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# Overexpression of Transcription Factor *WRKY76* in Rice Leaves Leads to Increased Photosynthesis and Plant Yield

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**Abstract:** The rate of leaf starch biosynthesis impacts overall plant growth and yield. Overexpression of ADP-glucose pyrophosphorylase (AGPase), the rate limiting enzyme in the starch pathway, has led to increased plant growth in multiple species. Our goal here was to identify and test transcription factors that themselves upregulate starch biosynthetic genes. To accomplish this objective, we examined rice genes upregulated in response to overexpression of leaf AGPase, and identified the transcription factor (TF) WRKY76 as a potential regulator of the rice leaf starch biosynthesis pathway. Overexpression of *WRKY76* in rice (*Oyrza sativa*) leaves led to a 27% increase in plant growth and seed yield. The enhanced productivity phenotype in rice overexpressing *WRKY76* was associated with a 40% increase in leaf starch levels at one month after planting and at anthesis. This was accompanied by a 16% increase in photosynthetic rate and 20% increase in net carbon metabolism, indicating that WRKY76 positively regulates leaf starch biosynthesis and carbon metabolism. Interestingly, increased expression of *WRKY76* led to changes in expression of other WRKY TFs, indicating that the mechanism by which WRKY76 regulates starch biosynthesis involves a complicated regulatory network. This research indicates that WRKY76 directly increases expression of genes involved in leaf starch biosynthesis.

**Keywords:** ADP-glucose Pyrophosphorylase (AGPase), Photosynthesis, Starch, Transcription Factor (TF), WRKY, Yield

## 1. Introduction

In attempts to increase cereal yield, many studies have focused on improving photosynthesis efficiency and carbon allocation. Starch biosynthesis in particular has been of interest, as starch impacts both sink and source strengths [1]. Transitory leaf starch is synthesized from excess photosynthates as they accumulate in leaves during the day and then serves as an energy source when it is broken down and transported to sink tissues during the dark period. Starch in seeds directly contributes to seed yield where it constitutes 65% of cereal grain weight and serves as an energy source for germination. Most research aimed at increasing cereal yield via starch biosynthesis has focused on targeting either individual enzymes within the starch biosynthesis pathway or upon increasing the rate limiting step in starch biosynthesis [2].

The rate limiting step in the starch biosynthesis pathway is controlled by the allosteric enzyme ADP-glucose pyrophosphorylase (AGPase) [3]. AGPase and leaf starch have been shown to be important contributors of crop yield as a transposon derived knock out mutation of AGPase, *agps-m1,* results in a 30% decrease in maize seed yield under field conditions [4]. Furthermore, overexpression of AGPase in leaf tissue has been associated with increased starch levels and yield increases across multiple species. A study in lettuce (*Lactuca sativa* L.) in which a modified potato AGPase large subunit, *upreg1* [5], was overexpressed in leaves resulted in an average 33% increase in starch content as well as an average increase of 21% in fresh weight at eight weeks after germination [6];

increases were attributed to greater 3-PGA activation along with lower Pi inhibition compared to wild type plants. Another study where *upreg1* was overexpressed in leaves resulted in increased starch levels in rice (*Oryza sativa* L.) leaves and increased grain yield [7]. Expression of a modified maize leaf AGPase subunit, *Sh2r6hs,* and native maize small subunit, *Bt2,* in rice leaves also led to an enhanced plant phenotype. Overexpression of AGPase led to significant increases in tiller number leading to a 25% increase in plant biomass [8, 9]. This was despite a decrease in photosynthesis on a per area basis [8, 9]. Additional analysis determined that yield increases were greatest when *Sh2r6hs* was overexpressed in both leaf and seed tissues [9]. However, these studies were conducted under non-limiting, controlled environment growth conditions and yield increases are likely environment dependent, as they are when AGPase is overexpressed in seeds [10].

An additional approach to increase yield via starch upregulation is to identify and overexpress a transcription factor that positively regulates starch biosynthesis. This approach may allow for more stable yield increases due to upregulation of the entire starch biosynthesis pathway. Transcription factors (TFs) are DNA binding proteins that regulate gene expression and are classified based on DNA binding motifs. They may act as gene activators and/or repressors and may act autonomously or with other TFs, enhancers, insulators, and/or tethering elements to form transcriptional complexes that often regulate gene expression through a cascade of biochemical reactions [11, 12].

Several studies have shown that transcription factors are in fact associated with the starch biosynthesis pathway. In seeds, OsbZIP58 has been shown to play a regulatory role in endosperm starch biosynthesis in rice, with binding sites in promoters of six starch biosynthesis genes [13], and Starch Regulator 1 (RSR1) has also been identified as a negative regulator of starch biosynthesis in seeds [14]. In leaves, a study in rice observed increased number of starch granules in flag leaf parenchyma cells as the result of constitutive overexpression of the Higher Yield Rice (HYR) TF [15]. HYR was found to directly activate several photosynthesis and carbon metabolism related processes resulting in a regulatory cascade. An additional study has linked a WRKY TF directly to starch biosynthesis in leaves, in which it has been suggested that AtWRKY20 functions directly as a transcriptional activator of the leaf AGPase large subunit *ApL3* promoter in Arabidopsis [16].

In this study, we identified WRKY76 as a possible positive regulator of rice leaf starch biosynthesis. Gene expression analysis of rice overexpressing AGPase subunits *Sh2r6hs* and *Bt2* [8] identified WRKY76 as a likely candidate to act as a positive regulator of starch biosynthetic genes since this TF was upregulated in rice overexpressing AGPase in leaves. Here we examined plant growth and metabolism after *WRKY76* was overexpressed in rice leaves under control of the RuBisCO small subunit promoter.

# 2. Materials and Methods

### *2.1. Transgene Selection and Creation of Transgenic Plants*

The transgene used in this study was identified in an expression dataset created in Schlosser et al. (2014) [8] which examined rice overexpressing the maize endosperm AGPase genes *Sh2r6hs* and *Bt2* in leaves. Expression analysis was performed as in Schlosser et al. (2014) [8] and *WRKY76* was identified as a candidate for starch biosynthesis regulation, as it was upregulated in plants overexpressing leaf AGPase by 4.30 fold at significance level $p$-value < 0.01.

The pRBC*WRKY76* construct was used for transformation of rice plants (Figure 1). This vector made use of the pTF101.1 binary vector [17], a derivative of the pPZP binary vector [18], that contains the *aadA* gene for bacterial selection of spectinomycin resistance as well as the *bar* gene for herbicide resistance to glufosinate (Bayer Crop Science, Kansas City, MO). The herbicide resistance selectable marker cassette also includes the 2X cauliflower mosaic virus 35S promoter (2X P35S) [19], tobacco etch virus translational enhancer (TEV) [20], and the soybean vegetative storage protein terminator (Tvsp) [21]. The 1,355 bp *WRKY76* coding sequence (NCBI accession AK068337), under control of the RuBisCO small subunit (*RBC*) promoter and NOS terminator, is flanked by SbfI and SpeI cloning sites. The tobacco RB7 matrix attachment region sequence (MARS) [22] is positioned between the NOS terminator and the herbicide selectable marker gene cassette.

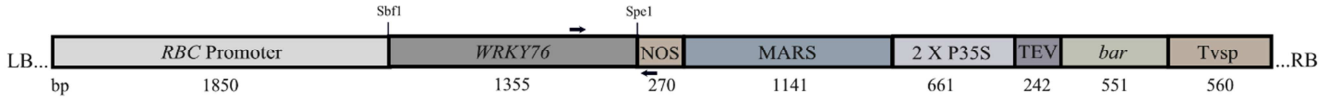


***Figure 1.*** *Structure of pRBCWRKY76. The 1,355 bp WRKY76 coding sequence (NCBI Accession AK068337) was inserted into the pTF101.1 binary vector under control of the rice RuBisCO small subunit (RBC) promoter and the NOS terminator. The tobacco RB7 matrix attachment region sequence (MARS) was also included. The pTF101.1 vector contains the bar gene selectable marker, conferring resistance to the herbicide glufosinate, under control of the cauliflower mosaic virus 35S promoter (2 x P35S). Arrows indicate PCR primer positions.*

Construct pRBC*WRKY76* was introduced into rice *japonica* variety Nipponbare calli at the Iowa State University Plant Transformation Facility via *Agrobacterium*-mediated transformation [23]. Sixteen independent transgenic events were received. Initial $T_0$ plantlets, hemizygous for the *WRKY76* transgene, followed by $T_1$ plants, segregating 1:2:1, were advanced to maturity in growth chambers. Plants self-pollinated and mature seed was harvested at 60 days after anthesis. Events WRKY 1, 5, and 12 were advanced for further analysis.

### *2.2. Preliminary Analysis of Transgenic Events*

Genotyping for the selectable marker (*bar*) was used to

identify homozygous positive and negative plants as well as heterozygotes. For progeny testing, 16 $T_2$ seeds from each $T_1$ plant were germinated on 1/2 x MS media (pH 5.8) in 7.5 x 7.5 x 8 cm Magenta GA-7 boxes (Magenta LLC, Chicago, IL). For each event, 12-33 $T_1$ plants were grown to the three-leaf stage and analyzed for the *bar* gene herbicide resistance selectable marker. Seedlings were sprayed with a 0.1% active ingredient glufosinate solution until runoff and individual plants were scored for herbicide resistance or susceptibility after 7 days. $T_1$ plants producing 12 or more consecutive herbicide resistant plants were classified as homozygous positive for the transgene. Those producing four or more consecutive herbicide susceptible plants were classified as homozygous negative for the transgene. $T_1$ sources producing seedlings segregating 3:1 herbicide resistant: susceptible were classified as heterozygous.

PCR was also performed on $T_1$ derived $T_2$ ($T_{1:2}$) plants for the *WRKY76* transgene to confirm co-segregation with *bar*. For this reaction, primers were designed specific to the pRBC*WRKY76* construct. The upstream primer, 5'-TCACGCTCGAACCTCACCAAGAACG-3' hybridizes to the *WRKY76* cDNA sequence. The downstream primer, 5'-GACACCGCGCGCGATAATTTATCCTAG-3', hybridizes within the NOS terminator. This primer pair produces a 727 bp product using GoTaq DNA polymerase (Promega, Madison, WI). The *WRKY76* primer pair was duplexed with actin as a control gene. This primer pair consisted of upstream PCR primer 5'-GAAGATCACTGCCTTGCTCC-3' and downstream PCR primer 5'-CGATAACAGCTCCTCTTGGC-3' and produced a 249 bp product [24]. Primers were diluted to 0.2 µg $\mu l^{-1}$ and incorporated into the master mix such that a 20 µl reaction contained 0.2 µl for each Actin primer and 0.4 µl for each *WRKY76* primer. PCR parameters were as follows: 94°C for 5 min, 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec, followed by 7 min at 72˚. Co-segregation of the *WRKY76* transgene and glufosinate resistance was confirmed for all events.

### *2.3. Sampling*

All leaf tissue was collected at one month after planting one hour prior to the end of the photoperiod and the terminal 10% was trimmed from each end of the leaf. Samples were collected from the uppermost fully expanded leaf of $T_1$ derived plants. Tissue was immediately frozen in liquid $N_2$ and ground to a fine powder.

### *2.4. Gene Expression*

Gene expression was examined at one month after planting. Samples were collected from $T_{1:2}$ plants segregating 3:1 for the *WRKY76* transgene with plants categorized as PCR positive or negative for the transgene. Three biological replicates were analyzed for each event and combined across events for a total sample size of n = 9, which is an adequate level of replication to detect important differences [25]. Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Sample RNA integrity (RIN) was determined using a Bioanalyzer (Agilent Technologies, Santa Clara, CA). All samples used for cDNA library creation had RIN > 7. Libraries were created using TruSeq RNA-SEQ library kits (Illumina Inc., San Diego, CA) and included the addition of six bp molecular identification tags for multiplexing. Amplicons were sequenced as single 50 bp reads on an Illumina High Scan-SQ platform.

Gene expression analysis was carried out with ArrayStar v. 12 within the DNASTAR software package (DNASTAR, Madison, WI). Sequence data was imported into ArrayStar with match settings set to 100% for 50 bp. All other settings were set to default with reads per kilobase of exon model per million mapped reads (RPKM) normalization [26]. Resultant expression values were normalized across samples to actin 1 (*ACT1*). For individual genes of interest, one-tailed t-tests with equal variance were used to determine significance between expression levels of *WRKY76* transgene positive and negative plants with the hypothesis that starch and carbon fixation genes would be upregulated in transgene positive plants.

Global expression analysis was performed for the entire *Oryza sativa* var. *japonica* genome, consisting of 30,524 genes downloaded from NCBI October 2017. Expression values were averaged across events and compared to the Nipponbare varietal control using two-tailed, equal variance t-tests for each gene. Functional annotation clustering was performed for genes that were significantly up or down regulated at a significance level of $p$-value < 0.001 using the Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics resource Functional Annotation tool [27].

### *2.5. Leaf Starch Quantification*

Leaf starch was examined at one month after planting and at anthesis for 10 homozygous transgene positive and negative plants. Starch was extracted from 7 mg DW powder according to Smith and Zeeman (2006) [28]. Free glucose was removed from ground leaf tissue by three incubations in 80% EtOH. Samples were incubated at 80°C for three minutes and centrifuged at 13,000 rpm for five min. Supernatants were discarded and extracted pellets were suspended in 100 mM sodium acetate (pH 4.8) and digested with 0.05 U α-amylase and 0.15 U amyloglucosidase $mg^{-1}$ DW. Starch was quantified using NADP as a substrate. The reaction mix consisted of 0.1M HEPES-KOH (pH 7.5), 5mM $MgCl_2$, 0.8mM ATP, and 1.6mM NADP [29]. Initial absorbances were recorded at 340 nm and 10µl 0.1M HEPES-KOH (pH 7.5) containing 0.5 U hexokinase and 0.5 U glucose 6-phosphate dehydrogenase was added to each sample. Absorbance was monitored and final, absorbances were recorded once after completion of the reaction (approx. 20 min). Leaf starch concentrations were determined by referencing a standard curve prepared with known amounts of purified wheat starch (Azure Farm, Dufur, OR). Significance was determined using one-tailed, equal variance

t-tests with the hypothesis that expression of the transgene would lead to leaf starch increases.

### *2.6. Metabolite Extraction and Analysis*

For metabolomic experiments, metabolites were quantified in leaves harvested at one month after planting with n = 3 samples bulked from five independent source leaves harvested from homozygous transgene positive and negative plants. Metabolites were extracted using a hot methanol extraction protocol in which 350 µl methanol (60°C) was added to each 30 mg FW sample [30]. Samples were incubated at 60°C for 10 min, vortexed, and placed in a sonicating water bath for 10 min. An equal volume of chloroform was added, samples were vortexed, and 300µl dH20 was added. Samples were vortexed and centrifuged for 5 min at full speed. Polar fractions were transferred to GC-MS glass vials in a volume dependent manner (150 µl/30 mg FW) and dried in a speed-vac concentrator. Samples were analyzed on an Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA) and data acquisition, metabolite identification, and normalization was performed as in Fiehn et al. (2008) [31]. Data processing was completed using ChromaTOF v. 2.32 (Leco, St. Joseph, MI) and raw values were obtained as peak heights and normalized to the total average mTIC (mean Total Ion Chromatogram, average of identified metabolites). Identified metabolites were classified into metabolic classes according to structure. Two-tailed, equal variance t-tests were used to determine significance between homozygous plants positive and negative for the *WRKY76* transgene.

### *2.7. Determination of Photosynthetic Carbon Fixation*

Photosynthetic measurements were collected at one month after planting with a CI-340 Photosynthesis meter (CID, Camas, WA). Measurements were collected on the widest portion of 10 uppermost, fully expanded leaves from homozygous transgene positive and negative plants. Plants were selected for analysis that reflected average measurements for chlorophyll, leaf length and width, and tiller number within transgene positive and negative genotypes. Data collection occurred over four days during the second half of the photoperiod. Data was collected as in Smidansky et al. (2007) [32] under ambient $CO_2$ with the meter zeroed to 402 µL $L^{-1}$ (http://co2now.org, accessed May 2015). Instrument settings consisted of an open system with a default flow rate of 0.3 $min^{-1}$, an added interval of 5 sec, and a 6.25 $cm^2$ leaf area chamber. Lighting conditions were held constant at 600 µE $m^{-2}$ $s^{-1}$ PAR. Temperature and humidity remained constant within the growth chamber at 26.5°C and 60% respectively. Comparisons were determined using two-tailed, paired t-tests.

### *2.8. Analysis of Plant Growth and Yield*

For each event, transgene positive and negative seed was de-hulled, surface sterilized, and germinated on 0.5 MS media, pH 5.8 in 7.5 x 7.5 x 8 cm Magenta GA-7 boxes (Magenta LLC, Chicago, IL) as in Smidansky et al. (2003) [33]. Magentas were placed in an incubator under artificial lights delivering 80 µE $m^{-2}$ $s^{-1}$ PAR with a 12 hour day length and temperature set at 25°C until plants were approximately six cm tall. Seedlings were transplanted into 3.8 cm diameter x 21 cm deep conetainers containing Profile Greens calcified clay (Profile Products LLC, Buffalo Grove, IL) with 4.93 cc of 1:0:1 Ironite (4.5% Fe) Mineral Supplement (Ironite, Walnut Creek, CA) mixed throughout. Transplanting occurred at a rate of one plant per conetainer. Conetainers were placed in growth chambers with a 12 hr photoperiod and an initial temperature regime of 25°C /24.5°C day/night temperature. After one week, temperature was adjusted to 26.5°C day/24.5°C night. Artificial lighting provided 750 µE $m^{-2}$ $s^{-1}$ PAR at mature canopy height. Once plants reached 15 cm in height, they were flood irrigated in 10 cm deep tubs. Plants received 100 g N $L^{-1}$ daily using Peter's Excel 15-5-15 Cal-Mag (Everris, Marysville, OH) [34]. Plants were allowed to self-pollinate and were harvested at maturity. Vegetative biomass was placed in a 37°C drying chamber for one week prior to weighing and seeds were placed in a 37°C drying chamber for two days prior to weighing.

Plant growth measurements were collected as in Oiestad et al. (2016) [9] at one month after planting, anthesis, and maturity (60 DPA). All measurements were collected on a per plant basis. Chlorophyll data was collected with a SPAD 502 chlorophyll meter (Minolta Co. Ltd, Tokyo, Japan) and represent three random readings per plant at one month after planting. At subsequent growth stages, chlorophyll values represent the average of three readings from the flag leaf associated with the tallest panicle. Plant height was measured to the top node of the tallest tiller at one month after planting, and to the base of the tallest panicle at subsequent growth stages. Leaf measurements were collected on the flag leaf associated with the uppermost node of the tallest tiller. Similar trends were observed across events, therefore data was combined for all events. Comparisons were determined using one-tailed, equal variance t-tests with the hypothesis that presence of the transgene would lead to increased plant vigor and yield.

## 3. Results

### *3.1. Analysis of WRKY76 Transgenic Lines*

*Agrobacterium* mediated transformation with the pRBC*WRKY76* construct (Figure 1) resulted in glufosinate resistant $T_0$ hemizygous plantlets from which $T_1$ seed segregating for glufosinate resistance and the *WRKY76* transgene was obtained. Progeny testing was carried out on $T_{1:2}$ plants. Herbicide screening coupled with PCR analysis indicate that the *bar* and *WRKY76* transgenes were co-integrated with all plants PCR positive for *WRKY76* also resistant to 0.1% glufosinate and PCR negative plants were glufosinate susceptible (Table 1). Chi-square analysis indicates that integration of the transgenes occurred at a single locus for each of the events (Table 1). Events WRKY 1, 5, and 12 were advanced

based on transgene expression.

***Table 1.*** *Transgene analysis of $T_{1:2}$ rice lines overexpressing WRKY76 and segregating 1:2:1 for the transgene.*

| WRKY Event | Progeny Test[a] | Chi Square | *P*-value |
|---|---|---|---|
| 1 | 10/21/5 | 2.38 | 0.30 |
| 5 | 5/19/13 | 2.18 | 0.17 |
| 12 | 14/19/8 | 1.98 | 0.37 |

[a] Number of $T_1$ derived $T_2$ progeny homozygous positive/heterozygous/homozygous negative for the *WRKY76* transgene. Progeny tests consisted of herbicide resistance screening of each line with a 0.1% active ingredient glufosinate solution for the *bar* gene, as well as PCR screening for the *WRKY76* transgene. In each event the *bar* herbicide resistance marker and *WRKY76* transgene co-segregated.

### *3.2. Gene Expression in Response to WRKY76 Overexpression*

Transcript abundance was examined via RNA-seq in source leaves harvested at the end of the photoperiod at one month after planting. On average, each sample yielded 535 Mb of sequence with 97.7% of reads with quality scores above Q30, indicating high sequence quality. After normalization across samples to *ACT1* (Os03g0718100), correlation expression between samples had an $r^2 > 0.98$ for the 30,524 *Oryza sativa* recognized genes. Average expression of *WRKY76* was 17,731 across transgene positive samples, compared to 72 for transgene negative samples (Table 2).

***Table 2.*** *Gene expression for starch biosynthesis and photosynthesis genes of interest in source leaves of WRKY76 transgene positive and negative plants[a].*

| | | | | ***WRKY76* Transgene Neg** | ***WRKY76* Transgene Pos** | **Pos/Neg** |
|---|---|---|---|---|---|---|
| Ensembl Gene | Protein | Gene symbol | Predominantly Expressed Tissue[b] | Ave ± SE[c] | Ave ± SE[c] | Ratio[c] |
| *Transgene* | | | | | | |
| Os09g0417600 | WRKY76 Transcription Factor | *WRKY76* | Leaf/ panicle/ seed/ carpel | 2.46 ± 0.51 | 606.5 ± 34.32 | 246.28*** |
| *Starch Biosynthesis* | | | | | | |
| Os05g0580000 | AGPase large subunit | *AGPL1* | All tissues (highest in post-flowering panicle) | 3.64 ± 0.02 | 10.90 ± 4.50 | 3.00 |
| Os01g0633100 | | *AGPL2* | Endosperm/ seed | 56.2 ± 0.6 | 57.4 ± 1.0 | 1.02 |
| Os03g0735000 | | *AGPL3* | Leaf/ panicle/ seed/ pistil/ endosperm | 348.6 ± 20.1 | 429.6 ± 22.1 | 1.23 |
| Os09g0298200 | AGPase small subunit | *AGPS1* | Panicle/ anther/ endosperm/ pistil/ embryo/seed | 29.3 ± 2.7 | 36.4 ± 2.1 | 1.24 |
| Os08g0345800 | | *AGPS2* | All tissues (highest in endosperm/ seed) | 294.8 ± 31.3 | 375.2 ± 20.2 | 1.27 |
| Os06g0160700 | Starch synthase (soluble) | *SSI* | All tissues (lowest in roots) | 36.6 ± 7.1 | 31.9 ± 4.0 | 0.87 |
| Os06g0229800 | | *SSIIa* | Panicle/ endosperm/ seed | 0.10 ± 0.02 | 0.02 ± 0.01 | 0.21** |
| Os02g0744700 | | *SSIIb* | Green tissues | 137.5 ± 7.3 | 137.9 ± 5.4 | 1.00 |
| Os10g0437600 | | *SSIIc* | All tissues (lowest in roots, anther, and carpel) | 12.2 ± 0.6 | 11.6 ± 0.4 | 0.95 |
| Os04g0624600 | | *SSIII* | All tissues (lowest in carpel, endosperm, and 10 DAF seed) | 107.6 ± 4.9 | 156.4 ± 9.3 | 1.45* |
| Os01g0720600 | | *SSIVa* | Leaf/ panicle/ pistil/ embryo | 16.1 ± 0.4 | 16.6 ± 0.9 | 1.03 |
| Os05g0533600 | | *SSIVb* | All tissues (lowest in carpel and pistil) | 59.9 ± 2.7 | 60.2 ± 1..8 | 1.00 |
| Os02g0807100 | | Probable *SSIV* | Panicle/ carpel/ seed | 27.8 ± 0.2 | 28.4 ± 1.0 | 1.02 |
| Os06g0133000 | Starch synthase (granule bound) | *GBSSI* | Endosperm/ seed | 4.64 ± 0.29 | 5.4 ± 0.47 | 1.16 |
| Os07g0412100 | | *GBSSII* | All tissues (lower in roots, endosperm, and 10 DAF seed) | 252.6 ± 18.2 | 443.9 ± 36.8 | 1.76* |
| Os06g0726400 | Starch branching enzyme | *SBE1* | Endosperm/ seed | 156.9 ± 4.6 | 144.9 ± 4.5 | 0.92 |
| Os02g0528200 | | *SBE3* | Endosperm/ seed | 3.91 ± 0.13 | 4.94 ± 0.38 | 1.26 |
| Os04g0409200 | | *SBE4* | All tissues | 273.9 ± 16.6 | 352.2 ± 17.8 | 1.29 |
| *Photosynthesis and Carbon Fixation* | | | | | | |
| Os03g0170900 | Sucrose Transporter | *SUT1* | All tissues (lowest in roots and carpel) | 56.8 ± 6.2 | 45.5 ± 2.1 | 0.80 |
| Os12g0641400 | | *SUT2* | All tissues (lowest in seeds) | 20.2 ± 0.4 | 22.2 ± 1.5 | 1.10 |
| Os10g0404500 | | *SUT3* | Anther (incomplete data set) | ND | ND | |
| Os02g0827200 | | *SUT4* | All tissues (lowest in endosperm, carpel, and seed) | 18.8 ± 0.3 | 19.4 ± 0.6 | 1.03 |
| Os02g0576600 | | *SUT5* | Panicle/ carpel/ pistil | ND | ND | |
| Os05g0168700 | Glucose-6-phosphate/phosphate-translocator | *GPT* | All tissue | 63.8 ± 1.8 | 56.0 ± 1.6 | 0.88* |
| Os08g0187800 | | *GPT2* | Endosperm/ seed | 28.5 ± 1.7 | 33.9 ± 1.4 | 1.19 |
| rbcL | RuBisCO large subunit | *RbcL* | Green tissues | 1,620 ± 251 | 1,997 ± 305 | 1.23 |
| Os12g0291100 | RuBisCO small subunit | *RbcS* | Green tissues | 34,444 ± 2,704 | 44,269 ± 2,530 | 1.29 |
| Os04g0486600 | Glyceraldehyde 3-phosphate dehydrogenase | *GAPDH* | Panicle/ pistil/ seed/ root | 347.5 ± 3.4 | 383.4 ± 8.6 | 1.10 |
| Os06g0136600 | | | Leaf/ panicle/ root | 886.4 ± 48.3 | 969 ± 37.9 | 1.09 |
| Os08g0440800 | NADP-dependent glyceraldehyde-3-phosphate | *NADP-GAPDH* | All tissues (lowest in endosperm and 10 DAF seed) | 111.8 ± 2.0 | 140.8 ± 5.2 | 1.26* |

| | | | | ***WRKY76* Transgene Neg** | ***WRKY76* Transgene Pos** | **Pos/Neg** |
|---|---|---|---|---|---|---|
| | dehydrogenase | | | | | |
| Os09g0535000 | Triose-phosphate isomerase | *TIM* | All tissues (lowest in endosperm) | 691.1 ± 77.8 | 867.1 ± 47.8 | 1.25 |
| Os01g0147900 | | *TPI* | All tissues (lowest in endosperm) | 1,683 ± 92 | 1,752 ± 70 | 1.04 |
| Os02g0698000 | Phosphoribulokinase | *Prk* | Leaf | 3,795 ± 268 | 4,987 ± 289 | 1.31 |
| *Housekeeping Genes* | | | | | | |
| Os03g0718100 | Actin 1 | *ACT1* | All tissues | 80.4 ± 0 | 80.4 ± 0 | 1.00 |
| Os02g0634800 | Ubiquitin conjugating enzyme | *UBC* | All tissues | 92.1 ± 3.1 | 86.8 ± 2.1 | 0.94 |

[a] Transgene positive samples were analyzed across WRKY events 1, 5 and 12 (n = 9) and compared to varietal control Nipponbare (n = 3). Samples were harvested from the uppermost, fully expanded leaf at the end of the photoperiod at one month after planting.
[b] Expression profiling of genes as described in Schlosser et al., (2014) and supported by RNA-seq data provided by Expression Atlas (http://, accessed October, 2017). Tissues listed indicate those in which expression levels are highest according to Expression Atlas.
[c] Expression on leaf tissue was not detected (ND).
*, **, *** Represent significance at $p$-value ≤ 0.05 and was determined using one-tailed, equal variance t-tests with the hypothesis that starch and carbon metabolism genes would be upregulated.

Expression of starch and photosynthetic genes was also examined in events overexpressing *WRKY76*. Although not significant, many genes for starch biosynthesis trended toward upregulation in transgene positive plants. These included subunits of AGPase, the rate-limiting enzyme in the starch biosynthesis pathway. Expression of *AGPL1* trended up by 300%, and *AGPL3,* the predominantly expressed large subunit in leaves, trended up by 23%. Small subunits *AGPL1* and *AGPL2* were both upregulated by an average of 25% (Table 2). Other starch genes with an upward trend in transcript abundance include *SSIII* (p-value = 0.04) and *GBSSII* (p-value = 0.04) which were upregulated by 45% and 76% respectively as well as *SBE3* (26%) and *SBE4* (29%) (Table 2). Many photosynthetic and carbon fixation genes also appear upregulated in *WRKY76* transgene positive plants, including both large and small RuBisCO subunits (approx. 26%), *NADP-GAPDH* (26%, significant at $p$-value ≤ 0.03), *TIM* (25%) and *Prk* (32%) (Table 2).

On a whole transcriptome level, three clusters of highly upregulated genes and 20 clusters of highly downregulated genes were identified via functional annotation clustering in response to overexpression of *WRKY76* in leaves (Table 3). Upregulated genes included those involved in protein biosynthesis, transcription regulation, and photosynthetic metal ion binding. Top downregulated clusters (Enrichment score > 1) include genes associated with serine/threonine protein kinases, ankyrin, sugar binding, membrane proteins, DNA binding proteins, signal response regulators, sugar transporters, and vesicle-mediated transport proteins. Of particular interest were clusters associated with transcription factor activity. Both upregulated Cluster 2 and downregulated Cluster 10 contained a member category titled WKRY, which included genes with WRKY domains (Table 4). WRKY genes that were upregulated include Os07g0111400, Os08g0499300, and Os12g0507300 (*WRKY30*). WRKY genes that were downregulated include Os01g0246700, Os01g0656400, Os02g0181300 (*WRKY71*), and Os09g0334500.

***Table 3.*** *Functional annotation clustering of highly up and down-regulated genes in response to overexpression of WRKY76 in leaves*[a].

| **Cluster** | **Function** | **Enrichment Score**[b] | **No. Member Categories** | **No. Genes** |
|---|---|---|---|---|
| *118 Genes upregulated in response to WRKY76 overexpression* | | | | |
| 1 | Protein Biosynthesis | 11.25 | 10 | ≥ 20 |
| 2 | Transcription Regulation | 0.62 | 14 | ≥ 9 |
| 3 | Photosynthetic Metal Ion Binding | 0.27 | 21 | ≥ 14 |
| *291 Genes downregulated in response to WRKY76 overexpression* | | | | |
| 1 | Serine/threonine protein kinase | 3.61 | 23 | ≥ 52 |
| 2 | Ankyrin | 2.31 | 3 | ≥ 7 |
| 3 | Sugar binding | 1.5 | 16 | ≥ 6 |
| 4 | Integral to membrane | 1.44 | 5 | ≥ 21 |
| 5 | DNA binding | 1.33 | 6 | ≥ 5 |
| 6 | Signal transduction response regulator | 1.23 | 4 | ≥ 3 |
| 7 | Sugar transporter | 1.11 | 5 | ≥ 9 |
| 8 | Vesicle-mediated transport | 1.02 | 8 | ≥ 4 |
| 9 | Potassium ion binding | 0.87 | 3 | ≥ 3 |
| 10 | Transcription factor activity | 0.8 | 16 | ≥ 19 |
| 11 | Armadillo | 0.78 | 3 | ≥ 4 |
| 12 | Membrane-bound vesicle | 0.76 | 4 | ≥ 17 |
| 13 | Cofactor binding | 0.68 | 3 | ≥ 10 |
| 14 | Leucine-rich repeat | 0.61 | 3 | ≥ 9 |

| Cluster | Function | Enrichment Score[b] | No. Member Categories | No. Genes |
|---|---|---|---|---|
| 15 | Ubiquitin ligase complex | 0.52 | 11 | ≥ 4 |
| 16 | Tyrosine protein kinase | 0.46 | 3 | ≥ 4 |
| 17 | Vitamin B6 binding | 0.36 | 4 | ≥ 3 |
| 18 | Cellular homeostasis | 0.32 | 3 | ≥ 3 |
| 19 | Peptide activity | 0.28 | 10 | ≥ 5 |
| 20 | Apoptosis | 0.26 | 6 | ≥ 6 |

[a] Genes found to be up or down-regulated at $p$-value ≤ 0.001 with two-tailed, equal variance t-tests were uploaded into the DAVID Functional Annotation Tool and clustered.
[b] Enrichment scores are the geometric mean (in –log scale) of $p$-values for member categories within each annotation cluster. Enrichment scores are used to rank biological significance. The higher the Enrichment Score, the lower the Fisher Exact $p$-value for each member category and the more biologically significant the annotation cluster. Functional annotation clusters with enrichment scores < 0.25 are not shown.

***Table 4.*** *WRKY genes with differential expression in response to WRKY76 overexpression in leaves[a].*

| Ensembl Gene | Name | *WRKY76* Transgene Neg Ave ± SE | *WRKY76* Transgene Pos Ave ± SE | Pos/Neg Ratio |
|---|---|---|---|---|
| *Upregulated in response to WRKY76* | | | | |
| Os07g0111400 | | 2.3 ± 0.3 | 4.5± 0.2 | 2.1 |
| Os08g0499300 | | 31.1 ± 0.9 | 69.3 ± 3.4 | 2.2 |
| Os12g0507300 | WRKY30 | 28.5 ± 0.5 | 41.0 ± 1.1 | 1.4 |
| *Downregulated in response to WRKY76* | | | | |
| Os01g0246700 | | 10.8 ± 0.8 | 5.6 ± 0.3 | 0.52 |
| Os01g0656400 | | 12.9 ± 1.0 | 4.8 ± 0.5 | 0.37 |
| Os02g0181300 | WRKY71 | 11.9 ± 1.2 | 3.9 ± 0.3 | 0.32 |
| Os09g0334500 | | 22.8 ± 1.1 | 9.1 ± 0.7 | 0.40 |

[a] Genes were significantly different between transgene positive and negative genotypes at a significance level of $p$-value ≤ 0.001.

### *3.3. Leaf Starch Levels*

At one month after planting, starch levels were significantly higher in WRKY events 5 and 12 transgene positive plants compared to transgene negative plants (Table 5). Although not significant, WRKY event 1 transgene positive plants trended 26% higher. When transgene positive and negative plants were combined and analyzed across events, plants overexpressing *WRKY76* were 44% ($p$-value = 0.001) higher in leaf starch compared to those with native starch levels.

***Table 5.*** *Leaf starch levels in response to increased WRKY76 expression at one month after planting[a].*

| | *WRKY76* Transgene Neg Ave ± SE | *WRKY76* Transgene Pos Ave ± SE | Pos/Neg Ratio | *P*-value[b] |
|---|---|---|---|---|
| WRKY 1 | 31.1 ± 5.4 | 39.3 ± 6.9 | 1.26 | 0.18 |
| WRKY 5 | 31.3 ± 4.4 | 43.5 ± 3.8 | 1.39 | 0.03 |
| WRKY 12 | 29.3 ± 4.4 | 54.5 ± 3.8 | 1.86 | 0.002 |
| Overall | 30.6 ± 2.6 | 44.0 ± 3.1 | 1.44 | 0.001 |

[a] Uppermost, fully expanded leaves were harvested at the end of the photoperiod at one month after planting.
[b] $P$-values are from one-tailed, equal variance t-tests with the hypothesis that expression of the *WRKY76* transgene would lead to increased leaf starch.

### *3.4. Metabolite Analysis*

Metabolite levels were examined at the end of the day in source leaves harvested one month after planting. A total of 145 metabolites were identified in an untargeted metabolomics study and classified into seven classes based on molecular structure. Metabolites that were significantly up or down regulated in response to *WRKY76* overexpression are plotted as a percentage of class in Figure 2. Carbohydrates were largely upregulated in plants overexpressing *WRKY76* across events. An average of 19% of the 34 identified carbohydrates were significantly higher in leaves from transgene positive plants while only 2% were downregulated. Organic acids also displayed net upregulation, with 10% of those identified being upregulated and 5% downregulated. Free amino acids were both up and downregulated while other nitrogen containing compounds were exclusively upregulated by an average of 9%.

### *3.5. Effect of WRKY76 Overexpression on Photosynthetic Rates*

Photosynthetic measurements were collected on source leaves during the second half of the photoperiod at one month after planting (Figure 3). Plants overexpressing *WRKY76* displayed significantly increased photosynthetic rates by an average of 15%. Increased photosynthetic rates corresponded with significant increases in transpiration and stomatal conductance in transgene positive plants by

averages of 21% and 31% respectively.

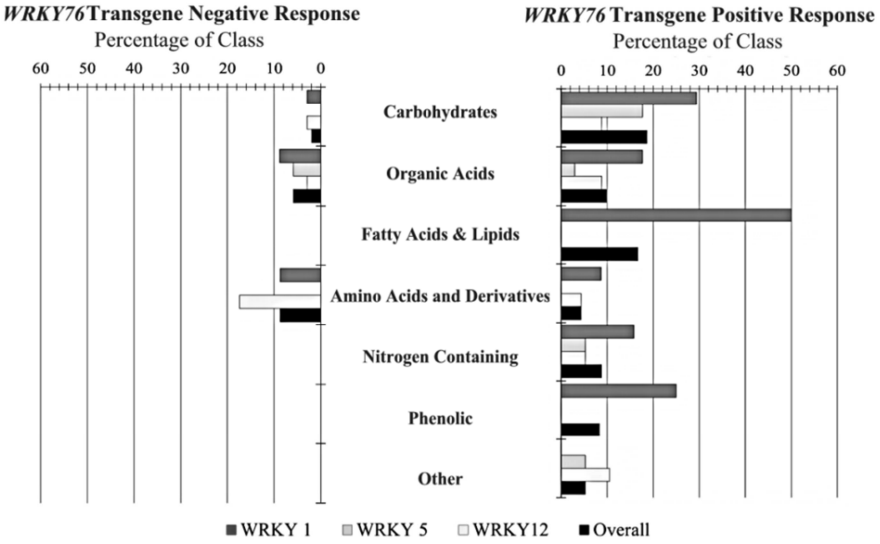


***Figure 2.*** *Metabolic response for plants with overexpression of WRKY76 in leaves. Metabolites were harvested from source leaves at one month after planting at the end of the photoperiod and the percentage of metabolites significantly up or down-regulated within each metabolic class was determined. Metabolites were identified via a GC-TOF MS/MS untargeted metabolomics study with a total of 145 identified metabolites. The total numbers of metabolites within each class are as follows: 34 carbohydrates, 34 organic acids, 8 fatty acids and lipids, 23 amino acids and derivatives, 19 nitrogen containing compounds, 8 phenolic compounds, and 19 metabolites classified as other.*

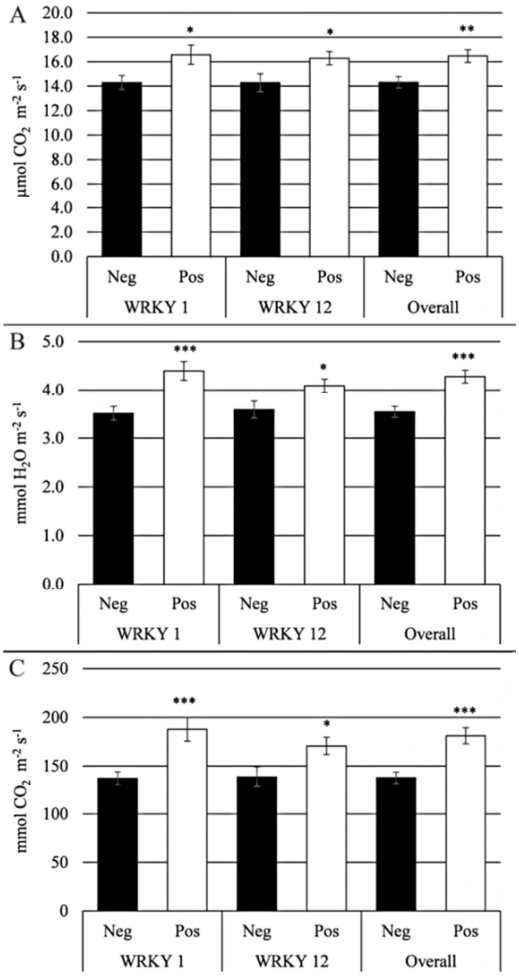


***Figure 3.*** *Photosynthetic gas exchange measurements collected on source leaves of WRKY76 transgene positive and negative plants at one month after planting during the second half of the photoperiod. Measurements included (A) photosynthetic rate, (B) transpiration, and (C) stomatal conductance. Significant differences are indicated: *,**,*** for p-values ≤ 0.05, 0.01, 0.001 respectively using two-tailed, paired t-tests.*

### *3.6. Plant Growth and Yield*

Plant growth measurements were collected at one month after planting, anthesis, and maturity for a NIL pair differing for the presence or absence of the *WRKY76* transgene (Figure 4). When data was averaged across events, the only trait that was significantly different at anthesis was leaf width (Figure 4). Plants overexpressing *WRKY76* had leaves that are approx. 5% wider. Once plants reached maturity, *WRKY76* transgene positive plants were higher yielding compared to transgene negative plants. Biomass was increased by 28% in transgene positive plants (3.23g vs. 2.53g) and seed weight per plant was increased by 26% (0.91g vs 0.73g). Individual seed weight was unchanged.

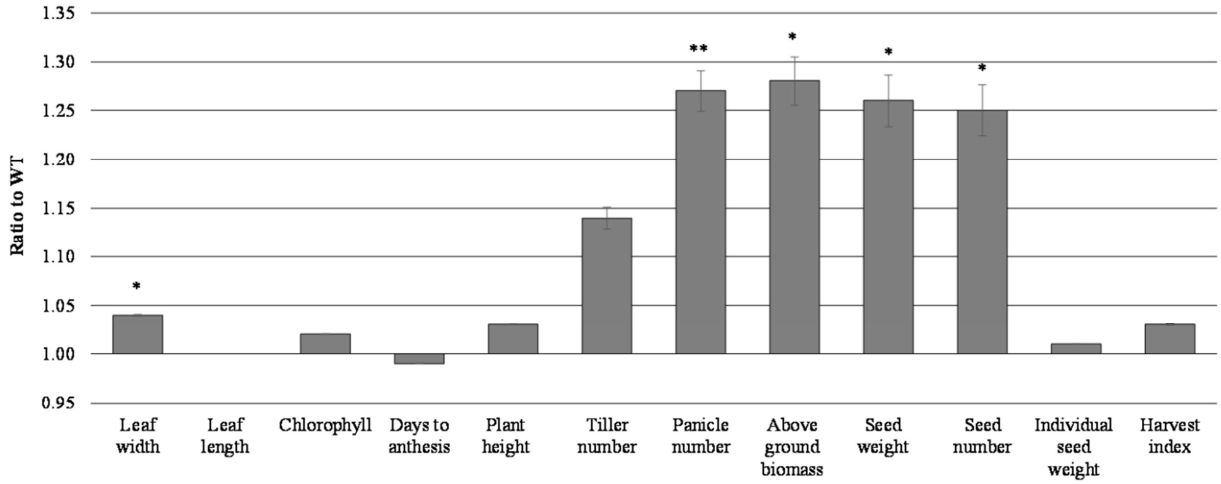


***Figure 4.*** *Influence of WRKY76 leaf overexpression on NIL plant phenotype. Sample size for homozygous negative plants was n = 11-15, for transgene positive plants n = 25-36. Leaf length, leaf width, chlorophyll, and days to anthesis were recorded at anthesis. All other traits were measured at maturity. Harvest index is the ratio of seed weight per plant to above ground biomass. *,**,*** Indicates significance at p-value ≤ 0.05, 0.01 and 0.001 respectively using one-tailed, equal variance t-tests with the hypothesis that presence of the transgene would lead to increased plant vigor and yield.*

## 4. Discussion

The starch biosynthesis pathway has been the focus of many studies as a means to increase cereal yields. Research has focused primarily on targeting individual enzymes within this pathway. AGPase in particular has received much attention, as it controls the rate limiting step of starch biosynthesis [3]. Although yield increases have been associated with upregulation of AGPase, it is likely that more stable yield increases would be observed via upregulation of the entire starch pathway. To this means, it would be advantageous to identify a transcription factor as a master controller for starch biosynthesis. Examination of highly upregulated genes in response to leaf AGPase overexpression (population developed by Schlosser et al. 2014 [8]) identified WRKY76 as a likely candidate for positive regulation of the leaf starch biosynthesis pathway. The WRKY TF family is a large family of plant specific proteins and it is believed that there are approximately 100 WRKY TFs within the rice genome [35, 36]. Most research regarding WRKY TFs has focused on their role in plant stress responses. There have been several WRKY TFs shown to function in metabolite biosynthesis, however much of this research has focused on alkaloid, terpene, and phenylpropanoid biosynthesis [12]. It is entirely possible that starch biosynthesis in leaves of rice may also be regulated by a member of this family.

In this study, *Agrobacterium*-mediated transformation resulted in integration of *WRKY76* at a single locus (Table 1) with large increases in gene expression (246X) (Table 2). Overexpression of *WRKY76* led to significant increases in leaf starch (40%) (Table 5). One of the underlying causes for enhanced leaf starch levels and plant yield observed in *WRKY76* transgene positive plants is likely the significant increase in photosynthetic gas exchange (Figure 3). Expectedly, increased photosynthetic rates were accompanied by significant increases in both transpiration and stomatal conductance (Figure 3), since these processes are closely linked [37]. It appears that overexpression of *WRKY76* resulted in photosynthetic gas exchange being more efficient on a per area basis since chlorophyll levels were unchanged between transgene positive and negative lines (Figure 4). The observed increased photosynthetic rates are in contrast to studies in which AGPase alone was upregulated in leaf tissue. In Schlosser et al. (2014) [8] and Oiestad et al. (2016) [9], overexpression of AGPase led to enhanced yield despite diminished photosynthetic rates. Here, increased photosynthetic rates along with the upregulation of carbon metabolites (Figure 2) provide evidence for a role of WRKY76 in regulating photosynthetic carbon fixation.

Although the precise mechanism of WRKY76 is unclear in this study, elevated photosynthesis and carbon metabolite pools would invoke a cascade effect among enzymes involved in carbon metabolism. This would include the starch biosynthesis pathway in leaves [2]. Contrary to our hypothesis, in this study increases in starch levels were not accompanied by significant increases in starch biosynthetic

gene expression (Table 2). Expression levels trended higher for *SSIII* and *GBSSII* ($p$-value = 0.04 for both) as well as for several AGPase subunits in response to *WRKY76* overexpression. Since AGPase controls the rate limiting step in starch biosynthesis, expression of the predominant leaf AGPase subunits were of particular interest. Both *AGPL3* and *AGPS2* trended 25% higher in transgene positive leaves. In fact, Nagata et al. (2012) [16] has shown that in Arabidopsis, the *AGPL3* promoter is positively regulated by the transcription factor AtWRKY20. Phylogenetic and comparative gene expression analysis of WRKY transcription factors support that WRKY speciation occurred prior to diversification of monocots and dicots. Therefore, WRKY function is retained across monocot and dicot species [38, 39, 40]. Unfortunately, BLAST results do not identify any homologous regions to *AtWRKY20* (At4g26640) within the rice genome.

WRKY76 in particular has been demonstrated to play a negative role in the plant response to rice blast disease as well as to positively regulate cold stress tolerance [41]. It is also possible that WKRY76 plays acts as a repressor of sugar transport in leaves. This would account for the accumulation of carbohydrates observed in response to *WRKY76* overexpression (Figure 2). Decrease in sugar transport activity can lead to a build up of sugars and starch. There are many sugar transporters involved in the transport of sugars between chloroplasts and the cytosol, including sucrose, glucose, triose phosphate/phosphate, and maltose transporters [42]. Knockdown in any one of these may result in altered leaf starch levels.

Interestingly, in this study overexpression of *WRKY76* led to significant changes in expression, both positive and negative, of other WRKY TFs. WRKY76 belongs to WRKY group IIa and possesses a single WRKY domain [43, 44, 45]; WRKYs in this group have been demonstrated to act as both positive and negative regulators of reactions [46, 47, 48]. Results demonstrated here indicate that WRKY76 plays a positive role in central carbon metabolism and leaf starch biosynthesis, though the mode of operation is presently unknown. Studies examining genome wide transcription, have shown that *WRKY76* has low native expression levels, with highest expression occurring in leaf and seed tissues [49, 50, 51, 52]. Our data supports these observations in leaves, with an expression value of 2.46 for *WRKY76* transgene negative plants (Table 4). If WRKY76 does not itself directly regulate the starch pathway, it is possible that it indirectly does via a network of other WRKY transcription factors. We found that overexpression of *WRKY76* led to significant upregulation of three WRKY TFs ($p$-value $\leq$ 0.001) as well as significant downregulation of four WRKY TFs. Of the seven WRKY TFs in which expression was significantly changed, all except one had higher base level expression values (Table 4). In fact, studies support the idea that WRKY TFs form regulatory networks involving other WRKY proteins [39, 53, 54]. Little is known regarding the interaction between WRKY TFs, however, this is an emerging area of interest. Research regarding the WRKY proteins with significant changes in expression show that WRKY30, which was upregulated, is involved in positive regulation of jasmonic acid and resistance to fungal pathogens in rice [55]. WRKY71, which was downregulated, is also involved in plant defense through gibberellic acid repression [56].

In agreement with the observation of higher photosynthetic rates, expression of many photosynthesis genes (Table 2, 3) trend up in response to *WRKY76* overexpression. For instance, photosynthetic metal ion binding was significantly upregulated on a global scale (Table 3), and expression of both RuBisCO subunits trended higher in response to the *WRKY76* transgene (Pos/Neg Ratios of 1.23 and 1.29) (Table 2). This is of interest because RuBisCO levels have been positively correlated with photosynthetic rate and starch level in leaves [57]. A 20% increase in net up-regulation of carbon metabolites was also observed in transgene positive plants at one month after planting (Figure 2). Broad increases in carbon metabolism were also observed in leaves when starch increases were the result of AGPase upregulation [9]. Here, it is interesting to note that 10% of the detected Organic Acids were upregulated in response to *WRKY76* overexpression (Figure 2). Organic acids serve as intermediates in many carbon fixation pathways and metabolism of organic acids has been associated with plant tolerance to environmental stress [58, 59]. It has been well established that WRKY proteins play important roles in plant stress responses [60]. The increase in organic acids due to overexpression of *WRKY76* may be beneficial in that the fate of individual metabolites of this class may be flexible and may contribute to plant growth and yield, or to stress tolerance.

Functional annotation clustering identified several clusters of genes related to signaling that were downregulated on a genome-wide level in *WRKY76* transgene positive leaves (Table 3). These clusters included genes with protein functions such as protein kinases, sugar binding, signal transduction response regulators, and sugar transporters. The most highly enriched cluster was for serine/threonine protein kinases, involved in protein phosphorylation. Alteration of the phosphorylation state of proteins is a post translational modification mechanism to regulate both plant signaling and metabolism [61, 62]. Interestingly, in the starch pathway SBEIIb and GBSSI have been identified as phosphoproteins in maize [63], and SSIIa, SSI, and GBSSI have been identified as phosphoproteins in wheat [64]. Furthermore, several studies have demonstrated that phosphorylation plays important roles in WRKY processes and may result in direct phosphorylation of WRKY TFs, cause changes in WRKY gene expression, and affect WRKY binding to promoter regions [65]. These results suggest that the mechanism by which WRKY76 functions results in a cascade effect for further regulatory control with broad implications for plant growth and development. Here, changes in plant phenotype were observed early in plant growth with overexpression of *WRKY76* resulting in significantly wider leaves (Figure 4).

In addition to changes in leaf width, overexpression of *WRKY76* resulted in a 28% and 26% increase in biomass and

seed yield respectively (Figure 4). There are several related factors that contribute to the enhanced phenotypes observed in *WRKY76* transgene positive plants. The first influencing factor is increased photosynthetic rates. Photosynthesis was increased on a per area basis (Figure 2), and although not measured would also be increased on a per plant basis as a combination of wider leaves, increased plant biomass, and elevated photosynthetic rate. In wheat, even small increases in net photosynthesis have been associated with increased biomass and yield [66]. In addition to increased photosynthetic rates, enhanced leaf starch levels in *WRKY76* transgene positive lines likely contributed to yield increases via enhanced source strength, as has been observed in multiple studies with overexpression of AGPase in rice leaves [6, 7, 8, 9]. This approach resulted in similar biomass increases in plants overexpressing *WRKY76* as in AGPase upregulation (approx. 25% increase). Interestingly, seed weight per plant was significantly increased by 26% in this study. Upregulation of AGPase alone led to a trend (not significant) of 10-25% increase in seed weight per plant in rice depending on which AGPase transgene was used [6, 7, 8, 9].

## 5. Conclusions

The results demonstrated here indicate that the transcription factor WRKY76 is involved in central carbon metabolism in leaves. Overexpression of *WRKY76* led to significant increases in photosynthetic carbon fixation, which was associated with a 40% increase in leaf starch as well as a 20% upregulation of carbon metabolism. At this point, it is unknown whether the observed leaf starch increases are the result of direct regulation by *WRKY76*, indirect regulation of overall carbon metabolism by WRKY76, or result from complex transcriptional regulation by multiple transcription factors. Here we provide evidence that *WRKY76* expression is associated with up and down regulation of gene expression of other WRKY TFs. The enhanced plant phenotypes observed here in association with overexpression of *WRKY76* and increased leaf starch provide further evidence of the complicated regulatory control surrounding photosynthesis and carbon metabolism, and provide insight into the importance of source strength for plant growth and yield.

## Abbreviations

AGPase, ADP-glucose Pyrophosphorylase; TF, Transcription Factor

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