TRANSITORY LEAF STARCH IS AN IMPORTANT DETERMINANT OF PLANT YIELD

by

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ABSTRACT

Efficient allocation of photoassimilates from source to sink tissues is important for optimal plant growth and yield as relative source and sink strength drives growth potential of plant organs. A common method aimed at improving plant yield has been to modify enzymes important to storage compound biosynthesis in sink tissues such as seeds. As the rate limiting step in starch biosynthesis, ADP-glucose pyrophosphorylase (AGPase) has received much attention in this regard. Previously, overexpression of AGPase in seeds resulted in an enhanced yield phenotype in which both plant yield and biomass were increased. However, yield advantages were only observed under non-limiting environmental resources. The objective of these studies was to 1) determine the importance of native leaf starch levels to the productivity and growth of maize and 2) target source strength by overexpressing AGPase in rice leaves. To determine the importance of native leaf starch levels in maize, field trials of $BC_4F_{2:3}$ plants segregating for the presence or absence of the *agps-m1* mutation and leaf starch were conducted in Citra, Florida. The results clearly demonstrate the importance of normal leaf starch levels to maize productivity. The starchless *agps-m1* plants were 6 to 13 cm shorter, flowered 2 to 3 days later, and had 30 percent lower seed yield than their wild type sister-lines. The impact of increased AGPase in rice leaves was then tested by overexpressing AGPase in rice leaves. Two expression constructs were used to transform rice cultivar Nipponbare, each containing a modified form of the maize endosperm AGPase large subunit sequence, *Sh2r6hs*, as well as the small subunit sequence, *Bt2*. Expression of the transgenes was under control of either the rice leaf AGPase small subunit promoter, *Ags1,* or native rice RuBisCO small subunit promoter, *RBC*. Expression of the transgenes under the *RBC* promoter is associated with significantly increased plant biomass. Our results indicate that it is possible to increase plant yield without increasing the rate of photosynthesis. Further, it indicates the possibility of manipulating plant yield through increasing AGPase activity in leaf tissue.

CHAPTER 1

THE MAIZE LEAF STARCH MUTATION, *agps-m1,* HAS DIMINISHED FIELD GROWTH AND PRODUCTIVITY

Introduction

Source and sink strength heavily influence production and allocation of photosynthate. Carbohydrates are produced in source tissues and translocated to sink tissues for growth, development, or storage (reviewed in Van Camp, 2005). In cereals, a primary sink storage product is the starchy endosperm which comprises 70-80% percent of cereal seed weight. In leaves, transitory starch accumulates as granules in the chloroplast in the light. These granules are then degraded and exported to sink tissues during the dark period (reviewed in Zeeman et al., 2007). Increasing sink strength has been of particular interest to researchers endeavoring to improve biological yield of cereals (reviewed in Van Camp, 2005; Araus et al. 2008). Numerous studies have attempted to increase the level of starch biosynthetic enzymes believed to limit plant productivity.

One starch biosynthetic enzyme that has received considerable attention is ADP-glucose pyrophosphorylase (AGPase). AGPase controls the rate limiting step in starch biosynthesis (Stark et al., 1992; Giroux et al., 1996; Smidansky et al., 2002, 2003). From the substrates glucose-1-phosphate (G-1-P) and ATP, AGPase produces pyrophosphate and ADP-glucose, the glucosyl donor for polymerization in starch biosynthesis (Espada, 1962). AGPase is an allosteric enzyme positively regulated by 3-phosphoglyceric acid

(3-PGA) and negatively regulated by orthophosphate (Pi) (reviewed in Hannah, 1997; Preiss, 1997). Plant AGPases are heterotetrameric and are assembled from two large and two small subunits with each encoded by separate genes (Hannah 1997; Preiss 1997). In cereals, there are seed and leaf specific genes for each AGPase subunit (Giroux and Hannah, 1994, Denyer et al., 1996; Thorbjornsen et al., 1996; Beckles et al., 2001). Unlike leaf AGPases, cereal endosperm AGPases lack transit peptides (Giroux and Hannah, 1994; Villand and Kleczkowski, 1994). Nearly all studies examining the degree to which plant yield is limited by native AGPase levels have focused upon increasing seed AGPase levels.

Transgenically increasing AGPase in seeds of wheat (Giroux et al., 1996; Meyer et al., 2004; Smidansky et al., 2007) and rice (Smidansky et al., 2003) leads to increases in seed yield. In these studies, higher seed yield resulted from increases in both heads per plant and number of seeds per head. Vegetative growth was also increased such that the harvest index remained unchanged. However, the yield advantages due to increased AGPase activity in wheat occurred only when resources were non-limiting (Meyer et al., 2007). Increases in seed yield and vegetative growth associated with seed-specific AGPase overexpression indicates metabolic alterations in both sink and source tissues.

In addition to studies where an AGPase with modified allosteric properties was overexpressed in seeds, a recent study targeted source strength by overexpressing AGPase in lettuce leaves. Lee et al. (2009) reported increased leaf starch content and fresh weight after 8 weeks of growth but did not report plant or seed weight at maturity. Several recent studies have investigated how leaf starch mutations impact plant

productivity. Transposon derived knockout mutations of leaf AGPases have been identified in rice (Rösti et al., 2007) and maize (Slewinski et al., 2008). In the rice leaf AGPase mutant, *apl1*, a mutation in the gene encoding the large subunit of leaf AGPase (*OsAPL1*) results in the near absence of starch in leaf blades (Rösti et al., 2007) while the maize leaf AGPase mutant, *agps-m1*, lacks transitory leaf starch (Slewinski et al., 2008). In both rice and maize, the loss of leaf starch did not result in significant decreases in productivity under the growth conditions tested. However, phenotypic yield characters are heavily influenced by the environment. It is possible that under different environmental conditions, differences in phenotype may be observed.

The objective of this study was to determine the importance of leaf starch to maize plant growth and productivity. This was accomplished by measuring the rate of leaf CO_2 carbon fixation and overall plant productivity under field conditions. The results indicate that under the conditions tested leaf starch is required for normal plant productivity.

Materials and Methods

Plant Material and Growth Conditions

The *agps-m1* allele, containing a *Mu* transposon insert in the leaf AGPase small subunit, was introgressed into inbred line B73 (Slewinski et al., 2008). Progeny from a BC_4F_1 plant heterozygous for *agps-m1* were grown to maturity and $BC_4F_{2:3}$ seed pools were genotyped for the *agps-m1* mutation as previously described (Slewinski et al., 2008). Five *agps-m1* and seven wild type independent $BC_4F_{2:3}$ seed pools homozygous

for the wild type or mutant *agps-m1* alleles were identified, combined, and used in all subsequent analyses.

All plants were grown at the University of Florida Plant Science Research and Education Unit, Citra, Florida during the Fall 2009 and the Spring 2010 growing seasons. At planting, $BC_4F_{2:3}$ plants homozygous for the mutant leaf starch allele, *agps-m1*, were alternated within individual rows with plants homozygous for the wild type allele, *Agpslzm*. Rows consisted of 15 positions 30 cm apart with two maize seeds planted at each position and thinned to one plant per position at the four leaf stage. Spacing between rows was 91 cm. The soil type at this location is Arredondo fine sand. Prior to planting, 4480 kg ha^{-1} chicken manure was applied and incorporated. At planting, 121.6 l ha^{-1} of 11-37-0 liquid starter fertilizer was applied to achieve 17.9 kg N and 61.6 kg P ha^{-1}. The plants were side-dressed three times, applying 84 kg of N ha^{-1} as NH_4NO_3 and 84 kg K ha^{-1} as KCl each time. The first, second, and third applications occurred when plants were 15 to 20, 30 to 36, and 46 to 51 cm tall, respectively. A total of 269 kg N, 62 kg P, and 252 kg K ha^{-1} were applied. For the Fall 2009 growing season four rows were planted and seeding occurred August 10. Plants were harvested in mid-December. The mean, high, and low temperatures for the Fall 2009 growing season months were as follows: August, 26°C mean, 35°C high, and 19°C low; September, 26°C mean, 33°C high, and 13°C low; October, 23°C mean, 33°C high, and 6°C low; November, 17°C mean 28°C high, and 3°C low; December, 15°C mean, 28°C high and the 0°C low. The Spring 2010 trial consisted of 30 rows and was planted April 8 and harvested July 21. The mean daily temperatures were: April, mean 21°C, 31°C high and 8°C low; May,

mean 26°C, 35°C high and 15°C low; June, mean 28°C, 37°C high, 19°C low; July, 28°C mean, 38°C high, 19°C low. Plots were irrigated and pest control measures were applied as needed.

Analysis of Development and Yield Characteristics

Plant height was measured from the soil surface to the top node at the base of the tassel. Days from planting to flowering was measured as days from planting to date of self-pollination. Only the first (top) ear on each plant was pollinated. All plants were self-pollinated on the first day that both pollen and first ear silks were abundant. Measurements on the leaf directly below the ear (ear leaf) were taken one week after pollination with width measured at the widest point and length as the distance from the collar to the leaf tip. Ear leaf chlorophyll measurements were collected using a SPAD-502 chlorophyll meter (Minolta Co., LTD, Japan) and are the average of three readings per leaf. Leaf parameters and chlorophyll quantification were measured only on the Spring 2010 trial. At maturity, ears were collected and placed in a 37°C forced air oven for one week. Ear length was measured from the base of the bottom row of kernels to the top of the uppermost layer of kernels. All filled kernels were shelled and counted with kernel weight measured as the average of 100 randomly selected kernels per ear. All weight and composition parameters are reported on a 10% moisture basis.

Photosynthetic Measurements

Photosynthetic measurements were taken two to five days after pollination for the Spring 2010 field trial. Measurements were taken over two days at two time points,

morning and afternoon. Morning measurements were recorded between 8 am and 12 pm and afternoon measurements between 1 and 4 pm. For wild-type and *agps-m1* genotypes 26 and 19 plants were measured for morning and afternoon, respectively. The data presented are the mean of all photosynthetic data. Photosynthetic CO_2 fixation was measured on the widest section of ear leaves using a CI-340 Photosynthesis System (CID, Camas, WA). Instrument settings were leaf area 6.25 cm^2, flow rate 0.3 min^{-1}, added interval of 15 sec, open system. Measurements were collected using ambient CO_2, and the instrument was zeroed using a value of 385 ppm CO_2. Lighting conditions were held constant at near ambient levels of 950 $\mu E\ m^{-2}s^{-1}$ PAR for morning, and 1050 $\mu E\ m^{-2}s^{-1}$ PAR for afternoon measurements using a CI-301LA (CID, Camas, WA). Temperature was set at 37°C using the CI-510CS temperature control module (CID, Camas, WA). The mean daily temperature during photosynthetic data collection was 28°C with a high of 37°C and low of 24°C. Mean relative humidity was 82 percent.

Biochemical Analysis

Ear leaf tissue (2 x 1 cm sections) was pooled from three plants into wild type and mutant bulks. Bulks were collected at four time points with four biological replicates. Collections occurred 30 min pre-sunrise (dark morning, DM), 30 min post-sunrise (morning, M), mid-photoperiod (mid-day, MD), and 30 min pre-sunset (end of day, ED). Ear leaf samples were collected and immediately frozen in liquid N_2. Samples were ground and used for protein extraction. Proteins were extracted from ground samples using 7.5 µl SDS Reducing Buffer (Laemmli, 1970) mg^{-1} FW leaf tissue with a final concentration of 0.066 M Tris-HCl pH 6.8, 10% (w/v) glycerol, 2.1% (w/v) SDS, 0.011%

(w/v) bromophenol blue, and 5% (v/v) β-mercaptoethanol. Samples were vortexed until suspended, boiled for 5 min, vortexed until suspended, centrifuged 1 min at 13,000 g, electrophoresed on 4-20% SDS acrylamide gels (Bio-Rad, Hercules, CA) and stained with coomassie according to standard methods. Protein bands were quantified using ImageJ (Rasband, 1997-2009). Bands corresponding to phosphoenolpyruvate carboxylase (PEPCase) and pyruvate orthophosphate dikinase (PPDK) at approximately 95 and 110 kDa respectively (Feller et al., 2008) as well as the large subunit of RuBisCO (RBCL), located at approximately 53 kDa (Feller et al., 2008), were quantified.

NIR

Near-infrared reflectance (NIR) (InfraTec GmbH, Dresden, Germany) was run on whole seeds to determine seed moisture, protein, oil, and starch content using a Foss Infratec 1241 Grain Analyzer (FOSS NIRSystems, Inc., Laurel, MD). Ten subsample readings were averaged per sample with the base equations and 10 calibration reference samples provided by the Iowa State University Grain Quality Laboratory.

Statistical Analysis

Data for each response variable were analyzed via analysis of variance including season, genotype, and the season by genotype interaction in the model using PROC GLM in SAS (SAS Institute, 2008). Genotype class means averaged over seasons were compared using the F ratio from the analysis of variance, and genotype class means for each season were compared using ESTIMATE in SAS with a *t* statistic.

Results

Plant Yield and Seed Parameters

Field trials were conducted during the Fall 2009 and Spring 2010 growing seasons at the University of Florida Plant Science Research & Education Unit, Citra, Florida to determine the effects of native leaf transitory starch levels on corn growth and yield. The corn leaf starch mutant *agps-m1* was compared to its B73 wild type $BC_4F_{2:3}$ sister-line for days to flowering, plant height, chlorophyll content, and ear and seed weight parameters (Table 1). The *agps-m1* plants averaged 30 percent lower seed weight per plant compared to their wild type sister plants, with significant reductions in individual seed weight across both environments as well as significantly lower seed number during the Spring 2010 growing season. No year by genotype effect was observed for yield characteristics, except for cob length. The *agps-m1* plants also flowered significantly later, were significantly shorter, and had 15 percent less chlorophyll at flowering than control plants. Seed from *agps-m1* plants had significantly higher seed protein and oil during the Spring 2010 growing season (Table 2). Additionally, *agps-m1* plants had significantly lower seed starch during the Spring 2010 season as well as across both environments (Table 2).

Table 1. Plant growth characteristics for wild type and *agps-m1* plants over two growing seasons.

		Wild type	***agps-m1***	***agps-m1*/ Wild type**[§]
Plant height (cm)	Fall 2009[†]	133.6 ± 3.4	120.7 ± 5.1	0.90
	Spring 2010[‡]	158.6 ± 1.7	152.9 ± 1.0**	0.96
	Mean	156.3 ± 1.6	150.9 ± 1.1**	0.97
Days to pollination (from planting)	Fall 2009	54.2 ± 0.9	57.7 ± 1.1**	1.06
	Spring 2010	58.6 ± 0.3	60.1 ± 0.1***	1.03
	Mean	58.1 ± 0.3	59.9 ± 0.1***	1.03
Cob length (cm)	Fall 2009	11.9 ± 0.5	9.8 ± 0.6*	0.82
	Spring 2010	13.5 ± 0.4	13.5 ± 0.3	1.00
	Mean[¶]	12.9 ± 0.4	12.6 ± 0.4*	0.97
Ear weight (g)	Fall 2009	75.0 ± 9.0	51.9 ± 6.0	0.69
	Spring 2010	93.1 ± 5.1	72.8 ± 5.6**	0.78
	Mean	87.7 ± 4.6	68.0 ± 4.7**	0.78
Cob weight (g)	Fall 2009	15.0 ± 1.1	5.6 ± 1.5*	0.64
	Spring 2010	24.4 ± 1.1	21.7 ± 1.1	0.89
	Mean	21.6 ± 1.1	18.9 ± 1.2**	0.88
Seed weight (g)	Fall 2009	60.1 ± 8.4	42.4 ± 5.4	0.71
	Spring 2010	70.8 ± 3.9	51.1 ± 4.8**	0.72
	Mean	67.5 ± 3.7	49.1 ± 3.9**	0.73
Seed number	Fall 2009	218.9 ± 34.0	207.6 ± 36.1	0.95
	Spring 2010	319.3 ± 17.0	242.8 ± 21.3**	0.76
	Mean	289.2 ± 16.8	234.4 ± 18.3	0.81
Individual seed weight (g)	Fall 2009	0.286 ± 0.009	0.235 ± 0.024***	0.82
	Spring 2010	0.224 ± 0.004	0.202 ± 0.005**	0.90
	Mean	0.242 ± 0.006	0.210 ± 0.007***	0.87
Chlorophyll	Spring 2010	59.0 ± 0.8	50.4 ± 1.6***	0.85

Table 1 continued

Leaf width (cm)	Spring 2010	9.2 ± 0.1	8.6 ± 0.2***	0.94
Leaf length (cm)	Spring 2010	78.1 ± 0.7	81.4 ± 1.0**	1.04

†For all measurements during the Fall 2009 growing season, wild type n = 15 and *agps-m1* n=10. Values are means ± standard error.
‡For Spring 2010 plant height n = 151 – 152, chlorophyll quantification n = 12, leaf length and width n = 21 – 22, days from planting to flowering n = 123 – 130 plants, and ear weight, cob weight, cob length, seed weight, seed number, and individual kernel weight n = 31 – 35 plants. Values are means ± standard error.
§Ratios were derived by dividing *agps-m1* homozygote means by wild type means.
¶Trait mean over growing seasons displays significant season x genotype interaction.
*, **, *** denote *agps-m1* mean value significantly different from wild type at α ≤ 0.05, 0.01, or 0.001 respectively using a two-tailed *t*-test.

Table 2. Wild type and *agps-m1* seed composition over two growing seasons.

Seed Trait	**Growing Season**	**Wild type**	***agps-m1***	***agps-m1*/ Wild type**§
Moisture (%)	Fall 2009†	11.66 ± 0.41	11.93 ± 0.71	1.02
	Spring 2010‡	14.55 ± 0.23	14.58 ± 0.33	1.00
	Mean	13.81 ± 0.28	13.92 ± 0.35	1.01
Protein (%)	Fall 2009	11.72 ± 0.31	11.77 ± 0.46	1.00
	Spring 2010	11.88 ± 0.11	12.49 ± 0.16**	1.05
	Mean	11.83 ± 0.11	12.31 ± 0.17	1.04
Oil (%)	Fall 2009	4.87 ± 0.11	4.87 ± 0.19	1.00
	Spring 2010	4.84 ± 0.06	5.12 ± 0.06**	1.06
	Mean	4.85 ± 0.05	5.06 ± 0.07	1.04
Starch (%)	Fall 2009	70.63 ± 0.23	70.17 ± 0.50	0.99
	Spring 2010	70.67 ± 0.12	69.84 ± 0.14**	0.99
	Mean	70.66 ± 0.13	69.92 ± 0.16**	0.99

†For Fall 2009, wild type n = 9, and *agps-m1* n=11
‡Spring 2010 wild type n = 32, and *agps-m1* n=27.
§Ratios were derived by dividing *agps-m1* homozygote means by wild type means
*, **, *** Value is significantly different from wild type at α ≤ 0.05, 0.01, or 0.001 respectively using a two-tailed *t*-test.

Photosynthesis

Photosynthetic CO_2 fixation rates for wild type and *agps-m1* were determined by measuring photosynthetic rates on the widest section of the ear leaf. Data presented in Table 3 was collected over the course of two days shortly after pollination during the Spring 2010 field season. Wild type and *agps-m1* photosynthetic rates were not different for the morning or afternoon time intervals (data not shown) or when averaged across the whole light period (Table 3). Likewise, transpiration and stomatal conductance did not differ between the wild type and *agps-m1* classes.

Table 3. Photosynthetic and gas exchange measurements for wild type and *agps-m1* plants.

Genotype	**Photosynthesis** $\mu mol\ m^{-2}\ s^{-1}$	**Transpiration** $mmol\ m^{-2}\ s^{-1}$	**Stomatal Conductance** $mmol\ m^{-2}\ s^{-1}$	***n***
Wild type	29.4 ± 1.2	4.6 ± 0.2	204.2 ± 10.7	43
agps-m1	28.4 ±1.4	4.6 ± 0.1	189.8 ± 8.9	44

Data represent means of measurements collected throughout the course of two days ± standard error. Photosynthetic CO_2 fixation was measured on the ear leaf 1-2 days after pollination during the Spring 2010 growing season.

Major Photosynthetic Protein Levels in Leaves

Leaf tissue was collected at four time points, dark morning (DM), morning (M), mid-day (MD), and end of day (ED). Leaf proteins were extracted and separated by SDS-PAGE. Band areas corresponding to PEPCase/PPDK as well as to RBCL (Figure 1) were quantified (Table 4). At the beginning of the day *agps-m1* leaves had only 69 percent of PEPCase and PPDK compared to wild type leaves, and only 61 percent of wild type RuBisCO large subunit levels. This trend continued and remained significant until the end of the light period. At that point (ED) *agps-m1* leaves contained more PEPCase

and PPDK as well as RuBisCO, but were not significantly different from wild type leaves.

Table 4. Levels of photosynthetic related proteins in wild type and *agps-m1* leaves[†].

	Dark morning[¶]	**Morning**[#]	**Mid-day**[††]	**End of day**[‡‡]
PEPCase + PPDK[‡]				
Wild type	1.00 ± 0.05	1.05 ± 0.02	1.12 ± 0.06	1.10 ± 0.08
agps-m1	0.69 ± 0.06	0.91± 0.06	0.94 ± 0.09	1.17 ± 0.05
agps-m1 / Wild type	0.69	0.87	0.84	1.06
P-value[¨]	0.00	0.03	0.07	0.24
RBCL[§]				
Wild type	1.00 ± 0.04	1.12 ± 0.04	1.02 ± 0.03	1.07 ± 0.06
agps-m1	0.61 ± 0.11	0.93 ± 0.07	0.75 ± 0.06	1.11 ± 0.08
agps-m1 / Wild type	0.61	0.83	0.74	1.04
P-value[¨]	0.01	0.07	0.01	0.67

[†]Tissue from ear leaf was collected at four time points and pooled from three plants into wild type and mutant bulks. Five biological replicates were performed.
[‡]Bands corresponding to PEPCase/PPDK and RBCL were quantified as a function of area using ImageJ v. 1.4.3.67. Values are means ± standard error.
[§]Bands corresponding to RBCL subunit were quantified using ImageJ v. 1.4.3.67. Values are means ± standard error.
[¶]*DM* dark morning collection occurred 30 min pre-sunrise.
[#]*M* morning collection occurred 30 min post-sunrise.
[††]*MD* mid-photoperiod collection time point.
[‡‡]*ED* end of day collection occurred 30 min pre-sunset.
[¨]Two-tailed *P*-values are from *t*-test assuming equal variance.

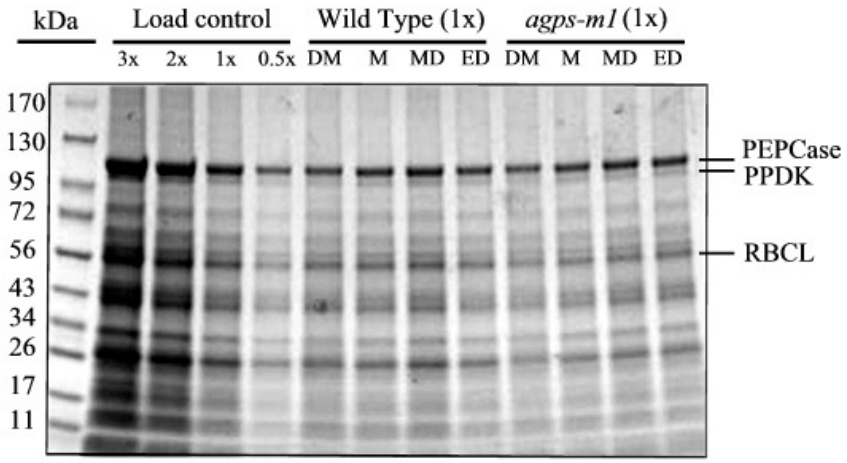

Figure 1. SDS-PAGE separation of leaf photosynthetic proteins at time points throughout the photoperiod. Tissue from three separate ear leaves was collected at 30 min pre-sunrise (DM); 30 min post-sunrise (M); mid-photoperiod (MD); and 30 min pre-sunset (ED) and pooled into wild type and *agps-m1* bulks with five biological replicates. Total extractable leaf proteins were separated using SDS-PAGE and phosphoenolpyruvate carboxylase (PEPCase), pyruvate orthophosphate dikinase (PPDK), and the RuBisCO large subunit (RBCL), located at approximately 110, 95, and 53 kDa respectively, were quantified. The load control represents the combination of equal parts from wild type mid-day collections.

Discussion

Through the processes of source and sink interaction, carbohydrates produced in source tissues are translocated to sink tissues for growth, development, or storage. One important carbohydrate that plays a role in both of these processes is starch. Starch is a major source of sink strength in the endosperm of developing seeds as well as an important transient storage product in source leaves (reviewed in Van Camp, 2005).

Previous studies focused on improving sink strength in corn and rice have demonstrated that seed-specific overexpression of starch biosynthesis increases seed weight as well as overall plant biomass (Giroux et al., 1996; Meyer et al., 2004; Smidansky et al., 2003, 2007). In these cases, it appears that the enhanced demand for

resources in sink tissue was met by production of additional resources in source tissue (Smidansky et al., 2007). The objective of the present work was to determine the importance of native leaf starch levels in maize by comparing growth and yield of the leaf starch mutant *agps-m1* to plants having wild type leaf starch levels under field conditions.

Under the conditions tested, *agps-m1* plants had reduced growth and seed yield. The leaf starch mutant group plants flowered significantly later, were significantly shorter, lower in chlorophyll, and 30% lower in seed yield than their wild type leaf starch sister plants (Table 1). Excepting cob length, there was no genotype by environment interaction for any yield or seed characteristic traits. Seed starch was also significantly reduced in *agps-m1* across environments and seed protein and oil were significantly increased during the Spring 2010 season. No data was obtained on plants heterozygous for the *agps-m1* mutation, but these findings demonstrate that total lack of leaf starch under field conditions limits plant growth.

Photosynthetic CO_2 fixation per unit area was relatively constant throughout the photoperiod between and within wild type and *agps-m1* genotypes (Table 3). It was surprising that there was no difference in photosynthetic rates, because *agps-m1* had significantly less chlorophyll than the wild type (Table 1). Despite not being measurably lower in photosynthetic rates, the *agps-m1* plants displayed reduced growth and were 6 to 13 cm shorter with longer narrower leaves than the wild type group depending on the growing season (Table 1). Although total plant biomass was not collected, it seems probable that *agps-m1* plants were decreased in total biomass due to reduced plant

growth. If this is so, net photosynthesis per plant is probably reduced as a result of the reduced net photosynthetic plant area in *agps-m1* plants.

Photosynthesis is a complex process that is dependent upon internal and external factors. One example of internal factors contributing to the rate of photosynthesis is repression of photosynthetic protein gene expression by increased leaf soluble sugars. Expression of many photosynthetic protein genes are known to be regulated through a carbon metabolite-mediated regulatory mechanism (reviewed in Pego et al., 2000). Depletion of sugars leads to activation of photosynthetic gene expression while increased sugar concentrations repress photosynthetic gene expression (Stitt, 1991; Krapp et al., 1993). Slewinski et al. (2008) demonstrated that sucrose and fructose levels are increased 84% and 75% respectively at the end of the day in *agps-m1* leaves compared to wild type leaves. They also observed a 12.5% decrease in glucose in *agps-m1* leaves. The fate of photosynthate in *agps-m1* plants remains unknown, however it is possible that the soluble sugar increases account for the lower level of leaf proteins during the day (Table 4). In maize, genes encoding PEPCase, PPDK, and RBCL are sugar repressible (Sheen, 1990) and are responsive to leaf nitrogen status (Brown, 1978; Suzuki et al. 1994). Additionally, the starchless maize leaves may not be efficiently exporting carbohydrates to sink tissues, explaining the lower yield.

It has been noted that photosynthetic rates are regulated by both carbon and nitrogen status and that plant growth reflects both source and sink interactions (Paul and Foyer, 2001). Another factor impacting the rate of carbon fixation is the level of photosynthetic leaf protein and chlorophyll in response to nitrogen. In general, when the

nitrogen supply is diminished there is a decrease in leaf protein and chlorophyll (Evans 1996). Expression of C4 photosynthetic enzymes positively respond to nitrogen status with increases in PEPcase and PPDK when nitrogen is prevalent (Suzuki et al., 1994). However, there may be a minimum threshold decrease in photosynthetic apparatus proteins before photosynthetic rates decrease.

As important photosynthetic proteins in maize, PEPCase/PPDK and RBCL were quantified in both wild type and *agps-m1* plants (Table 4). In both the wild type and *agps-m1* plants, RBCL and PEPCase/PPDK levels remained fairly constant throughout the photoperiod. The small differences in leaf photosynthetic proteins may account for the observation that CO_2 fixation rates remained constant between morning and afternoon measurements. However, the wild type group retained significantly higher PEPCase/PPDK and RBCL levels until the end of the photoperiod. At the pre-sunrise time point (end of the night) *apgs-m1* leaves had 31 % less PEPCase/PPDK and 39 % less RBCL than wild type leaves. However, over the course of the photoperiod both PEPCase/PPDK and RBCL increased in the leaf starch mutant while remaining essentially unchanged in the wild type starch group such that *agps-m1* and wild type leaf photosynthetic protein levels were about equal at the end of the day (pre-sunset). The observation that the leaf starch mutant group is lower in leaf photosynthetic protein levels at the end of the night may reflect a lower rate of protein synthesis during the dark period, which may be accounted for by repression of photosynthetic protein gene expression by sugars (Pego et al., 2000). Another explanation may be that the increased amount of photosynthetic apparatus proteins in *agps-m1* leaves at the end of the day is broken down

during the dark period. In *Arabidopsis*, chloroplasts can be partially mobilized to the vacuole via RuBisCO containing bodies (RBCs) and degraded through autophagy, to release nitrogen and carbon for new growth (Ishida et al., 2008; Wada et al., 2009). Izumi et al. (2010) found that degradation of chloroplasts is dependent upon carbon status in leaves with *Arabidopsis* leaf starch mutants having increased RBCs and decreased RBCs in excess starch mutants. Autophagy is a mechanism used under nutrient deficient conditions and most plant autophagy research has been conducted using *Arabidopsis*. Orthologous loci to *Arabidopsis* autophagy related (ATG) genes have been identified in rice and maize (Chung et al., 2009). It is possible that ATG in *agps-m1* plants produces carbon or nitrogen products from leaf photosynthetic proteins. This mechanism would be especially useful in the dark period when transitory starch would normally be broken down.

In summary, our results indicate that native leaf starch levels are important to maize plant growth and agronomic yield. Despite having the same photosynthetic rate as the wild type starch plants, *agps-m1* plants lacking leaf starch flowered later, were shorter, and had decreased chlorophyll and seed yield under the field conditions tested. The reduced level of photosynthetic apparatus in *agps-m1* leaves indicate a link between carbon and nitrogen metabolism. These findings give further support to the idea that source strength, along with sink strength, plays an important role in determining plant growth and yield.

Acknowledgements

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CHAPTER 2

ENHANCED PLANT GROWTH IN RICE IS CONFERRED BY INCREASED EXPRESSION OF ADP-GLUCOSE PYROPHOSPHORYLASE IN LEAVES

Introduction

Rice is one of the most important cereal crops in the world, second only to wheat in metric tons produced (http://faostat.fao.org/site/339/default.aspx, accessed September 2, 2011). Much of the global population relies on rice as a source of calories, most of which comes from rice seed's starchy endosperm. The number of rice consumers is advancing at a rate of 1.5% annually, higher than the 1% annual increase in rice production (reviewed in Jeon et al., 2011). Therefore, developing an understanding of genetic factors limiting rice agronomic yield is important to ensuring future global food supplies.

Agronomic yield is a quantitative trait, highly impacted by the environment (Annicchiario et al., 2002). There is no dominant yield gene and productivity is dependent upon biochemical and physiological processes with underlying genetic control (reviewed in Ashraf et al., 1994). Grain yield is dependent upon photosynthesis, and thus upon photoassimilate production in source tissues (source strength) and efficient carbon allocation of assimilate to developing or storage tissues (sink strength) (reviewed in Van Camp, 2005; Smith and Stitt, 2007). Increasing plant productivity may be achieved through increased photosynthetic rates (reviewed in Murchie et al., 2009). Growth potential of different plant organs is largely driven by relative strength of different source

and sink tissues, which determines production and flow of photoassimilates and nutrients inside the plant (reviewed in Van Camp, 2005). There have been many studies aimed at increasing yield by targeting increased sink strength (Paul and Foyer, 2001; Araus et al., 2008), the most common method being to increase activity of carbohydrate metabolic pathway enzymes in sink tissues. For example, downregulating activity of the plastidial isoform of adenylate kinase in potato results in an increased adenylate pool leading to increased tuber weight compared to wild type potatoes (Regierer et al., 2002). Increased expression of ADP-glucose pyrophosphorylase in rice seeds has been associated with increased seed yield (Smidansky et al., 2003), and overexpression of a sucrose-phosphate synthase gene in potato tubers has been associated with increase potato tuber yield under field conditions (Ishimaru et al., 2008).

One important carbohydrate metabolism pathway involved in sink strength in developing seeds is starch biosynthesis. As the enzyme controlling the rate limiting step in starch biosynthesis, ADP-glucose pyrophosphorylase (AGPase) has received considerable attention (Stark et al., 1992; Giroux et al., 1996; Greene and Hannah, 1998). In plants, AGPase is a heterotetramer consisting of two large and two small subunits (reviewed in Hannah, 1997; Preiss, 1997). In the presence of ATP, AGPase acts upon the substrates glucose 1-phosphate and ATP to produce ADP-glucose (Espada, 1962). ADP-glucose pyrophosphorylase is also an allosteric enzyme; the positive regulator is 3-PGA and the negative regulator is inorganic phosphate (Pi) (Hannah, 1997; Preiss, 1997). Additionally, there are seed and leaf specific isoforms of AGPase (Giroux and Hannah, 1994; Denyer et al., 1996; Thorbjornsen et al., 1996; Beckles et al., 2001). Cereal

endosperm AGPases lack transit peptides and thus there are both seed (endosperm cytoplasm localized) and leaf specific (chloroplast localized) forms of both the large and small AGPase subunits (Giroux and Hannah, 1994; Villand and Kleczkowski, 1994).

Several studies have focused on increasing sink strength by overexpressing AGPase in seeds. In maize (Giroux et al., 1996), wheat (Meyer et al., 2004; Smidansky et al., 2007) and rice (Smidansky et al., 2003) increased expression of a deregulated form of the maize large subunit of AGPase under an endosperm-specific promoter led to increases in seed weight per plant and plant biomass. Increases in both seed yield and plant biomass, such that harvest index remained unchanged, indicate up-regulation of metabolism in both sink and source tissues. However, this complex enhanced yield phenotype was not observed under field conditions (Meyer et al., 2007).

A less studied area involving carbon metabolism has been leaf starch as a source strength limiting factor. Recently, a study in rice (Rösti et al., 2007) investigated how a transposon derived leaf-specific AGPase knockout mutation impacted plant productivity. Although the rice mutation resulted in near absence of leaf starch, no differences in plant growth or yield were observed under low light growth conditions (Rösti et al., 2007). A similar mutation was reported in maize in which the leaves lacked leaf starch due a transposon insertion in the small leaf AGPase subunit (Slewinski et al., 2008). We tested the impact of the lack of leaf starch upon corn productivity by examining the growth of *agps-m1* plants under field conditions. The starchless *agps-m1* plants flowered 2 days later, were 5 cm shorter, and had 30% lower seed yield relative to wild type isogenic sister-line plants (Schlosser et al., in press), indicating the importance of leaf starch under

field conditions. In addition to reverse genetic studies where leaf starch has been removed, other studies have examined whether native leaf starch levels limit plant growth by over-expressing AGPase in leaves. Expression of a modified potato AGPase large subunit, *upreg1,* in lettuce increased fresh weight and starch content at 8 weeks after germination in lettuce (Lee et al. 2009). However, the lettuce plants were not taken to maturity and so plant development and seed yield data were not reported. An additional study in rice utilizing leaf specific overexpression of *upreg1* showed a trend of increased seed yield in comparison to the varietal control when grown under high light conditions (Gibson et al., 2011).

Here we tested the role of leaf AGPase in rice productivity by overexpressing AGPase in leaves. Rice was transformed with the maize endosperm AGPase subunits modified to have the leaf AGPase chloroplast transit peptide. The AGPase transgenes were under the control of either a native rice leaf AGPase or a RuBisCO promoter. The results indicate that increased leaf AGPase is associated with increased plant biomass.

Materials and Methods

Plasmid Constructs Used for Transformation

Two plasmid constructs, pABSSh2Bt2 and pRBCSh2Bt2, were used for transformation. Both vectors were identical with the exception of the leaf-specific promoter used. Both make use of the pTF101.1 binary vector (Paz et al., 2004) that contains the *aad*A gene, for bacterial selection of spectinomycin resistance, and the phosphinothricin acetyl transferase (*bar*) gene controlled by the cauliflower mosaic virus

35S promoter (2x CaMV 35S) and the nopaline synthase (NOS) terminator (Paz et al., 2004). The *bar* gene confers resistance to the herbicide glufosinate (Bayer CropScience, Kansas City, MO). The AGPase subunits in both vectors were the large and small subunits of maize endosperm, *Sh2* and *Bt2*, respectively. The wild type *Sh2* coding sequence (Shaw and Hannah, 1992) was modified as previously described (Smidansky et al., 2002). The resulting *Sh2r6hs*, coding sequence contains two alterations. The (*r6*) modification is a two amino acid insertion conferring reduced AGPase phosphate inhibition (Giroux et al., 1996). The *hs* alteration is a single amino acid substitution that confers more stable AGPase subunit interactions *in vitro* (Greene and Hannah 1998). The wild type *Bt2* coding sequence (Bhave et al., 1990) was also included in both constructs. The tobacco RB7 matrix attachment region sequence (MARS) (reviewed in Allen et al., 2000) was included between the NOS terminator of the *Sh2r6hs* coding sequence and the promoter of the *Bt2* gene. Additionally, both the *Sh2r6hs* and *Bt2* coding sequences were modified to include rice leaf AGPase chloroplast specific signal peptides (Figure 2). The signal peptide was 153 and 141 bp in pAPSSh2Bt2 and pRBCSh2Bt2, respectively. In construct pAPSSh2Bt2, *Sh2r6hs* and *Bt2* were under control of the native rice leaf AGPase small subunit promoter (*Aps1*), while construct pRBCSh2Bt2 contained the native rice RuBisCO small subunit promoter (*RBC*) (Figure 2).

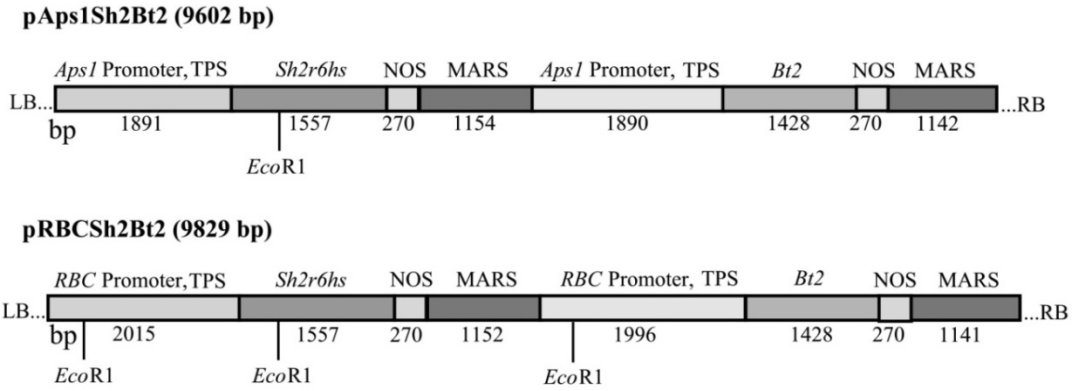

Figure 2. Structure of the pAPSSh2Bt2 and pRBCSh2r6hs AGPase transgenes. The *Sh2r6hs* and *Bt2* coding sequence were combined into the pTF101.1 binary vector under control of either the rice leaf AGPase (*Aps1*) or rice RuBisCO small subunit (*RBC)* promoter and the NOS terminator. The tobacco RB7 matrix attachment region sequence (MARS), transit peptide sequences (TPS), and position of *Eco*R1 restriction sites is as noted.

Production of Transgenic Plants and
Preliminary Analysis of Transgenic Lines

Constructs pAPSSh2Bt2 and pRBCSh2Bt2 were introduced into rice *japonica* cultivar Nipponbare calli at the Iowa State University Plant Transformation Facility according to Toki et al. (1997) via an *Agrobacterium*-mediated transformation system. Depending on T_0 yield, 24 or 36 T_1 seeds from 17 pRBCSh2Bt2 (NR) events and 17 pAPSSh2Bt2 (NA) events were planted and grown in a greenhouse. The six most vigorous events from each promoter were selected based upon plant height of the first sequential plants at one month after planting and moved to a growth chamber. Growth chamber conditions were set at 28 °C day/22 °C night with a 12 h photoperiod. Plants were allowed to self-pollinate and mature and were harvested 60 days after flowering. For progeny testing, 16 T_2 seeds from each T_1 plant were planted in Sunshine soilless mix #1 (Sun Gro Horticulture, Vancouver, British Columbia, Canada) and grown to the 2-3

leaf stage. The seedlings were then sprayed with a solution of 0.1% glufosinate until runoff and scored for herbicide resistance or susceptibility after 7 days. T_1 plants giving greater than 10 consecutive positive or negative plants were classified as homozygous for the presence or absence of the transgene, respectively. T_1 derived homozygous transgene positive and negative plants with overexpression of the transgenes were used for further study. Genomic DNA was isolated and PCR was conducted on T_1 plants to identify transgenic lines containing the *Sh2r6hs* sequence. PCR reactions were conducted using an upstream PCR primer, 5'- ACCATCAACGATGGGTCTGT-3', which hybridizes to the *Sh2r6hs* cDNA beginning 24 base pairs upstream of the stop codon, and downstream primer, 5'-TTGCGCGCTATATTTTGTTTT-3', complementary to base pairs 204-264 of the NOS terminator. This primer pair produces a 210 base pair amplified product. PCR parameters are as follows: 94°C for 5 min, 35 cycles of 94°C 30 sec, 51°C 30 sec, 72°C 45 sec, followed by 72°C for 7 min. Co-segregation of the AGPase transgenes and glufosinate resistance was confirmed for all events.

Integration of the *Sh2r6hs* Transgene

For Southern blot analysis, genomic DNA from homozygous $T_{1:3}$ plants was isolated, digested with *Eco*R1 (Promega, Madison, WI), fractionated on a 0.6% agarose gel, and transferred to a Hybond-XL nylon membrane (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions. The membrane was probed with the ^{32}P random primer labeled (Takara Bio Inc., Otsu, Shiga, Japan) *Sh2r6hs* coding sequence and hybridized overnight at 65°C in a solution containing 0.5 M sodium phosphate, pH 7.2. Hybridized membranes were washed five times in 2X sodium

chloride/ sodium phosphate/ EDTA (SSPE), from a 20X SSPE stock with concentration of 3.0 M NaCl, 0.2 M NaH_2PO_4 and 0.02 M EDTA, pH 7.3. Membranes were washed an additional two times in 0.2X SSPE and dried at 37°C for 40 min. Hybridized membranes were exposed to Kodak (Rochester, NY) Biomax MS film with an intensifying screen at -80°C.

Transgene Expression Analysis

T_1 seed was planted and tissue was collected at the 1-2 leaf stage from 3-5 plants within each genotype and combined according to genotype. Tissue was directly frozen in liquid N_2, ground, and total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions, with the following alterations: 500 µl extraction buffer was added directly to ground powder, followed by 250 µl of a 1:1 v/v phenol-chloroform mixture. Samples were inverted to mix and then centrifuged at 13,000 g for 15 min and RNA was extracted from upper aqueous layer following manufacturer's instructions. Total RNA was quantified on a Bioanalyzer (Agilent Technologies, Santa Clara, CA) and electrophoresed on formaldehyde containing agarose gels with equal portions of each sample electrophoresed on an ethidium bromide stained agarose gel without formaldehyde to ensure equal formaldehyde gel loading. RNA was transferred to a Hybond-XL membrane and probed with ^{32}P-random primer labeled *Sh2* and *Bt2* coding sequences as described above.

Whole transcriptome shotgun sequencing (RNA-seq) technology was employed to determine transgene and genome wide expression levels for NR16 and NR31. Leaf tissue from randomly selected AGPase transgene homozygous positive and negative T_3 plants

was collected at the 3 to 4 leaf stage for RNA extraction. The terminal 3 cm of the uppermost erect leaf at 30 days post emergence was collected and immediately frozen. Three biological replicates were collected per AGPase genotype, each consisting of two plant bulks of harvested leaf tissue from 2 randomly selected plants within each genotype. Tissue was ground in liquid N_2 and total RNA extracted as described above. For RNA-seq analysis, 1 μg of total RNA was used for the creation of cDNA libraries using TruSeq RNA-SEQ library kits (Illumina Inc., San Diego, CA) with six bp molecular identification tags added for multiplexing. Amplicons from cDNA libraries were sequenced as single 50 bp reads using an Illumina High Scan-SQ platform. RNA-seq data was analyzed using QSeq and ArrayStar v4.1 (DNASTAR, Madison, WI). Sequence data was imported and genes of interest selected for analysis with the match settings set to 100% for at least 40 bp and all other settings left at default with reads per kilobase of exon model per million mapped reads (RPKM) normalization (Mortazavi et al., 2008). Resultant expression data was converted to raw counts and normalized to eukaryotic elongation factor 1-alpha (*eEl-1α*). Student *t*-tests were performed to determine if significant changes in expression existed between positive and negative AGPase transgene genotypes.

AGPase Activity Assays

Assays were performed on leaf tissue harvested at the four leaf stage at mid-photoperiod. The leaf tip (3 x 1 cm) of the most erect leaf was collected from three randomly selected T_3 plants within each genotype and combined into homozygous transgene positive or negative bulks with three biological replicates and directly frozen.

Leaf tissue was then ground to a powder without allowing samples to thaw. Five µl mg^{-1} of leaf tissue fresh weight of extraction buffer containing 80 mM HEPES, 1 mM EDTA, 0.1 mM DTT, 2 mM $MgCl_2$, and 10 µl ml^{-1} Halt protease inhibitor (ThermoScientific, Rockford, IL) was added. Samples were vortexed and centrifuged at 13,000 g for 2 min and 5 µl of supernatant was transferred to new tubes with 20 µl extraction buffer containing 2 mM ATP, 2 mM glucose-1-phosphate, 1 nCi ^{14}C-labeled glucose-1-phosphate (PerkinElmer, Boston, MA, USA) and 0, 0.5, 2, or 4 mM 3-PGA. Samples were incubated at 37°C for 10 min, boiled for 5 min and then treated with 3 U alkaline phosphatase (Promega, Madison, WI, USA). Reactions were transferred to DE81 disks (Whatman, Buckinghamshire, UK), which were then washed 4 times in H_2O and dried. Total activity (measured in counts per minute) was then measured with a Tri-Carb 1905AB/LA liquid scintillation counter (Packard BioScience, Meriden, CT). Enzyme activity (min^{-1} mg^{-1} FW) was calculated as in Meyer et al., 2004 substituting mg^{-1} FW for mg^{-1} total protein (Table 9).

Leaf Protein Analysis

Leaf tissue was collected at three time points throughout the photoperiod, 30 min pre-lights on (morning), mid-photoperiod (mid-day), and 30 min pre-lights off (end of day) at the four leaf stage. The uppermost, erect leaves were selected and 4 x 1 cm sections were pooled from three plants into positive and negative transgene bulks with three biological replicates. Tissue was directly frozen in liquid N_2 and total soluble proteins extracted and the large subunit of RuBisCo (RBCL) was analyzed by SDS-

PAGE as in Schlosser et al. (in press). Relative protein abundance was quantified by comparing to a standard curve of UT Nipponbare RBCL levels.

Photosynthetic Carbon Fixation Measurements

Measurement of photosynthetic rates were conducted as in Schlosser et al., (in press). The CI-340 machine was zeroed using ambient CO_2 with the value set at 389 ppm. Measurements were conducted over the course of the photoperiod on the uppermost flag leaf of $T_{1:3}$ plants at anthesis. Data presented are the means of all data collected from $T_{1:3}$ plants grown in a paired and randomized growth chamber yield trial.

Analysis of Yield Phenotype Parameters

The three most vigorous T_1 events from each construct were advanced for a $T_{1:2}$ yield trial. Seed was direct seeded in 7.5 x 10 x 13 cm pots filled with Sunshine soilless mix #1 (Sun Gro Horticulture, Vancouver, British Columbia, Canada) containing 4.93 cc each of 30:10:10 Osmocote (The Scotts Company LLC, Marysville, OH) and Ironite Mineral Supplement 1-0-1 (Ironite, Walnut Creek, CA). Growth conditions consisted of a 28°C day/22°C night temperature regime and a 12 hr photoperiod. Artificial lighting provided 600 $\mu E\ m^{-2}\ s^{-1}$ PAR at canopy height. Homozygous positive and negative plants were paired within each genotype and randomized. Plants were bottom irrigated daily in tubs, delivering 132 ppm 30-10-10 plant food daily (Miracle-Gro Azalea Camellia Rhododendron, Scotts Miracle-Gro Products, Marysville, Ohio, USA). Plants were allowed to self-pollinate and were watered daily until 57 days after flowering. Mature plants were harvested, and biomass was weighed after one week in a drying

chamber. Biomass, panicles, seeds per panicle, filled seeds and unfilled seeds per panicle, total number of seeds, seed weight, and individual seed weight were recorded on a per plant basis. Leaf chlorophyll measurements were collected using a SPAD-502 chlorophyll meter (Minolta Co., LTD, Japan).

Results

Preliminary Analysis of Transgenic Lines

The rice variety Nipponbare was transformed with Sh2r6hs and *Bt2* under the control of either the *Aps1* or *RBC* promoter (Figure 2). T_1 seed from 34 spectinomycin resistant T_0 transgenic rice lines were obtained through *Agrobacterium* mediated transformation, 17 having the AGPase transgenes under control of the *Aps1* promoter, and 17 under the *RBC* promoter. T_1 seed from each event was planted and grown in a greenhouse. Plant height was recorded one month after planting, and the six tallest events for each of the two promoters were selected for further study.

Northern blot analysis of RNA from T_1 seedling leaf tissue revealed 11 of the 17 *RBC* events exhibited a range of *Shr6hs* and *Bt2* transgene expression with both transgenes expressed at similar levels (Figure 3). Expression of *Sh2r6hs* and *Bt2* under the *Aps1* promoter was not detected with northern blotting. Staining of agarose gel fractionated rRNA with ethidium bromide indicated similar loading between lanes and lack of RNA degradation. Lines NR40, NR26, NR7, and NR1 had the highest levels of expression, whereas lines NR6, NR16, and NR31 had the lowest levels of expression of positively expressing events. These three events were advanced for further study.

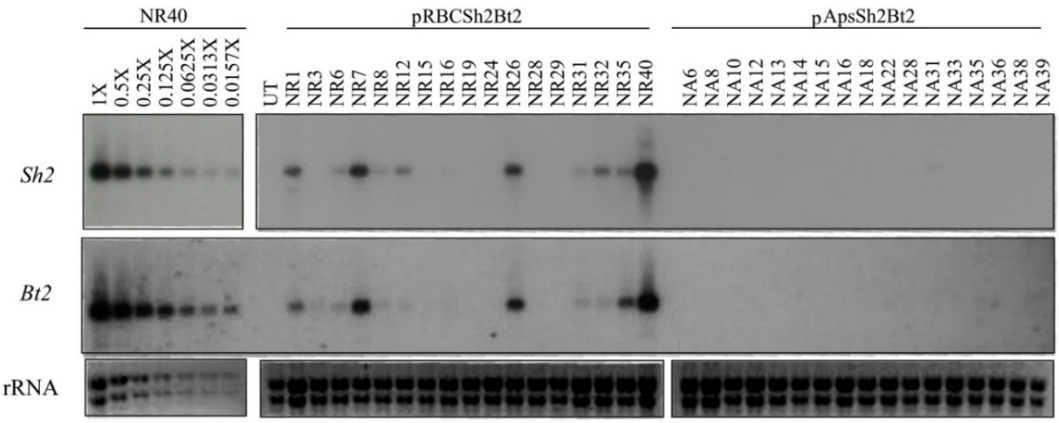

Figure 3. Northern blot analysis of 17 pRBCSh2Bt2 and 17 pAPSSh2Bt2 transgenic rice lines and untransformed Nipponbare (UT). Leaf tissue was bulked from 3-5 T_1 plants segregating 1:2:1 for the transgenes. Aliquots of 5 µg total RNA were fractionated on a formaldehyde agarose gel, blotted to a nylon membrane and probed with ^{32}P-labelled *Bt2* and *Sh2* cDNA. Ethidium bromide staining of rRNA fractionated on agarose gels indicate similar load concentration between lanes and lack of RNA degradation. A loading curve for NR40 was included to quantify expression of the transgenes in lines expressing *Sh2r6hs* and *Bt2*. Expression of NR40 at 1X represents 5 µg total RNA.

Transgene Integration and Segregation

Southern blot analysis was conducted on AGPase transgene homozygous T_3 seedling leaf tissue of each of the three selected AGPase expression positive events. Figure 4 shows a representative sample of each event and an untransformed Nipponbare varietal control (UT) in which genomic DNA was *Eco*R1 digested and probed with *Sh2*. The variable signal strength between transgenic lines indicate transgene copy number was highest in NR16 and lowest in NR31. PCR specific to the *Sh2r6hs* and *Bt2* transgenes along with herbicide screening of T_1 plants indicate that the *bar* transgene and the *Sh2r6hs* transgene co-integrated, as all plants PCR positive for *Shr6hs* also showed resistance to 0.1% glufosinate with all Sh2 PCR negative plants also glufosinate susceptible (Table 5). Chi-square analysis is consistent with integration of the transgenes at a single locus in each of the three events (Table 5).

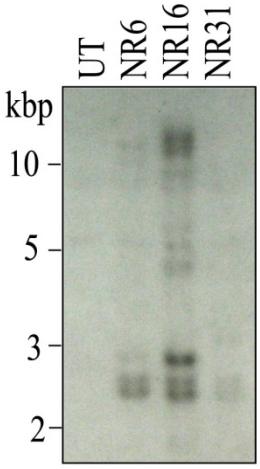

Figure 4. Southern blot analysis of three transgenic rice lines and untransformed Nipponbare (UT). Twenty µg aliquots of *Eco*R1 digested total rice leaf DNA was fractionated on a 0.6 % agarose gel, blotted to nylon, and probed with ^{32}P-labelled *Sh2*.

Table 5. Transgene analysis of three T_1 rice lines overexpressing *Sh2r6hs* and *Bt2*.

Rice Line	Southern[a]	Progeny Test[b]	Chi-Square *P*-value
NR6	+	44/7	0.06
NR16	+	9/3	1.00
NR31	+	17/3	0.30

[a]+ Presence of *Sh2r6hs* coding region according to Southern blotting.
[b]Number of T_1 progeny positive/negative for *Sh2r6hs*. Progeny tests consisted of herbicide resistance screening of each plant with 0.1% glufosinate along with PCR for *Sh2r6hs*. In each event the herbicide resistance marker and AGP transgene co-segregated.

Expression Analysis

Using next generation sequencing technology, over 150 million reads were obtained for twelve samples (Table 6). Raw sequence counts were normalized to eukaryotic elongation factor-1alpha (*eEl-1α*) and correlations between biological reps for both transgene positive and negative genotypes had an $r^2 > 0.9$. In homozygous positive plants there was on average a 3,300 fold increase of both the *Sh2r6hs* and *Bt2* transgenes

compared to the negative controls (Table 7). The sequence hits in the transgene negative plants are likely due to cross contamination during tissue harvesting. Native leaf AGPase expression was approximately 1/120th that of the *Bt2* transgene and 1/20th that of the *Sh2r6hs* transgene (Table 7). Besides the transgenes, the only other leaf starch synthesis gene with significantly different expression between positive and negative plants was starch synthase II-B (*SSIIb*), which was up-regulated in transgene positive plants. When analyzed against the whole rice genome, which contains approximately 30,000 genes, there were 84 genes that were significantly down-regulated ($p < 0.01$) in transgene positive genotypes and 50 genes that were significantly up-regulated ($p < 0.01$). The top 25 genes with significant increased or decreased expression at $p < 0.01$ in the positive transgenic lines versus the negative lines are listed in Table 8. In addition, northern blot analysis was conducted on RNA used for sequencing to confirm increased expression of the *Sh2r6hs* and *Bt2* transgenes (Figure 5). NR16 positive replicate 3 was replaced with a fourth biological replicate for RNA sequencing.

Total AGPase activity in seedling leaf tissue at the four leaf stage was analyzed in NR6, NR16, and NR31 homozygous transgene positive and negative sister-lines at varying concentrations of negative regulator, Pi (Table 9). In each event, AGPase activity of the positive line trended higher than that of the negative sister-line. At 0 mM Pi, enzyme activity was highest and lines NR16 and NR31 AGPase transgene positives had significantly higher enzyme activity, 28% and 29% respectively, than their negative sister-lines. This trend remained throughout all concentrations, though not all comparisons were significant. Positive line NR6 did not have increased activity at 0 mM

Pi, however it trended higher at all other concentrations, and was significantly higher at 0.5 mM Pi. AGPase activity data was consistent with northern blot and RNA sequencing data indicating that the Sh2R6HS and BT2 proteins were expressed in leaf tissue.

Table 6. Number and quality of reads used for RNA-seq analysis.

ID	Yield (Mb)	# Reads	Mean Quality Score (PF)[a]
NR16 Negative 1	557	12,041,852	37.03
NR16 Negative 2	605	13,183,980	36.68
NR16 Negative 3	595	12,783,360	37.29
NR16 Positive 1	703	15,183,032	37.08
NR16 Positive 2	550	11,865,464	37.23
NR16 Positive 3	504	10,835,220	37.17
NR31 Negative 1	654	14,107,974	37.27
NR31 Negative 2	481	10,338,742	37.31
NR31 Negative 3	546	11,763,444	37.26
NR31 Positive 1	659	14,194,410	37.19
NR31 Positive 2	584	12,577,242	37.17
NR31 Positive 3	526	11,389,162	37.03

[a]Per base sequence quality Phred score. Values > 27 indicate high sequence quality.

Table 7. RNA-seq expression data for genes involved in starch synthesis and housekeeping genes in positive and negative plants transformed with AGPase.

GenBank Accession no.	Protein	Gene	Predominantly expressed tissue	Transgene negative average[a]	Transgene positive average[a]	Pos/neg fold	*P*-value[b]
Transgenes							
NM_001127632	Shrunken2	*Sh2*	Maize endosperm	20.3 ± 2.9	67506 ± 9269	3331	0.0008
AF330035	Brittle2	*Bt2*	Maize endosperm	28.9 ± 4.2	98097 ± 18036	3392	0.0029
Starch Biosynthesis[c]							
AK100910	ADP-glucose pyrophosphorylase large subunit	*AGPL1*	Leaf sheath	35.2 ± 6.9	27.2 ± 5.1	0.77	0.3576
AK071497		*AGPL2*	Endosperm	264 ± 35.5	323 ± 36.6	1.23	0.3873
AK069296		*AGPL3*[h]	Leaf tissue	2988 ± 156	3191 ± 165	1.07	0.4520
AK121036		*AGPL4*	Roots	60.7 ± 6.0	55.9 ± 6.8	0.92	0.6944
AK073146		*AGPS1*	Leaf sheaths	101 ± 13.8	95.8 ± 12.4	0.95	0.8516
AK071826	ADP-glucose pyrophosphorylase small subunit	*AGPS2a*[i]	Leaf tissue	857 ± 96.4	764 ± 101	0.89	0.1758
AK103906		*AGPS2b*	Endosperm	2555 ± 156	2167 ± 282	0.85	0.3410
AK109458	Starch synthase (soluble)	*SS1*	Endosperm	499 ± 26.2	552 ± 58.6	1.11	0.3987
AK101978		*SSIIa*	Endosperm	-	-		
AK066446		*SSIIb*	Leaf tissue	1498 ± 86.8	1876 ± 117	1.25	0.0225
AK072339		*SSIIc*	Leaf tissue and roots	122 ± 8.7	108 ± 4.6	0.89	0.0830
AK061604		*SSIIIa*	Endosperm	-	-		
AK122098		*SSIIIb*	Leaf tissue	1963 ± 130	1991 ± 114	1.01	0.8570
AK067577		*SSIVb*	Leaf tissue	171 ± 18.5	182 ± 14.5	1.07	0.6371
AK070431	Starch synthase (granule bound)	*GBSSI*	Endosperm	25.2 ± 5.3	36.8 ± 6.0	1.46	0.2479
AK067654		*GBSSII*	Leaf tissue	10938 ± 775	12556 ± 702	1.15	0.2048
AK065121	Branching enzyme	*BEI*	Endosperm	857 ± 70.4	945 ± 81.5	1.10	0.5415
AB023498		*BEIIa*	Leaf tissue	3195 ± 211	3527 ± 152	1.10	0.2466
D16201		*RICBCE3*	Endosperm	33.0 ± 5.2	34.2 ± 5.4	1.04	0.9040
AK060577	Glucose 6-phosphate/phosphate translocator	*GPT1*	Endosperm	287 ± 9.1	353.5 ± 28.8	1.23	0.0425
AK059906		*GPT2*	Leaf tissue	831 ± 138	824 ± 106	0.99	0.9652

Table 7 Continued

Photosynthesis[d] and Carbon Fixation[e]							
AY445627	RuBisCO small subunit	*RbcS*	Leaf tissue	600007 ± 33217	640394 ± 7820	1.07	0.6574
D00207	RuBisCO large subunit	*RbcL*	Leaf tissue	1047 ± 128	2693 ± 1630	2.577	0.3311
GQ848049	Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	Leaf tissue	3208 ± 86.6	3287 ± 234	1.027	0.7882
NM_001070312	Triose-phosphate isomerase	*TIM*	Leaf tissue	11878 ± 1160	13404 ± 1145	1.13	0.2831
NM_001054360	Phosphoribulokinase	*PRK*	Leaf tissue	70575 ± 3820	77490 ± 3967	1.10	0.2083
D87819	Sucrose Transporter	*SUT1*	Above ground tissue	171 ± 9.8	191 ± 40.9	1.12	0.6701
AB091672		*SUT2*	All tissue	770 ± 33.0	754 ± 42.6	0.98	0.8259
AB071809		*SUT3*	Primarily sink leaves	-	-		
AB091673		*SUT4*	Sink leaves	126 ± 6.8	133 ± 11.0	1.06	0.5911
AB091674		*SUT5*	Primarily sink leaves	-	-		
Nitrogen Metabolism[f]							
NM_001060668	Glutamine synthetase	*GS2*	Leaf tissue	41405 ± 1941	45473 ± 3513	1.10	0.3009
AJ132280	Ferredoxin-dependant glutamate synthase	*Fd-GOGAT*	Leaf tissue	5152 ± 648	5160 ± 801	1.00	0.9939
NM_001059992	Glutamate dehydrogenase2	*GDH2*	Leaf tissue	60.7 ± 5.3	60.5 ± 9.2	1.00	0.9880
Housekeeping Genes[g]							
AK059694	Ubiquitin-conjugating enzyme E2	*UBC*		890 ± 37.0	853 ± 55.7	0.96	0.6119
AK061988	Ubiquitin 5	*UBQ5*		1100 ± 36.4	1216 ± 83.0	1.11	0.1997
AK072502	Tubulin beta-4 chain	*β-TUB*		92.5 ± 12.5	76.8 ± 11.6	0.83	0.3632
AK100267	Actin1	*ACT1*		700 ± 33.4	693 ± 39.9	0.99	0.9136
AK061464	Eukaryotic elongation factor 1-alpha	*eEI-1α*[b]		18334 ± 0.0	18334 ± 0.0	1.00	0.8752

[a]Raw counts. Data from NR16 and NR31 was combined (n=6). Data was normalized to *eEl-1α*.
[b]*P*-value is from a two-tailed, paired *t*-test.
[c]Expression profiling of genes related to starch synthesis determined by Hirose et al., (2006).
[d,e,f]Important C3 photosynthesis (Malkin and Niyogi, 2000), rice carbon assimilation *SUT* genes (Aoki et al., 2003) and nitrogen metabolism genes (Malkin and Niyogi, 2000).
[g]Housekeeping genes determined by Jain et al., (2006).
[h,i]Native rice leaf AGPase large and small subunits respectively.

Table 8. Top 25 genes with significant expression changes.

Name	Protein	Transgene negative average	Transgene positive average	Pos/neg fold	Direction	*P*-value
Os02g0759000	DNA binding protein, putatative, expressed	1.57	0.34	4.62	down	0.002
Os08g0521200	Receptor-like protein kinase 1, putative, expressed	0.12	0.55	4.61	up	0.008
Os03g0440200	DNA helicase, putative	1.93	8.75	4.54	up	0.005
Os11g0117500	DNA-binding WRKY domain containing protein, putative, expressed	0.72	0.16	4.47	down	0.002
Os02g0779000	Proteinase inhibitor, propeptide domain containing protein, putative, expressed	0.08	0.33	4.33	up	0.005
Os12g0239300	Putative uncharacterized protein	1.15	4.43	3.86	up	0.008
Os02g0453800	Putative uncharacterized protein	0.75	2.71	3.62	up	0.003
Os02g0711900	Putative uncharacterized protein	0.27	0.96	3.61	up	0.007
Os03g0261800	Protein of unknown function (DUF3049); pfam11250	0.34	1.16	3.47	up	0.007
Os12g0543600	Similar to Potential sarcosine oxidase, putative, expressed	0.21	0.67	3.15	up	0.006
Os01g0937366	Putative uncharacterized protein	0.91	2.84	3.14	up	0.010
Os02g0221900	Cytochrome P450 family protein, putative, expressed	0.31	0.97	3.12	up	0.006
Os05g0280700	ATP binding protein, putative, expressed	0.17	0.49	2.90	up	0.002
Os07g0541000	Receptor-like serine-threonine protein kinase, putative, expressed	0.08	0.22	2.89	up	0.000
Os01g0616800	Transposon protein, putative, unclassified, expressed	0.33	0.89	2.74	up	0.008
Os07g0530100	Tyrosyl-DNA phosphodiesterase 1, putative, expressed	0.82	0.31	2.61	down	0.002
Os02g0558300	Molybdopterin converting factor, subunit 1 family protein, putative, expressed	0.50	1.24	2.47	up	0.002
Os11g0249000	Resistance protein LR10, putative, expressed	0.45	0.18	2.47	down	0.003
Os03g0129400	Putative uncharacterized protein	115.83	46.95	2.47	down	0.009
Os12g0283300	Putative uncharacterized protein	3.08	7.42	2.40	up	0.001
Os02g0667500	Tetracycline transporter protein, putative, expressed	5.17	2.25	2.30	down	0.008
Os06g0711200	Putative uncharacterized protein	0.78	0.35	2.28	down	0.010
Os06g0687000	Protein of unknown function DUF292, eukaryotic domain containing protein	0.15	0.07	2.21	down	0.006
Os05g0122700	Similar to Low-temperature induced protein lt101.2, putative, expressed	2.02	0.92	2.20	down	0.002
Os03g0284400	50S ribosomal protein L10, putative, expressed	28.53	59.92	2.10	up	0.009

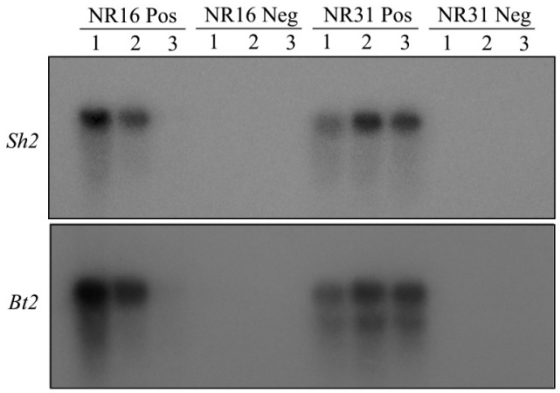

Figure 5. Northern blot expression analysis of RNA used for RNA sequencing. Three biological replicates were collected of T_3 homozygote transgene positive or negative plants of NR16 and NR31. Each replication consisted of a three plant bulk of leaf tissue collected at the 3-4 leaf stage.

Table 9. AGPase Activity of three transgenic rice lines in response to phosphate inhibition[a].

	0 mM	0.5 mM	2 mM	4 mM
NR6 negative	1095.2 ± 60.7	962.9 ± 35.4	975.1 ± 56.8	862.0 ± 68.6
NR6 positive	1099.3 ± 7.5	1020.1 ± 3.9	1004.0 ± 23.0	917.3 ± 45.2
Pos/negratio[c]	1.00	1.06	1.03	1.06
P-value[d]	0.95	0.02	0.73	0.57
NR16 negative	1071.5 ± 36.1	951.5 ± 47.2	932.2 ± 18.5	920.1 ± 87.9
NR16 positive	1373.0 ± 79.0	1223.5 ± 143.5	1162.4 ± 146.8	1065.7 ± 124.5
Pos/neg ratio[c]	1.28	1.29	1.25	1.16
P-value[d]	0.06	0.42	0.26	0.44
NR31 negative	1083.3 ± 43.4	944.9 ± 5.4	886.2 ± 51.2	864.0 ± 2.7
NR31 positive	1392.5 ± 5.6	1194.6 ± 18.6	1091.0 ± 74.7	978.3 ± 17.2
Pos/neg ratio[c]	1.29	1.26	1.23	1.13
P-value[d]	0.003	0.002	0.14	0.01

[a]AGPase activity (nmol ADP glucose mg^{-1} FW) was measured on T_3 flag leaf tissue bulked from three homozygous transgene positive and negative plants with two to three biological replicates.
[b]Values represent mean obtained when 5 µl solubilized protein was added to 20 µl extraction buffer containing 2 mM ATP, Glc-1-P, and 3-PGA and 0, 0.5, 2, or 4 mM Pi respectively.
[c]Positive/negative ratio
[d]One-tailed *p*-values are from *t*-tests assuming equal variance.

Effect of the *Sh2r6hs* and *Bt2*
Transgenes on Photosynthetic Rates

Photosynthetic CO_2 fixation rates were measured on NR16 and NR31, as these two events had higher AGPase activity than NR6. Measurements were collected from the top flag leaf and are presented in Table 10. The data represent photosynthetic and gas exchange measurements throughout the photoperiod of a single day shortly after the majority of plants reached anthesis. Photosynthetic rates were not significantly different between AGPase transgene homozygote positive and negative sister-lines, however the positive plants trended lower. The positive transgenic plants also trended lower in transpiration and stomatal conductance, with NR16 positive plants exhibiting significantly lower stomatal conductance values (Table 10).

Table 10. Photosynthetic and gas exchange measurements for transgenic rice lines NR16 and NR31[a].

	Photosynthesis µmol CO_2 $m^{-2}s^{-1}$	**Transpiration** mmol H_2O $m^{-2}s^{-1}$	**Stomatal Conductance** mmol H_2O $m^{-2}s^{-1}$	n
NR16 Negative	11.58 ± 1.09	4.53 ± 0.11	202.7 ± 10.6	4
NR16 Positive	9.55 ± 1.56	3.62 ± 0.36	136.8 ± 19.6	5
Positive/negative ratio	0.82	0.80	0.67	
P-value[b]	0.35	0.07	0.03	
NR31 Negative	12.05 ± 1.01	3.87 ± 0.17	151.4 ± 10.1	10
NR31 Positive	11.26 ± 1.05	3.43 ± 0.23	129.3 ± 12.6	10
Positive/negative ratio	0.93	0.89	0.85	
P-value[b]	0.59	0.15	0.19	

[a] Data represent means of measurements collected throughout the photoperiod ± standard error. Photosynthetic CO_2 fixation was measured on the top flag leaf at anthesis.
[b] One-tailed *p*-value is from a paired *t*-test.

Major Leaf Photosynthetic Protein Levels

Leaf tissue was collected at three time points throughout the photoperiod, morning, mid-day, and end of day. Total SDS-PAGE buffer soluble proteins were extracted and separated by SDS-PAGE and band areas corresponding to RBCL were quantified against a standard curve. At the beginning of the day, both NR31 and NR6 AGPase transgene positive plants were significantly lower in RBCL levels (Table 11). At mid-day, RBCL levels remained 17% to 25% lower in transgene positive NR31 and NR6, respectively, and at the end of the day, transgene positive NR31 RBCL levels increased by 6% while transgene positive NR6 RBCL levels decreased, becoming significant again at 60% of NR6 negative levels. NR16 transgene positive levels of RBCL trended higher than the NR16 negative sister-line throughout the photoperiod.

Table 11. Levels of RBCL protein in three transgenic lines throughout the photoperiod.

	Morning[a]	**Mid-Day**[a]	**End of Day**[a]
NR6 positive	0.50 ± 0.03	0.69 ± 0.07	0.48 ± 0.04
NR6 negative	0.95 ± 0.03	0.92 ± 0.33	0.75 ± 0.03
Pos/neg ratio[b]	0.53	0.75	0.60
P-value[c]	0.00	0.55	0.01
NR16 positive	0.90 ± 0.06	0.88 ± 0.11	0.63 ± 0.06
NR16 negative	0.80 ± 0.03	0.83 ± 0.02	0.61 ± 0.07
Pos/neg ratio[b]	1.13	1.1	1.09
P-value[c]	0.20	0.2	0.82
NR31 positive	0.62 ± 0.06	0.88 ± 0.02	0.92 ± 0.16
NR31negative	0.87 ± 0.04	1.07 ± 0.11	1.01 ± 0.12
Pos/neg ratio[b]	0.71	0.83	0.89
P-value[c]	0.03	0.17	0.66

[a]Leaf tissue was collected at three time points and pooled from three plans into transgene positive and negative bulks. Three biological replicates were collected. Values are means ± standard error.
[b]Positive/negative ratio
[c]Two-tailed *P*-values are from *t*-tests assuming equal variance.

Plant Growth and Yield Analysis

NR6, NR16, and NR31, were the highest yielding lines with expression of the AGPase transgenes in a T_1 yield trial (data not shown), and were therefore advanced for a T_2 yield trial (Table 12). Paired, and randomized yield trials were conducted under high light conditions. Individually, all three T_2 transgene positive lines significantly outperformed their negative sister-lines in total number of stems per plant by 40% or more. Additionally, panicles per plant were significantly higher in NR6 and NR16 transgene positive plants and trended higher for NR31. Seed number per plant also trended higher in the positive plants by 7%, 30%, and 100% for NR31, NR6, and NR16 respectively, although this parameter was only significant in NR16. Increase in seed weight per plant is most likely attributed to the increase in stem number, as seed weight per panicle and individual seed weight were only increased in NR16. Additionally because NR6, NR16, and NR31 have similar transgene expression, these lines were combined and yield and phenotype traits were analyzed. Combined data showed that the positive transgenic plants flowered significantly earlier and contained 7% more chlorophyll at anthesis. Additionally, seed number and seed weight per plant were increased 30%, and total biomass was increased by 40% in the transgene positive plants.

Table 12. Influence of the *Sh2r6hs* and *Bt2* transgenes on T_2 plant phenotype[a].

	NR6 Neg[a]	NR6 Pos[a]	NR16 Neg[a]	NR16 Pos[a]	NR31 Neg[a]	NR31 Pos[a]	Overall Neg[a]	Overall Pos[a]
Days from planting to flowering	72.7 ± 1.0	70.6 ± 1.0	74.0 ± 1.5	71.6 ± 1.1	73.9 ± 0.8	72.6 ± 0.8	73.8 ± 0.6	71.6 ± 0.6*
Height (cm)[b]	75.4 ± 2.0	73.7 ± 7.1	67.8 ± 3.0	80.3 ± 1.8**	71.6 ± 2.0	69.3 ± 3.0	71.9 ± 1.5	74.4 ± 2.5
Panicles/plant	7.8 ± 0.9	10.7 ± 1.2**	5.2 ± 1.0	9.7 ± 1.3**	5.9 ± 0.7	7.1 ± 0.9	6.5 ± 0.5	9.2 ± 0.7***
Seed number/panicle	23.4 ± 3.3	21.0 ± 2.0	13.6 ± 2.6	14.6 ± 1.9	23.8 ± 2.4	24.3 ± 2.3	20.9 ± 1.7	20.0 ± 1.3
Seed weight/panicle (g)	467.7 ± 62.2	420.8 ± 42.8	262.8 ± 46.2	286.9 ± 38.0	466.0 ± 48.4	450.3 ± 45.6	403.1 ± 34.5	386.0 ± 26.8
Seed number/plant	115.2 ± 14.9	148.8 ± 28.0	34.7 ± 7.5	69.1 ± 11.8*	103.4 ± 17.9	114.2 ± 15.6	87.1 ± 10.0	110.7 ± 12.4
Seed weight/plant (g)	2.3 ± 0.3	3.0 ± 0.6	0.6 ± 0.1	1.4 ± 0.3*	2.0 ± 0.3	2.1 ± 0.3	1.7 ± 0.2	2.2 ± 0.3
Unfilled seed number/plant	211.5 ± 26.6	309.2 ± 33.4**	125.6 ± 24.4	250.6 ± 35.4**	155.7 ± 18.7	203.8 ± 30.5	169.4 ± 14.1	254.5 ± 20.0***
Mean individual seed weight (mg)	20.9 ± 1.9	20.0 ± 0.4	15.6 ± 1.6	19.6 ± 0.4*	19.7 ± 1.3	18.4 ± 0.5	19.3 ± 0.8	19.3 ± 0.3
Number filled panicles/plant	5.0 ± 0.4	6.6 ± 0.8	2.5 ± 0.4	4.7 ± 0.6**	4.2 ± 0.4	4.6 ± 0.5	4.0 ± 0.3	5.3 ± 0.4**
Number unfilled panicles/plant	2.8 ± 0.8	4.1 ± 1.2	2.7 ± 0.8	5.0 ± 1.1	1.7 ± 0.6	2.5 ± 0.7	2.5 ± 0.4	3.8 ± 0.6*
Unfilled seed number/panicle	41.4 ± 2.5	47.8 ± 2.3*	48.8 ± 4.1	53.4 ± 4.6	37.9 ± 3.9	42.67 ± 2.9	42.5 ± 2.2	48.0 ± 2.1*
Number non-panicle bearing stems/plant	3.7 ± 1.1	4.8 ± 1.3	2.9 ± 0.6	5.5 ± 0.8*	2.5 ± 0.8	4.6 ± 1.0*	3.1 ± 0.5	4.9 ± 0.6**
Total number stems/plant	11.6 ± 1.4	15.6 ± 1.8*	8.1 ± 1.4	15.2 ± 1.5***	8.4 ± 0.9	11.6 ± 1.3*	9.6 ± 0.7	14.1 ± 0.9***
Chlorophyll at anthesis[c]	40.2 ± 1.1	43.2 ± 0.8	39.1 ± 0.8	41.3 ± 0.6*	38.7 ± 0.6	41.7 ± 0.9*	39.2 ± 0.5	42.0 ± 0.5***
Chlorophyll at harvest[c]	37.0 ± 2.0	39.9 ± 1.7	33.9 ± 1.9	33.4 ± 2.7	33.0 ± 1.7	36.0 ± 1.4	34.5 ± 1.1	36.4 ± 1.2
Total biomass/plant (g)	12.4 ± 1.7	15.3 ± 2.2	5.4 ± 1.0	12.1 ± 1.7***	8.1 ± 0.8	10.4 ± 1.3	8.9 ± 0.7	12.6 ± 0.9***
Harvest Index[d]	0.19 ± 0.03	0.19 ± 0.03	0.13 ± 0.04	0.1 ± 0.02	0.23 ± 0.03	0.20 ± 0.02	0.19 ± 0.02	0.17 ± 0.01

[a]Values are means ± standard errors.All positive (pos) and negative (neg) plants were paired within genotypes to give n=11.
[b]Height was measured to the base of the tallest panicle.
[c]Chlorophyll values represent the mean of 5 random measurements throughout the plant.
[d]Harvest index is the ratio of seed weight to total plant biomass.
*,**,*** Homozygote transgene positive is significantly different from homozygote negative sister-line at $\alpha < 0.05$, 0.01, or 0.001 respectively, using a two-tailed, paired *t*-test.

Discussion

Grain yield is dependent upon efficient allocation of photoassimilates from source to sink tissues, with growth potential of plant organs being largely driven by relative strength of different source and sink tissues (reviewed in Van Camp, 2005; Smith and Stitt, 2007). Increasing plant yield through increases in sink strength has been the topic of much research (reviewed in Paul and Foyer, 2001; Araus et al., 2008). A common approach used to increase sink strength that has been associated with increases in seed yield (Regierer et al., 2002; Smidansky et al., 2003; Heyer et al., 2004) is to increase activity of enzymes involved in carbon metabolism in sink tissue. As the enzyme controlling the rate limiting step in starch biosynthesis, AGPase has been the focus of much attention (Stark et al., 1992; Giroux et al., 1996; Greene and Hannah, 1998). Several studies have observed increases in seed yield as well as plant biomass associated with overexpression of AGPase under control of an endosperm specific promoter (Giroux et al., 1996; Smidansky et al., 2003; Meyer et al., 2004; Smidansky et al., 2007). The increases in both plant biomass as well as seed yield indicate that metabolism was not only up-regulated in sink tissue, but in source tissues as well. Here we examine rice productivity of plants overexpressing AGPase in leaves, i.e. in source organs.

In this study *Sh2r6hs* and *Bt2* were overexpressed in rice leaves under control of either the rice *Aps1* or *RBC* leaf-specific promoters. The *RBC* promoter conferred a much higher transgene expression level *Aps1,* as transcripts under the *Aps1* promoter were undetectable with northern blotting (Figure 3). However, the RNA-seq results indicate that the three RBC lines have AGP transgene expression levels roughly 100 x that of

native leaf AGPase genes. Therefore, the *Aps1* lines may in fact have AGPase transgene expression levels comparable to those of the native genes. Future use of RNA-sequencing on the *Aps1* lines may provide a much more accurate means for determining relative expression. For all tested events, progeny test data (Table 5) indicate single locus inheritance. The northern and RNA sequencing results indicate that both transgenes were actively transcribed into mRNA at high levels relative to native leaf starch biosynthetic genes (Figure 3 and Table 7). Additionally, increased AGPase activity in leaf extracts from transgene positive relative to control leaves indicate that SH2R6HS and BT2 are actively expressed (Table 9). The AGPase activity assay also indicates that there were no changes in allosteric properties of the enzyme conferred by the R6 and HS alterations present in SH2R6HS. This observation is in contrast to findings by Meyer et al., (2004) in which overexpression of SH2R6HS in seeds was found to decrease AGPase phosphate inhibition and increase heat stability. It is generally believed that AGPase's allosteric properties are key to the increased seed (Giroux et al., 1996) or plant (Smidansky et al., 2002, 2003) size seen in previous studies. However, no previous study has examined whether simple overexpression of native AGPase impacts plant productivity. Therefore, it remains possible that simple AGPase overexpression alone would be sufficient to modify plant growth. However, there have been no studies directly comparing transformation with *Sh2r6hs* versus native *Sh2*.

In our experiments, elevated activity of AGPase in leaves was associated with increased plant vigor (Table 12), but did not lead to changes in photosynthetic rates (Table 10). This is surprising, as increased expression of seed specific AGPase led to

enhanced photosynthesis in flag leaves of wheat shortly after anthesis (Smidansky et al., 2007). It is also interesting to note that each of the transgenic lines had varying levels of Rubisco compared to their negative controls (Table11), though photosynthetic rates were unchanged. However, photosynthesis is a complex process, with complex regulation (Malkin and Niyogi, 2000). Although a relationship between leaf starch and photosynthesis has been observed in *Arabidopsis* (Sun et al., 1999), it appears that rice plant biomass may be increased without increasing the rate of photosynthesis per unit area.

Expression analysis of photosynthetic protein transcripts revealed that there were no obvious differences between transcript levels of transgene positive and negative plants (Table 7). This supports our observation that photosynthetic rates were constant between genotypes (Table 10). It also indicates that levels of the large subunit of RuBisCo trended higher in the transgene positives, similar to what was observed in NR16. It is interesting that the ratio of transcript levels of glucose 6-phosphate/phosphate translocator (*GPT1*) were significant at $\alpha < 0.05$ considering that GPT1 is expressed predominantly in the endosperm while GPT2 is expressed in vegetative tissue (Hirose et al., 2006). Another interesting observation was overexpression of 50S ribosomal protein transcripts (Table 8) which would indicate that protein synthesis was deregulated in the positive lines. This would also suggest a link between carbon and nitrogen metabolism. Here we report that overexpression of the *Sh2r6hs* and *Bt2* transgenes in leaves (Figure 2) is associated with an enhanced plant phenotype. Seed weight and seed number per plant trended higher across the three transgenic lines, and the AGPase transgene positive group

flowered significantly earlier and contained significantly more chlorophyll in leaves at anthesis. The most striking observation is that plant biomass was significantly increased in all three transgenic lines by an average of 40% (Table 12). These findings are consistent with observations in lettuce and rice in which a modified potato AGPase was overexpressed in leaves. In lettuce, overexpression of AGPase resulted in increased fresh weight at 8 weeks post germination (Lee et al., 2009) and Gibson et al., (2011) observed a trend of increased leaf dry weight in rice overexpressing AGPase in leaves compared to the varietal control.

The results shown here indicate that source strength is an important factor governing plant yield since increasing the expression of AGPase in leaves results in an enhanced plant phenotype. Our results indicate that it is possible to increase plant yield without increasing rate of photosynthesis per unit area. These results provide insight into the complex nature of plant yield and indicate that it is possible to increase plant yield through manipulation of leaf source strength.

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