Title: Novel transcription factors *PvBMY1* and *PvBMY3* increase biomass yield in greenhouse-grown switchgrass (*Panicum virgatum* L.)

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Abstract

Increasing crop yield requires the coordination of multiple metabolic pathways spanning photosynthetic carbon fixation, central carbon metabolism, and finally targeted carbon deposition to end product. In this study, we used a transcriptome-based gene regulatory association network to search for transcription factor genes that could play a role in increasing carbon flow through pathways associated with these processes to increase biomass yield in switchgrass. Two novel switchgrass transcription factors, PvBMY1 (*BioMass Yield 1*, belonging to the APETALA2/Ethylene Response Factor family of transcription factors) and PvBMY3 (*BioMass Yield 3*, a member of the Nuclear-Factor Y family of transcription factors), with predicted roles in the regulation of photosynthesis and related metabolism were identified. These genes were overexpressed in switchgrass to determine their impact on biomass yield. A significant increase in both aboveground and root biomass was observed in transgenic greenhouse grown plants compared to wild-type control plants with the best line producing 160% more aboveground biomass than controls. Transgenic lines with elevated electron transport rate of photosystems I and II as well as increased levels of starch and soluble sugars were identified.

Keywords: transcription factor; biomass; crop yield; switchgrass; photosynthesis

Abbreviations: TF, transcription factor

1. Introduction

The global population is expected to reach a staggering total of 9.6 billion people by year 2050 [1] which will require food production to minimally increase by at least 60-70% [2] and possibly double over the next 35 years [3]. This combined with the use of crops to produce renewable biofuels and biobased materials has placed pressure on agriculture to deliver greater outputs. Increased pressure on the availability of arable land due to increased infrastructure and already limited water resources in some geographies will require significant improvement in crop productivity to meet these demands [2, 4]. New approaches are required to meet these challenges and will be based on integrating current state of the art plant breeding with advanced biotechnology and systems biology that incorporate metabolic and other quantitative models for gene discovery ([5], this issue).

Achieving the necessary step change increases in crop yield to meet this demand will require building better plants with increased carbon fixation where the additional fixed carbon is targeted to the desired organ, either harvestable seed or biomass depending on the crop. There have been significant efforts to increase the efficiency of photosynthesis including for example improving the activity of the carbon fixation enzyme RuBisCo (reviewed in [6-9]), reducing the impact of photorespiration [10], manipulation of photoprotection [11], and expressing genes to increase the activities of key enzymes within the Calvin Cycle (reviewed in [8]). These prior efforts to improve photosynthesis have had some success in increasing plant yield, depending on the method, but may not have realized their full potential since only limitations in the front end of the system, carbon fixation, are being addressed. The complexity of multiple plant regulatory mechanisms and/or rate limiting metabolic steps makes debottlenecking the flow of carbon from

photosynthesis to harvestable organ a daunting task that will likely require targeted manipulation of multiple metabolic enzymes.

The use of transcription factors (TFs) is a promising approach for increasing crop productivity since multiple pathways can be regulated by manipulating the expression of one gene ([12]; [5], this issue). With the identification of the right transcription factor(s), one could potentially address the need to both improve photosynthesis and address multiple downstream bottlenecks that prevent the increased flow of carbon from reaching the intended harvestable product. A great amount of effort has already been devoted to genomics and bioinformatics approaches to identify transcription factors by both academia and industry. This work has often included large scale plant screening to characterize the genes [13]. However only a limited number of transcription factors have been subjected to extensive functional characterization and, for the most part, their postulated functions are based on DNA or protein sequence homology. This is a major limitation of modern genomics.

In this study, we searched for candidate transcription factors to increase plant yield through the analysis of a transcriptome-based gene regulatory association network. Similar networks have been used previously to identify transcription factors associated with starch biosynthesis [14], higher grain yield and stress tolerance [15], seed desiccation tolerance [16], and wheat spike architecture [17] (reviewed [5], this issue). We looked for genes associated with photosynthesis and related carbon metabolism with the goal of identifying candidate genes that would enable increased carbon fixation and the efficient flow of this carbon to the production of biomass. For this work, we chose switchgrass (*Panicum virgatum* L.), a perennial C_4 grass

native to North America that is considered a leading candidate among potential dedicated bioenergy crops due to its high biomass production on marginal land and tolerance to a wide range of climate and soil conditions [18]. We have developed an efficient *Agrobacterium*-mediated transformation procedure [19, 20] allowing us to use switchgrass as a robust C_4 monocot system for analyzing the function of genes. The orthologous gene sequences of two previously uncharacterized transcription factors, PvBMY1 and PvBMY3, were identified using a rice transcriptome-based gene regulatory association network [15]. These genes were cloned behind a heterologous promoter with high activity in green tissue and introduced into switchgrass for functional characterization. An increase in several parameters of photosynthesis as well as total biomass production was observed. These findings suggest that PvBMY1 and PvBMY3 are transcription factors that have a role in increasing carbon fixation and converting it to harvestable biomass.

2. Materials and Methods

2.1. Identification of transcription factor genes

2.1.1. Identification of PvBMY1 and PvBMY3

Candidate rice transcription factors that potentially play a role in photosynthetic processes were identified using the rice regulatory association network of Ambavaram et al. [15], essentially as previously described [15]. Switchgrass orthologous TFs were identified using reciprocal BLAST searches between the rice and switchgrass genomes (Fig. 1A). To predict specific key biological pathways of interest in switchgrass (for example, central carbon metabolism), we used rice co-expression network modules, which can infer causal relationships

(i.e., directionality of interaction), to scale down the number of genes in a specific module of interest (for example, photosynthesis related processes, carbohydrate metabolism, etc). Using a subset of functional modules, the function of uncharacterized TF genes was predicted by associating them with genes with known function based on a "guilt by association approach", where genes (homologous and/or orthologous) that have similar mRNA expression profiles are often functionally related. The pool of genes was further reduced by examining *in silico* expression patterns of the rice TFs. The expression pattern of a select number of candidate switchgrass TF genes was experimentally determined (Section 2.4.2) and PvBMY1 and PvBMY3 (Table S1) were chosen for functional characterization. PvBMY1 has been previously referred to as PvERF164 in a study where 207 members of the switchgrass AP2/ERF family of transcription factors were identified, but PvBMY1 was not functionally characterized [21].

2.1.2. Identification of orthologs of PvBMY1 and PvBMY3 in multiple crops

Orthologs of PvBMY1 and PvBMY3 were identified by performing reciprocal BLAST searches against proteins encoded by genes in the plant genome of interest. The National Center for Biotechnology Information (NCBI) database (https://blast.ncbi.nlm.nih.gov), The Phytozome (database from the Joint Genome Institute (https://phytozome.jgi.doe.gov)), The Michigan State University Rice Genome Annotation Project database (http://rice.plantbiology.msu.edu), and The Plant Transcription Factor Database (http://planttfdb.cbi.pku.edu.cn) were used for the BLAST searches and to extract the orthologous gene sequences and gene ID's (Table S2). Multiple sequence alignments in the vicinity around the transcription factor binding domain for the orthologs from select plants was performed using the clustal omega multiple sequence alignment web based tool (https://www.ebi.ac.uk/Tools/msa/clustalo/) (Figure S1).

2.2. Construction of plant transformation vectors

Binary vectors pMBXS809 and pMBXS855, derivatives of pCAMBIA3300 (Centre for Application of Molecular Biology to International Agriculture, Canberra, Australia), contain the switchgrass cDNAs for the *PvBMY1* and *PvBMY3* genes, respectively, under the control of the promoter from the maize chlorophyll a/b-binding protein [22]. This promoter is equivalent to the *cab-m5* promoter described in other work [23, 24]. The *cab-m5* promoter is fused to the maize heat shock protein 70 gene (*hsp70*) intron [25] for enhanced expression in monocots. The *PvBMY1* or *PvBMY3* gene is flanked at the 3' end by the *nos* terminator of the nopaline synthase gene [26]. The cDNA sequences of the *PvBMY1* and *PvBMY3* genes were synthesized by a commercial vendor and contain the native codon usage of the genes. These vectors also contain the pCAMBIA3300 expression cassette for the *bar* gene (encoding phosphinothricin acetyltransferase which confers resistance to bialaphos) that contains the cauliflower mosaic virus (CaMV) *e35S* promoter (contains a duplicated enhancer region), the *bar* gene, and the CaMV polyadenylation signal, for callus and plant selection. An *hsp70* intron was inserted between the *e35S* promoter and the *bar* gene to increase expression of the selectable marker in monocots as previously described [19].

2.3. Plant material and transformation

Switchgrass genotype 56 [19] from the lowland cultivar Alamo was used for transformation experiments. Immature inflorescence callus cultures were initiated and propagated as described previously [27, 28]. *Agrobacterium*-mediated transformation of these highly embryogenic cultures with the vectors pMBXS809 and pMBXS855 was performed using

strain AGL1 and subsequent selection of cultures and plants with 10 mg l^{-1} bialaphos was performed according to previously published protocols [19, 27, 29]. Multiple plants originating from a single bialaphos-resistant callus line (Table S3) were considered a transgenic line. Wild-type plants obtained from untransformed callus cultures were transferred to soil and used as controls. The growth conditions of plants in tissue culture and soil were as described previously [19].

2.4. Molecular analyses

2.4.1. PCR

Primary switchgrass transformants were identified in tissue culture by PCR as described previously [19] using the following pairs of primers: 5'- CTCTCCTTACCTCCTGATGGTATC-3' and 5'-GTTTCTCTCCCATCAGCTTCAG-3' for the *PvBMY1* gene and 5'-GCACAGGCTTCATACTACATGGGT-3' and 5'-ACTTGTGGAGGTAGAGCTTGAGTG-3' for the *PvBMY3* gene.

2.4.2. Gene expression analyses in switchgrass

Gene expression levels in transgenic and wild-type switchgrass plants were assessed by RT-PCR and qRT-PCR using β-actin as a reference. Total RNA was isolated from different switchgrass tissues using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. DNase treatment and column purification were performed as described previously [19] and RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technolgies, Santa Clara, CA, USA) according to the manufacturer's instructions.

The levels of the *PvBMY1* and *PvBMY3* transcripts (Figure 1B) were determined by RT-PCR from five month old greenhouse grown plants in different organs from reproductive R1 tillers [30]: young and mature leaves (phytomers 2 and 4, respectively, according to [31]), leaf sheath, internode (phytomer 4), root, and panicle. The RT-PCR analysis was performed with 50 ng of total RNA using One Step RT-PCR Kit (Qiagen, Valencia, CA, USA) as described previously [28] under the following amplification conditions: 50°C for 30 min; 95°C for 15 min; 94°C for 1 min, 55°C for 30 sec, 72°C for 1 min (30 cycles); extension at 72°C for 15 min, using the following pairs of primers: 5' CACAGTCACCAGCTTCTCCA 3' and 5' TCAGTAAACACCACGGTCCA 3' for PvBMY1; 5' AGGAGTGCGTCTCCGAGT 3' and 5' GACGGCGTTGTCTCTCTCTG 3' for PvBMY3.

For identification of TF-overexpressing lines in tissue culture, RNA was isolated from the second youngest leaf from primary transformants prior to transfer to soil and the quantitative differences in the expression levels of *PvBMY1* and *PvBMY3* genes in transgenic and WT plants were determined by qRT-PCR (Table S4). Three independent biological replicates for each transcription factor gene with two technical replicates of each biological replicate were used for expression analysis. For each biological replicate, total RNA (500 ng) was converted into cDNA using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The cDNA was diluted to 250 µL and subjected to real-time PCR using Fast SYBR® Green Master Mix (Life Technologies, Carlsbad, CA, USA) in an Applied Biosystems 7500 Fast Real-Time PCR system. Validation experiments were performed on five to six log dilutions of each of the target genes together with the β-actin reference to test if the amplification efficiencies were equal. To determine relative fold differences for each sample in each experiment, the threshold cycle (Ct) value for each gene

was normalized to the Ct value for β-actin and was calculated relative to a calibrator using the equation $2^{-\Delta\Delta Ct}$ [32].

2.4.3. Gene expression analyses in maize

The expression pattern of the maize orthologs of *PvBMY1* (Gene ID: GRMZM2G110333) and *PvBMY3* (Gene ID: GRMZM2G384528) were determined by RT-PCR analysis. Maize plants (inbred line B73 obtained from The North Central Regional Plant Introduction Station, Iowa State University) were grown in a greenhouse and tissue at different developmental stages was harvested. The levels of amplification products (Figure 5C) were measured in 50 ng of total RNA using One Step RT-PCR Kit (Qiagen, Valencia, CA, USA) as described previously [28] using the following pairs of primers: 5'CGTGTTTGGCTTGGTACTTTC3' and 5'GGAAGTGATGTCTGGTGTCTT3' for the maize ortholog *PvBMY1*; 5'GTCGGAGTTCATCTCCTTCATC3' and 5' TCATCATGATCATACCGCTTCC3' for the maize ortholog of *PvBMY3*. Amplification conditions were as follows: 50°C for 30 min; 95°C for 15 min; 94°C for 1 min, 55°C for 30 sec, 72°C for 1 min (30 cycles); extension at 72°C for 15 min.

2.5. Measurements of photosynthetic activity

For analyses of the photosynthesis rate in plants overexpressing the transcription factors, various parameters were measured in light adapted leaves using a Dual-PAM-100 Measuring System (Heinz Walz GmbH, Effeltrich, Germany). All measurements were performed with the phytomer 2 leaf of 2-3 E2 tillers [30, 31] per plant in the morning on a sunny day. Experiments were performed using the automated light intensity response curve with repetitive saturation

pulses for assessment of chlorophyll fluorescence and P700 parameters. The photochemical quantum yields (Y) and the electron transport rates (ETR) of photosystem I (PSI) and photosystem II (PSII) were derived from these measurements by the software [33, 34].

2.6. Biochemical analyses

All measurements were performed with the phytomer 2 leaf of 2-3 E2 tillers per plant (see above). Leaf blades were sampled around noon on a sunny day, snap frozen in liquid nitrogen and used for determination of the contents of products of primary metabolism and photosynthetic pigments.

2.6.1. Starch and soluble sugars

The leaf tissue was ground in liquid nitrogen, freeze-dried for 3 days, and the resulting leaf powder was used for measurements of the levels of starch, sucrose and glucose as described previously [35]. Briefly, starch content was measured in 40 mg of tissue after extraction with 80% ethanol to remove soluble sugars. The percentage of starch per dry weight was determined as glucose using a quantitative, enzymatic Starch Assay Kit (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. Sucrose and glucose contents were determined in water extracts of 50 mg of leaf tissue by HPLC using a Shodex KS-801 column and a refractive index detector. The HPLC was run with a mobile phase of DI water, a column temperature of 65°C, and a flow rate of 0.9 mL/min. Preliminary experiments showed that no other monosaccharides were detectable in switchgrass leaves at this developmental stage under the conditions used.

2.6.2. Photosynthetic pigments

The contents of chlorophyll a, chlorophyll b, and carotenoids were determined in 30 mg (fresh weight) of leaf tissue following a previously described procedure [36].

2.7. Biomass yield

Growth of switchgrass for the biomass measurements described in Table 3 and Figure 4 were conducted in two separate growing seasons in two different greenhouses.

In the first greenhouse growth experiment, biomass measurements were performed with primary transformants (Table 3, Table S3). Transgenic and wild-type plants (see Table 3 for sample size) were grown in 3-gallon pots filled with Sunshine MVP soil mix (Sun Gro Horticulture; http://www.sungro.com/files/catalogues/SunshineMMTechno_Catalogue_2013.pdf) in a greenhouse set at 28^{o}C with supplemental lighting (16-h photoperiod, sodium halide lamps) for five months. For each transgenic and wild-type plant, all vegetative and reproductive tillers at different developmental stages were counted and cut below the basal node. Leaf blades and stem tissues (nodes, internodes, leaf sheaths, and panicles) were separated, cut into smaller pieces, air-dried at 27°C for 12-14 days and dry weight measurements were obtained.

For biomass measurements in the second growth experiment (Figure 4), multiple transgenic plants were obtained from immature inflorescence derived cultures obtained from individual primary transformants. These cultures were initiated and propagated as described previously [27, 28]. Cloned plants for each line and wild-type controls (n=4) were grown in 3-gallon pots filled with Sunshine MVP soil mix in a greenhouse set at 28^{o}C with supplemental lighting (16-h photoperiod, sodium halide lamps) for five months. Due to gene containment

regulations in the greenhouse, the developing panicles (out of the boot) were removed before anthesis. To measure the production of aboveground biomass, all tillers were counted, cut, and dried as described above. The roots were washed and air dried prior to the measurements of their weight.

2.8. Statistical analyses

For biomass measurements in the second growth experiment (Fig. 4), four plants for each line and wild-type controls were analyzed using Student's t-test. All t-tests performed were two sided.

3. Results

3.1. Expression and in silico analyses of candidate transcription factor genes in switchgrass

A previously published rice regulatory association network, developed based on genome wide expression profiles [15, 37], was used as a query to identify rice genes with a predicted role in photosynthesis and related primary metabolic processes using methods similar to those described previously [15]. Reciprocal BlastN and BlastP searches between the genomes of rice and switchgrass resulted in the identification of approximately five hundred orthologous switchgrass candidate TF genes with a predicted involvement in these processes (Fig. 1A). To further narrow down the selection of candidate switchgrass TFs for functional analysis, we used rice co-expression network modules to predict the function of uncharacterized TF genes and examined the *in silico* expression pattern of their rice orthologs using the rice expression atlas [38]. These analyses restricted our list to approximately twenty candidate genes that were preferentially expressed in green tissues and/or that had a positive association to photosynthesis.

The expression pattern of select genes was experimentally verified using RT-PCR to determine transcript accumulation in leaves at different developmental stages, as well as stem tissues and roots from wild-type switchgrass plants. Two orthologous genes, Pavirv00046166m and Pavirv00029298m (Table S1), that showed strong association to 'photosynthetic carbon metabolic processes' in the rice co-expression network and possessed the desired expression profile were selected for further study. *PvBMY1* expression was strongest in the leaves and panicles and low in the internode and leaf sheath (Figure 1B). It had little to no detectable expression in roots. In contrast, *PvBMY3* was expressed in all tissues examined with slightly higher levels observed in mature leaves and panicles (Figure 1B). Based on the predicted functions of these genes and subsequent functional characterization studies that demonstrated increased biomass formation upon heterologous expression (Section 3.4), genes Pavirv00046166m and Pavirv00029298m were named *PvBMY1* (*BioMass Yield 1*) and *PvBMY3* (*BioMass Yield 3*), respectively. A multiple amino acid sequence alignment (Figure S1) and domain search analysis across a plant transcription factor database (PlnTFDB; http://plntfdb.bio.uni-potsdam.de) revealed that PvBMY1 belongs to APETALA2/Ethylene Response Factor (AP2/ERF) family of transcription factors [39] whereas PvBMY3 has a NFYB-HAP3 domain that is typical for members of the Nuclear-Factor Y (NF-Y) family of TFs [40]. Orthologs of *PvBMY1* and *PvBMY3* were found to be widespread in terrestrial plants (Table S2).

3.2. Generation of switchgrass lines overexpressing PvBMY1 and PvBMY3

The full-length switchgrass cDNA coding sequences of PvBMY1 and PvBMY3 were used to produce genetic constructs pMBXS809 and pMBXS855, respectively, for *Agrobacterium*-mediated transformation of switchgrass. These vectors contained the

transcription factor of interest driven by the maize *cab-m5* promoter of the chlorophyll a/b-binding protein gene [22-24] fused to the maize heat shock protein 70 gene (*hsp70*) intron [25]. The *cab-m5* promoter has been previously shown to be light inducible and expressed in both mesophyll and bundlesheath cells of maize, with some preference for mesophyll [24]. This promoter and intron combination has previously worked well in our laboratory for engineering switchgrass for the production of the biopolymer polyhydroxybutyrate (PHB) [19]. A genotype from the lowland switchgrass cultivar Alamo, genotype 56, was used to generate transgenic lines for functional analyses of PvBMY1 and PvBMY3 through *Agrobacteirum*-mediated transformation of highly embryogenic callus cultures [19, 27, 29]. *PvBMY1* T_0 plants were obtained from eight independent bialaphos-resistant callus lines (Table S3) approximately three to five months after transformation. Similarly, *PvMBY3* T_0 plants originating from nine independent bialaphos-resistant callus lines were generated. Prior to transfer to soil, the primary transformants were tested for stable transgene expression by qRT-PCR. Transgenic plants that expressed the introduced TF gene at levels significantly higher (2 to 7.9 fold increase) than the background levels detected in the wild-type (WT) plants were identified (Table S4) and transferred to soil and grown in a greenhouse for further analyses. Non-transgenic, wild-type plants (regenerated from untransformed callus cultures) grown under the same conditions were used as controls.

3.3. Biochemical and physiological analyses of transgenic lines

Transgenic plants and the wild type controls were grown in a greenhouse for two months prior to the biochemical and physiological analyses. At this stage of rapid growth, the plants contain only vegetative tillers at different stages of leaf development and stem elongation. This

experimental design allowed for the elimination of the influence of changes in source-sink relationships and metabolite allocation that accompany reproductive development and senescence in perennial turf grasses [41]. In addition, the natural variation of biochemical processes among the phytomers of the same tiller [42] was minimized by using leaves at the same developmental stage.

Comparative measurements of various photosynthetic parameters were conducted with light adapted leaves. The functioning of photosystem I (PSI) and photosystem II (PSII) was primarily studied in terms of photochemical quantum yield (Y) and electron transport rate (ETR). In most of the transgenic plants analyzed, the effective quantum yield of PSII [Y(II)], which represents the portion of absorbed quanta that is converted into chemically fixed energy by the PSII reaction centers, was increased at a photosynthetic photon flux density (PPFD) in the range of 46-849 µmol photons m^{-2} s^{-1} (results for select transgenic lines shown in Figure 2). Increases in the effective quantum yield of PSI [Y(I)] primarily occurred in *PvBMY1* lines (up to 185% compared to the control). Notable increases in the electron transport rate of PSII (the rate of non-cyclic electron transfer), and the electron transport rate of PSI (both cyclic and non-cyclic electron transfer), were detected in all transgenic lines analyzed at PPFD $\geq$77 µmol m^{-2} s^{-1} with the greatest increases (up to 160% and 165% to the control) in the *PvBMY1* lines.

Transgenic lines were also characterized by measuring starch and soluble sugars (sucrose and glucose) in leaf tissue (Table 1). These non-structural carbohydrates were found to be present at increased levels in some of the lines analyzed. The *PvBMY1* line 7 accumulated the highest levels of starch and soluble sugars producing 9.77$\pm$1.80% DW and 4.87$\pm$1.17% DW,

respectively. These values are 4.8 times and 2.5 times the levels observed in the wild-type control. The *PvBMY3* lines 2 and 6 produced 5.00±1.02% DW and 6.41±5.32% DW starch, respectively compared to the control value of 2.88 ± 0.99% DW. PvBMY3 lines 5 and 6 showed the highest levels of soluble sugars producing 3.18±1.24% DW and 3.57±3.06% DW, respectively compared to the control value of 1.86±0.26% DW. The high standard deviations observed in some of the measurements (Table 1) could be due in part to the transient nature of starch and sugar turnover in plants.

The levels of photosynthetic pigments chlorophyll and carotenoids were also increased in most of the lines analyzed (Table 2).

3.4. Biomass production in transgenic lines

Biomass production of select transgenic lines was assessed in plants grown in soil for 5 months. The phenotype of individual representative transgenic plants before biomass harvest is shown in Figure 3. About 50% of the tillers in both the transgenic lines and the wild-type controls were at different stages of reproductive development. The contribution of leaf (dry weight of leaf blades) and stem biomass (dry weight of leaf sheaths, nodes, internodes, and panicles) to the total biomass yield was determined. Although standard deviations of some lines including the wild-type controls were high, all of the transgenic lines produced more biomass than the wild-type controls (Table 3), which was mainly based on the increased tillering capacity. *PvBMY1* line 4 and the *PvBMY3* line 5 accumulated the highest amounts of biomass, producing 139% and 133% to the control, respectively. The highest numbers of tillers were detected in

PvBMY1 line 6, which possessed 52% more tillers than WT, and in *PvBMY3* line 2, which had 62% more tillers than the control.

A similar experiment was performed with plants regenerated from immature inflorescence derived cultures obtained from some of the transgenic lines with high biomass production. In this growth experiment, the developing panicles emerged from the boot were removed before anthesis due to gene containment regulations of the greenhouse used for the experiments. After five months of growth, the aboveground biomass and the roots (free of soil) were separated, dried and measurements were obtained. All three of the PvBMY1 lines tested had a significantly increased ($p\ value \leq 0.01$) total biomass compared to WT (Figure 4), producing 75-100% more aboveground biomass and 85-140% more root biomass than WT. Two out of three PvBMY3 lines showed a significantly higher biomass production compared to WT with 100-160% more leaves and stems (*p values of* ≤ 0.01 *or* ≤ 0.05*, depending on the line*) and approximately 40% more root biomass ($p\ value \leq 0.01$).

3.5. Expression pattern of maize orthologs of PvBMY1 and PvBMY3

Increasing biomass yield is important for crops such as switchgrass, biomass sorghum, silage corn, or forage crops such as alfalfa, but not for commodity grain crops such as corn and soybean. Since orthologs of *PvBMY1* and *PvBMY3* are present in crops where grain is the primary product (Table S2), we were interested in determining the expression pattern of these orthologs in developing seed tissue. *In silico* analysis of gene expression from the maize Electronic Fluorescent Pictograph browser [43] was performed and maize orthologs of both *PvBMY1* and *PvBMY3* were found to be expressed in a broad range of maize tissues including roots, leaves, silks, developing seed and whole seed (Figure 5 A, B). The highest levels of

expression were found in the endosperm tissue of seeds 12 and 16 days after pollination (DAP). The *in silico* analysis was followed with measurements of the expression levels of the transcription factor genes in greenhouse grown maize (inbred line B73) by RT-PCR. Our experimental data was similar to the *in silico* analysis, with observed expression of the *PvBMY1* and *PvBMY3* maize orthologs in leaves at different stages, the pre-pollination cob, and the whole seed 12 DAP (Figure 5C).

4. Discussion

The need to enhance crop productivity to provide food, fiber, and fuel for a growing global population is a well recognized critical task [4, 8, 44, 45]. Improved farming and crop management practices as well as increased stress tolerance achieved by genetic engineering or conventional breeding have been the primary sources of enhanced yield in recent decades. However, these traditional approaches appear to have reached their biological limit [4, 44, 46]. The identification of transcription factors controlling the expression of genes in multiple biosynthetic pathways spanning carbon fixation to harvestable end product, either biomass or seed depending on the crop, is a promising strategy to remove biological barriers currently limiting crop productivity.

In this study, we used transcriptome-based gene regulatory association networks to search for genes predicted to improve photosynthesis and other pathways of central carbon metabolism with the goal of increasing yield. Switchgrass was chosen for this work since, in our hands, it is a good model system for C_4 monocots that allows routine testing of genes due to a highly efficient *Agrobacterium*-mediated transformation system (reviewed in [20]). Switchgrass is also a promising, low input, high biomass yielding, perennial bioenergy crop that can be used for the

production of fuels through lignocellulosic conversion, pyrolysis, gasification, or simple combustion [47] as well as for the production of polymers [19, 20, 35] and commodity chemicals [20, 48]. Due to the limited availability of switchgrass genomic information when our study was initiated, a rice gene network [15] was used to identify candidate transcription factor genes with a predicted role in photosynthesis and central carbon metabolism. This rice gene network was used previously to identify the HYR transcription factor that, when transformed back into rice, produced plants with higher grain yield and improved environmental stress tolerance [15]. To identify switchgrass orthologs for genes selected from the rice gene network, we performed reciprocal BLAST searches.

Two candidate genes from this gene network analysis, *PvBMY1* and *PvBMY3*, were chosen for functional analysis. Transgenic plants overexpressing these genes were generated and thoroughly analyzed. Comparative measurements of the effective photochemical quantum yield of photosystems I and II [Y(I) and Y(II), respectively] and electron transport rate (ETR) of photosystems I and II [ETR(I) and ETR(II), respectively] demonstrated that the overexpression of the TFs resulted in an overall improvement of photosynthetic activity under normal growth conditions. The biggest changes were found in ETR(I) and ETR(II). The enhanced ETR(I) observed in all of the transgenic lines analyzed could indicate increased cyclic electron transport around PSI which provides the additional ATP needed for the CO_2-concentrating mechanism [49]. The increased Y(II) and the higher rates of electron transport detected in the transgenic plants may have contributed to the increased accumulation of starch and soluble sugars (Table 1) in some lines. Although little is known about transitory starch metabolism in C_4 plants, it has been proposed that starch synthesis may be crucial as an over flow mechanism allowing the

plants to maintain a photosynthetic rate higher than the rate of sucrose synthesis and export [50], potentially reducing inhibitory feedback on photosynthesis [51].

Consistent with the observed increase in photosynthesis and higher levels of sugars and starch in some plants, the transgenic lines produced more biomass than the wild-type control plants (Table 3). This was especially apparent in the growth of lines obtained from immature inflorescence derived cultures of the primary transformants (Figure 4). In these lines, very large increases in aboveground and root biomass were observed with the best plants producing up to 160% more aboveground biomass than controls. These results indicate a significant improvement in the efficiency of the conversion of solar energy to biomass, which suggests that PvBMY1 and PvBMY3 also regulate genes involved in other biological processes that help enable carbon deposition into end product. The faster growth, higher rate of biomass accumulation, and increased tiller formation capacity, all represent powerful sinks for photosynthates which could reduce feedback inhibition of photosynthesis.

Although overexpression of *PvBMY1* and *PvBMY3* has similar promotive effects on photosynthesis and carbohydrate metabolism leading to enhanced biomass production in switchgrass, they are members of different families of transcription factors, belonging to the APETALA2/Ethylene Response Factor (AP2/ERF) and the Nuclear-Factor Y (NF-Y) families, respectively. Genes encoding 207 members of the AP2/ERF family have previously been identified in the switchgrass genome using protein sequences of rice AP2/ERF orthologs, but to date only one of these transcription factors, named PvERF001, has been functionally characterized [21]. To our knowledge, no members of the switchgrass NF-Y family have been functionally characterized. Further studies on the gene expression perturbations induced by the overexpression of PvBMY1 and PvBMY3 will help identify their downstream targets and

provide a systems global view of the multiple layers of regulation and control underlying the achieved trait enhancement.

Orthologous genes were found in many other crops (Table S2) including maize and soybean, the two major commodity crops in North America, and rice, the staple food for half of the world's population [52]. To determine the pattern of expression of similar genes in a grain crop, we chose to examine the *in silico* expression pattern of the orthologous genes in maize. Surprisingly, these genes had their highest level of expression in seed tissue (Fig. 5). Our experimental examination of the expression pattern of the genes confirmed that the *PvBMY1* and *PvBMY3* maize orthologs were expressed in leaves important for providing photoassimilates during seed formation, as well as in the pre-pollination cob and the whole seed 12 days after pollination (Fig. 5). This could suggest a possible role for the transcription factor genes in regulating processes during seed formation and warrants further study.

Our use of a transcriptome-based gene regulatory association network to identify candidate regulatory genes predicted to play a role in photosynthesis and related metabolism has resulted in the identification of two unique transcription factors, *PvBMY1* and *PvBMY3*, that when overexpressed in switchgrass, led to significantly increased biomass formation. These genes are distinct from the switchgrass transcription factors that have previously been functionally characterized [21, 53-56] and we are not aware of reports on experimental validation of the function of orthologs of PvBMY1 and PvBMY3 from other crops. The work presented here is another example of the power of the transcriptome-based gene regulatory association network approach ([5], this issue) to discover regulatory genes that are associated with multiple metabolic pathways to contribute to the improvement of a crop trait.

Author contributions. M.S., M.A., K.S., & O.P. conceived the project and original research plans; M.A., M.S., A. A., and K. R. performed the experiments; M.A., M.S., A. A., K. R., and K.S. analyzed the data; K.S., M.A. and M.S. wrote the paper. All authors read and approved the final version of the paper.

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Tables

Table 1. Products of primary carbon metabolism in transgenic lines overexpressing *PvBMY1* and *PvBMY3*

Gene	Line	Starch content			Soluble sugars		
		Range [% DW]	Average [% DW]	No. plants	Range [% DW]	Average [% DW]	No. plants
PvBMY1	1	1.38-1.57	1.49 ± 0.14	2	1.59-2.30	1.98 ± 0.36	3
PvBMY1	2	0.95-1.94	1.44 ± 0.70	2	1.87-2.06	1.95 ± 0.10	3
PvBMY1	3	1.31-1.53	1.42 ± 0.15	2	2.12-4.49	3.09 ± 1.24	3
PvBMY1	4*	4.49-5.95	5.07 ± 0.78	3	2.50-3.07	2.75 ± 0.29	3
PvBMY1	5*	1.82-4.97	3.67 ± 1.65	3	2.32-2.52	2.40 ± 0.11	3
PvBMY1	6*	4.31-4.75	4.48 ± 0.24	3	2.39-2.66	2.50 ± 0.14	3
PvBMY1	7*	8.06-11.66	9.77 ± 1.80	3	4.04-5.69	4.87 ± 1.17	2
WT		1.03-3.58	2.02 ± 1.15	4	1.58-2.41	1.91 ± 0.29	8
PvBMY3	1	2.39 - 2.78	2.53 ± 0.22	3	1.55 - 2.03	1.78 ± 0.24	3
PvBMY3	2*	4.28 - 5.72	5.00 ± 1.02	2	1.56 - 2.15	1.91 ± 0.31	3
PvBMY3	3*	2.73 - 5.12	3.93 ± 1.69	2	2.12 - 2.96	2.54 ± 0.60	2
PvBMY3	4*	2.66 - 4.19	3.54 ± 0.79	3	1.97 - 2.60	2.36 ± 0.34	3
PvBMY3	5*	2.13 - 9.20	4.90 ± 3.78	3	1.76 - 4.09	3.18 ± 1.24	3
PvBMY3	6*	1.90 - 12.27	6.41 ± 5.32	3	1.66 - 7.10	3.57 ± 3.06	3
WT		2.18 - 3.58	2.88 ± 0.99	2	1.58 - 2.13	1.86 ± 0.26	4

Average values are presented as the mean ± standard deviation. For each transgenic and control line, data were obtained from the indicated number of plants. Abbreviations are as follows: WT, wild-type plant; % DW, percentage of leaf dry weight. *Lines used for biomass analysis (Table 3).

Table 2. Leaf content of photosynthetic pigments in transgenic switchgrass lines overexpressing the transcription factor genes *PvBMY1* and *PvBMY3*

Gene	Line	Chlorophyll [mg g^{-1} FW]	No. plants	Carotenoids [mg g^{-1} FW]	No. plants
PvBMY1	1	1.65 ± 0.25	3	0.238 ± 0.025	3
PvBMY1	2	1.57 ± 0.03	2	0.22 4± 0.004	2
PvBMY1	3	1.02 ± 0.08	2	0.141 ± 0.003	2
PvBMY1	4*	1.66 ± 0.57	3	0.252 ± 0.072	3
PvBMY1	5*	1.34 ± 0.16	3	0.199 ± 0.024	3
PvBMY1	6*	2.31 ± 0.03	2	0.324 ± 0.016	2
PvBMY1	7*	1.25 ± 0.09	2	0.190 ± 0.029	2
WT		0.97 ± 0.22	4	0.148 ± 0.042	4
PvBMY3	1	1.30 ± 0.19	4	0.202 ± 0.030	4
PvBMY3	2*	1.22 ± 0.20	3	0.183 ± 0.024	3
PvBMY3	3*	1.29 ± 0.03	2	0.175 ± 0.004	2
PvBMY3	4*	1.35 ± 0.06	4	0.210 ± 0.008	4
PvBMY3	5*	1.25 ± 0.06	3	0.189 ± 0.012	3
PvBMY3	6*	1.15 ± 0.14	2	0.132 ± 0.005	2
WT		0.97 ± 0.22	4	0.148 ± 0.042	4

Values are presented as the mean value ± standard deviation of milligrams of pigment per gram of leaf fresh weight. For each transgenic and control line, data was obtained from the indicated number of plants. Abbreviations are as follows: WT, wild-type plant; FW, fresh weight. *Lines used for biomass analysis (Table 3).

Table 3. Biomass yield of transgenic switchgrass lines overexpressing *PvBMY1* and *PvBMY3*

Gene	Line	Sample size (*No. of plants*)	Biomass [g DW]			Number of tillers[1]
			total	leaf	stem	
PvBMY1	4	3	77.99 ± 10.99	20.51 ± 1.17	57.49 ± 12.14	31.0 ± 7.0
PvBMY1	5	3	70.14 ± 8.36	19.04 ± 3.45	50.89 ± 5.36	30.3 ± 8.7
PvBMY1	6	3	73.01 ± 7.17	22.17 ± 3.57	50.71 ± 3.61	36.0 ± 8.5
PvBMY1	7	2*	63.49 ± 7.25	18.95 ± 0.32	44.25 ± 7.99	26.5 ± 0.7
WT		3	56.09 ± 18.42	17.32 ± 3.56	38.77 ± 15.75	23.7 ± 3.5
PvBMY3	2	2	64.70 ± 1.22	22.68 ± 0.40	42.03 ± 1.62	38.5 ± 3.5
PvBMY3	3	2	68.94 ± 2.18	20.98 ± 0.67	47.97 ± 2.85	32.0 ± 2.8
PvBMY3	4	4	69.23 ± 7.06	21.47 + 1.87	47.76 + 6.15	36.5 ± 10.7
PvBMY3	5	3	74.71 ± 4.64	21.58 ± 1.81	53.13 ± 3.05	37.7 ± 4.2
PvBMY3	6	3	69.84 ± 7.07	22.21 ± 2.90	47.63 ± 4.25	37.3 ± 1.5
WT		3	56.09 ± 18.42	17.32 ± 3.56	38.77 ± 15.75	23.7 ± 3.5

All measurements represent the mean value of the indicated number of plants ± the standard deviation. *WT*, wild-type control plants. [1]Total number of vegetative and reproductive tillers at different developmental stages (emerging tillers not included). *One plant dried out during experiment and was not included.

Figure legends

Figure 1. Identification of candidate TFs predicted to increase yield in switchgrass. (A) Flow chart describing the main steps used for gene identification in this study. (B) RT-PCR analysis of the expression levels of the *PvBMY1*, *PvBMY3*, and β-actin (control) genes in switchgrass wild-type plants. The tissue samples for RNA isolation were collected from a single tiller at anthesis.

Figure 2. Photosynthetic activity in transgenic switchgrass lines overexpressing the *PvBMY1 and PvBMY3* transgenes. For each line, values are derived from two plants and are presented as mean ± standard deviation. *WT*, wild type (non-transformed) plants; *Y(II)*, effective photochemical quantum yield of photosystem II (PSII); *ETR(II)*, electron transfer of PSII; *Y(I)*, effective photochemical quantum yield of photosystem I (PSI); *ETR(I)*, electron transfer of PSI; *PPFD*, photosynthetic photon flux density (μmol photons m^{-2} s^{-1}).

Figure 3. **Phenotype of transgenic and control plants.** Individual plants were grown under greenhouse conditions for five months. Aboveground biomass harvested from the plants shown is as follows: PvBMY1-6 (65.95 g); PvBMY3-2 (65.56 g); wild-type (59.80 g).

Figure 4. Biomass production in *PvBMY1* and *PvBMY3* lines. Plants were generated from immature inflorescence derived cultures of primary transformants (the control plants were obtained from immature inflorescence cultures from non-transformed plants) and grown in soil for 5 months before harvest. Individual plants from *PvBMY1* lines (A) and their roots (B). (C) Total biomass in *PvBMY1* lines. Bars represent mean values ± standard deviation from

measurements of 4 plants per line (n=4). The same number of wild-type plants served as control. Individual plants (D) from *PvBMY3* lines and their roots (E). (F) Total biomass production in *PvBMY3* lines (n=4). Asterisks indicate levels of significance; * $P \leq 0.01$, ** $P \leq 0.05$)

Figure 5. Expression pattern of the maize orthologs of *PvBMY1* and *PvBMY3*. *In silico* analysis of the expression pattern of the maize orthologs *PvBMY1* (A) and *PvBMY3* (B) in different organs and developmental stages in maize. Data was retrieved from the maize Electronic Fluorescent Pictograph browser (http://bar.utoronto.ca/efp_maize/cgi-bin/efpWeb.cgi). Levels of expression signals are in FPKM units (Fragment Per Kilobase of exon per Million fragments mapped). FPKM estimates the relative transcript abundance of each gene by combining the expression of all the transcripts of a gene. (C) RT-PCR analysis of different organs at different developmental stages of greenhouse grown maize plants (inbred line B73). *Z. mays* GRMZM2G110333 is the maize ortholog of *PvBMY1*. *Z. mays* GRMZM2G384528 is the maize ortholog of *PvBMY3*.

References

[1] T. Searchinger, C. Hanson, J. Ranganathan, B. Lipinski, R. Waite, R. Winterbottom, A. Dinshaw, R. Heimlich, Creating a sustainable food future: A menu of solutions to sustainably feed more than 9 Billion people by 2050. World Resources Institute Report 2013-2014: Interim Findings, 14 (2013).

[2] R.Q. Grafton, C. Daugbjerg, M.E. Qureshi, Towards food security by 2050, Food Secur., 7 (2015) 179-183.

[3] D.K. Ray, N.D. Mueller, P.C. West, J.A. Foley, Yield trends are insufficient to double global crop production by 2050, PLoS One, 8 (2013) e66428.

[4] Stephen P. Long, A. Marshall-Colon, X.-G. Zhu, Meeting the global food demand of the future by engineering crop photosynthesis and yield potential, Cell, 161 (2015) 56-66.

[5] F.A. Skraly, M.R. Ambavaram, O.P. Peoples, K.D. Snell, Metabolic engineering to increase crop yield: From concept to execution, Plant Sci. (submitted), (2018).

[6] M.A.J. Parry, P.J. Andralojc, J.C. Scales, M.E. Salvucci, A.E. Carmo-Silva, H. Alonso, S.M. Whitney, Rubisco activity and regulation as targets for crop improvement, J. Exp. Bot., 64 (2013) 717-730.

[7] J. Singh, P. Pandey, D. James, K. Chandrasekhar, V.M.M. Achary, T. Kaul, B.C. Tripathy, M.K. Reddy, Enhancing C3 photosynthesis: an outlook on feasible interventions for crop improvement, Plant Biotechnol. J., 12 (2014) 1217-1230.

[8] W. Yamori, Improving photosynthesis to increase food and fuel production by biotechnological strategies in crops, J. Plant Biochem. Physiol. , 1 (2013) doi:10.4172/2329-9029.1000113.

[9] Ü. Niinemets, J.A. Berry, S. von Caemmerer, D.R. Ort, M.A.J. Parry, H. Poorter, Photosynthesis: ancient, essential, complex, diverse … and in need of improvement in a changing world, New Phytol., 213 (2017) 43-47.

[10] M. Hodges, Y. Dellero, O. Keech, M. Betti, A.S. Raghavendra, R. Sage, X.-G. Zhu, D.K. Allen, A.P.M. Weber, Perspectives for a better understanding of the metabolic integration of photorespiration within a complex plant primary metabolism network, J. Exp. Bot., 67 (2016) 3015-3026.

[11] J. Kromdijk, K. Głowacka, L. Leonelli, S.T. Gabilly, M. Iwai, K.K. Niyogi, S.P. Long, Improving photosynthesis and crop productivity by accelerating recovery from photoprotection, Science, 354 (2016) 857-861.

[12] E. Grotewold, Transcription factors for predictive plant metabolic engineering: are we there yet?, Curr. Opin. Biotech., 19 (2008) 138-144.

[13] S. Sivasankar, R.W. Williams, T.W. Greene, Abiotic Stress Tolerance in Plants: An Industry Perspective, in: Improving Crop Resistance to Abiotic Stress, Wiley-VCH Verlag GmbH & Co. KGaA, 2012, pp. 27-47.

[14] F.-F. Fu, H.-W. Xue, Coexpression analysis identifies Rice Starch Regulator1, a rice AP2/EREBP family transcription factor, as a novel rice starch biosynthesis regulator, Plant Physiol., 154 (2010) 927-938.

[15] M. Ambavaram, S. Basu, A. Krishnan, V. Ramegowda, U. Batlang, L. Rahman, N. Baisakh, A. Pereira, Coordinated regulation of photosynthesis in rice increases yield and tolerance to environmental stress, Nat. Commun., 5 (2014) 5302.

[16] S.I. González-Morales, R.A. Chávez-Montes, C. Hayano-Kanashiro, G. Alejo-Jacuinde, T.Y. Rico-Cambron, S. de Folter, L. Herrera-Estrella, Regulatory network analysis reveals novel regulators of seed desiccation tolerance in *Arabidopsis thaliana*, P. Natl. Acad. Sci. USA, 113 (2016) E5232-E5241.

[17] Y. Wang, H. Yu, C. Tian, M. Sajjad, C. Gao, Y. Tong, X. Wang, Y. Jiao, Transcriptome association identifies regulators of wheat spike architecture, Plant Physiol., (2017) DOI: https://doi.org/10.1104/pp.1117.00694.
[18] A. Monti, Switchgrass: A Valuable Biomass Crop for Energy, in: Green Energy and Technology, Springer, London, 2012.
[19] M.N. Somleva, K.D. Snell, J.J. Beaulieu, O.P. Peoples, B.R. Garrison, N.A. Patterson, Production of polyhydroxybutyrate in switchgrass, a value-added co-product in an important lignocellulosic biomass crop, Plant Biotechnol. J., 6 (2008) 663-678.
[20] M.N. Somleva, O.P. Peoples, K.D. Snell, PHA bioplastics, biochemicals, and energy from crops, Plant Biotechnol. J., 11 (2013) 233-252.
[21] W. Wuddineh, M. Mazarei, G. Turner, R. Sykes, S. Decker, M. Davis, N. Stewart, Identification and molecular characterization of the switchgrass AP2/ERF transcription factor superfamily, and overexpression of PvERF001 for improvement of biomass characteristics for biofuel, Front. Bioeng. Biotechnol., 3 (2015) 101.
[22] T. Sullivan, A. Christensen, P. Quail, Isolation and characterization of a maize chlorophyll a/b binding protein gene that produces high levels of mRNA in the dark., Mol. Gen. Genet., 215 (1989) 431-440.
[23] T. Becker, T. Templeman, J. Viret, L. Bogorad, The cab-m7 gene: a light-inducible, mesophyll-specific gene of maize., Plant Mol. Biol., 20 (1992) 49-60.
[24] J.-Y. Sheen, L. Bogorad, Differential expression of six light-harvesting chlorophyll a/b binding protein genes in maize leaf cell types, P Natl Acad Sci USA, 83 (1986) 7811-7815.
[25] S.M. Brown, C.G. Santino, Enhanced expression in plants, US Patent 05593874, (1997).
[26] M. Bevan, W.M. Barnes, M.-D. Chilton, Structure and transcription of the nopaline synthase gene region of T-DNA, Nucleic Acids Res., 11 (1983) 369-385.
[27] M.N. Somleva, Switchgrass, in: K. Wang (Ed.) *Agrobacterium* Protocols, Humana Press, Totowa, NJ, 2006, pp. 65-74.
[28] M.N. Somleva, C.A. Xu, K.P. Ryan, R. Thilmony, O. Peoples, K.D. Snell, J. Thomson, Transgene autoexcision in switchgrass pollen mediated by the Bxb1 recombinase, BMC Biotechnol., 14 (2014) 79.
[29] M.N. Somleva, Z. Tomaszewski, B.V. Conger, *Agrobacterium*-mediated genetic transformation of switchgrass, Crop Sci., 42 (2002) 2080-2087.
[30] K.J. Moore, L.E. Moser, K.P. Vogel, S.S. Waller, B.E. Johnson, J.F. Pedersen, Describing and quantifying growth stages of perennial forage grasses, Agron. J., 83 (1991) 1073-1077.
[31] K.J. Moore, L.E. Moser, Quantifying developmental morphology of perennial grasses, Crop Sci., 35 (1995) 37-43.
[32] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method, Methods, 25 (2001) 402-408.
[33] C. Klughammer, U. Schreiber, Complementary PS II quantum yields calculated from simple flourescence parameters measured by PAM fluorometry and the saturation pulse method, PAM Application Notes, 1 (2008) 27-35.
[34] E. Pfundel, C. Klughammer, U. Schreiber, Monitoring the effects of reduced PS II antenna size on quantum yields of photosystems I and II using the Dual-PAM-100 measuring system, PAM Application Notes, 1 (2008) 21-24.
[35] R. McQualter, M. Somleva, L. Gebbie, X. Li, L. Petrasovits, K. Snell, L. Nielsen, S. Brumbley, Factors affecting polyhydroxybutyrate accumulation in mesophyll cells of sugarcane and switchgrass, BMC Biotechnol., 14 (2014) 83.
[36] H.K. Lichtenthaler, Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes, in: Methods Enzymol., Academic Press, 1987, pp. 350-382.
[37] M. Ambavaram, A. Krishnan, K.R. Trijatmiko, A. Pereira, Coordinated activation of cellulose and repression of lignin biosynthesis pathways in rice, Plant Physiol., 155 (2011) 916-931.

[38] Y. Jiao, S.L. Tausta, N. Gandotra, N. Sun, T. Liu, N.K. Clay, T. Ceserani, M. Chen, L. Ma, M. Holford, H.Y. Zhang, H. Zhao, X.W. Deng, T. Nelson, A transcriptome atlas of rice cell types uncovers cellular, functional and developmental hierarchies, Nat. Genet., 41 (2009) 258-263.
[39] E. Magnani, K. Sjölander, S. Hake, From endonucleases to transcription factors: Evolution of the AP2 DNA binding domain in plants, Plant Cell, 16 (2004) 2265-2277.
[40] X.-Y. Li, R. Mantovani, R. Hooft van Huijsduijnen, I. Andre, C. Benoist, D. Mathis, Evolutionary variation of the CCAAT-binding transcription factor NF-Y, Nucleic Acids Res., 20 (1992) 1087-1091.
[41] N. Palmer, A. Saathoff, B. Waters, T. Donze, T. Heng-Moss, P. Twigg, C. Tobias, G. Sarath, Global changes in mineral transporters in tetraploid switchgrasses (*Panicum virgatum* L.), Front. Plant Sci., 4 (2014) 549.
[42] C. Hardin, C. Fu, H. Hisano, X. Xiao, H. Shen, C. Stewart, W. Parrott, R. Dixon, Z.-Y. Wang, Standardization of switchgrass sample collection for cell wall and biomass trait analysis, BioEnergy Res., (2013) 1 - 8.
[43] P. Li, L. Ponnala, N. Gandotra, L. Wang, Y. Si, S.L. Tausta, T.H. Kebrom, N. Provart, R. Patel, C.R. Myers, E.J. Reidel, R. Turgeon, P. Liu, Q. Sun, T. Nelson, T.P. Brutnell, The developmental dynamics of the maize leaf transcriptome, Nat Genet, 42 (2010) 1060-1067.
[44] D.R. Ort, S.S. Merchant, J. Alric, A. Barkan, R.E. Blankenship, R. Bock, R. Croce, M.R. Hanson, J.M. Hibberd, S.P. Long, T.A. Moore, J. Moroney, K.K. Niyogi, M.A. Parry, P.P. Peralta-Yahya, R.C. Prince, K.E. Redding, M.H. Spalding, K.J. van Wijk, W.F. Vermaas, S. von Caemmerer, A.P. Weber, T.O. Yeates, J.S. Yuan, X.G. Zhu, Redesigning photosynthesis to sustainably meet global food and bioenergy demand, Proc. Natl. Acad. Sci. USA, 112 (2015) 8529-8536.
[45] S. de Bossoreille de Ribou, F. Douam, O. Hamant, M.W. Frohlich, I. Negrutiu, Plant science and agricultural productivity: why are we hitting the yield ceiling?, Plant Sci., 210 (2013) 159-176.
[46] V. Srinivasan, P. Kumar, S.P. Long, Decreasing, not increasing, leaf area will raise crop yields under global atmospheric change, Glob. Change Biol., 23 (2017) 1626-1635.
[47] K.D. Snell, O.P. Peoples, PHA bioplastic: A value-added coproduct for biomass biorefineries, Biofuels, Bioprod. Bioref., 3 (2009) 456-467.
[48] C.A. Mullen, A.A. Boateng, D. Schweitzer, K. Sparks, K.D. Snell, Mild pyrolysis of P3HB/switchgrass blends for the production of bio-oil enriched with crotonic acid, J. Anal. Appl. Pyrol., 107 (2014) 40-45.
[49] O. Kiirats, D.M. Kramer, G.E. Edwards, Co-regulation of dark and light reactions in three biochemical subtypes of C_4 species, Photosynth. Res., 105 (2010) 89-99.
[50] S.E. Weise, K.J. van Wijk, T.D. Sharkey, The role of transitory starch in C(3), CAM, and C(4) metabolism and opportunities for engineering leaf starch accumulation, J. Exp. Bot., 62 (2011) 3109-3118.
[51] F. Rolland, E. Baena-Gonzalez, J. Sheen, Sugar sensing and signaling in plants: conserved and novel mechanisms, Annu. Rev. Plant Biol., 57 (2006) 675-709.
[52] S. Muthayya, J.D. Sugimoto, S. Montgomery, G.F. Maberly, An overview of global rice production, supply, trade, and consumption, Ann. N Y Acad. Sci., 1324 (2014) 7-14.
[53] H. Shen, X. He, C.R. Poovaiah, W.A. Wuddineh, J. Ma, D.G.J. Mann, H. Wang, L. Jackson, Y. Tang, C. Neal Stewart, F. Chen, R.A. Dixon, Functional characterization of the switchgrass (*Panicum virgatum*) R2R3-MYB transcription factor PvMYB4 for improvement of lignocellulosic feedstocks, New. Phytol., 193 (2012) 121-136.
[54] W.A. Wuddineh, M. Mazarei, J.-Y. Zhang, G.B. Turner, R.W. Sykes, S.R. Decker, M.F. Davis, M.K. Udvardi, C.N. Stewart, Identification and Overexpression of a Knotted1-Like Transcription Factor in Switchgrass (Panicum virgatum L.) for Lignocellulosic Feedstock Improvement, Front. Plant Sci., 7 (2016) 520.

[55] Z. Wu, Y. Cao, R. Yang, T. Qi, Y. Hang, H. Lin, G. Zhou, Z.-Y. Wang, C. Fu, Switchgrass SBP-box transcription factors PvSPL1 and 2 function redundantly to initiate side tillers and affect biomass yield of energy crop, Biotechnol. Biofuels, 9 (2016) 101.
[56] R. Zhong, Y. Yuan, J.J. Spiekerman, J.T. Guley, J.C. Egbosiuba, Z.H. Ye, Functional Characterization of NAC and MYB Transcription Factors Involved in Regulation of Biomass Production in Switchgrass (Panicum virgatum), PLoS One, 10 (2015) e0134611.

Figure 5.

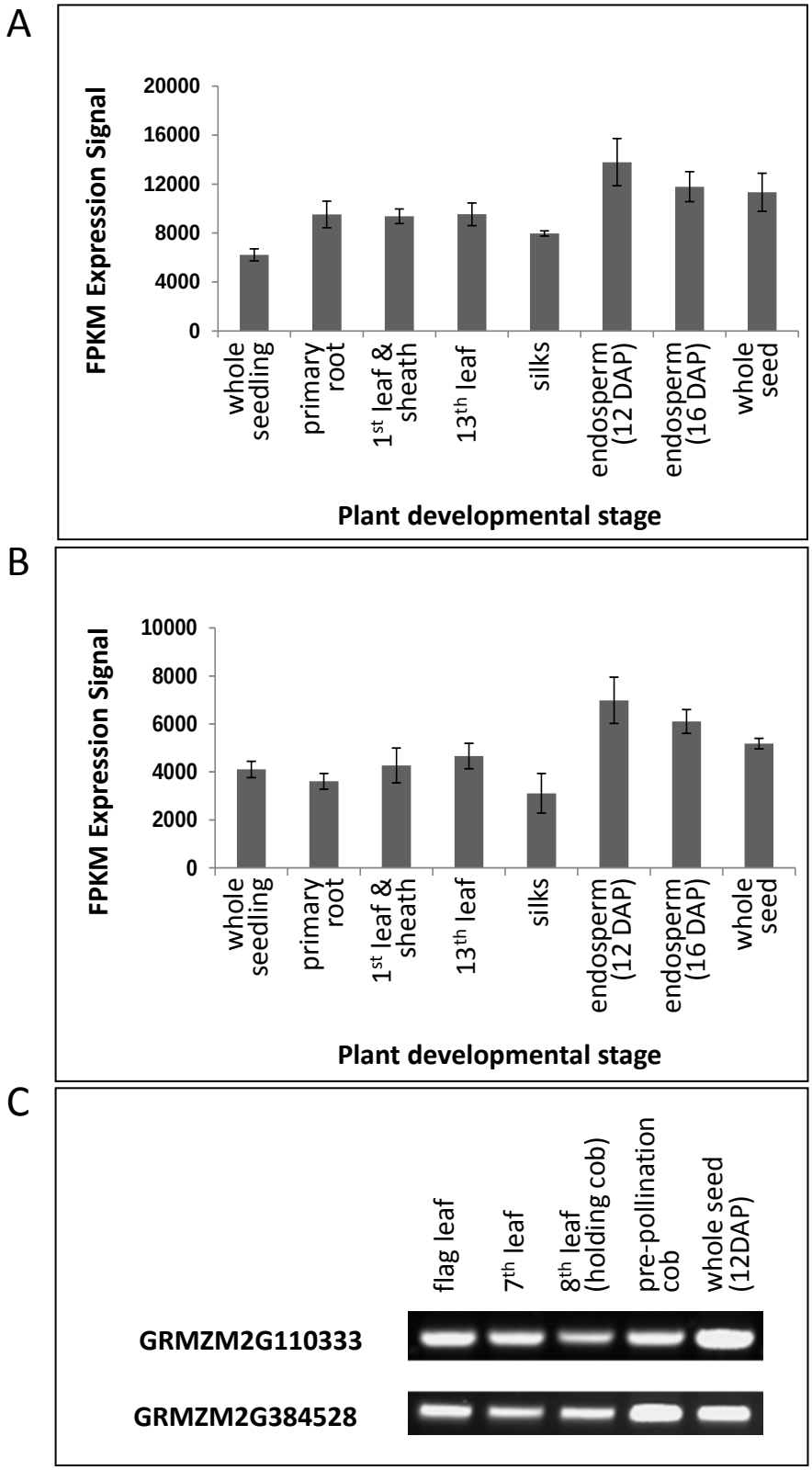

A
FPKM Expression Signal
20000
16000
12000
8000
4000
0
whole seedling
primary root
1st leaf & sheath
13th leaf
silks
endosperm (12 DAP)
endosperm (16 DAP)
whole seed
Plant developmental stage
B
FPKM Expression Signal
10000
8000
6000
4000
2000
0
whole seedling
primary root
1st leaf & sheath
13th leaf
silks
endosperm (12 DAP)
endosperm (16 DAP)
whole seed
Plant developmental stage
C
flag leaf
7th leaf
8th leaf (holding cob)
pre-pollination cob
whole seed (12DAP)
GRMZM2G110333
GRMZM2G384528

Figure 4.

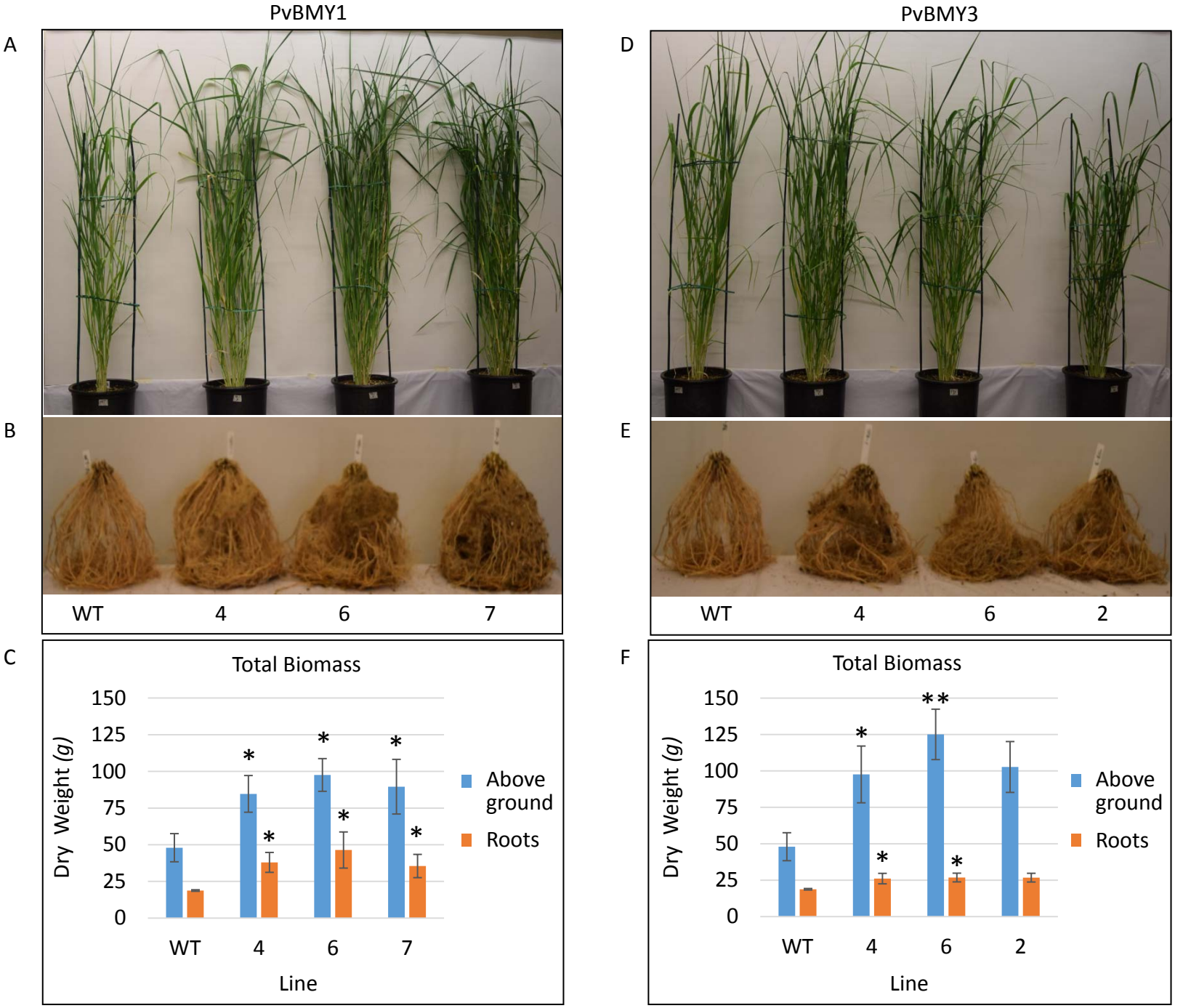

PvBMY1
PvBMY3
A
B
C
D
E
F
WT
4
6
7
2
Total Biomass
Dry Weight (g)
150
125
100
75
50
25
0
Above ground
Roots
Line

Figure 3

PvBMY1-6

PvBMY3-2

Wild-type

Figure 2.

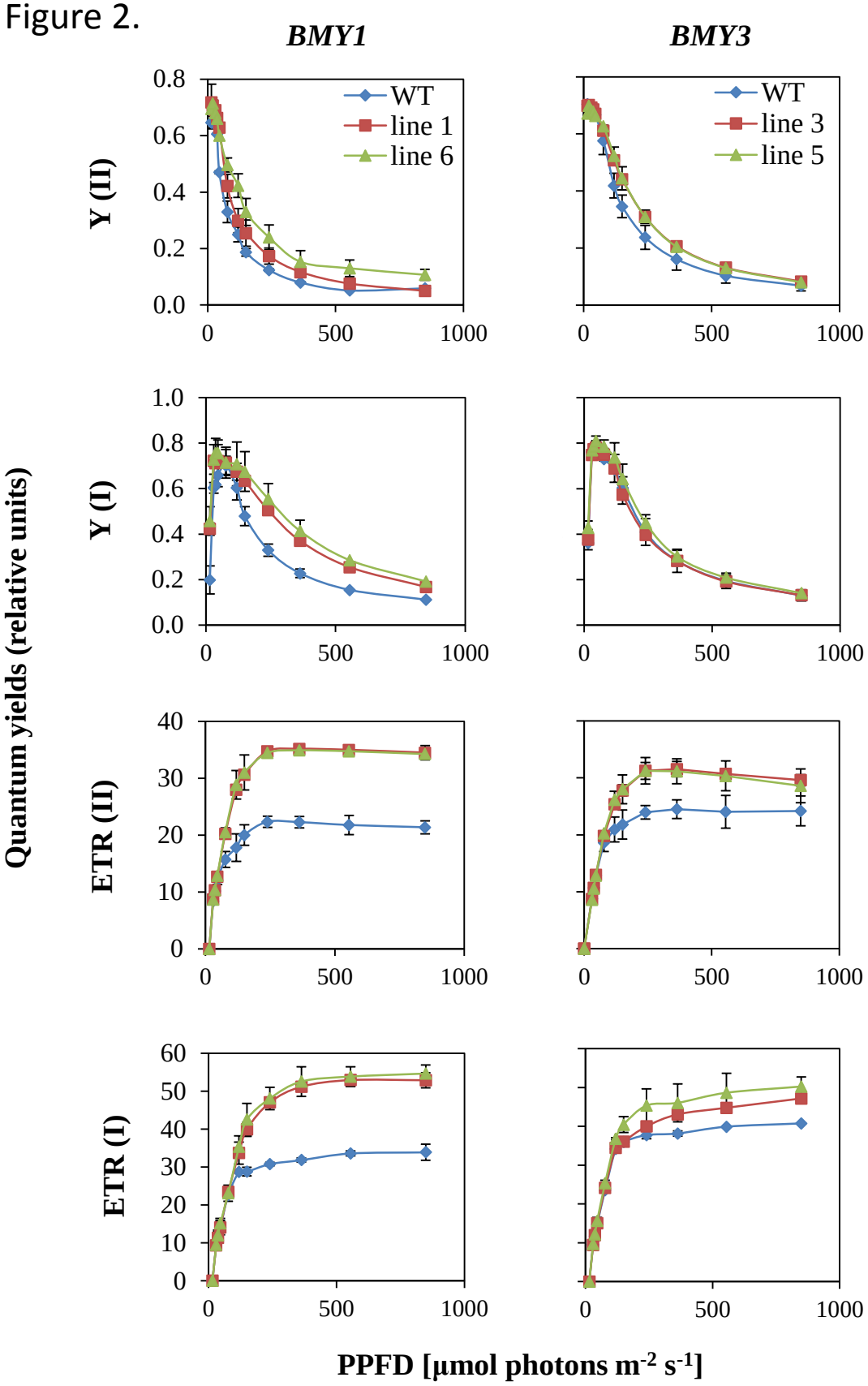
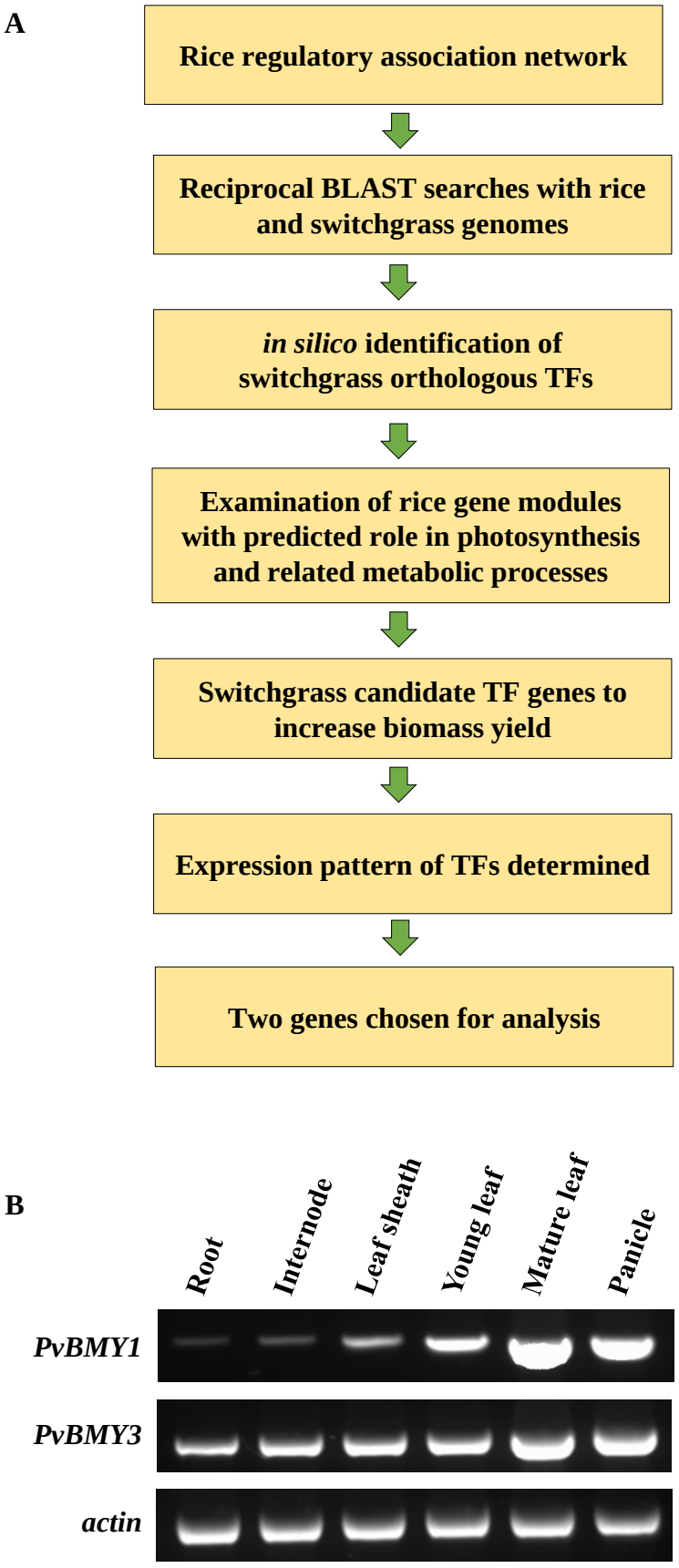

BMY1
BMY3
WT
line 1
line 6
line 3
line 5
Y (II)
Y (I)
ETR (II)
ETR (I)
Quantum yields (relative units)
PPFD [µmol photons m-2 s-1]

Figure 1


A
Rice regulatory association network
Reciprocal BLAST searches with rice and switchgrass genomes
in silico identification of switchgrass orthologous TFs
Examination of rice gene modules with predicted role in photosynthesis and related metabolic processes
Switchgrass candidate TF genes to increase biomass yield
Expression pattern of TFs determined
Two genes chosen for analysis
B
Root
Internode
Leaf sheath
Young leaf
Mature leaf
Panicle
PvBMY1
PvBMY3
actin

Gene regulatory association network

Genes with predicted association to photosynthesis and related metabolism

Switchgrass with

- ***photosynthesis***
- ***biomass yield***