utilized by starch synthases (SSys).

The catalytic activity of AGPase is governed by the effectors 3-phosphoglyceric acid (3-PGA), which activates the enzyme activity, and Pi which serves as an inhibitor (Hwang and Okita 2012). Many AGPases are also regulated by redox potential. These regulatory properties limit the maximum flow of carbon into starch (Sakulsingharoj et al 2004). In the following sections, we will briefly describe recent studies on manipulating AGPase activity to control transient leaf starch synthesis and then focus on the efforts to improve sink-strength and overall yields in wheat, maize and rice.

A. Studies on source tissue

The importance of leaf starch for overall plant productivity and seed yield is supported by our studies of the *Arabidopsis* leaf starch mutants where a close relationship in the capacities for photosynthesis, plant growth and leaf starch were readily evident.

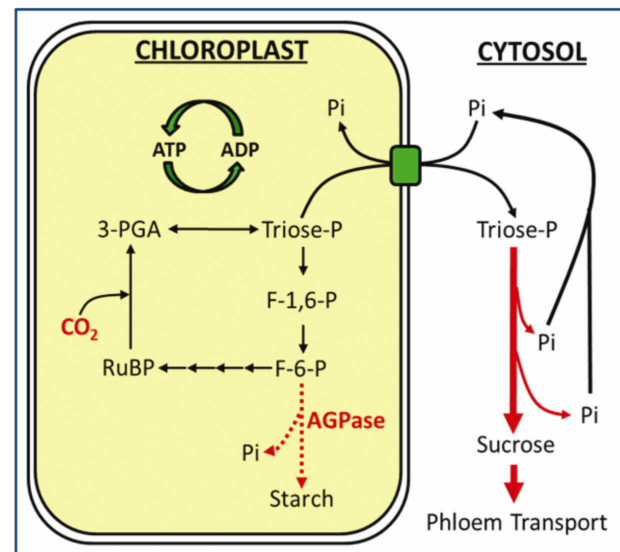

Figure 1. Primary carbon metabolism in photosynthetic plant cell. CO_2 assimilation produces triose-phosphates (triose-P) which are exported into the cytoplasm for sucrose synthesis. Alternatively, triose-P can be retained within the chloroplast to support starch synthesis. In both instances, synthesis of these carbohydrates releases Pi which is recycled to support photophosphorylation and the C_3 cycle.

Similarly, maize plants with a mutation in leaf AGPase SS *agps-m1* allele (Slewinski et al 2008) were smaller in length and had 30% less total seed weight with 15% decrease in individual seed weight (Schlosser et al 2012). In contrast, a rice mutant lacking leaf starch grew normally with no apparent differences seen in yield components compared to wild type (Rösti et al 2007). However, rice is a poor leaf starch accumulator and the relatively small carbohydrate reserve, *i.e.* transient starch, may not provide a sufficient sink size to recycle Pi especially in rice plants grown under low light growth chamber conditions. Indeed, the inability to utilize leaf starch as a mechanism to recycle Pi is likely responsible for pronounced susceptibility of this plant to photosynthetic feedback inhibition even under moderate temperature and at atmospheric levels of CO_2(Sun et al 1999; Winder et al 1998). The role of leaf starch as a transient sink to recycle Pi was directly tested by expression of up-regulated AGPase activities in *Arabidopsis*. The transgenic plants displayed higher AGPase activity and increased content and turnover of transitory leaf starch than wild type plants (Obana et al 2006). They also exhibited enhanced photosynthetic capacity which led to increased plant growth and leaf biomass (Gibson et al 2011).

A similar strategy was implemented for rice. *UpReg-1*, the potato up-regulatory LS gene, was introduced into wild type plant and the homozygous plants were selected and tested for their agronomic traits after grown under natural environmental conditions (Gibson et al 2011) (Table 1). The transgenic lines showed elevated AGPase activity and increased leaf starch content which is utilized by plant as a larger transient reserve to support heterotrophic growth in the dark. The increased starch also enhanced the plant growth but no difference in seed weight was observed. Rather, seed yields were increased up to 39% due to stimulatory effects on reproductive development, such as increase in the number of panicles per plant or more grains per panicle (Gibson et al 2011) (Table 1). Overall, these studies show a direct relationship between leaf starch, photosynthesis and plant growth.

B. Studies on sink-strength

An equally viable and alternative approach to increase the yield is to enhance photosynthate utilizing capacity of the sink organs. This can again be achieved by taking advantage of the properties of AGPase as a rate-determining enzyme in the starch biosynthetic pathway. Interestingly, although significant increases in seed yields were obtained by the introduction and expression of novel AGPase activities, the mechanisms by which these yields increases were attained, differed considerably as described in the following.

i) Enhancing productivity by total seed weight and biomass

Expression of the maize endosperm AGPase containing *Sh2r6hs,* a LS double mutant which was less sensitive to P_i inhibition and more heat resistant (Giroux et al 1996; Greene and Hannah 1998), had a large global effect on plant growth and yields (Smidansky et al 2002). The transgenic wheat lines showed an average increase of 38% more total seed weight per plant and 31% more total plant biomass in comparison to wild-type. Interestingly, individual seed weights remained relatively unchanged. They suggested that the elevated seed number was due to the increased survival of fertilization events as a result of AGPase over-expression and enhanced activity. Such an explanation would require that the *Sh2* promoter, normally thought to be endosperm-specific, be active before and/or during fertilization. Moreover, the lack of increase grain weight indicates that endosperm-specific expression did not enhance starch biosynthesis. It is not clear whether this failure to increase starch synthesis was due to the lack of increase in synthesis and/or utilization of ADPglucose or some other unknown unrelated process. The same *Sh2r6hs* gene was also expressed under the control of the wheat *glutenin* promoter (Blechl and Anderson 1996; Meyer et al 2004).

Table 1. Properties of wildtype (WT) and transgenic rice plants expressing potato LS UpReg1

Plant	Culm length (cm) (CL)	Panicle length (cm) (PL)	Panicle number/hill	Spikelet number /panicle	Grain ripening (%)	1000-grain wt. (g)	Grain yield (kg/100 m^2)
WT	57	13.4	8.0	43.6	69.9	29.0	178 (100%)
UpReg1-16	57	13.8	12.3	40.0	72.4	28.5	248 (139%)
UpReg2-3	53	14.2	11.0	46.4	59.8	26.5	199 (112%)
UpReg2-19	58	14.6	8.7	46.5	74.9	29.7	244 (137%)

The rice plant was engineered for temporal and tissue-specific expression of the up-regulatory potato large subunit in the source tissues using Rubisco small subunit promoter and transit peptide. All parameters but grain yield were measured from 20 plants. Grain yield represents the total dry weight of seeds from 80 plants. These data are from Gibson et al (2011)

Although *Sh2r6hs* RNA expression level was 20-fold higher at the RNA levels and 5-fold higher at the protein level, there was no further improvement in seed number compared to those generated by the *Sh2* promoter driven *Sh2r6hs* lines, 161-12 and 152-7 (Smidansky et al 2002). The *Sh2r6hs* mutant AGPase gene was also over-expressed in rice resulting in more than 20% increase in total seed weight per plant and total plant biomass with no significant effect on the harvest index (Smidansky et al 2003). The transgenic lines expressing *Sh2r6hs* also showed a significant increase in the number of unfilled seeds per panicle over transgenic negative lines. Further improvement in seed yields could be obtained if the number of aborted seed events were lessened.

Two *Sh2r6hs* transgenic lines, 161-12 and GS8, were assessed for their photosynthetic rates and metabolite levels (Smidansky et al 2007). Both lines showed higher CO_2 assimilation rates than the transgenic negative lines at 5 days before fertilization and during early and mid-stages of seed development. Such stimulation in photosynthesis was not seen at the prehead stage. The observed increases in photosynthesis during and after the heading stage are consistent with the increases in sink demand dictated by increases in the number of florets per wheat head. When evaluated under field conditions, yield increases by these transgenic lines was only observed under non-limiting environmental conditions (Meyer et al 2007). The effect on total seed number was also evident when the potato AGPase LS *UpReg-1* (Greene et al 1996) under the control of the storage protein *glutelin* promoter was expressed in rice (Lee et al 2010). Unlike the other studies where the AGPase subunit was targeted to the cytoplasm, the *Arabidopsis rbcS* transit peptide was employed to target the *UpReg-1* encoded AGPase LS to amyloplast and, therefore, stimulate the AGPase reaction in this organelle instead of the cytoplasm. Increases in grains/panicle and total seed weight/plant were evident similar to the yield increases seen for the maize *Sh2r6h.*

ii) Enhancing productivity by individual seed weight

Expression of the *Sh2r6hs* stimulates the formation of florets in the reproductive organ but does not elevate starch synthesis in developing seeds and, in turn, individual seed weight as expected. Other studies have documented that the seed-specific AGPase reaction limits starch production. This condition was first demonstrated for maize plants expressing the *Sh2 Rev6* mutation formed by excision of transposable element *dissociation (Ds).* The *Ds* induced mutation resulted in a insertion of two extra residues, tyrosine and serine, close to the 3PGA binding site of the AGPase SH2 LS. The resulting mutant enzyme was less sensitive to P_i inhibition. Maize lines that harbor the *rev6* allele showed 11-18% increased weight per seed. Our own studies showed that the weight of individual rice seeds were elevated by expression of a *Escherichia coli glcC* triple mutant (TM) gene (Preiss and Romeo 1994), which encodes a highly active and allosterically insensitive AGPase, under control by the *glutelin* promoter (Sakulsingharoj et al 2004) (Fig. 2).

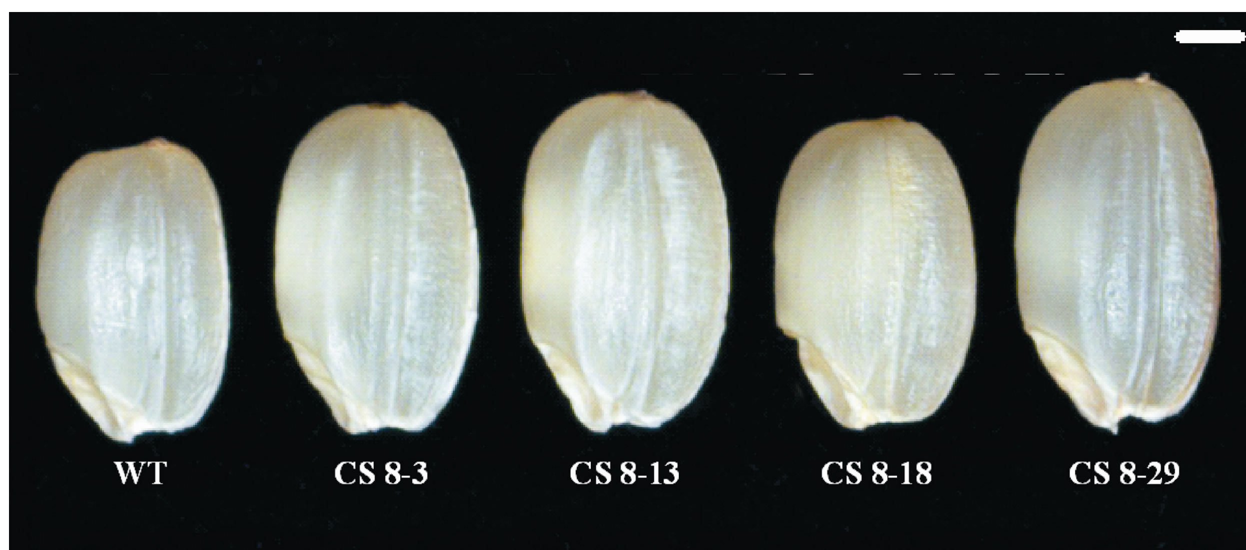

Figure 2. Dried mature rice seeds from wild type (WT) and transgenic plants expressing E. coli glgC-TM.CS 8-3, CS8-13, CS 8-18, and CS 8-29 are the engineered rice plants which express high levels of up-regulatory E. coli AGPase (glgC-TM) in cytosols of the endosperm cells in developing seeds. These data are from Nagai et al (2009)

Rice endosperm have multiple SSy isoforms and mutations in SSy1, the major isoform, have no significant effect on grain weight (Fujita et al 2006). Therefore, the limitation is most probably at the ADPglucose transport step. This hypothesis is further supported by mutations in ADPglucose transporter genes of maize and barley. Maize *bt1* mutant kernels were shown to accumulate 80% less starch than wild-type and similar results were observed in barley *lys5* mutant (Patron et al 2004; Tobias et al 1992). To test if transport of ADPglucose into amyloplast is a rate limiting step we have transformed wild-type rice (cv. Kitaake) with the *bt1* gene from *Zea mays* and obtained homozygous transgenic lines recently. Our next aim is to cross these homozygous lines with the transgenic lines which already over-express the *E. coli glgC-TM* gene to see the combined effect on overall yield.

Engineering AGPase with improved quantitative traits

As briefly described above, starch metabolism plays an important role in source-sink relationships. This view is more conspicuous in developing rice seeds where the rate of starch synthesis and, in turn, seed weight, is controlled by the rate-limiting AGPase activity. As demonstrated by expression of *Sh2r6hs* in transgenic wheat and rice (Smidansky et al. 2002; Smidansky et al 2003), AGPase also is essential for overall plant productivity and yields, although the basis for this stimulation remains unresolved. Nevertheless, the introduction of AGPase activities with novel enzyme properties has the potential to further enhance rice productivity and yields. In the following sections, we describe the properties of AGPase that can be manipulated and the general strategy in generating these traits.

What to change?

The catalytic activity of AGPases is dependent on the relative ratio of activator (3-PGA) and inhibitor (Pi). In the presence of equal amounts of these effectors, the catalytic activity is only about 20-50% of maximum. Thus, an obvious strategy is to improve the enzyme's performance in the transgenic plants by modifying the enzyme's allosteric regulatory properties by increasing its sensitivity to activation by 3-PGA and/or increasing its resistance to Pi inhibition. The second strategy is to improve the heat stability of AGPase. Heat stress is an important factor limiting grain yield of maize, wheat, and rice since exposure of these cereal crop plants to an impermissibly high temperature greatly impairs starch synthesis and thus reduces grain filling. AGPase is among the starch biosynthetic enzymes that are severely affected by the elevated temperature. The maize enzyme, for example, loses 97% of its catalytic activity after incubation at 57°C for 5 min and our recent study with the purified recombinant rice endosperm AGPase also showed poor heat stability (unpublished data). Thus, engineering of the enzyme for increased heat tolerance would result in higher grain yields in years that experience abnormally elevated temperatures. Collectively, these approaches, alone or combined, could dramatically improve plant growth and productivity.

How to change?

Protein engineering of AGPase not only generates enzyme activities with novel functions but also provides a powerful tool in elucidating the structure-function relationship of the plant AGPase subunits. There are two general approaches to modify the enzymatic properties of AGPase: 'Luck of the draw' (or random mutagenesis) and knowledge-based modification (or site-specific mutagenesis). A prerequisite for both approaches is to develop a facile and fast screening procedure that can quantitatively assess the properties of AGPase activity. Such a tool has been developed in *Escherichia coli.*

- ***Semi-quantitative screening system using E. coli host cells and plasmid vectors***

In the random mutagenesis approach, a large number of mutants must be screened for their acquired traits by mutations. Hence, the identification of a mutant harboring desired traits from a large mutant library is challenging unless a high-throughput screening system is available. Such a system has been successfully devised and implemented for the identification of numerous AGPase mutants having up-regulatory or down-regulatory properties. Like other bacteria, *E. coli* synthesizes glycogen, which can be easily stained with iodine which results in a color intensity proportional to the amount of α-glucan accumulated within the cell and, in turn, the extent of AGPase activity. The bacterial host strain AC70R1-504 contains a point mutation in the AGPase structural gene, *glgC*, which results in a truncated, inactive enzyme form. We also generated a new *glgC* strain EA345, which has several notable advantages over AC70R1-504 in being deficient in endogenous endonuclease and proteinase activities (Hwang et al 2007). In addition to the host strain, expression plasmids are required. The higher plant AGPase is a heterotetramer containing a pair of LSs and a pair of small subunits. We originally co-expressed the potato subunit cDNAs using pMON17335 and pMON17336 (Iglesias et al 1993). These plasmids contain compatible origins of replication (Ballicora et al 1995).

In order to avoid the use of nalidixic acid, a toxic inducer, and to enhance the control of protein expression, we constructed two new plasmids, pSH275 and pSH228. Expression of AGPase from these plasmids can be stimulated by a single inducer, IPTG, and more tightly controlled by the LacI repressor produced from pSH228. pSH228 also produces the lambda Q protein which activates the lambda $P_{R'}$ promoter to enable the expression of glycogen synthase and glycogen phosphorylase in EA345 cells. pSH228 was further manipulated to generate pSH597. This plasmid expresses elevated copy numbers of tRNAs which are rare in *E. coli*, to alleviate the codon usage bias problem which potentially hinders the efficient production of higher plant AGPases in the bacterial host. The EA345 cell together with pSH275 and pSH228 (or pSH597) has been regularly used in this laboratory for screening of AGPase mutants and their expression (Hwang et al 2007; Hwang et al 2008; Kim et al 2007).

- ***Engineering AGPase via molecular evolution***

The ultimate goal for engineering AGPase is to increase the potential of the enzyme to more efficiently synthesize starch and, in turn, elevate the plant's capacity to utilize more photoassimilates. Introduction of a mutation at random positions of the enzyme can be accomplished by many different ways and combination of two or more methods may enhance the odds for creating the desired novel trait. These approaches include UV mutagenesis (C-to-T transition), chemical mutagenesis using an alkylating agent ethylmethanesulfonate (EMS; C-to-T or G-to-A transition), hydroxylamine HCl (C-to-T or G-to-A transitions), nitrous acid (C-to-T or G-to-A transition), transversion-causing purine analogues, error-prone PCR, rolling circle error-prone PCR, mutator strains, and deletion/insertion mutagenesis.

In the following sections, we summarize the approaches used to identify novel AGPase mutants possessing desirable regulatory/catalytic properties and thermostability.

Allosteric regulation of AGPase

Treatment of the potato AGPase LS cDNA with hydroxylamine-HCl followed by co-expression with SS cDNA in *E. coli* cells revealed many mutant colonies which failed to accumulate glycogen as viewed by iodine staining. One of the first AGPase mutant enzymes, P52L, contained a replacement of a proline residue at position 52 to leucine in the LS. This enzyme showed down-regulatory properties in requiring much higher 3-PGA levels for activation than the wildtype enzyme. The enzyme was also inhibited by Pi at much lower levels than the wildtype enzyme. By employing a suppressor mutation approach, second site mutations, which suppressed the initial P52L phenotype, in the LS were identified. By expressing these suppressor mutations in the absence of the P52L mutation, several mutants, which displayed an excess glycogen phenotype and stained intensely with iodine, were generated. These included UpReg-1 containing a E38K substitution and UpReg-2 containing a G101N replacement (Greene and Hannah 1998). Heterotetrameric enzymes containing these mutations in the LS exhibited up-regulatory properties in requiring substantially reduced amounts of 3-PGA for maximum enzyme activity and displayed increased resistance to Pi inhibition.

As hydroxylamine creates only C-to-T or G-to-A transitions, a more general mutagenesis approach has been employed using error-prone PCR. Unlike the E38K mutation, E38A results in a down-regulation of the potato tuber AGPase. Error-prone PCR using LS[E38A] generated a pool of secondary mutations on the LS and positive selection of the mutation(s) reversing the effect of the E38A mutation on the regulatory properties allowed us to isolate eleven different mutants accumulating high amount of glycogen (Kavakli et al 2001).

A similar strategy was also employed for the SS. The SS was treated with hydroxylamine and co-expressed with LS[P52L] in the *glgC$^-$ E. coli* cells to identify AGPase mutants that showed normal iodine staining phenotype. This approach resulted in the identification of SUP-1 [L48F], which suppressed the LS [P52L] phenotype. When further mutagenesis was performed on this mutant form, TG-15[L48F, V59I] was isolated. Interestingly, the homotetramer of TG-15 exhibited regulatory properties similar to those of wild-type AGPaseheterotetramer (Salamone et al 2000). DNA shuffling was used to further improve the enzymatic (regulatory) properties. This method generated several mutant forms showing up-regulatory properties better than TG-15. As an example, devo330 has additional Y317C and L380S mutations and exhibited higher sensitivity to 3-PGA (12-fold), increased resistance to Pi inhibition (19-fold), and enhanced affinity for ATP (2.4-fold) than TG-15 (Salamone et al 2002).

Based on the previous results with potato tuber AGPase mutants, site-specific substitutions have been made on the homologous residues on the *Arabidopsis* L1 (ApL1) subunit: Ala33 to Lys (A33K) and Gly96 to Asn (G96N). AGPases containing ApL1[A33K] and

ApL1[G96N] showed significantly increased 3-PGA sensitivity while the double mutant ApL1[A33K,G96N] showed more tolerance to Pi inhibition than the single mutant types (Kavakli et al 2002; Obana et al 2006).

Site-specific mutagenesis introduced three mutations (R67K, P295D, and G366D) in the *E. coli glgC* gene to yield *glgC* triple mutant (TM). Glc-TM possesses up to 90% of the enzyme activity observed for the fully activated wild-type enzyme even in the absence of any activators and interestingly is resistant to Pi inhibition. These traits can be very useful when introduced into plants since GlgC-TM is catalytically active and sustain its catalytic activity independently of 3-PGA or Pi (Sakulsingharoj et al 2004).

Stability of AGPase

Several hybrid mosaic SS proteins were constructed using different parts of the maize endosperm SS, BT2, and potato tuber SS (Cross et al 2004). One mosaic maize-potato (MP) SS, comprised of the N-terminal amino acids from position 1 to 199 of maize BT2 and the C-terminal amino acids from position 200 to 475 of the potato tuber SS had pronounced effects on enzyme function. When assembled with maize wildtype L subunit, SH2, the resulting enzyme displayed higher catalytic activity, 3-PGA sensitivity and Pi tolerance as well as heat stability (see following section) compared to AGPases containing wild type BT2 or potato SS (Boehlein et al 2005).

Wild type maize AGPase retains less than 2.5 % of the catalytic activity when incubated at 58°C for 6 min. Substitution of Tyr12 to Cys on the SS (BT2) significantly elevated the enzyme's thermostability by enabling the formation of an interchain disulfide bond between the small subunits (Linebarger et al 2005). Half-life ($t_{1/2}$) of the hetero tetrameric AGPase mutant containing BT2-QTCL (S10Q and Y12C substitutions on BT2) at 42°C was more than 8-fold longer than for the wild type enzyme. Half-life of the heterotetrameric AGPases containing SH2 assembled with wild-type BT2, MP, BT2-QTCL, and MP-QTCL at 55°C were 0.4 min, 1.4 min, 24.9 min, and 109.6 min, respectively, indicating both Cys12 and the C-terminal region from potato enzyme potentiated the enzyme's tolerance to heat as well as its catalytic activity.

In an effort to render the maize endosperm AGPase more heat stable, the BT2 sequence was subjected to an error-prone PCR. The Bt2 mutants were co-expressed with a wild type SH2 in *E. coli* and the two darkest colonies were selected after iodine staining for glycogen production. Both mutants had a T462I mutation. Although the kinetic properties of the mutant enzyme were very similar to wild type AGPase, it was much more heat stable than the wild type (Georgelis et al 2008).

Perspectives: challenging with new traits

Grain yields of many important cereal crops are highly susceptible to extreme temperature. In maize, AGPase expression levels and enzyme activity levels are most profoundly affected by elevated temperature (Singletary et al 1994) and have been a target for enhancing starch synthesis, in turn, seed yield. Several maize AGPase variants with enhanced heat stability have been already produced by mutagenesis studies. When expressed in cereal endosperms, these AGPase variants increase plant growth and seed yields (Hannah 2011).

It is still worthwhile to obtain additional AGPase mutants. Since we still do not have sufficient information to rationally design a thermostable AGPase, random mutagenesis study using a heat labile AGPase, such as those from rice, wheat, and maize, affords the possibility of surveying a larger number of mutations that could result in a more heat stable, highly catalytic enzyme. Thermostable AGPases can be readily generated and identified by the methods described here. A large plasmid library of AGPase LS or SS mutants containing single and multiple amino acid substitutions can be generated and transformed into *glgC*$^-$ *E. coli* cells expressing the counterpart subunit cDNA and grown at 45°C, a permissible growth temperature for the bacterium (Heitzer et al 1991). Since heat labile AGPase would be quickly denatured after expression, only AGPase which acquired heat stability will produce sufficient ADP-glucose for glycogen production. The degree of iodine staining would be a direct reflection of the extent of glycogen produced and, in turn, the extent of catalytic activity at elevated growth temperature. It may be worthwhile to pyramid several amino acid substitutions that induce heat stability to generate a more thermostable enzyme.

Rice grown at elevated temperatures have smaller grain size, chalky appearance, and lower amylose content (Yamakawa et al 2007). A comprehensive study of the metabolome and transcriptome of developing rice grains exposed to elevated temperatures identified a number of different processes that limit starch synthesis (Yamakawa and Hakata 2010). In addition to AGPase, others include sucrose loading into endosperm, sucrose hydrolysis, ADPglucose transport into amyloplast and starch synthases and branching enzymes. The genes involved in many of these processes have been defined and, hence, can be manipulated to more desirable properties using the same general experimental approaches describe here.

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