THERMOTOLERANT VARIANTS OF MAIZE ENDOSPERM ADENOSINE DIPHOSPHATE GLUCOSE PYROPHOSPHORYLASE

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
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To my mother and father, and to the memory of my grandfather, Charles William Burger.

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Adenosine diphosphate glucose pyrophosphorylase (AGPase) has received considerable study because of its allosteric regulation and rate-limiting role in starch biosynthesis. Further, heat lability of AGPase has been implicated in heat-induced yield loss in cereals. A previous study in this laboratory identified a heat-stable mutant (*Sh2hs33*) with a single amino acid substitution in the large subunit of maize endosperm AGPase. In this study bacterial expression of AGPase combined with a novel mutagenesis scheme allowed us to identify temperature sensitive mutants of the large subunit of maize endosperm AGPase. Two such mutants, *Sh2ts48* and *Sh2ts60*, fully complement the *E. coli glgC*- (AGPase) mutation at 37°C, but not at 30°C. We mutagenized these mutants and isolated second-site reversion mutants (*Sh2rts60-1* and *Sh2rts48-2*) with restored glycogen synthesis at 30°C. The second-site reversion mutations, separated from their respective parental mutations, confer limited heat stability

to the enzyme. However, combining the mutation of *Sh2hs33* with the second-site reversion mutation of *Sh2rts48-2* results in an enzyme with 83% retention of activity after heat treatment and a nearly three-fold increase in activity compared to wild-type. This study shows the feasibility of isolating mutations affecting enzyme stability, and allows a more focused approach to understanding regions important in the structure-function relationship of maize endosperm AGPase.

CHAPTER 1
INTRODUCTION

Starch

Importance

In terms of its benefit to humankind, starch can be considered one of the most important products synthesized by plants. Starch constitutes most of the dry matter in the harvested organs of crop plants, and thus serves as the major source of calories in both human and animal diets. That synthesized in the harvested organs of wheat, rice, maize and potato alone exceeds 10^9 ton yr^{-1} (Kossman and Lloyd, 2000). Additionally, starch can be considered a renewable resource used in many industrial applications.

In the U.S., corn is the major cereal crop, planted on 70 to 80 million acres annually and appearing in more than 1200 items in a typical grocery store (Hallauer, 2001). In addition to foodstuffs, corn is used predominantly as livestock feed, but also in wet and dry milling, and as an export product. In recent years, corn has also been used to produce ethanol for corn-based fuels. The mature corn kernel is approximately 70% starch, and thus its importance is inextricably linked to its starch content.

Structure

Starch exists in a semi-crystalline state composed predominantly of the glucose polymers amylose and amylopectin. Amylose is an essentially linear polymer whose approximate 1000 glucose units are joined through α-1,4-glycosidic bonds. It does

contain roughly 0.1% α-1,6-glycosidic branchpoints. Amylose comprises approximately 30% of the composition of starch, but varies among species, varieties, plant organs, developmental age of the plant, and growth conditions. Detherage et al. (1955) found that amylose content ranged from 11to 35% in 51 species studied, and from 20 to 36% in a survey of 399 maize varieties. Amylopectin comprises the remaining approximate 70% of starch. This polymer also consists of α-1,4-linked glucan chains, but contains approximately 4% α-1,6-glycosidic branchpoints. Amylopectin (10^7-10^9 Da) is larger than amylose (10^5-10^6 Da), and is sufficient to form starch granules in the absence of amylose (Ball et al., 1996). The remaining components of starch include proteins (0.5% in cereal endosperm and 0.05% in potato tuber), including the enzymes of the starch biosynthetic pathway, and lipids (1% in cereal endosperm and 0.1% in potato tuber) (Martin and Smith, 1995). A highly branched (10%) glucose polymer called phytoglycogen is also present in maize lacking a functional *sugary1* (*Su*) allele.

Biosynthesis

As reviewed in Hannah (1997), elucidation of the starch biosynthetic pathway, particularly in maize, has benefited from the abundance of mutants, their easily scoreable phenotypes, and the large size of the maize seed. Additionally, investigators have gained information about starch biosynthesis through the discovery and subsequent study of transposable elements, through studies brought about by the advent of gene cloning technologies, and through projects aimed at modification of the end product. Despite a large knowledge base, it is unclear whether all starch biosynthesis occurs via the pathway proposed in Figure 1.

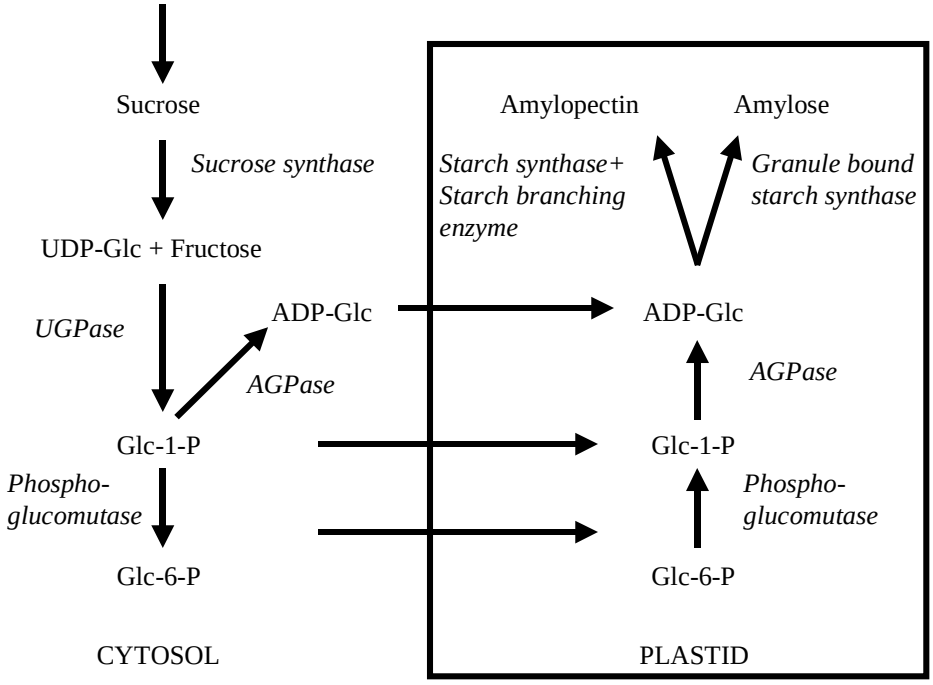

Figure 1. Proposed Starch Biosynthetic Pathway. Adapted from Kossman and Lloyd (2000).

However, the required steps committed to starch biosynthesis are known. They are provided by the following enzymes: adenosine diphosphate glucose pyrophosphorylase (AGPase; EC 2.7.7.27), starch synthase (SS; EC 2.4.1.21), starch branching enzyme (SBE; EC 2.4.1.28), and starch debranching enzyme (SDE; EC 3.2.1.68).

Adenosine diphosphate glucose pyrophosphorylase catalyzes the synthesis of ADP-glucose and pyrophosphate from glucose-1-phosphate and ATP. This reaction represents the first dedicated step in starch biosynthesis and results in the activated glucosyl donor, ADP-glucose. Pyrophosphate produced in the reaction is metabolized by pyrophosphatases, shifting equilibrium of the AGPase reaction in the direction of ADP-glucose synthesis.

Starch synthases represent the second committed step in starch biosynthesis, catalyzing the formation of α-1,4 linkages between the nonreducing end of a glucan chain and the glucosyl moiety of ADP-glucose. These enzymes can be classified in at least four groups with respect to sequence similarity. Collectively, the starch synthases can utilize amylose or amylopectin as substrates *in vitro*. However the specific role of each class *in vivo* is unknown.

The third dedicated reaction in starch biosynthesis is catalyzed by starch branching enzyme. SBE creates α-1,6 linkages between a glucose residue and the reducing end of a previously hydrolyzed α-1,4 linkage within a chain. Branching is not random, and enzymes exhibit specificity for glucan chain lengths and for amylose or amylopectin substrates.

Starch debranching enzyme plays a role in the final starch structure. By enzymatically cleaving α-1,6 linkages, SDE likely strikes a balance between SBEs to produce the starch granule.

Adenosine Diphosphate Glucose Pyrophosphorylase

Enzymology

Adenosine diphosphate glucose pyrophosphorylase catalyzes the reaction resulting in the activated glucosyl donor used to extend the polysaccharide polymer. Historically, this enzyme has received much attention because of its position as the first committed step in starch biosynthesis and its allosteric regulation, reviewed below. Its importance in starch biosynthesis has been confirmed by a number of mutants in the structural genes of AGPase that greatly reduce starch content in mature seeds (reviewed in Nelson and Pan, 1995). Its role as the rate-limiting step in starch biosynthesis and the

physiological relevance of its allosteric regulation, was demonstrated most convincingly by two experiments. Stark et al. (1992) expressed an allosterically altered AGPase from bacteria in potato tuber and increased starch content 30%. Giroux et al. (1996) used a *Ds*-induced insertion in an allosterically important region of the maize endosperm-specific gene encoding the large subunit of AGPase to condition an 11-18% increase in seed weight.

A genetic lesion later shown to affect this enzyme was first described by Mains (1949) in maize endosperm. The mutant gene was designated *shrunken-2* (*sh2*) because of the collapsed endosperm resulting from the presence of the mutant allele in a homozygous state, but distinguishing it from *sh* described by Hutchinson (1921). The enzymic activity was first isolated from wheat flour by Espada (1962). Early study by Laughnan (1953) noted an unusually sweet flavor associated with the *sh2* kernels. An 11-fold increase in sucrose concentration was accompanied by a nearly 75% decrease in starch content in *sh2* kernels as compared to wild-type. Tsai and Nelson (1966) first showed a lack of AGPase activity in *sh2* mutants synthesizing only 25-30% as much starch as wild type. Further study by Dickinson and Preiss (1969) qualitatively confirmed the findings of Tsai and Nelson, but detected low but measurable amounts of AGPase activity in *sh2* and *bt2* mutants. Adenosine diphosphate glucose pyrophosphorylase from *sh2* and *bt2* mutants differed from wild type and from each other with respect to extent of urea denaturation, K_m for Glc-1-P, and types of Glc-1-P saturation curves. This fact led to the hypothesis that *sh2* and *bt2* were structural genes for the enzyme (Hannah and Nelson, 1976). The fact that *E. coli* AGPase was known to

be a homotetramer (reviewed in Preiss and Levi, 1980) provided cause for debate on the existence of dissimilar subunits for AGPase in plants.

Structure

Definitive evidence for the authenticity of dissimilar subunits in plants was provided by the cloning of *Sh2* and *Bt2* from maize endosperm (Bhave et al., 1990; Bae et al., 1990), and the homologs from other plants, including potato tuber (Okita et al., 1990), rice (Anderson et al., 1991), and pea (Burgess et al., 1997) and reviewed in Smith-White and Preiss (1992). *Sh2* encodes the large (54 kDa) subunit and *Bt2* encodes the small (51 kDa) subunit of an $\alpha_2\beta_2$ heterotetramer. Sequence similarity among the large and small subunits of AGPase and between the *E. coli* AGPase subunit *glgC* indicates a shared evolutionary origin. Duplication followed by independent mutations within the *Sh2* and *Bt2* coding regions has resulted in noninterchangeable proteins that have nonetheless retained the ability to interact. That *Sh2* and *Bt2* are functionally nonduplicate genes is evidenced by complementation studies, in which either protein cannot substitute for loss of function in the other. Despite common ancestry, the rates of divergence among *Sh2* and *Bt2* homologs vary. *Bt2* retains a large degree of sequence similarity in comparison with the small subunits from other AGPases, while large subunits of AGPases have diverged such that many probes do not cross hybridize.

The $\alpha_2\beta_2$ heterotetrameric structure of plant AGPases is more complex than that of the homotetrameric AGPases of prokaryotes, and the dynamics involved in subunit assembly, interaction, and stability are largely unknown. Several studies have indicated that both subunits must be present for maximum stability and enzymic activity (Giroux and Hannah, 1994; Wang et al., 1997; Greene and Hannah, 1998b). Evidence gathered

using a yeast-two hybrid system to monitor subunit interactions between SH2 and BT2 suggests that individual subunits do not interact, and that polymerization involves formation of a heterodimer intermediate (Greene and Hannah, 1998b). Furthermore, yeast-two hybrid analysis showed that the N-terminal region of SH2 and the C-terminal regions of both SH2 and BT2 are required for subunit interaction.

Regulation

Adenosine diphosphate glucose pyrophosphorylase is an allosteric enzyme in virtually all organisms studied to date, although the specific effectors vary among organisms. *E. coli* AGPase is activated by fructose-1,6-bisphosphate and inhibited by cAMP, while plant AGPases are activated by 3-PGA and inhibited by phosphate. The extent of regulation differs greatly among plant species, and has defined a clear dichotomy. Leaf and tuber AGPases are quite sensitive to 3-PGA activation, while those of seed origin show variable response to the effectors. Endosperm AGPases from maize (Dickinson and Preiss, 1969; Hannah and Nelson, 1975; Hannah and Nelson, 1976), wheat (Olive et al., 1989), and barley (Kleczkowski et al., 1993; Doan et al., 1999), and developing seeds from pea (Hylton and Smith, 1992) and bean (Weber et al., 1995) show little to no 3-PGA activation. However, AGPase activity from developing rice seeds is dependent on 3-PGA for activity (Sikka et al., 2001). The difference between leaf and endosperm AGPases can be neatly explained by photosynthetic capabilities of the organs. Unlike the situation in leaves, the major carbon assimilatory pathway in developing seeds is likely glycolysis and the pentose phosphate pathway, thus excluding 3-PGA as a metabolite in the cellular environment. The dependence of potato tuber and maize embryo AGPases on 3-PGA for activity are interesting exceptions. As discussed below,

emerging data implicate a relationship between cellular location of AGPase and sensitivity to the allosteric effectors.

Localization

With an established plastidal localization of AGPase for spinach leaf (Okita et al., 1979) and potato tuber (Kim et al., 1989), it was assumed that endosperm AGPases were localized to the amyloplast. Mounting evidence over the years, however, has pointed to a cytosolic location for endosperm AGPases. Giroux and Hannah (1994) reported that SH2 and BT2 synthesized in a rabbit reticulocyte system and that synthesized in the maize endosperm were indistinguishable in size. Further studies of AGPase processing (Villand et al., 1992; Villand and Kleczowski, 1994), and the studies of transporter mutants (Cao et al., 1995; Shannon et al., 1996), cell fractionation (Thorbjornsen et al., 1996; Denyer et al., 1996), immunocytological localization (Brangeon et al., 1997), and metabolic profiling (Beckles et al., 2001) all argue for cytosolic localization of endosperm AGPases. Most recently, targeting of a GFP protein containing the N-terminus of *Bt2* also showed cytosolic localization (Choi et al., 2001).

Cytoplasmic AGPase localization would facilitate starch synthesis while conserving energy and carbon. Imported sucrose would be metabolized by sucrose synthase in the presence of UDP to form UDP-Glc and Fruc. UDP-Glc, in the presence of PPi, would form UTP and Glc-1-P, the latter being a substrate (+ ATP) to form ADP-Glc. In this scenario, the number of high-energy phosphate bonds is conserved. Therefore, it is possible that differences in sensitivity to allosteric effectors are the result of evolution in the presence of different environments of the plastid and cytoplasm.

Heat Stability

Background

Limited by high summer temperatures to the south and shorter growing seasons to the north, most of the world's grain is produced in the middle latitudes where average summer temperatures range from 21°C to 24°C (Thompson, 1975). In a study of corn yield for the years 1975 to 1977, Chang (1981) found the highest yields (kg ha $^{-1}$) to be at latitudes between 45 and 55°. However, variable weather patterns can have a drastic effect on yield. In particular, reduced grain yields due to elevated temperatures have been well documented in a number of historical and climatological studies (Thompson, 1975; Chang, 1981; Thompson, 1986; and Conroy et al., 1994).

Studies using growth chamber conditions (Hunter et al., 1977) showed the effect of elevated temperatures on maize grain yield. Despite a higher rate of dry matter accumulation, grain yield was reduced for plants grown at 30°C, compared to those grown at 20°C, because of a shorter grain-filling period. The work of Tollenaar and Bruulsema (1988) also showed a decrease in kernel weight due to a shortened grain-filling period in plants grown at a day/night temperature regime of 28/18 vs. 21/15°C.

In vitro studies have corroborated the *in planta* effects of elevated temperatures on grain yield. Jones et al. (1981), using an *in vitro* kernel culture system, showed that the increased dry matter accumulation at elevated temperatures is insufficient to compensate for the shortened grain-filling period. Further, the study indicated that adequate sucrose was taken up from the media by the developing kernels at elevated temperatures, thus implicating starch synthesis or sucrose unloading from the pedicel into the basal endosperm transfer cells as the likely cause of decreased yield. Using an in vitro kernel culture system and [^{14}C]sucrose, Cheikh and Jones (1995) showed that

incubation at elevated temperatures resulted in kernels with higher levels of radiolabeled sucroses and hexoses. The results point to carbon utilization, rather than carbon uptake, as the perturbation in kernel growth.

In an effort to characterize the effect of increased temperature on starch biosynthetic enzymes, Ou-Lee and Setter (1985) found less AGPase activity in the apical kernels than in the basal kernels of tip-heated ears during the time when most of starch synthesis occurred. Subsequent work on reduced grain yields due to elevated temperatures focused on soluble starch (SSS) synthase in wheat. Rivjen (1986) found that heat treatment reduced the conversion of sucrose to starch in wheat endosperm in vitro, with SSS activity declining rapidly at temperatures above 30°C. In contrast, activity of SSS from rice was thermotolerant at 30°C, perhaps reflecting a mechanism for the higher temperature optimum for grain development in this cereal. In a broader study of the effect of elevated temperature on starch deposition in wheat endosperm, Keeling et al. (1993) reported the activity of SSS to be the only enzymatic activity affected by temperatures above 20°C. Activities of AGPase, UDP-glucose pyrophosphorylase, sucrose synthase, phosphoglucomutase, phosphoglucose isomerase, bound starch synthase, and hexokinase remained constant despite elevated temperature. Further studies in wheat (Jenner, 1994; Keeling et al., 1994; Jenner et al., 1995) corroborated the heat lability of SSS, but also recognized the heat lability of AGPase. Further, AGPase activity was partially recoverable after heat treatment by transfer to unheated conditions, indicating the direct effect of temperature on the enzyme rather than that of advancing development. The work of Duke and Doehlert (1996) focused not only on the effect of heat stress on enzyme activities in maize kernels in vitro, but on transcript levels of these

enzymes as well. Decline in AGPase activity was most apparent and corresponded with the decline in the mRNA levels for its respective subunits.

Heat-Stable Mutant *Sh2hs33*

A bacterial expression system developed by Iglesias et al. (1993) allows subunits for AGPase to be expressed on compatible vectors in an AGPase deficient *E. coli* strain to produce a functional enzyme able to complement the mutant phenotype. This bacterial expression system has been used in a number of studies aimed at elucidation of the structure-function relationship of AGPase. Using this system, Ballicora et al. (1995) determined that a 10 amino acid region of the N-terminus of the potato tuber small subunit is important for heat stability. The role in heat stability of the Cys residue at position 12 in the small subunit of potato tuber was also determined through the bacterial expression system (Ballicora et al., 1999). This residue is the location of a disulfide bridge implicated in enzyme stability.

However, one mutant in particular has shed insight into the mechanisms involved in heat lability in maize endosperm AGPase. Using this system in conjunction with chemical mutagenesis, Greene and Hannah (1998a) were able to isolate mutations in the large subunit of maize endosperm AGPase that conferred heat stability to the enzyme *in vitro*. A single base pair mutation, arising repeatedly in the study and conferring the highest degree of heat stability, resulted in an amino acid change from His to Tyr at position 333 in the large subunit of maize endosperm AGPase. This mutant was designated *Sh2hs33*. Interestingly, the large subunit of heat-stable potato tuber AGPase contains a Tyr residue at the corresponding location. *Sh2hs33* retained 76% activity after heat treatment at 60°C for 5 min, compared to 25% for wild-type and 90% for heat-stable potato tuber AGPase. Further, specific activity of AGPase in crude extracts of *Sh2hs33*

was 2-fold higher than wild-type before heat treatment. Glycerol density gradient analysis of heated and nonheated *Sh2hs33* and wild-type crude samples showed that the mutation in *Sh2hs33* stabilizes heterotetrameric AGPase formation. Results of this study suggest a single amino acid change is sufficient to condition heat stability in the maize endosperm, and increased stability may be modulated through enhanced subunit interactions.

The objective of this project is to identify additional mutations in the large subunit of maize endosperm AGP that alter enzyme stability. Mutants isolated in a previous study in this lab (Greene and Hannah, 1998a) repeatedly contained the His-to-Tyr mutation of *Sh2hs33*. The protocol employed in this study varies from the one used previously, and should yield different mutants. In brief, the methodology consists of using chemical mutagenesis in conjunction with a bacterial expression system to first identify temperature sensitive mutants. Specifically, these mutants have lost the ability to produce glycogen at 30ºC, but retain AGPase activity at 37ºC. These mutants were then used to screen for second-site revertants following subsequent rounds of chemical mutagenesis. Isolation of second-site reversion mutations, in the absence of the negative parental mutations, confers varying degrees of heat stability to the enzyme and increase activity of the enzyme in the absence of heat. However, the second-site reversion mutations are inferior to the mutation identified in *Sh2hs33*. By combining the mutations discovered in this study with the mutation arising in *Sh2hs33*, heat stability and pre-heat treatment activity are increased more than *Sh2hs33* alone.

CHAPTER 2
MATERIALS AND METHODS

Mutagenesis and Mutant Selection

Mutagenesis of plasmid DNA containing the coding region of wild-type *Sh2* cDNA was performed for 48 hours as described by Greene et al. (1996). Treated *Sh2* plasmid DNA was then electroporated into *Escherichia coli* strain AC70R1-504 containing the wild-type *Bt2* coding region on a compatible vector. Antibiotic resistance conditioned by plasmid DNA containing *Sh2* and *Bt2* cDNA allowed for selection of putative transformants on agar plates containing 75 mg mL^{-1} spectinomycin and 50 mg mL^{-1} kanamycin.

Colonies were incubated at 30°C on enriched medium plates (0.85% [w/v] KH_2PO_4, 1.1% [w/v] K_2HPO_4, 0.6% [w/v] yeast extract, 1% [w/v] glucose, and 1.5% [w/v] agar) containing 75 mg mL^{-1} spectinomycin and 50 mg mL^{-1} kanamycin and stained with iodine. Approximately 6,000 colonies were screened. The original screen to isolate colonies with activity as evidenced by iodine staining at 30°C but not at 37°C proved unsuccessful. Instead 66 colonies with varying degrees of abnormal activity at 30°C were isolated. These colonies were streaked in duplicate at 30°C and 37°C on enriched medium plates. Of these, twenty colonies were identified with activity at 37°C but not at 30°C. Based on reproducibility and intensity of staining patterns six mutants were selected by their inability to complement the *glgC*- mutation at 30ºC. Wild-type is fully able to complement at both temperatures. That the six mutants

exhibited positive iodine staining at 37ºC indicates that we have isolated temperature sensitive mutants of maize endosperm AGPase. Two of the temperature sensitive mutants, *Sh2ts60* and *Sh2ts48*, were randomly selected for further analysis.

Coding regions of *Sh2ts60* and *Sh2ts48* were subcloned into unmutated vectors as 1553 bp *Nco* I/*Sst* I fragments. The use of *Nco* I and *Sst* I allows isolation of the coding region in its entirety, without vector (Figure 2).

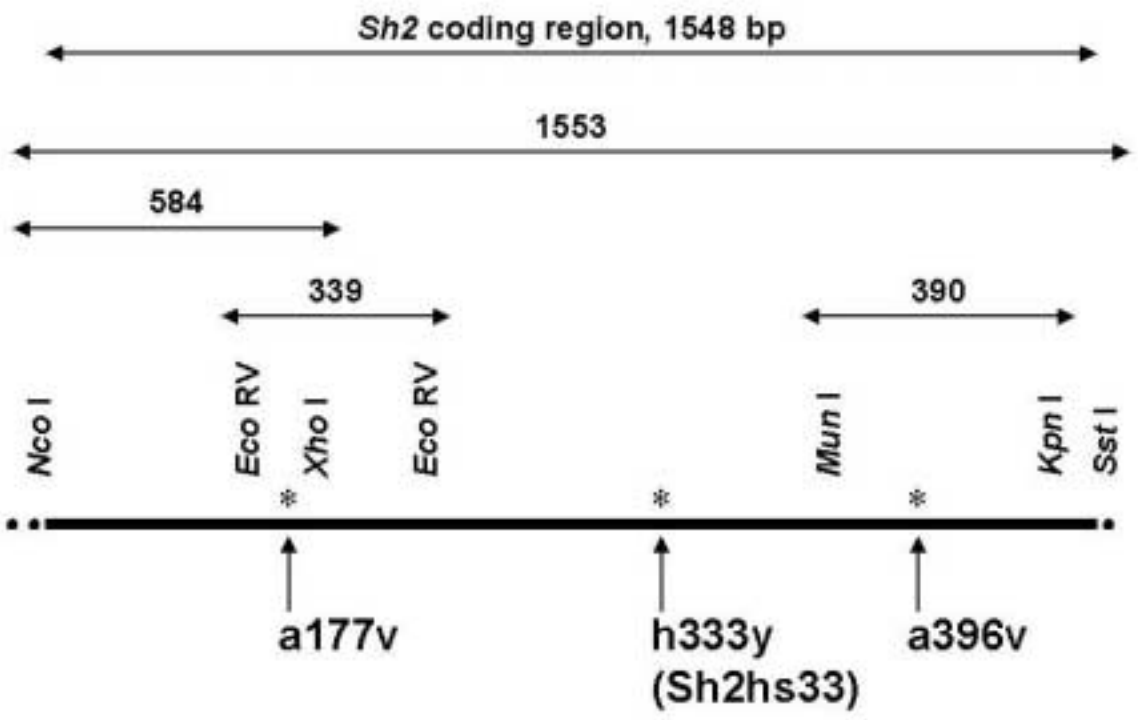

Figure 2. Restriction map of *Sh2* coding region. Restriction enzymes shown are those used in isolation of entire coding region and in creation of double and triple mutants. Mutations are indicated with asterisks (*).

At this point, plasmids were single-pass sequenced at the University of Florida DNA Sequencing Core Facility, with the following primers:

G10-2: 5'-CCCTCTAGAAATAATTTTG-3';

LH28: 5'-GAGTGGCGATCAGCTT-3';

LH21: 5’-TCACGTGTCAGCTCT-3’.

Sequence alignments with wild-type were performed using MultAlin (Corpet, 1988).

To isolate second-site revertants of *Sh2ts60* and *Sh2ts48*, the plasmids were treated with hydroxylamine, transformed into *E. coli* AC70R1-504 cells containing wild-type *Bt2*, and selected for antibiotic resistance on LB plates as described above. Colonies were streaked in duplicate on enriched medium plates containing 75 mg mL^{-1} spectinomycin and 50 mg mL^{-1} kanamycin, and incubated at 30°C and 37°C. Mutants were selected by their restored ability to complement the *glgC*- mutation at 30ºC. Five second-site revertants of *Sh2ts60* and three second-site revertants of *Sh2ts48* were isolated. Focus was restricted to only one randomly selected revertant each for *Sh2ts60* and *Sh2ts48*: *Sh2rts60-1* and *Sh2rts48-2*. Coding regions from *Sh2rts60-1* and *Sh2rts48-2* were subcloned into unmutated vectors as *Nco* I/*Sst* I fragments, sequenced, and aligned as described above.

Sequence analysis of *Sh2ts60* revealed two point mutations: one mutation generated a Glu to Lys change at amino acid 324, while an additional mutation resulted in an Ala to Val substitution at amino acid 359. It was desirable to separate the two mutations of *Sh2ts60* to determine the effect of the single mutations. However, the absence of a unique cloning site between the mutations necessitated use of site-directed mutagenesis. The Transformer Site-Directed Mutagenesis kit (Clontech, Palo Alto, CA) and protocol were used with the following primers (5’ phosphorylated):

TS60E324K: 5’-CATGACTTTGGATCTAAAATCCTCCCAAGAGC-3’;

TS60A359V: 5’-CTTTGATGCAAACTTGGTCCTCACTGAGCAGCC-3’;

JSSh2mutSstI: 5’-GGGTCTGTCATATAGTGAGCACGGTACCCGGGG-3’.

The resulting plasmids containing the separated mutations of *Sh2ts60* were designated *Sh2e324k* and *Sh2a359v*.

Site-directed mutagenesis was also employed to separate the second-site reversion mutation present in *Sh2rts60-1* from the parental mutations of *Sh2ts60*, using the primers (5' phosphorylated):

RTS60-1A396V: 5'-GCAAGATGAAATATGTATTTATCTCAGATGGTTGC-3', and JSSh2mutSstI: 5'-GGGTCTGTCATATAGTGAGCACGGTACCCGGGG-3'. The resulting plasmid containing only the reversion mutation of *Sh2rts60-1* was designated *Sh2a396v*.

The second-site reversion mutation of *Sh2rts48-2* was subcloned from the parental mutation of *Sh2ts48* as a 584 bp *Nco* I/*Xho* I fragment into an unmutated vector (Figure 2). The resulting plasmid containing only the reversion mutation of *Sh2rts48-2* was designated *Sh2a177v*.

A subcloning strategy was designed to study the effects of the mutations in combination with *Sh2hs33*, and with each other. To combine the mutations of *Sh2a177v* and *Sh2a396v*, the plasmids were digested with *Eco* RV and a 339 bp fragment of *Sh2a177v* was exchanged for the corresponding fragment of *Sh2a396v* (Figure 2). The resulting plasmid was designated *Sh2-177-396*. A similar strategy was used to combine the mutation of *Sh2a177v* with the mutation identified in *Sh2hs33*. Plasmids were digested with *Eco* RV and a 339 bp fragment of *Sh2a177v* was exchanged for the corresponding fragment of *Sh2hs33*. The resulting plasmid was designated *Sh2-177-33*. To combine the mutation of *Sh2a396v* with the mutation identified in *Sh2hs33*, the plasmids were digested with *Mun* I/*Kpn* I and a 390 bp fragment of *Sh2a396v* was

exchanged for the corresponding fragment of *Sh2hs33* (Figure 2). The resulting plasmid was designated *Sh2-396-33*. In order to combine the mutations of *Sh2a396v* and *Sh2a177v* with the mutation identified in *Sh2hs33*, *Sh2-396-33* and *Sh2a177v* were digested with *Eco* RV and a 339 bp fragment of *Sh2a177v* was exchanged for the corresponding fragment of *Sh2-396-33*. The resulting plasmid was designated *Sh2-177-396-33*.

Final sequencing of all plasmids was performed using six primers to cover the entire *Sh2* coding region in both directions. Primers used are as follows:

LHBB1 (5'→3'): 5'-CGACTCACTATAGGGAGACC-3';

LH27 (5'→3'): 5'-CCCTATGAGTAACTG-3';

LH9 (5'→3'): 5'-TATACTCAATTACAT-3';

LHBB2 (3'→5'): 5'-GTGCCACCTGACGTCTAAG-3';

LH2135 (3'→5'): 5'-CAGAGCTGACACGTG-3';

LH32 (3'→5'): 5'-AAGCTGATCGCCACTC-3'.

Enzymology

To obtain quantitative data for the mutants described above, activity was measured with the synthesis (forward) assay that measures incorporation of [^{14}C]Glc-1-P into the sugar nucleotide ADP-Glc. Assays were performed on crude enzyme extracts prepared as described below.

Aliquots (10 mL) of LB containing 75 mg ml^{-1} of spectinomycin and 50 mg mL^{-1} of kanamycin were inoculated from glycerol stocks of *E. coli* AC70R1-504 cells expressing mutant or wild-type AGPase, and grown overnight at 37°C with shaking at 225 rpm. These cultures were used to inoculate 250 mL of LB containing 75 mg mL^{-1} of

spectinomycin and 50 mg mL^{-1} kanamycin. Cultures were grown to an OD_{600}= 0.5-0.6 at 37°C with shaking at 225 rpm, then induced with 0.2 mM isopropyl B-D-thiogalactoside and 0.02 mg mL^{-1} nalidixic acid for 6.5 h at 23°C with shaking at 200 rpm. Cells were harvested by centrifugation at 3500 x g for 10 min at 4°C, and stored at –80°C. Cell pellets were resuspended in 1mL of extraction buffer: 10mM KPO_4, pH 7.5, 50 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 5 mM EDTA, 30% (w/v) ammonium sulfate, and 20% (w/v) sucrose. DTT (1 mM), 50 mg mL^{-1} lysozyme, 1 mg mL^{-1} pepstatin, 1 mg mL^{-1} leupeptin, 1 mg mL^{-1} antipain, 1 mg mL^{-1} aprotinin, 10 mg mL^{-1} chymostatin, 1mM phenylmethylsulonyl fluoride, and 1 mM benzamidine were added to extraction buffer just before use. Resuspended cells were sonicated 3 times with a Branson 450 Sonifier (Branson Ultrasonics Corporation, Danbury, CT) for 7 seconds at 60% duty cycle and output control level 3, with incubation on ice between sonications. Samples were centrifuged for 1 min at 13,000 rpm at 4°C, and supernatants were removed and used for assays. Heat treatment consisted of incubation at 60°C for 5 min.

The ADP-Glc synthesis reaction measures incorporation of [^{14}C]Glc-1-P into ADP-Glc. The reaction mixture contained 80 mM HEPES, pH 7.5m, 1 mM Glc-1-P, 4 mM $MgCl_2$, 0.5 mg mL^{-1} bovine serum albumin, 10 mM 3-PGA, and 15,000 cpm of [^{14}C]Glc-1-P. Reaction volume was 50 mL. Assays were initiated by addition of 1.5 mM ATP. Reaction was incubated for 30 min at 37°C and terminated by boiling for 2 min. Unincorporated Glc-1-P was cleaved by addition of 0.3 U of bacterial alkaline phosphatase (Worthington Biochemical Corporation, Lakewood, NJ) and incubation for 2.5 h at 37°C. A 20 mL aliquot of the reaction mixture was spotted on DEAE paper,

washed with distilled water three times, dried, and quantified in a liquid scintillation counter.

Gel Filtration Chromatography

In order to assess the effect of mutations on enzyme assembly and/or subunit interactions, it was necessary to separate the enzymes by size. Crude extract, prepared as above except that extraction buffer also contained 10 mM $MgCl_2$, 5% (v/v) glycerol, and 200 mM KCl, was loaded on a Pharmacia Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech, Piscataway, NJ) connected to an FPLC system (Amersham Pharmacia Biotech) at 4°C. The column was previously equilibrated with 2 volumes of buffer containing 10 mM $MgCl_2$, 5 mM EDTA, 5% (w/v) sucrose, 50 mM HEPES, pH 7.5, and 200 mM KCl and filtered through a 0.45 μM filter (Gelman Sciences, Ann Arbor, MI). Flow rate was 0.5 mL min^{-1} and loading volume was 200 mL. Fractions of 250 mL were collected. The column was calibrated with the following markers, dissolved at 1 mg mL^{-1} in equilibration buffer (50 mM Tris-Cl, pH 7.5, 100 mM KCl, and 5% (v/v) glycerol), and filtered at 0.45 μM: apoferritin (443 kD), β-amylase (200 kD), alcohol dehydrogenase (150 kD), BSA (66 kD), and carbonic anhydrase (29 kD). Flow rate was 0.5 mL min^{-1} and loading volume was 200 mL. A void volume of 8 mL was determined using blue dextran.

To verify size and quantify relative amounts of AGPase present, proteins were separated on a Novex NuPAGE 7% Tris-Acetate polyacrylamide gel (Invitrogen, Carlsbad, CA) using a Novex XCell II Mini-Cell electrophoresis unit (Invitrogen). SDS-PAGE standards for SYPRO Orange Stain-Broad Range were used in conjunction with SYPRO Orange Stain (Bio Rad, Hercules, CA). Gel was stained for 0.5 h. Protein was

visualized with UV light and an AlphaImager 2200 digital imaging system (Alpha Innotech Corporation, San Leandro, CA).

Proteins were transferred to nitrocellulose (Micron Separations Inc., Westborough, MA) with a Hoefer TE 70 Series SemiPhor semi-dry transfer unit (Amersham Pharmacia Biotech). Membrane and filter paper used in transfer were soaked in Towbin transfer buffer: 25 mM Tris, pH 8.3, 192 mM glycine, 20% (v/v) methanol, and 0.1% (w/v) SDS. Transfer time was one hour. Membranes were washed three times for ten minutes each in Tris-buffered saline Tween (TTBS) solution: 100 mM Tris-Cl, pH 6.8, 150 mM NaCl, and 0.1% (v/v) Tween 20. Membranes were blocked for one hour in TTBS containing 5% (w/v) BSA. Membranes were incubated for one h with primary antibodies (1:1000) directed against BT2 (Giroux and Hannah, 1994). Excess antibodies were removed by washing membrane three times for ten min each in TTBS solution. Primary antibodies were detected by incubation for one h with 1:5000 dilution of horseradish peroxidase conjugated to donkey anti-rabbit IgG (Amersham Pharmacia Biotech) in TTBS solution containing 5% (w/v) BSA. Excess antibodies were removed by washing three times for ten min each in TTBS solution. Blots were visualized by ECL chemiluminescent detection (Amersham Pharmacia Biotech).

To quantify relative amounts of AGPase present, films were scanned using a Hewlett Packard (Palo Alto, CA) scanner in conjunction with Alpha Ease software (Alpha Innotech Corporation). Densitometry was determined using auto background settings.

CHAPTER 3
RESULTS

Sequencing

The mutagenesis and mutant selection strategy made use of hydroxylamine and a bacterial expression system. Hydroxylamine preferentially hydroxylates the amino nitrogen at the C-4 position of cytosine, resulting in a GC to AT transition (Suzuki et al., 1989). While hydroxylamine can induce only two of the possible twelve base pair substitutions, the nature of the mutagen ensures no direct base pair reversions, an advantage utilized in searches for second site suppressors. The bacterial expression system uses *E. coli* strain AC70R1-504, a strain lacking endogenous bacterial AGPase activity (*glg C*-). Using cDNA clones of the large and small subunits of potato tuber AGPase, Iglesias et al. (1993) showed that expression of both subunits complements the mutation, while either subunit alone is unable to do so. Complementation is easily visualized by staining colonies with iodine. Colonies with restored glycogen production stain brown, as iodine chelates with glycogen, to produce an easily detectable indication of complementation. Complementation of the *E. coli* AC70R1-504 mutation with cDNA clones of the large and small subunits of maize endosperm AGPase has also been demonstrated (Giroux et al., 1996).

Sequencing of *Sh2ts48* identified a single mutation at amino acid 426, a Leu to Phe substitution. The Leu residue is conserved among the large subunits of rice

endosperm, developing wheat grain, barley endosperm, sorghum, and potato tuber AGPases (Figure 3).

```
                                        F 426
[Zea]     S V I G V C S R V S S G C E L K D S V M M G A D I
[Sorghum] S V I G V C S R V S Y G C E L K D C V M M G A D I
[Rice]    S V I G I S S R V S I G C E L K D T M M M G A D Q
[Barley]  S I I G V R S R L N S G S E L K N A M M M G A D S
[Wheat]   S I I G V R S R L N S G S E L K N A M M M G A D S
[Potato]  S I V G E R S R L D C G V E L K D T F M M G A D Y
consensus S . i G v r S R . s s G c E L K d . m M M G A D .
```

Figure 3. Mutation identified in *Sh2ts48*. Mutation is a Leu to Phe substitution at amino acid 426.

Two point mutations were identified in *Sh2ts60* (Figure 4). One mutation generated a Glu to Lys change at amino acid 324. This Glu is conserved in the large subunits of rice, wheat, barley, sorghum, and potato AGPases. An additional mutation in *Sh2ts60* resulted in an Ala to Val substitution at amino acid 359. This Ala is conserved in the large subunits of rice, wheat, barley, sorghum, and potato AGPases. Of significance, the mutations of *Sh2ts60* flank the mutation (His to Tyr at amino acid 333) arising in *Sh2hs33*, a heat-stable variant of maize endosperm AGPase (Greene and Hannah, 1998a).

Sequencing of the *Sh2ts48* second-site revertant, *Sh2rts48-2*, identified an Ala to Val substitution at amino acid 177 (Figure 5). The Ala is conserved in the large subunits of rice, wheat, and barley AGPases. The mutation is 249 amino acids in the N-terminal direction from the parental mutation, and maps to the same site as the mutation in *Sh2hs13* (Greene and Hannah, 1998a). The mutation in *Sh2hs13* is an Ala to Pro substitution, and confers some degree of heat stability, indicating this region is important in AGPase heat stability.

A.

```
                                        K 324
[Zea]     L K S K Y T Q L H D F G S E I L P R A V L D H S V
[Sorghum] L K S K Y T Q L H D F G S E I L P R A V L E H N V
[Rice]    L K S K Y A H L Q D F G S E I L P R A V L E H N V
[Barley]  L K S R Y A E L H D F G S E I L P R A L H D H N V
[Wheat]   L K S R Y A E L H D F G S E I L P R A L H D H N V
[Potato]  L K W S Y P T S N D F G S E I I P A A I D D Y N V
consensus L K s k Y a . l h D F G S E I l P r A v l d h n V
```

B.

```
                                        V 359
[Zea]     D V G T I K S F F D A N L A L T E Q P S K F D F Y
[Sorghum] D V G T I K S F F D A N L A L T E Q P S K F D F Y
[Rice]    D I G T I K S F F D A N L A L T E Q P P K F E F Y
[Barley]  D I G T I R S F F D A N M A L C E Q P P K F E F Y
[Wheat]   D I G T I R S F F D A N M A L C E Q P P K F E F Y
[Potato]  D I G T I K S F Y N A S L A L T Q E F P E F Q F Y
consensus D i G T I k S F f d A n l A L t e q p p k F e F Y
```

Figure 4. Mutations identified in *Sh2ts60*. A. One mutation is a Glu to Lys substitution at amino acid 324. B. The second mutation is an Ala to Val substitution at amino acid 359.

```
                                    V 177
[Zea]     V L A A T Q M P E E P A G . W F Q G T A D S I R K
[Sorghum] V L A D T Q M P E E P D G . W F Q G T A D S V R K
[Rice]    V L A A T Q M P D E P A G . W F Q G T A D A I R K
[Barley]  V L A A T Q M P G E A A G . W F R G T A D A V R K
[Wheat]   V L A A T Q M P G E A A G . W F R G T A D A V R K
[Potato]  V L A A T Q T P G E A G K K W F Q G T A D A V R K
consensus V L A a T Q m P g E . a g . W F q G T A D a v R K
```

Figure 5. Mutation identified in *Sh2rts48-2*. Mutation is an Ala to Val substitution at amino acid 177.

Sequencing of the *Sh2ts60* second-site revertant, *Sh2rts60-1*, identified an Ala to Val mutation at amino acid 396 (Figure 6). This Ala is conserved in the large subunits of rice, wheat, barley, sorghum, and potato AGPases. This mutation is 37 and 42 amino acids in the C-terminal direction from the parental *Sh2ts60* mutations. In addition, the

mutation is four amino acids away from one of two mutations found in *Sh2hs14* (Greene and Hannah, 1998a). All mutations identified in this study, and the mutation in *Sh2hs33*, are shown in Figure 7.

```
                                        V 396
[Zea]     L P P T Q L D K C K M K Y A F I S D G C L L R E C
[Sorghum] L P P T Q L D K C K I K D A S I S D G C L L R E C
[Rice]    L P P A R L E K C K I K D A I I S D G C S F S E C
[Barley]  L P P T K S D K C R I K E A I I S H G C F L R E C
[Wheat]   L P P T K S D K C R I K E A I I S H G C F L R E C
[Potato]  L P P T K I D N C K I K D A I I S H G C F L R D C
consensus L P P t k l d k C k i K d A i I S . G C f l r e C
```

Figure 6. Mutation identified in *Sh2rts60-1*. Mutation is an Ala to Val substitution at amino acid 396.

```
Sh2       TYLEGGINFA DGSVQVLAAT QMPEEPAGWF QGTADSIRKF
Sh2rts48-2                            V(177)

Sh2       IWVLEDYYSH KSIDNIVILS GDQLYRMNYM ELVQKHVEDD

Sh2       ADITISCAPV DESRASKNGL VKIDHTGRVL QFFEKPKGAD

Sh2       LNSMRVETNF LSYAIDDAQK YPYLASMGIY VFKKDALLDL

Sh2       LKSKYTQLHD FGSEILPRAV LDHSVQACIF TGYWEDVGTI
Sh2ts60                K(324)
Sh2hs33                             Y(333)

Sh2       KSFFDANLAL TEQPSKFDFY DPKTPFFTAP RCLPPTQLDK
Sh2ts60           V(359)

Sh2       CKMKYAFISD GCLLRECNIE HSVIGVCSRV SSGCELKDSV
Sh2rts60-1     V(396)
Sh2ts48                                             F(426)
```

Figure 7. Mutations identified from sequence analysis in temperature sensitive mutants, revertants, and *Sh2hs33*.

In vitro Enzyme Assays

The ADP-Glc synthesis (forward) reaction provided quantitative data on the activity of *Sh2ts48*, *Sh2rts48-2*, and *Sh2a177v* as compared to wild-type (Table 1). The assay was performed with undiluted, 1:2 diluted, and 1:4 diluted samples. All dilutions were assayed in duplicate. Controls (minus ATP) were subtracted before multiplication by dilution factors and averaging.

Table 1. Activity (AGPase) in *Sh2* wild-type, *Sh2ts48*, *Sh2rts48-2*, and *Sh2a177v*. *Sh2ts48* is the original temperature sensitive mutant. *Sh2rts48-2* is a revertant of *Sh2ts48* and contains a second-site suppressor mutation, in addition to the mutation found in *Sh2ts48*. *Sh2a177v* contains only the second-site suppressor mutation from *Sh2rts48-2*. Activity is assayed at 37C.

Enzyme	Activity	SEM[a]	N[b]
Sh2 wt	2088	377	6
Sh2ts48	1128	270	6
Sh2rts48-2	1246	455	6
Sh2a177v	2103	393	6

[a] standard error of the mean
[b] number of experimental replicates

This data set clearly illustrates the effectiveness of the screening procedure used to identify mutants. A single mutation (*Sh2ts48*) causes a loss of activity when compared to wild-type. It is interesting to note that while activity of the enzyme containing a second-site reversion mutation (*Sh2rts48-2*) does not return to wild-type levels with regards to the *in vitro* enzyme assay, iodine staining in *Sh2rts48-2* is restored to wild-type or near wild-type levels. Isolation of the second-site reversion mutation in the absence of the parental mutation (*Sh2a177v*) does not significantly increase activity of the enzyme compared to wild-type. The data not only provide quantitative data supporting earlier qualitative data, but also show the ability of the screening procedure to effectively identify mutations altering enzyme activity.

The ADP-Glc synthesis (forward) reaction also provided quantitative data on the activity of *Sh2ts60*, *Sh2rts60-1*, *Sh2e324k*, *Sh2a359v*, and *Sh2a396v* as compared to wild-type (Table 2). The assay was performed with undiluted, 1:3 diluted, and 1:6 diluted samples. All dilutions were assayed in duplicate. Controls (minus ATP) were subtracted before multiplication by dilution factors and averaging. The data presented also illustrate the effectiveness of the protocol in identifying mutants affecting enzyme activity. Here, the mutations in *Sh2ts60* clearly affect the activity of AGPase. Upon separation of the two mutations present in *Sh2ts60*, the mutation present in *Sh2e324k* appears to be responsible for the phenotype of *Sh2ts60*.

Table 2. Activity (AGPase) in *Sh2* wild-type, *Sh2ts60*, *Sh2e324k*, *Sh2a359v*, *Sh2rts60-1*, and *Sh2a396v*. *Sh2ts60* is the original temperature sensitive mutant. *Sh2e324k* contains only one of two mutations found in *Sh2ts60*. *Sh2a359v* contains the second of two mutations found in *Sh2ts60*. *Sh2rts60-1* is a revertant of *Sh2ts60* and contains a second-site suppressor mutation, in addition to the mutations found in *Sh2ts60*. *Sh2a177v* contains only the second-site suppressor mutation found in *Sh2rts60-1*. Activity is assayed at 37C.

Enzyme	Activity	SEM[a]	N[b]
Sh2 wt	1398	520	6
Sh2ts60	84	49	6
Sh2rts60-1	109	43	6
Sh2e324k	67	42	6
Sh2a359v	996	241	6
Sh2a396v	2410	421	6

[a] standard error of the mean
[b] number of experimental replicates

The percentage of activity remaining after heat treatment at 60°C for 5 min is presented in Table 3. Genotypes in the data set are *Sh2* wild-type, *Sh2hs33*, *Sh2a396v*, *Sh2a177v*, *Sh2-396-177*, *Sh2-396-33*, *Sh2-177-33*, and *Sh2-396-177-33*. Activity of *Sh2* wild-type and *Sh2hs33* are in agreement with earlier reports (Greene and Hannah, 1998a).

Sh2a396v, *Sh2a177v*, *Sh2-396-177*, *Sh2-396-33*, *Sh2-177-33*, and *Sh2-396-177-33* are not significantly different in terms of heat stability.

Table 3. Percent Activity Remaining After Heat Treatment.
Heat treatment consists of incubation at 60°C for 5 min. *Sh2hs33* is a heat stable mutant (Greene and Hannah, 1998). Sh2a396v is the second-site reversion mutation found in *Sh2rts60-1*. *Sh2a177v* is the second-site reversion mutation found in *Sh2rts48-2*. *Sh2-396-177* is a double mutant of *Sh2a396v* and *Sh2a177v*. *Sh2-396-33* is a double mutant of *Sh2a396v* and *Sh2hs33*. *Sh2-177-33* is a double mutant of *Sh2a177v* and *Sh2hs33*. *Sh2-396-177-33* is a triple mutant of *Sh2a396v*, *Sh2a177v*, and *Sh2hs33*.

Enzyme	% Activity	SEM[a]	N[b]
Sh2 wt	32	11	3
Sh2hs33	69	7	7
Sh2a396v	61	13*	2
Sh2a177v	64	6	3
Sh2-396-177	77	21*	2
Sh2-396-33	69	9	3
Sh2-177-33	83	8	3
Sh2-396-177-33	72	11	3

[a] standard error of the mean
[b] number of experimental replicates
* represents range, rather than S.E.M.

Activity before heat treatment for *Sh2* wild-type, *Sh2hs33*, *Sh2a396v*, *Sh2a177v*, *Sh2-396-177*, *Sh2-396-33*, *Sh2-177-33*, and *Sh2-396-177-33* is shown in Table 4. *Sh2hs33* has 2.1 fold more activity than does *Sh2* wild-type. This is in agreement with previous data (Greene and Hannah, 1998a). Both *Sh2a177v* and *Sh2a396v* show a 1.4 fold increase in activity. Their double mutant contains a 1.9 fold increase in activity. While the combination of the two mutants increases activity, the double mutant does not experience synergistic effects. The mutation of *Sh2a396v* when combined with that of *Sh2hs33* experiences an additive effect, raising activity 3.4 fold compared to *Sh2* wild-type. The mutation of *Sh2a177v* in combination with that of *Sh2hs33* exhibits a slightly

smaller increase to 2.9 fold. Interestingly, the triple mutant shows a slightly greater increase than either second-site reversion mutation alone, but less than the double mutant between second-site revertants.

Table 4. Fold Increase in Activity.
Sh2hs33 is a heat stable mutant (Greene and Hannah, 1998). *Sh2a396v* is the second-site reversion mutation found in *Sh2rts60-1*. *Sh2a177v* is the second-site reversion mutation found in *Sh2rts48-2*. *Sh2-396-177* is a double mutant of *Sh2a396v* and *Sh2a177v*. *Sh2-396-33* is a double mutant of *Sh2a396v* and *Sh2hs33*. *Sh2-177-33* is a double mutant of *Sh2a177v* and *Sh2hs33*. *Sh2-396-177-33* is a triple mutant of *Sh2a396v*, *Sh2a177v*, and *Sh2hs33*.

Enzyme	Fold increase	Range	N[b]
Sh2 wt	n/a	n/a	n/a
Sh2hs33	2.1	0.2[a]	3
Sh2a396v	1.4	0	1
Sh2a177v	1.4	0	1
Sh2-396-177	1.9	0.2	2
Sh2-396-33	3.4	0	1
Sh2-177-33	2.9	0.1	2
Sh2-396-177-33	1.8	0.1	2

[a] standard error of the mean
[b] number of experimental replicates
n.a. not applicable

Relative Specific Activities of Heat-Sensitive and Heat-Stable Mutants

Gel filtration chromatography was used to isolate heterotetrameric AGPase from other size fractions of its subunits. To test the effectiveness of the column for separation, individually collected fractions were subjected to SDS-PAGE and western blotting. A western blot of Fractions 1-14 is shown in Figure 8. Low levels of BT2 protein are detected in Fractions 1 –8. Beginning with Fraction 9 there is a steady increase in the amount of BT2 present. Higher molecular weight proteins present in Lanes 1-6 are possibly due to transcriptional run-on and have been observed previously (Hannah, personal communication). Fraction 11 is considered to be the heterotetrameric fraction as

predicted by molecular mass markers. The presence of BT2 protein in Fractions 10 and 12-14 suggest that the heterotetrameric peak is split among fractions. However, by using Fraction 11 as the heterotetrameric fraction, contamination by AGPase dimers is unlikely as higher numbered fractions contain lower molecular weight molecules.

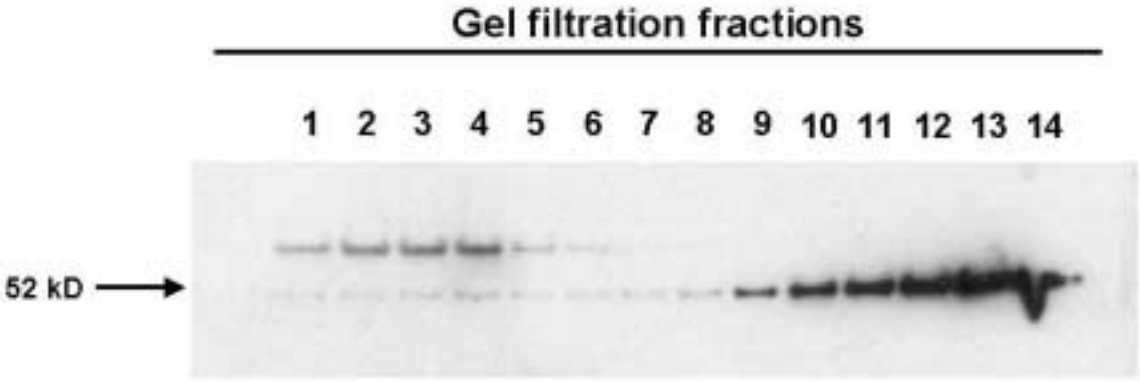

Figure 8. Western blot of *Sh2hs33* gel filtration fractions. Fractions 1-14 of Sh2hs33 were subjected to SDS-PAGE, transferred to nitrocellulose, and hybridized with antibody to BT2. Fraction 11 is the heterotetrameric fraction as determined by molecular mass markers.

Isolation of the heterotetrameric fraction, followed by relative quantitation of active enzyme through SDS-PAGE and western blotting, allowed an estimate of specific activities. The first of two western blots used to estimate specific activities is shown in Figure 9. The specific activities calculated using in vitro enzyme assays and relative amounts of AGPase present in Figure 9 are shown in Table 5. The second of two western blots used to estimate specific activities is shown in Figure 10. Specific activities calculated using the forward assay from gel filtration purification fractions and relative amounts of AGPase protein present in Figure 10 are shown in Table 6.

Following gel filtration chromatography of heated and nonheated samples of *Sh2* wild-type, *Sh2hs33*, *Sh2-177-33*, and *Sh-2177-396-33* aliquots of the heterotetrameric

fraction were subjected to SDS-PAGE. SDS gels were transferred to nitrocellulose and probed with antibody to BT2. Films were digitally scanned and relative amounts of protein determined using densitometry software. We loaded 12 and 6 μL to monitor linearity of AGPase signal. Lack of detectable protein for both heated and nonheated samples of *Sh2* wild-type made determination of relative protein amounts, and subsequent specific activity calculations, impossible. Specific activities decrease as stability increases (Table 5), and although no specific activity for *Sh2* wild-type is given, enzyme assays of *Sh2* wild-type suggest the highest specific activity in the group.

To verify results shown in Figure 9 and Table 5, a slightly modified version of the experiment was performed. Enzymes used were *Sh2* wild-type, *Sh2hs33*, and *Sh2-177-33*. Heat-treatment was excluded from the experiment. The heterotetrameric gel filtration fractions and the succeeding fraction were used. Two fractions were subjected to SDS-PAGE in Figure 10 to evaluate contamination by dimers. That specific activities of both fractions from each enzyme are similar suggests no contamination by dimeric AGPase subunits. In contrast to the previous experiment, specific activities increase with increasing stability, with *Sh2* wild-type exhibiting the lowest specific activity and *Sh2-177-33* exhibiting the greatest specific activity. The discrepancy between experiments is discussed below.

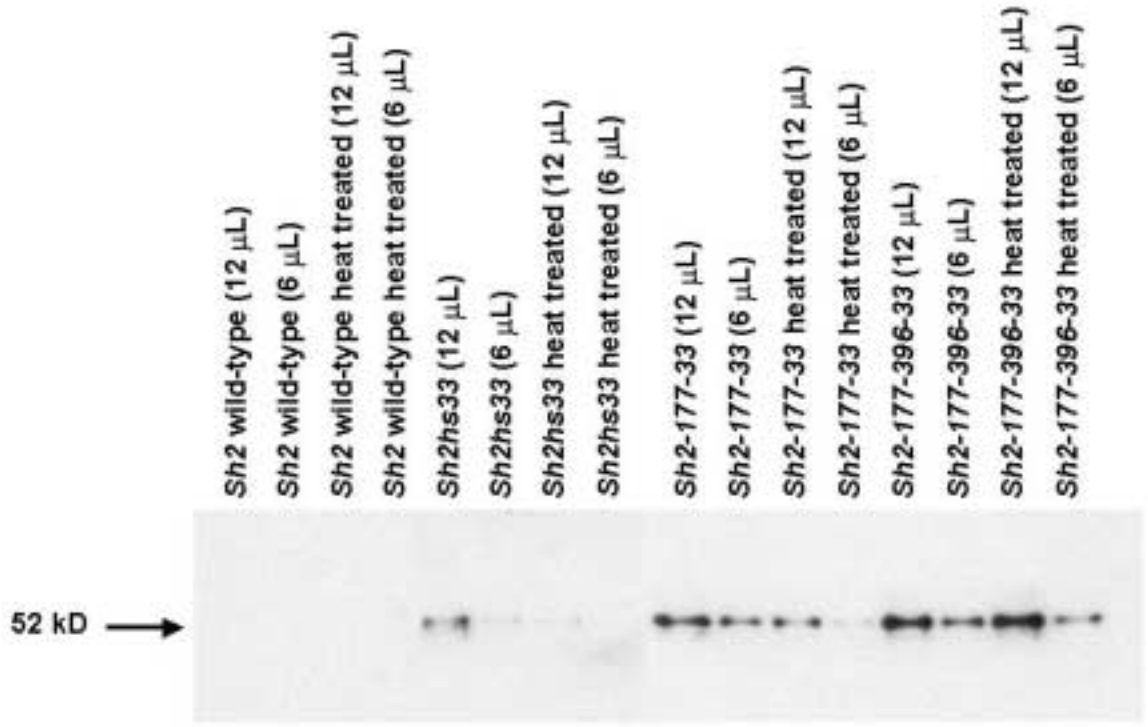

Figure 9. Western blot of gel filtration fractions to calculate specific activities. Samples of heterotetrameric fraction, separated by SDS-PAGE, transferred to nitrocellulose, and hybridized with antibody to BT2 protein. Lanes 1 and 2: *Sh2* wild-type, 12 µL and 6 µL, respectively. Lanes 3 and 4: heat-treated *Sh2* wild-type, 12 µL and 6 µL, respectively. Lanes 5 and 6: *Sh2hs33*, 12 µL and 6 µL, respectively. Lanes 7 and 8: heat-treated *Sh2hs33*, 12 µL and 6 µL, respectively. Lanes 9 and 10: *Sh2-177-33*, 12 µL and 6 µL, respectively. Lanes 11 and 12: heat-treated *Sh2-177-33*, 12 µL and 6 µL, respectively. Lanes 13 and 14: *Sh2-177-396-33*, 12 µL and 6 µL, respectively. Lanes 15 and 16: heat-treated *Sh2-177-396-33*, 12 µL and 6 µL, respectively.

Table 5. Specific Activities of *Sh2* wild-type, *Sh2hs33*, *Sh2-177-33*, and *Sh2-177-396-33* *Sh2hs33* is a heat stable mutant (Greene and Hannah, 1998). *Sh2-177-33* is a double mutant of *Sh2a177v* (second-site reversion mutation) and *Sh2hs33*. *Sh2-396-177-33* is a triple mutant of *Sh2a396v* (second-site reversion mutation), *Sh2a177v*, and *Sh2hs33*.

Enzyme	Heat treat	Activity	Protein amt.	Sp. activity
Sh2 wt	no	2291	n.d.	n.d.
Sh2hs33	no	8455	22	384
Sh2-177-33	no	7071	38.5	184
Sh2-177-396-33	no	7131	49.5	144
Sh2 wt	yes	7709	n.d.	n.d.
Sh2hs33	yes	7888	6.8	1160
Sh2-177-33	yes	8927	20.5	435
Sh2-177-396-33	yes	7878	47	168

n.d. not determined

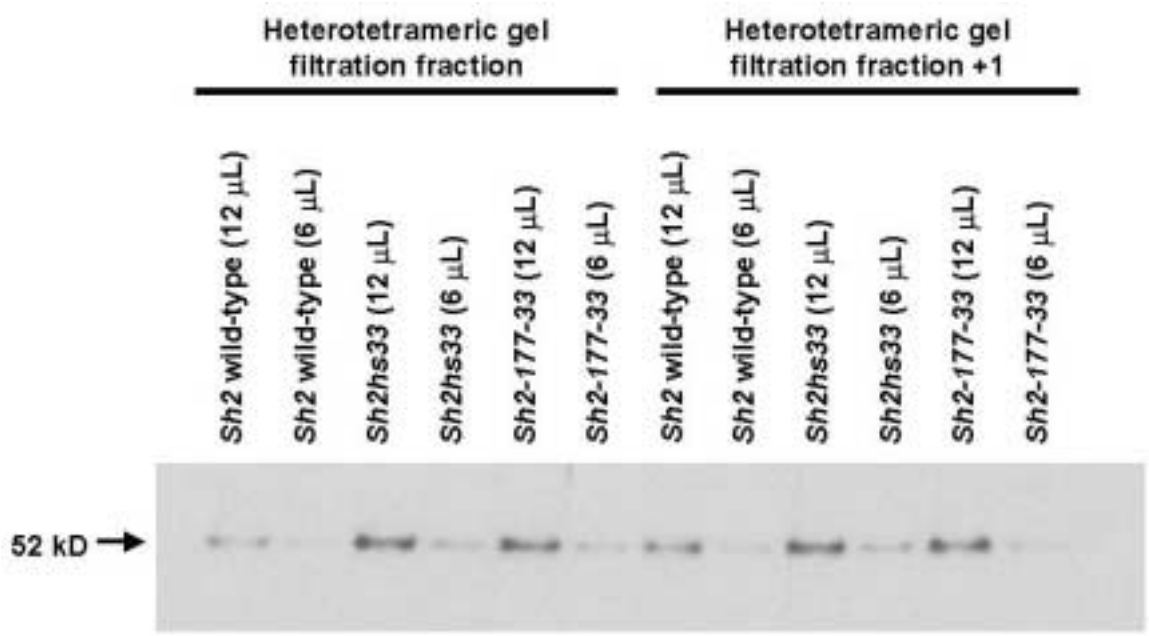

Figure 10. Western blot of *Sh2* wild-type, *Sh2hs33* and *Sh2-177-33* gel filtration fractions. Lanes1-6: gel filtration fractions corresponding to the heterotetrameric fraction. Lanes 7-12: gel filtration fractions corresponding to the succeeding heterotetrameric fraction. Fractions separated by SDS-PAGE, transferred to nitrocellulose, and hybridized with antibody to BT2 protein. Lanes 1, 2, 7, and 8: *Sh2* wild-type, 12 µL and 6 µL, respectively. Lanes 3, 4, 9, and 10: *Sh2hs33*, 12 µL and 6 µL, respectively. Lanes 5, 6, 11, and 12: *Sh2-177-33*, 12 µL and 6 µL, respectively.

Table 6. Specific Activities for *Sh2* wild-type, *Sh2hs33*, and *Sh2-177-33*
Sh2hs33 is a heat stable mutant (Greene and Hannah, 1998). *Sh2-177-33* is a double mutant of *Sh2a177v* (second-site reversion mutation) and *Sh2hs33*.

Enzyme	Fraction	Activity	Protein amt.	Sp. activity
Sh2 wt	12	586	13	45
Sh2hs33	12	1843	26	71
Sh2-177-33	12	2188	23	95
Sh2 wt	13	750	16	47
Sh2hs33	13	1870	22.5	83
Sh2-177-33	13	2190	21.5	102

CHAPTER 4
DISCUSSION

It is well known that single amino acid polymorphisms in the coding region of enzymes can alter properties. The work of Greene and Hannah (1998a) showed that a single amino acid change in the large subunit of maize endosperm AGPase can affect subunit interactions and heat stability. The aim of this project was to identify other mutations affecting enzyme stability. Through sequence comparison of mutants from this study and mutants identified by Greene and Hannah (1998a), regions important in enzyme stability both in the presence and absence of increased heat are becoming apparent. Because the coding region of *Sh2* is by no means mutation saturated, at this point only two regions of importance can be tentatively identified. The first region spans 113 amino acids, from residue 104 to residue 217. Amino acid 217 was first identified by Greene and Hannah (1998a) as one (Arg to Pro) of two mutations in *Sh2hs47*, a heat-stable mutant retaining approximately 60% activity after incubation at 60°C for 5 min. However, because the two mutations in *Sh2hs47* were not separated to determine their individual effect on stability, no definitive conclusions about this amino acid can be drawn. Also identified by Greene and Hannah (1998a) was residue 114 in *Sh2hs16*. An Arg to Thr substitution at this position conditions retention of approximately 40% activity after heat treatment. Amino acid 177 is the site of the mutation (Ala to Pro) identified in *Sh2hs13* (Greene and Hannah, 1998a). This mutant was isolated in the screening procedure and presumably confers some degree of heat stability, although no quantitative data were given. Amino acid 177 is also the location of the second-site reversion

mutation present in *Sh2rts48-2*. An Ala to Val substitution at this residue confers retention of approximately 64% activity after heat treatment. That residue 177 has been identified in two studies, lends credence to the importance of this residue in stability of maize endosperm AGPase.

The second region of importance in terms of AGPase stability is a 136 amino acid stretch between residues 324 and 460. Mutations in this study include those identified in *Sh2ts48* and *Sh2ts60* at residues 324, 359, and 426. Data presented illustrate the importance of these residues, particularly 324 and 426, in enzyme stability as evidenced by the concomitant conditional phenotype upon mutation of these residues. Also identified in this study is residue 396, the location of the second-site reversion mutation of *Sh2ts60*. An Ala to Val substitution at this residue confers approximately 61% retention of activity following heat treatment. The remaining residues that define this region were identified in Greene and Hannah (1998a) and include residues 333 (His to Tyr), 400 (Asp to His), 454 (Val to Ile), and 460 (Thr to Ile). The His to Tyr mutation at residue 333 was repeatedly encountered in the study, and its importance in stability has been shown in both the original and the present study.

Comparison of the mutations identified here show an interesting pattern. Of the five mutations, three are Ala to Val substitutions. Further, both second-site reversion mutations are Ala to Val substitutions. Whether the over-representation of Ala to Val substitutions is because of the relatedness of the codons for these amino acids, the limited range of substitutions possible through hydroxylamine mutagenesis, or reflects the accessibility of these particular Ala codons to the mutagen is unclear.

As stated above, with the limited number of mutations in the coding region of *Sh2* it is difficult to identify regions of importance with any certainty. In fact, there is no compelling reason to include residue 217 with residues 177 and 104 rather than with the remaining mutations other than distance between the next closest mutations is smaller. It remains to be seen whether the residues identified can in fact be divided into two regions or whether the mutations are isolated. The two tentatively identified regions may be divided further, their domains expanded or contracted, and their relative importance verified as more mutants become available. However, the mutations have given us a starting point to further study the interactions of the subunits and their role in the stability of this important enzyme.

The mechanism by which the mutation of *Sh2hs33* enhances heat stability is not definitively known. Glycerol density gradient centrifugation and SDS-PAGE/western blotting provided evidence that *Sh2hs33* increases heat stability through stabilizing heterotetrameric AGPase formation (Greene and Hannah, 1998a). Certainly this result is in agreement with earlier reports (Giroux and Hannah, 1994; Wang et al., 1997) in which both subunits must be present for maximum stability. Perhaps *Sh2hs33* stabilizes heterotetrameric AGPase formation, thus forming an enzyme more resistant to stress and with more activity in the absence of stress, because of an increased affinity for heterotetrameric formation. This scenario presents a testable hypothesis. If Sh2hs33 and heat-stable variants identified in this study enhance stability by shifting equilibrium of subunit interaction in the direction of heterotetrameric formation, activity per heterotetramer should be equal to wild-type and to each other. Using techniques developed previously in this lab the question has been addressed.

Using heated and nonheated samples of *Sh2*, *Sh2hs33*, *Sh2-177-33*, and *Sh2-177-396-33* (Figure 9), specific activities were calculated (Table 5). Specific activities decrease as stability increases. Although no numerical values were given for *Sh2* wild-type because of undetectable amounts of protein, enzyme assays suggest a higher specific activity than the other enzymes. Heat treatment increases specific activities but does not change the underlying observation. In a slightly modified experiment to confirm earlier results, differing results were obtained. Using *Sh2*, *Sh2hs33*, and *Sh2-177-33* (Figure 10) specific activities were calculated (Table 6). In contrast to the previous experiment, specific activities increase with increasing stability. Without further experimentation, no definitive conclusions can be drawn.

Activity levels in the first experiment were higher than the second experiment (Table 5 vs. Table 6). Whether this reflects differences in enzyme preparations, enzyme integrity during gel filtration chromatography, assay conditions, or other phenomena is unknown. The differing data do provide an interesting opportunity to speculate about the reasons behind the possible final outcomes. As discussed above, if the mechanism by which the various mutants increase stability occurs via a shift in equilibrium of subunit interactions towards heterotetrameric AGPase formation, specific activities per heterotetramer should be equal. Existing data for *Sh2hs33* (Greene and Hannah, 1998a) suggest that heterotetrameric AGPase formation is increased. In order to explain differences in specific activities per heterotetramer, however, other mechanisms must be at work. According to data presented in Figure 10 and Table 6, it appears possible that mutations have rendered the enzymes more efficient. By increasing subunit interactions equilibrium has not only shifted in favor of heterotetramers, but the mechanisms by

which substrates are converted to product are altered. A change in conformation of active site due to conformational changes in enzyme structure is but one explanation. Undoubtedly, 3-D structure of the enzyme would offer insight as to the placement of the amino acids and the plausibility of their effect on enzyme structure. Lack of crystal and 3-D structure, and the unequivocal data presented in this study, relegate these musings to mental exercises.

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BIOGRAPHICAL SKETCH

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