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Research Article

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Enhanced Rice Growth is Conferred by Increased Leaf ADP-Glucose Pyrophosphorylase Activity

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Abstract

Modification of leaf starch levels may be employed in attempts to increase cereal yield. Few studies have examined leaf starch as a plant biomass limiting factor. Here we test the hypothesis that rice plant productivity may be increased by increasing leaf starch. Starch biosynthesis is controlled by the heterotetrameric rate-limiting enzyme ADP-glucose pyrophosphorylase (AGPase). Rice variety Nipponbare was transformed with a modified form of the maize endosperm AGPase large subunit gene, *Sh2r6hs,* as well as with the small subunit gene, *Bt2,* under control of a rice RuBisCO small subunit promoter. RNA sequencing results indicated that *Sh2r6hs* and *Bt2* transcript levels were each greater than 20 times that of the native genes. Increased total AGPase activity was correlated with higher leaf starch accumulation at the end of the day. Yield trials of T_1 derived homozygous plants indicate that increased leaf AGPase leads to a 29% increase in plant biomass under the conditions tested without changing the rate of photosynthesis while significantly reducing leaf transpiration and conductance. Additionally, functional annotation clustering of significantly up and down regulated transcripts reveals areas of protein metabolism, specifically protein biosynthesis, transport, and localization, that were altered in response to increased leaf starch. Together, these findings indicate plant growth is limited by native levels of leaf starch and that it is possible to increase plant yield via the starch biosynthesis pathway.

Keywords

Rice; Leaf starch; Biomass; Photosynthesis

Abbreviations

AGPase: ADP-glucose pyrophosphorylase

Introduction

Agronomic yield is a quantitative trait, highly impacted by the environment [1]. Productivity is reliant upon biochemical and physiological processes with underlying genetic control [2]. Grain yield is ultimately dependent upon photosynthesis, and thus upon photoassimilate production and efficient carbon allocation of assimilate to sinks which are developing or storage tissues [3,4]. Growth potential of different plant organs is largely driven by production and flow of photoassimilates and nutrients inside the plant [3]. There have been many studies aimed at increasing yield by targeting increased production and storage of photoassimilates [5,6], the most common method being to increase activity of carbohydrate metabolic pathway enzymes in sink tissues such as seeds. For example, increased expression of ADP-glucose pyrophosphorylase in rice seeds is associated with increased seed yield [7], and over expression of a sucrose-phosphate synthase gene in potato tubers is associated with increased potato tuber yield [8].

The most important metabolic 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 [9]. In plants, AGPase is a heterotetramer consisting of two large and two small subunits [10,11]. In the presence of ATP, AGPase acts upon the substrates glucose 1-phosphate and ATP to produce ADP-glucose [12]. ADP-glucose pyrophosphorylase is also an allosteric enzyme; the positive regulator is 3-PGA and the negative regulator is inorganic orthophosphate (Pi) [10,11]. Additionally, there are seed and leaf specific isoforms of AGPase [13-16]. 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 [13,17].

Several studies have focused on increasing seed sink strength by over expressing AGPase in seeds. In wheat [18,19] and rice [7] increased expression of a deregulated form of the maize large subunit of AGPase under an endosperm-specific promoter led to increased seed weight per plant and higher plant biomass. Increases in both seed yield and plant biomass, such that harvest index remained unchanged, indicated up-regulation of metabolism in both sink and source tissues. However, this complex enhanced yield phenotype was not observed under field conditions [20].

A less studied area involving carbon metabolism has been leaf starch as a plant productivity limiting factor. A study in rice [21] 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 chamber conditions [21]. A similar mutation, *agps-m1*, was reported in maize in which the leaves lacked leaf starch due to a transposon insertion in the leaf AGPase small subunit [22]. We tested the impact of the lack of leaf starch upon maize productivity by examining the growth of *agps-m1* plants under field conditions. The *agps-m1* plants flowered two days later, were five cm shorter, and had 30% lower seed yield relative to wild type leaf starch isogenic plants [23], 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* [24], in lettuce increased fresh weight and starch content at eight weeks after germination [25]. However, the lettuce plants were not taken to maturity thus plant development and seed yield data were not reported. An additional study in rice utilizing leaf

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specific over expression of *upreg1* showed a trend of increased seed yield in comparison to the varietal control [26].

In this study, we have tested the role of leaf AGPase in rice productivity by over expressing AGPase in leaves. Unlike the approach used in [26], rice was transformed with maize endosperm AGPase large and small subunits modified to have the rice leaf AGPase chloroplast transit peptide. The AGPase transgenes were under the control of the native rice RuBisCO small subunit promoter. Under the conditions tested, over-expression of both AGPase subunits lead to significantly increased biomass without changing photosynthetic rates while significantly reducing leaf transpiration and conductance. Global changes in transcript levels provide insight into molecular processes associated with increased starch biosynthesis.

Materials and Methods

Plasmid construct used for transformation

The pRBC*Sh2Bt2* plasmid construct was used for transformation (Figure 1). The vector made use of the pTF101.1 binary vector [27] 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. The *bar* gene confers resistance to the herbicide glufosinate (Bayer Crop Science, Kansas City, MO). The AGPase subunits were the large and small subunits of maize endosperm, *Sh2* and *Bt2*, respectively. The wild type *Sh2* coding sequence [28] was modified as previously described [29]. The resulting *Sh2r6hs* coding sequence contains two alterations. The (*r6*) modification is a two amino acid insertion conferring reduced AGPase phosphate inhibition *in vitro* [30]. The *hs* alteration is a single amino acid substitution that confers more stable AGPase subunit interactions *in vitro* [31]. The wild type *Bt2* coding sequence [32] was also included along with the tobacco RB7 matrix attachment region sequence (MARS) [33] which was positioned 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 a 141 bp segment encoding a rice leaf AGPase chloroplast specific signal peptide. *Sh2r6hs* and *Bt2* were under control of the native rice RuBisCO small subunit promoter (*RBC*).

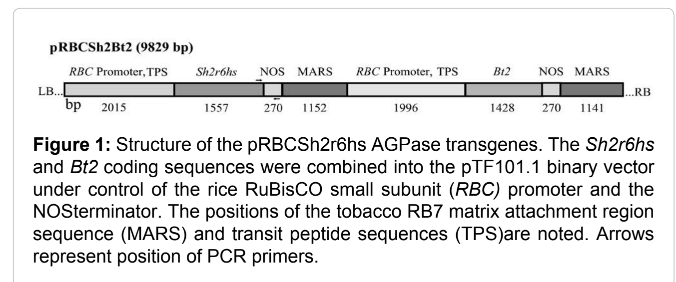

Figure 1: Structure of the pRBCSh2r6hs AGPase transgenes. The *Sh2r6hs* and *Bt2* coding sequences were combined into the pTF101.1 binary vector under control of the rice RuBisCO small subunit (*RBC)* promoter and the NOSterminator. The positions of the tobacco RB7 matrix attachment region sequence (MARS) and transit peptide sequences (TPS)are noted. Arrows represent position of PCR primers.

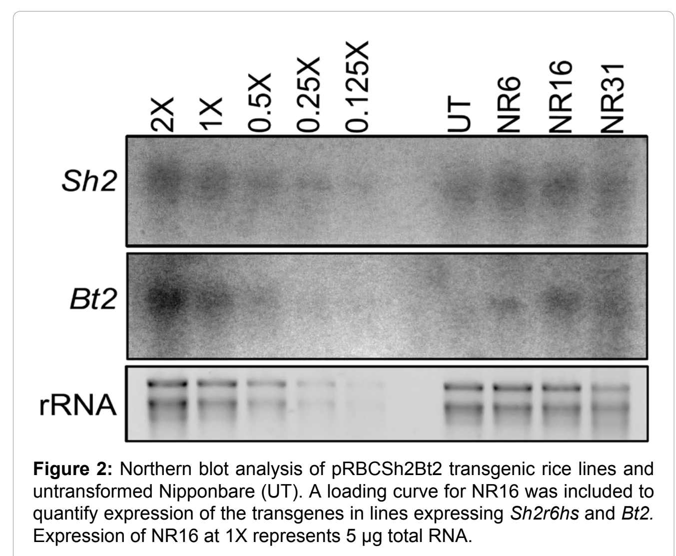

Figure 2: Northern blot analysis of pRBCSh2Bt2 transgenic rice lines and untransformed Nipponbare (UT). A loading curve for NR16 was included to quantify expression of the transgenes in lines expressing *Sh2r6hs* and *Bt2*. Expression of NR16 at 1X represents 5 µg total RNA.

Production of transgenic plants and preliminary analysis of transgenic lines

Construct pRBC*Sh2Bt2* was introduced into rice *japonica* variety Nipponbare calli at the Iowa State University Plant Transformation Facility via an *Agrobacterium*-mediated transformation system [34]. The initial T_0 plantlets, hemizygous for the transgenes, were advanced to maturity in growth chambers with conditions 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. T_1 plants segregating 1:2:1 for the transgenes were advanced and seed was harvested.

For selection of homozygotes 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 producing 12 or more consecutive resistant plants were classified as homozygous for the presence of the transgenes. Plants producing 4 or more consecutive susceptible seedlings were classified as homozygous for the absence of the transgenes. Three events with high over expression of the transgenes were chosen for further study. T_1 derived homozygous transgenes positive and negative plants ($T_{1:2}$ and $T_{1:3}$) with over expression of both AGPase transgenes were used for further study, such that negative and positive sister lines were derived from the same T_1 source material within each event.

Genomic DNA was isolated and PCR was conducted on T_1 glufosinate resistant and susceptible plants to confirm that the *Sh2* and *Bt2* transgenes co-segregated with glufosinate resistance. 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 204-224 of the NOS terminator. This primer pair produces a 210 base pair amplified product using GoTaq DNA polymerase (Promega, Madison, WI, USA). PCR parameters were 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.

Transgene expression analysis

Tissue was collected near the end of the day (1.5 hr pre-lights off) from three individual $T_{1:3}$ plants at anthesis. The terminal 7.5 cm of a leaf, located two leaves down from the tallest panicle, was collected from each plant and bulked within genotype. Tissue was

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directly frozen in liquid N_2, ground into a powder, and total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Total RNA was quantified on a Bioanalyzer (Agilent Technologies, Santa Clara, CA) and electrophoresed on formaldehyde containing agarose gels with an equal portion 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 nylon membrane (Amersham Biosciences, Uppsala, Sweden), probed with ^{32}P-random primer labeled (Takara Bio Inc., Otsu, Shiga, Japan) *Sh2* and *Bt2* coding sequences, and hybridized overnight at 65 ˚C in a solution that was 7% SDS and 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 at 65˚C. Membranes were washed an additional two times in 0.2X SSPE at 65˚C and dried at 37˚C for 40 min. Hybridized membranes were exposed to Kodak Biomax MS film (Rochester, NY) with an intensifying screen at -80˚C.

Whole transcriptome shotgun sequencing (RNA-seq) technology was employed to determine transgene and genome wide expression levels for NR16 and NR31. *Sh2r6hs* expression of samples utilized in RNA-seq was confirmed with northern blot analysis (data not shown). Leaf tissue from randomly selected AGPase transgene homozygous positive and negative $T_{1:3}$ plants were collected at the 3-4 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 creation of cDNA libraries using TruSeq RNA-SEQ library kits (Illumina Inc., San Diego, CA) with 6 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 analysis was carried out with QSeq and ArrayStar v4.1 (DNASTAR, Madison, WI). Sequence data were 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 [35]. Resultant expression data were converted to linear counts and normalized to eukaryotic elongation factor 1-alpha (*eEl-1α*). Two-tailed *t*-tests were performed to determine if significant changes in expression existed between positive and negative AGPase transgene genotypes.

The most highly up (54) or down-regulated (84) genes throughout the genome (*P*-value < 0.01) were identified using ArrayStar v4.1 (DNASTAR, Madison, WI). Functional annotation clustering of these genes was performed using the DAVID Bioinformatics Resources Functional Annotation tool [36].

AGPase activity assays

Assays were performed on leaf tissue harvested at the four leaf stage at mid-day. The terminal 3 cm of the uppermost erect leaf was collected from three randomly selected $T_{1:3}$ plants within each genotype and combined into homozygous transgenes positive or negative bulks with three biological replicates and directly frozen. Leaf tissue was then ground to a powder without allowing samples to thaw and 5 μl mg $^{-1}$ fresh weight leaf tissue of extraction buffer with a concentration of 80 mM HEPES, 1 mM EDTA, 0.1 mM DTT, 2 mM $MgCl_2$ and10 μl ml^{-1} Halt protease inhibitor (ThermoScientific, Rockford, IL) was added to samples. Samples were vortexed and centrifuged at 13,000 g for 2 min then 5 μl of supernatant was transferred to new tubes containing 20 μl extraction buffer with 2 mM ATP, 2 mM glucose-1-phosphate, 0, 0.5, 2, or 4 mM Pi and contained 1 nCi ^{14}C-labeled glucose-1-phosphate (PerkinElmer, Boston, MA, USA). Reactions 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 four times in H_2O and dried. Total activity (measured in counts per minute) was 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 [18] substituting mg^{-1} FW for mg^{-1} total protein (Figure 3A).

Leaf starch quantification

Leaf tissue was collected near the end of the day (1.5 hr pre-lights off) from the largest fully expanded leaf at the four leaf stage. The terminal 10 cm was collected and bulked between two samples. Three biological replicates were collected and tissue was ground. Leaf starch was extracted from 20 mg of ground powder according to [37]. Free glucose was removed from ground leaf powder by incubating in 80% EtOH for 3 min at 80˚C. This step was repeated twice. Extracted starch pellets were resuspended in 1:1 dH_2O:200 mM sodium acetate (pH 4.8) and starch was digested with 0.2U α-amylase and 0.6U amyloglucosidase mg^{-1} DW. Samples were assayed according to [38] using NADP in place of NAD. Starch concentration was determined by including a standard curve in assays prepared with known amounts of wheat starch (Azure Farm, Dufur, OR).

Photosynthetic carbon fixation measurements

Measurements of photosynthetic rates were conducted as in [23]. The CI-340 machine (CID, Camas, WA) was zeroed using ambient CO_2 with the value set at 389 ppm (http://co2now.org, accessed October 11, 2011). Measurements were conducted over the course of the photoperiod on the uppermost flag leaf of $T_{1:3}$ plants at 1-3 days after flowering (DAF). Values presented are the means of all data collected from $T_{1:3}$ plants grown in a paired and randomized growth chamber yield trial though out the photoperiod.

Analysis of yield phenotype parameters

$T_{1:3}$ seed was pooled from parent plants into homozygous positive or negative bulks. Seed was de-hulled and soaked in deionized water overnight. Five seeds were planted per 7.5 x 10 x 13 cm pot and direct seeded into Profile Greens Grade calcified clay (Profile Products LLC, Buffalo Grove, IL) containing 4.93 cc each of 30:10:10 Osmocote (The Scotts Company LLC, Marysville, OH) and 1:0:1 Ironite (4.5% Fe) Mineral Supplement (Ironite, Walnut Creek, CA). Pots were placed in an incubator under artificial lights delivering 100 photosynthetically active radiation (PAR) and incubated at 25˚C until plants were approx. 15 cm tall. Pots were thinned to two plants and homozygous positive and negative plants were paired and randomized within each genotype for a sample size of n = 12-15. Plants were moved to a growth chamber with a 24˚C day/22˚C night temperature regime and a 12 hr photoperiod. Artificial lighting provided 600 μE m^{-2} s^{-1} PAR at canopy height. Day temperature was increased to 28˚C at the five tiller

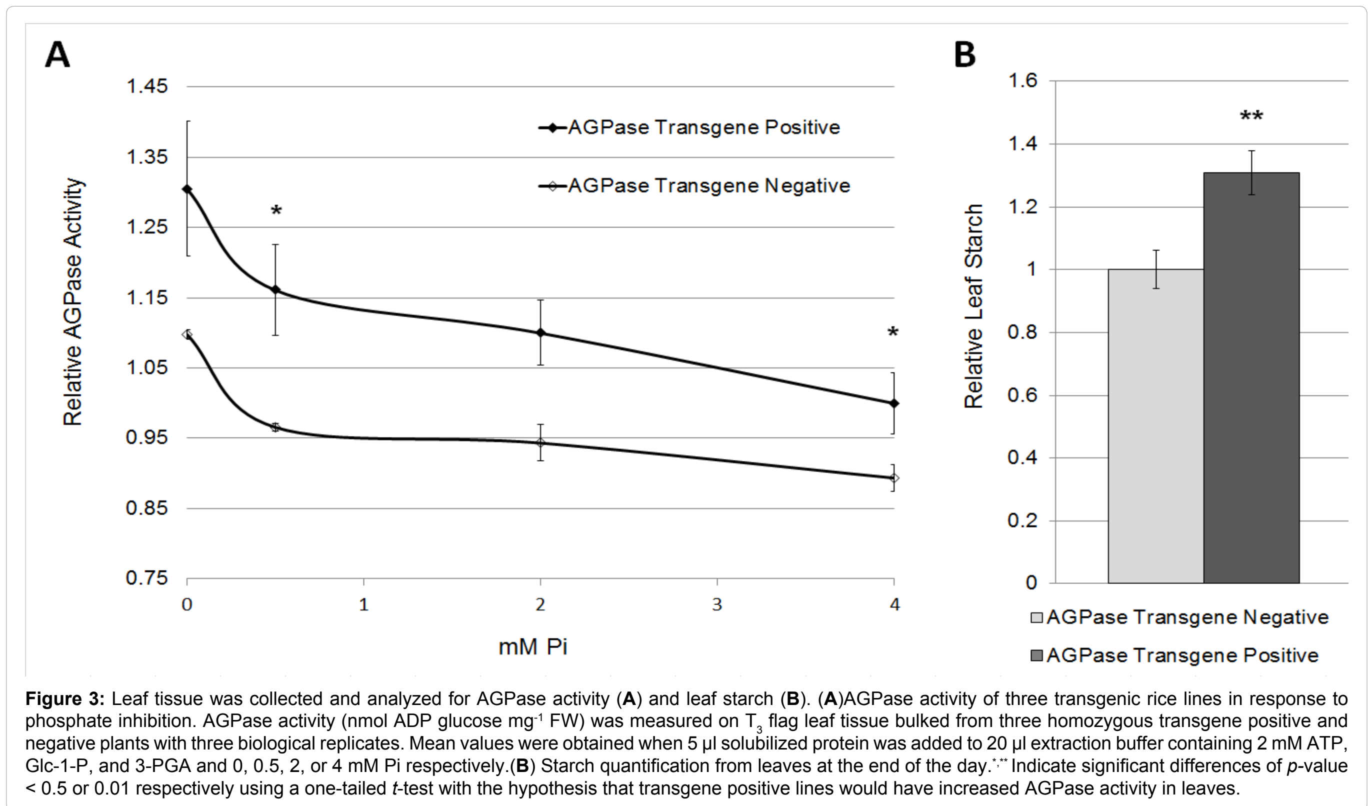

Figure 3: Leaf tissue was collected and analyzed for AGPase activity (**A**) and leaf starch (**B**). (**A**)AGPase activity of three transgenic rice lines in response to phosphate inhibition. AGPase activity (nmol ADP glucose mg^{-1} FW) was measured on T_3 flag leaf tissue bulked from three homozygous transgene positive and negative plants with three biological replicates. Mean values were 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.(**B**) Starch quantification from leaves at the end of the day.*,** Indicate significant differences of *p*-value < 0.5 or 0.01 respectively using a one-tailed *t*-test with the hypothesis that transgene positive lines would have increased AGPase activity in leaves.

stage and pots were thinned to one plant. Plants were bottom irrigated daily in tubs delivering 57 ppm N prepared using Peters Excel 15-5-15 Cal-Mag (Everris, Marysville, OH) [39]. Plants were allowed to self-pollinate and watered daily until 60 days after flowering. Mature plants were harvested, and biomass was weighed after one week in a 37°C drying chamber. Biomass and all yield parameters were recorded on a per plant basis. Leaf chlorophyll values were collected using a SPAD-502 chlorophyll meter (Minolta Co., LTD, Japan) and represent the mean of five leaves per plant. Height was measured to the base of the tallest panicle.

Analysis of results

Transgene positive and negative mean values were compared using a two-tailed, two independent sample *t* statistic or a one-tailed, two independent sample *t* statistic with the hypothesis that transgene positive plants would be more vigorous than transgene negative plants. For the yield trials, positive/negative ratio was calculated between each genotypic pair. Standard error is representative of the ratios of each pair within the trial.

Results

Analysis of transgenic lines

The rice variety Nipponbare was transformed with *Sh2r6hs* and *Bt2* under the control of the *RBC* promoter (Figure 1). T_1 seed from glufosinate resistant T_0 transgenic rice lines were obtained through *Agrobacterium* mediated transformation. Lines NR6, NR16, and NR31 were advanced for further study as they each had expression of both AGP transgenes (Figure 2).

PCR specific to the *Sh2r6hs* and *Bt2* transgenes along with

Table 1: Transgene analysis of three T_1 rice lines segregating 3:1 for over expression of *Sh2r6hs* and *Bt2*.

Transgenic Event	Progeny Test[a]	Chi-Square Value	P-value
NR6	10/3	0.03	0.87
NR16	9/2	0.27	0.60
NR31	16/2	1.85	0.17

[a]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.

herbicide screening of $T_{1:2}$ 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 *Sh2r6hs* PCR negative plants also glufosinate susceptible (Table 1). Chi-square analysis is consistent with integration of the transgenes at a single locus in each of the three events (Table 1).

Expression analysis

Northern blot and RNA Seq analysis indicate *Bt2* is expressed at a higher level than *Sh2r6hs* (Table 2). The high homology between native genes and transgenes was apparent in the northern blot signal associated with the untransformed control when probed with *Sh2*. Staining of agarose gel fractionated rRNA with ethidium bromide indicated similar loading between lanes. Northern blot analysis revealed similar over expression levels across events (Figure 2), therefore NR16 and NR31 were selected as representatives for RNA sequencing.

Using next generation sequencing technology, over 150 million reads were obtained for the twelve samples (3 biological reps of two

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Table 2: RNA-seq expression data for genes of interest and housekeeping genes in positive and negative plants transformed with AGPase.

GenBank Accession no.	Protein	Gene	Predominantly expressed tissue	AGPase transgene negative average[a]	AGPase transgene positive average[a]	Pos/neg fold	*P*-value[b]
Transgenes							
NM_001127632	Shrunken2	*Sh2*	Maize endosperm	20.3 ± 2.9	67,506 ± 9,269	NA	
AF330035	Brittle2	*Bt2*	Maize endosperm	28.9 ± 4.2	98,097 ± 18,036	NA	
Starch Biosynthesis[c]							
AK100910	ADP-glucose pyrophosphorylase large subunit	*AGPL1*	Leaf sheath	35.2 ± 6.9	27.2 ± 5.1	0.77	0.36
AK071497		*AGPL2*	Endosperm	264 ± 35.5	323.2 ± 36.6	1.23	0.39
AK069296		***AGPL3*[h]**	**Leaf tissue**	2,988 ± 156	3,191.4 ± 164.5	1.07	0.45
AK121036		*AGPL4*	Roots	60.7 ± 6.0	55.9 ± 6.8	0.92	0.69
AK073146	ADP-glucose pyrophosphorylase small subunit	*AGPS1*	Leaf sheaths	101 ± 13.8	95.8 ± 12.4	0.95	0.85
AK071826		***AGPS2a*[i]**	**Leaf tissue**	857 ± 96.4	764 ± 101	0.89	0.18
AK103906		*AGPS2b*	Endosperm	2,555 ± 156	2,167 ± 282	0.85	0.34
AK109458	Starch synthase (soluble)	*SS1*	Endosperm	499 ± 26.2	552 ± 58.6	1.11	0.40
AK101978		*SSIIa*	Endosperm	-	-		
AK066446		*SSIIb*	Leaf tissue	1,498 ± 86.8	1,876 ± 117	1.25	**0.02**
AK072339		*SSIIc*	Leaf tissue and roots	122 ± 8.7	108 ± 4.6	0.89	0.08
AK061604		*SSIIIa*	Endosperm	-	-		
AK122098		*SSIIIb*	Leaf tissue	1,963 ± 130	1,991 ± 114	1.01	0.86
AK067577		*SSIVb*	Leaf tissue	171 ± 18.5	182 ± 14.5	1.07	0.64
AK070431	Starch synthase (granule bound)	*GBSSI*	Endosperm	25.2 ± 5.3	36.8 ± 6.0	1.46	0.25
AK067654		*GBSSII*	Leaf tissue	10,938 ± 775	12,556 ± 702	1.15	0.20
AK065121	Branching enzyme	*BEI*	Endosperm	857 ± 70.4	945 ± 81.5	1.10	0.54
AB023498		*BEIIa*	Leaf tissue	3,195 ± 211	3,527 ± 152	1.10	0.25
D16201		*RICBCE3*	Endosperm	33.0 ± 5.2	34.2 ± 5.4	1.04	0.90
AK060577	Glucose 6-phosphate/ phosphate translocator	*GPT1*	Endosperm	287 ± 9.1	353.5 ± 28.8	1.23	**0.04**
AK059906		*GPT2*	Leaf tissue	831 ± 138	824 ± 106	0.99	0.97
Photosynthesis[d] and Carbon Fixation[e]							
AY445627	RuBisCO small subunit	*RbcS*	Leaf tissue	600,007 ± 33217	640,394 ± 7820	1.07	0.66
D00207	RuBisCO large subunit	*RbcL*	Leaf tissue	1,047 ± 128	2,693 ± 1630	2.57	0.33
GQ848049	Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	Leaf tissue	3,208 ± 86.6	3,287 ± 234	1.02	0.79
NM_001070312	Triose-phosphate isomerase	*TIM*	Leaf tissue	11,878 ± 1160	13,404 ± 1145	1.13	0.28
NM_001054360	Phosphoribulokinase	*PRK*	Leaf tissue	70,575 ± 3820	77,490 ± 3967	1.10	0.21
D87819	Sucrose Transporter	*SUT1*	Above ground tissue	171 ± 9.8	191 ± 40.9	1.12	0.67
AB091672		*SUT2*	All tissue	770 ± 33.0	754 ± 42.6	0.98	0.83
AB071809		*SUT3*	Primarily sink leaves	-	-		
AB091673		*SUT4*	Sink leaves	126 ± 6.8	133 ± 11.0	1.06	0.59
AB091674		*SUT5*	Primarily sink leaves	-	-		
Nitrogen Metabolism[f]							
NM_001060668	Glutamine synthetase	*GS2*	Leaf tissue	41,405 ± 1941	45,473 ± 3513	1.10	0.30
AJ132280	Ferredoxin-dependant glutamate synthase	*Fd-GOGAT*	Leaf tissue	5,152 ± 648	5,160 ± 801	1.00	0.99
NM_001059992	Glutamate dehydrogenase2	*GDH2*	Leaf tissue	60.7 ± 5.3	60.5 ± 9.2	1.00	0.990
Housekeeping Genes[g]							
AK059694	Ubiquitin-conjugating enzyme E2	*UBC*		890 ± 37.0	853 ± 55.7	0.96	0.61
AK061988	Ubiquitin 5	*UBQ5*		1,100 ± 36.4	1,216 ± 83.0	1.11	0.20
AK072502	Tubulin beta-4 chain	*β-TUB*		92.5 ± 12.5	76.8 ± 11.6	0.83	0.36
AK100267	Actin1	*ACT1*		700 ± 33.4	693 ± 39.9	0.99	0.91
AK061464	Eukaryotic elongation factor 1-alpha	*eEl-1α*		18,334 ± 0.0	18,334 ± 0.0	1.00	0.88

[a]Raw counts. Data from NR16 and NR31 were combined (n=6) and normalized to *eEl-1α*.
[b]*P*-value is from a two-tailed, paired *t*-test.
[c]Expression profiling of genes related to starch synthesis with tissue specificity as determined previously [51].
[d,e,f]C3 photosynthesis genes [52], rice carbon assimilation *SUT* genes [53] and nitrogen metabolism genes [52].
[g]Housekeeping genes represent genes with stable expression across all genotypes and were determined previously [54].
[h,i]Native rice leaf AGPase large and small subunits respectively.

transgene positive and negatives for each of the two events). On average each sample yielded 580 Mb of sequence with approximately 12,500,000 total 50 bp reads generated from each cDNA library. The average per base sequence quality Phred scores from a RNA sample was 37.1, indicating high sequence quality. Linear counts were normalized to eukaryotic elongation factor-1alpha (*eEl-1α*) and correlations between biological replicates for both transgene positive and negative genotypes had an r^2> 0.9 for the 21,000 recognized genes. Expression of the native leaf AGPase small subunit, *AGPS2a,* was approximately 1/120th that of the *Bt2* transgene and the native leaf large subunit, *AGPL3*, was 1/20th that of the *Sh2r6hs* transgene (Table 2). Besides the transgenes, the only other leaf starch synthesis gene with a significant difference in expression between positive and negative plants was starch synthase II-B (*SSIIb*), which was up-regulated in transgene positive plants (Table 2).

Functional annotation clustering identified 3 clusters of highly up-regulated genes and 6 clusters of highly down-regulated genes in lines over expressing leaf starch biosynthesis (Table 3). Groups that were both up and down regulated include transcripts associated with iron and metal binding proteins and protein kinases. An up-regulated functional group that was not found to also be down-regulated was associated with ribosomal proteins and protein biosynthesis. Additional down-regulated groups also associated with protein metabolism were protein transport and localization and amino acid binding.

Enzyme activity and leaf starch levels

Total AGPase enzyme activity in leaf tissue was analyzed in NR6, NR16, and NR31 homozygous transgene positive and negative sister-lines at varying concentrations of negative regulator, Pi (Figure 3A). In each event, AGPase enzyme activity of the positive line trended higher than that of the negative sister-line. Data were combined for homozygous positive and negative lines. At 0 mM Pi, enzyme activity was highest and transgenes positives were higher in total AGP activity by 19%. This trend remained throughout all concentrations, with positive transgenic lines with enzyme activity significantly higher at 0.5 mM and 4 mM Pi. No differences were seen in phosphate inhibition between the enzyme extracted from transgenes positive and negative plants. However, AGPase activity results were consistent with northern blot and RNA sequencing data, which demonstrates that the Sh2R6HS and BT2 proteins were expressed in leaf tissue.

Leaf starch was measured on fully expanded leaves collected from the four leaf stage at the end of the light period. All transgenes positive plants were higher in leaf starch compared to their negative sister lines (Figure 3B). Plants over expressing AGPase had a 31% increase in leaf starch (p-value > 0.01) near the end of the day (Figure 3B). Lines negative for the transgenes had starch levels similar to starch levels of the 'Nipponbare' varietal control (data not shown).

Effect of the *Sh2r6hs* and *Bt2* transgenes on photosynthetic rates

Photosynthetic CO_2 fixation rates were measured on NR16 and NR31 on flag leaves at mid-day and throughout the photoperiod (Table 4). There was no difference between morning or afternoon measurements, therefore data represent photosynthetic and gas exchange measurements taken throughout the photoperiod of a single day shortly after plants reached anthesis. Photosynthetic rates were not significantly different between AGPase transgene homozygote positive and negative sister-lines. However, transgenes positive transgenic plants were significantly lower in transpiration and stomatal conductance (Table 4).

Table 3: Functional annotation gene clustering of highly up-regulated and down-regulated genes in response to over-expression of AGPase in leaves[a].

Functional Group of Differentially Expressed Genes	No. genes	*P*-value[b]
Up-regulated by leaf AGPase transgene expression		
Cluster 1		
Ribosomal protein	3	0.07
Non-membrane-bounded organelle	4	0.14
Cluster 2		
Ion binding	6	0.52
Transition metal ion binding	5	0.48
Iron ion binding	4	0.09
Cluster 3		
Phosphate metabolic process	5	0.38
Protein tyrosine kinase activity	3	**0.03**
ATP binding	4	0.74
Down-regulated by leaf AGPase transgene expression		
Cluster 1		
Amino acid binding	3	**0.01**
Kinase	8	**0.01**
Cluster 2		
Intracellular protein transport	3	0.10
Cellular protein localization	3	0.10
Cluster 3		
Integral to membrane	6	0.10
Transmembrane	4	0.62
Cluster 4		
Kinase	8	**0.01**
Serine/threonine-protein kinase	6	0.11
Phosphorylation	8	0.17
ATP binding	10	0.62
Cluster 5		
Electron carrier activity	3	0.79
Transition metal ion binding	7	0.92
Cluster 6		
Zinc	3	0.72
Zinc ion binding	5	0.77
Metal ion binding	9	0.91

[a]Gene lists consisting of all genes found to be significantly up or down regulated at *P*- value < 0.01 with two-tailed, equal variance t-tests were uploaded into the DAVID Functional Annotation Tool where they were clustered based on gene similarity. There were 54 up-regulated and 84 down-regulated genes included in analysis.

[b]EASE score *P*-values were generated during DAVID analysis and are a more conservative form of Fisher Exact P-values.

Plant growth and yield analysis

NR6, NR16, and NR31 were the transgenic events with the highest transgene expression levels and were therefore advanced for a T_3 yield trial (Figure 4). A paired and randomized yield trial was conducted under 600 PAR growth conditions. Similar trends were observed between events, therefore data was combined. There was no difference between AGP transgenes positive or negative lines for days to anthesis or chlorophyll at anthesis, with negative lines flowering 86 days after planting and chlorophyll levels at 45. AGP transgene negative plants were significantly shorter (6%) and measured 38 cm tall. Seed weight per plant was not significantly different with AGP transgene negative plants having 2.9

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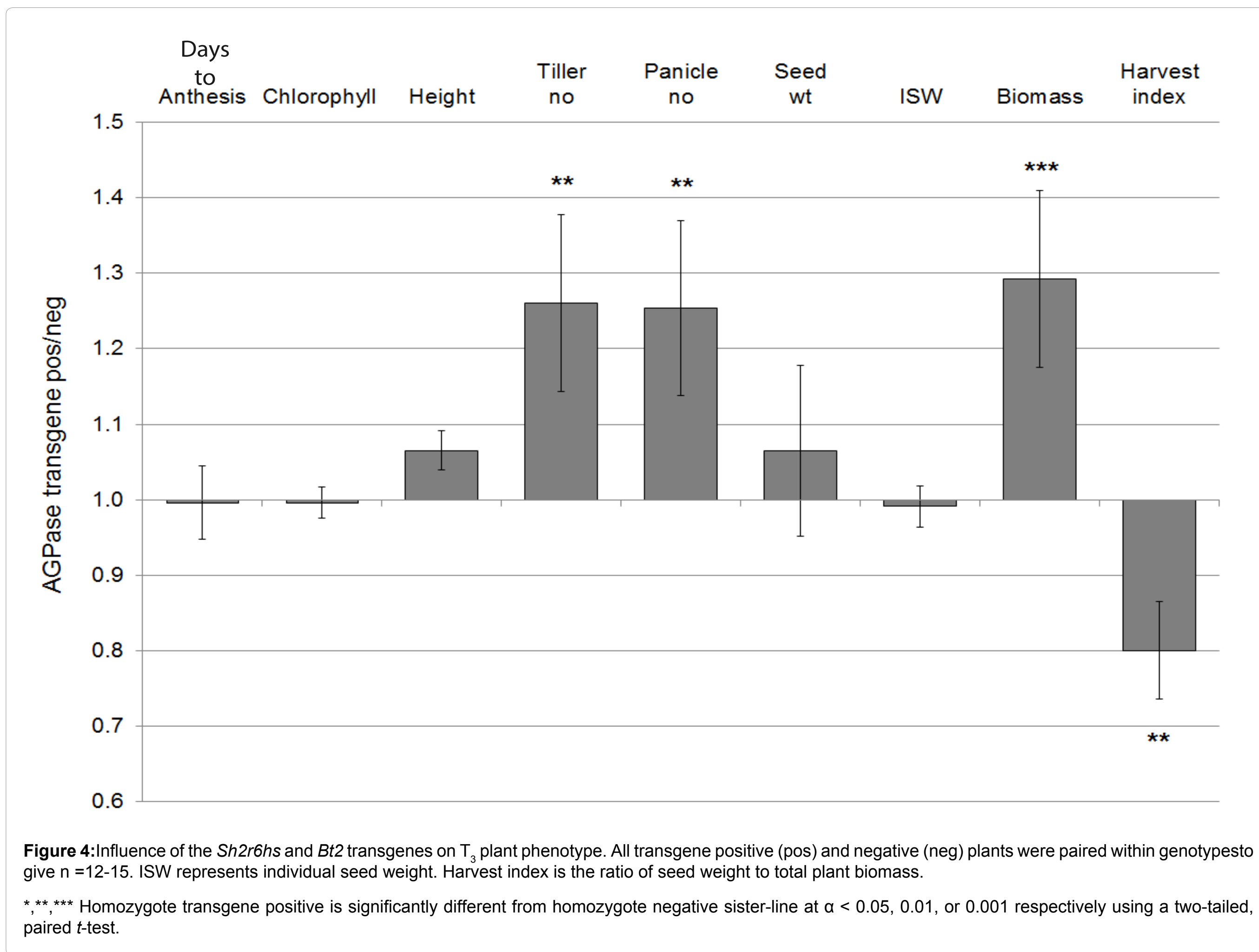

Figure 4: Influence of the *Sh2r6hs* and *Bt2* transgenes on T_3 plant phenotype. All transgene positive (pos) and negative (neg) plants were paired within genotypesto give n =12-15. ISW represents individual seed weight. Harvest index is the ratio of seed weight to total plant biomass.

*,**,*** Homozygote transgene positive is significantly different from homozygote negative sister-line at α < 0.05, 0.01, or 0.001 respectively using a two-tailed, paired *t*-test.

Table 4: Photosynthetic and gas exchange measurements for rice lines over expressing starch biosynthesis in leaves[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
AGPase Transgene Negative	11.91 ± 0.76	4.06 ± 0.15	166.1 ± 10.0	15
AGPase Transgene Positive	10.69 ± 0.90	3.49 ± 0.19	131.8 ± 10.3	14
Positive/negative ratio	0.90	0.86	0.79	
P-value	0.30	**0.03**	**0.02**	

[a]Data represent means of measurements collected from NR16 and NR31 lines throughout the photoperiod ± standard error. Photosynthetic CO_2 fixation was measured on the top flag leaf of T_3 plants at 1-3 DAF.

[b]Two-tailed *p*-value is from a paired *t*-test.

grams, although plants over -expressing AGPase trended 9% higher (3.2 g) (Figure 4). This is attributed to increase in seed number, as individual seed weight was unchanged between genotypes (18.5mg). AGP transgene negative plants had 19 panicles, 22 tillers, and 15 g plant biomass. Each of these traits was significantly increased in the homozygous positive group by 25-30% (Figure 4).

Discussion

Grain yield is dependent upon efficient allocation of photoassimilates, with growth potential of plant organs being largely driven by relative strength of different source and sink tissues [3,4]. Increasing plant yield through increases in sink strength such as by increasing seed starch biosynthesis has been the topic of much research [5,6], however fewer studies have focused upon leaf starch biosynthesis. Here we examine the role that leaf starch plays in rice growth by over expressing AGPase in rice leaves.

In this study *Sh2r6hs* and *Bt2* were over-expressed in rice leaves under control of the native rice *RBC* green tissue-specific promoter. The transgenes were selected for transformation due to high sequence similarity to the native genes and for the genetic alterations found in *Sh2r6hs* [29,30]. For all tested events, progeny test data indicate single locus inheritance (Table 1). Northern blot and RNA-seq results indicate that both transgenes were actively transcribed into mRNA (Figure 2 and Table 2). RNA-seq was better able to differentiate between native gene and transgene isoforms of AGPase transcripts compared to northern blot analysis. RNA-seq also indicated that the

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transgenes were expressed at high levels relative to native leaf starch biosynthetic genes (Table 2). Additionally, increased total AGPase enzyme activity in leaf extracts from transgenes positive relative to control leaves indicate that *SH2R6HS* and *BT2* are actively expressed (Figure 3A) and starch assays indicate that leaf starch levels at the end of the day correspond with AGPase protein over expression (Figure 3B). While increased *Sh2r6hs* and *Bt2* expression did not lead to very large increases in AGPase enzyme activity or leaf starch, we previously observed that over expression of *Sh2r6hs* in wheat seeds led to only 5% to 20% increases in seed AGPase activity [18]. The AGPase activity assay indicated 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 our previous findings [18] in which over expression of *SH2R6HS* in seeds was found to decrease AGPase phosphate inhibition and increase heat stability. The lack of an observed difference in AGPase allosteric properties may reflect differences in AGPase subunit processing and assembly in leaves relative to seeds. Another possibility is that the transgenic maize endosperm AGPase subunits are interacting with the native rice leaf subunits to form mosaic heterotetramers, changing the allosteric properties of the enzyme. It has been demonstrated that the maize endosperm subunits, encoded by the *Sh2* and *Bt2* genes, have the ability to bind with the potato tuber large and small subunits [40].

It is generally assumed that AGPase's allosteric properties are key to the increased seed [30] or plant size [7,29] seen in previous studies. There have been no studies examining whether simple over expression of an unmodified AGPase in leaves impacts plant productivity. However, over expression of native *Sh2* and *Bt2* in corn endosperm lead to a 9% increase in starch to give a total starch content of 74% of seed composition. This led to a 15% increase in 100-grain weight compared to the WT [41].Therefore it seems likely that over expression of native AGPase alone in leaves would be sufficient to modify plant growth.

In our experiments, elevated activity of AGPase in leaves was associated with significantly increased plant vigor (Figure 4), but did not lead to a difference in seed size. This observation may indicate that carbon metabolism in the plant is not a limiting factor in seed production. However the positive trend in overall seed weight reflects the significant increase in tiller and panicle number in plants over expressing starch biosynthesis. Therefore this trait may be of interest to cereal breeders in their efforts to increase overall seed yield.

Although we observed a positive association between elevated activity of AGPase in leaves and increased plant vigor (Figure 4), we did not observe differences in photosynthetic rates (Table 3). This observation is supported by our RNA-seq results where important photosynthetic transcripts were unchanged between transgenes positive and negative plants (Table 4). This is interesting, as increased expression of seed specific AGPase led to enhanced photosynthesis in flag leaves of wheat shortly after anthesis [19]. Photosynthesis is however a complex process, with complex regulation [42]. Although a relationship between leaf starch and photosynthesis has been observed in *Arabidopsis* [43], it appears that rice plant biomass may be increased without increasing the rate of photosynthesis per unit area.

It is also interesting that stomatal conductance and transpiration were significantly lower in transgene positive plants while photosynthetic rates were unchanged, though also trended lower. Several studies have shown that it is possible to uncouple the positive correlation between photosynthetic rate and stomatal conductance [44-46]. However, the pathways associated with changes in stomatal regulation are not fully understood [47]. Of course whole plant photosynthetic rates would in fact be higher in AGPase transgene positive plants given that total plant biomass was substantially increased by AGPase over expression.

RNA-seq identified 134 genes with highly significant changes in expression level. Functional annotation clustering of these genes identified several areas in plant metabolism that were previously unknown to have functions relating to starch biosynthesis. Over expression of leaf starch biosynthesis lead to transcript changes in several functional groups. Up and down regulation of transcripts for protein kinases most likely represent changes to amino acid processes and cellular signaling (Table 3). Also in support of this, ribosomal transcripts were up-regulated indicating deregulation of protein metabolism (Table 3).

Carbon metabolism is intrinsically linked with nitrogen and lipid metabolism, as they are central to whole plant growth and metabolism. However, few studies have looked directly at the relationship between leaf starch and whole plant metabolism. In attempts to increase oil production by diverting carbon from starch biosynthesis, studies have shown that altering levels of leaf starch leads to changes in oil content [48,49]. In [48] repressing AGPase activity in conjunction with increased triacylglycerate biosynthesis led to decreased leaf starch and increased oil levels in vegetative tissues. Another study shows the importance of carbohydrate supply from vegetative tissue during the dark period; both an excess leaf starch phenotype in which starch is not degraded as well as a near starchless phenotype lead to significantly decreased lipid content in seeds [49]. Along with altering oil metabolism, starch is also linked to nitrogen metabolism. A study in maize, demonstrates that lack of leaf starch leads to significant decreases in photosynthetic proteins throughout the light cycle [23]. Additionally, a study on transgenic potato with inhibited leaf and tuber AGPase activity found that a decrease in expression of major proteins in the tuber accompanied the inhibition of starch biosynthesis [50]. Together, these studies provide a glimpse into the complex nature of starch biosynthesis and its role in metabolism. Further study on the impact of starch levels on carbon and nitrogen metabolism in various tissues throughout development would provide important information regarding how starch biosynthesis impacts plant growth and would provide insight into the nature of starch metabolism and its role in source-sink regulation.

Here we report that over expression of AGPase in leaves (Figure 2 and Table 2) is associated with an enhanced plant growth phenotype under the conditions tested. The most striking observation is that plant biomass was increased 29% in transgene positive plants (Figure 4). These findings are consistent with observations in lettuce and rice in which a modified potato AGPase was over expressed in leaves. In lettuce, over expression of AGPase resulted in increased fresh weight at 8 weeks post germination and a trend of increased panicle number in rice over expressing AGPase in leaves compared to the varietal control [25,26].

The results shown here indicate that leaf starch is an important factor governing plant yield. Increasing leaf starch via increased expression of AGPase in leaves results in an enhanced plant productivity phenotype. Our results indicate that it is possible to increase plant yield without detectable changes in the rate of photosynthesis per unit area. These findings provide insight into the complex nature of plant yield and

provide insight into the potential of increasing plant yield through manipulation of leaf starch biosynthesis.

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