Transcriptional Analysis of Flowering Time in Switchgrass

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Abstract Over the past two decades, switchgrass (*Panicum virgatum*) has emerged as a priority biofuel feedstock. The bulk of switchgrass biomass is in the vegetative portion of the plant; therefore, increasing the length of vegetative growth will lead to an increase in overall biomass yield. The goal of this study was to gain insight into the control of flowering time in switchgrass that would assist in development of cultivars with longer vegetative phases through delayed flowering. RNA sequencing was used to assess genome-wide expression profiles across a developmental series between switchgrass genotypes belonging to the two main ecotypes: upland, typically early flowering, and lowland, typically late flowering. Leaf blades and tissues enriched for the shoot apical meristem (SAM) were collected in a developmental series from emergence through anthesis for RNA extraction. RNA from samples that flanked the SAM transition stage was sequenced for expression analyses. The analyses revealed differential expression patterns between early- and late-flowering genotypes for known flowering time orthologs. Namely, genes shown to

play roles in photoperiod response and the circadian clock in other species were identified as potential candidates for regulating flowering time in the switchgrass genotypes analyzed. Based on their expression patterns, many of the differentially expressed genes could also be classified as putative promoters or repressors of flowering. The candidate genes presented here may be used to guide switchgrass improvement through marker-assisted breeding and/or transgenic or gene editing approaches.

Keywords Switchgrass · Biomass · Flowering time · RNAseq · Differential expression

Introduction

Switchgrass (*Panicum virgatum*) is a warm season, C4 perennial grass that is drought-tolerant and requires little fertilizer inputs to thrive [1]. A prairie grass native to the USA, with a wide adaptation range east of the Rocky Mountains, extending from the Gulf of Mexico to Canada, switchgrass has historically been used as a forage crop [2]. It was not until the early 1990s that the US Department of Energy (DOE) chose switchgrass as a model biofuel feedstock worthy of intensive research with goals for improvement [3].

There are two main ecotypes of switchgrass: lowland and upland. In general, lowland varieties are tall, have wide leaves with a bluish hue, grow upright, flower late, and are not cold-tolerant. Uplands, on the other hand, are cold-hardy, early-flowering, and, in general, have narrower leaves and smaller stature than lowlands. In switchgrass, as with many crops, later-flowering genotypes accumulate more biomass [4]. To make switchgrass cultivation an economically feasible endeavor for farmers as a biofuel feedstock, a minimum biomass yield must be achievable [4]. In the southern USA, there are

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many late-flowering lowland switchgrass varieties that yield high amounts of biomass to allow for efficient switchgrass cultivation [3]. In the North Central USA, however, the lowland varieties would not be reliable due to reduced winter survival. Therefore, the aim of this study was to identify flowering time gene candidates that could be employed to manipulate flowering time in upland, cold-tolerant cultivars.

From a developmental standpoint, the key event in determining flowering time is the transition of the shoot apical meristem (SAM) from the vegetative to reproductive phase. Several input pathways feed into determination of the flowering transition, with mechanisms that either promote or repress flowering. Ultimately, in the case of flowering activation, a signaling cascade results in gene expression and physiological changes in the SAM, transitioning it from a vegetative to a reproductive meristem. The signaling cascades converge on genes classified as floral pathway integrator (FPI) and floral meristem identity (FMI) genes [5], which act as hubs to sense inputs and to activate the meristem transition, respectively. Once the FMI genes are activated, the SAM begins the transition to reproductive development. This transition in switchgrass may occur several weeks before heading is observed, which is the exertion of the panicle from the surrounding leaves; heading date is a practical measure of flowering time. After the SAM has transitioned to reproductive growth, the remaining floral development is dependent, for the most part, on developmental processes running their course. Therefore, the SAM transition is the key molecular process defining flowering time.

The genetic pathway of flowering time has been studied extensively in model species, particularly the dicot *Arabidopsis* (*Arabidopsis thaliana*), in which many mechanisms have been largely elucidated [5, 6]. The genetics of flowering time has also been studied intensely in monocots such as maize (*Zea mays*) (e.g., [7]), rice (*Oryza sativa*) (e.g., [5, 8]), and *Brachypodium* (*Brachypodium distachyon*) (e.g., [5, 9]). Some aspects of the flowering time pathway in *Arabidopsis* are shared in monocots, as evidenced by the many orthologs of *Arabidopsis* genes found in such species [5].

The main flowering time input pathways are photoperiod, circadian clock, hormone, vernalization, and autonomous. The genes in these pathways function to perceive day length, internal rhythms, hormones, temperature, and developmental maturity, respectively. The photoperiod and circadian clock pathways work in tandem through the so-called external coincidence model, in which diurnal rhythms of circadian clock gene expression induce expression of downstream genes depending on the light cycle [10]. The outcomes of these pathways act on the FPIs to either enhance or suppress FPI and subsequent FMI gene expression.

In this study, an ortholog search using flowering time genes from other species was employed to identify putative

flowering time genes in switchgrass. By comparing flowering-associated gene expression between upland and lowland ecotypes or between early- and late-flowering genotypes, this study aimed to elucidate differences due to ecotype- and flowering time-specific expression patterns. Results of this investigation will inform breeding efforts and/or advance genome engineering of switchgrass for improved biomass yield.

Materials and Methods

Plant Material

Switchgrass plants were grown at the University of Wisconsin Agricultural Research Station in Arlington, Wisconsin. The switchgrass genotypes used in this study were grown in a common garden that had been established 5 years prior to this study. Plants were established from vegetative ramets in May 2008 on a spacing of 0.9 m between adjacent plants. There were three clonal replicates of each of two upland genotypes, from the cultivar Cave-in-Rock, and two clonal replicates of each of the lowland genotypes, one from the cultivar High Tide and the other from the cultivar Kanlow (Table 1). The switchgrass genotypes used in this study broke dormancy in early May 2013, and tissue collection began shortly thereafter. In this study, flowering date was defined as the day of the year when at least 50% of the panicles on a plant reached R3, the fully expanded stage.

Sample Collection

Samples of three different tissues were taken at growth stages determined by leaf number, as defined in Moore et al. [11], and following a developmental series from emergence through anthesis. The tissues included the youngest emerging leaf blade, the entire blade of the second leaf at stage V2, a 2–3-cm section of the stem containing the shoot apical meristem (SAM), and, for stages after V2, the basal third of the blade of the fully collared leaf subtending the meristem (Fig. 1). For leaves at stages after V2, samples were collected twice: at the “early” stage, when the distance from the collar of the youngest fully collared leaf to the next emerging leaf tip was 15–30 cm and at the “late” stage, when the distance from the collar to the next emerging leaf tip was double that of the “early” stage. “Early”-stage samples for V3 and V4 are denoted as V3.1 and V4.1, respectively. “Late”-stage samples for V3 and V4 are denoted as V3.2 and V4.2, respectively. An additional SAM-enriched sample at stage V5 was taken from each lowland genotype (5305-7 and 5300-8).

For each sample, tissue was pooled from four tillers per plant, with three replicate plants for each upland genotype (WS08-R3 and WS08-U1) and two for each lowland (5305-

Table 1 Switchgrass genotype information, meristem transition stage, and flowering date

Genotype	WS08-R3	WS08-U1	5305-7	5300-8
Cultivar	Cave-in-Rock	Cave-in-Rock	High Tide	Kanlow
Ecotype	Upland	Upland	Lowland	Lowland
Flowering	Early	Early	Early	Late
Source	Illinois	Illinois	Maryland	Oklahoma
Replicates	3	3	2	2
SAM transition ^a	June 7 (158 ^b)	June 7 (158 ^b)	June 20 (171 ^c)	June 28 (179 ^d)
Flowering date ^a	August 8 (220)	August 8 (220)	August 15 (227)	September 5 (248)

^a Number in parentheses is day of year^b Stage is V3.1^c Stage is V3.2^d Stage is V4.1

7 and 5300-8), as follows. For each replicate, after taking measurements and determining which tillers to collect tissue, four tillers were removed from the plant and all leaves below the uppermost collared leaf were removed from each tiller. Then, the basal third of the blade of the uppermost collared leaf from each tiller was collected and the pooled samples were immediately flash frozen in liquid nitrogen. Next, a 3-cm-long section (containing the SAM) of the internode adjacent to and above the uppermost collared leaf was dissected from each tiller, pooled, and flash frozen. Lastly, the emerging leaf tissue above the uppermost collared leaf was collected from each tiller, pooled, and flash frozen. Dissection of samples from the tillers was done rapidly, to limit the time between tiller harvest and sample freezing to less than 2 min. All samples were kept in liquid nitrogen after flash freezing and during transport back to the laboratory in Madison, Wisconsin. Tissues were stored at -80°C until RNA extraction.

Morphological Analysis of Shoot Apical Meristems

For each stage at which samples were taken for possible RNA extraction, fresh tillers were also collected for analysis of the SAM morphology. At least three whole tillers at the corresponding stage were collected and kept on ice during transport back to the laboratory. Fresh tillers were stored for up to 3 days at 4°C , before microscopic analysis. Shoot apical meristems were dissected from surrounding sheath tissue, using a razor blade and an 18-gauge needle (Becton Dickinson, Franklin Lakes, NJ). Meristems were visualized under a Leica MZ16F stereomicroscope (Leica Microsystems, Switzerland) at various magnifications.

RNA Extraction and Quantification

RNA was extracted from selected samples taken at stages flanking and including meristem transition (Table 1), using the TRIzol (Life Technologies, Carlsbad, CA) method, following the manufacturer's protocol. Briefly, plant material

was pulverized in liquid nitrogen, using a mortar and pestle to produce a fine powder, and approximately 100 mg of powdered tissue was then used for RNA extraction in 1 mL of TRIzol. The RNA pellet was resuspended in 50 μL RNase-free water and the concentration checked on a NanoDrop, ND 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). After determining the concentration, RNA integrity was checked via gel electrophoresis. RNA samples without degradation were purified using RNeasy spin columns (Qiagen, Valencia, CA) and eluted in 30 μL RNase-free water. Sample integrity and quality were further verified with an Agilent 2100 Bioanalyzer (Agilent Technologies, Germany), at the University of Wisconsin Biotechnology Center.

RNA Sequencing and Sequence Processing

Illumina TruSeq Stranded libraries were constructed and sequenced in paired end-mode at the DOE Joint Genome Institute (JGI) on an Illumina HiSeq 2000. One of the lowland samples, 5305-7 stage V4.2 SAM replicate 1, failed the final quality control and was not sequenced. However, the remaining 83 samples each produced an average of 25 million reads. Libraries from Cave-in-Rock were contaminated with switchgrass mosaic virus. To remove virus sequences, all libraries were aligned to the switchgrass mosaic virus genome (NC_015522.1) using TopHat v1.4.1 [12] with a minimum intron size of 5, maximum intron size of 5000, and the initial read mismatches ($-N$) set to 7; all other parameters were set to default. All sequence reads were then cleaned using Cutadapt v1.4.1 [13] setting the quality score cutoff to 20 ($-q$) and a minimum read length of 50. After cleaning, reads were assessed for quality using FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and then trimmed to 150 nucleotides using FASTX_TRIMMER from the FASTX-Toolkit v0.0.14 (http://hannonlab.cshl.edu/fastx_toolkit/commandline.html). Cleaned reads were then aligned to the switchgrass reference genome sequence (*P. virgatum*

Fig. 1 Representative tissue types sampled. Shown are images of samples from two developmental stages. WS08-U1 is an early-flowering, upland genotype. 5300-8 is a late-flowering, lowland genotype. SAM shoot apical meristem-enriched stem. V3B (or V4B) basal third of leaf 3 (or 4). Dashed line marks the distal boundary of the sample collected. Bar = 2 cm

v1.1, DOE-JGI, <http://phytozome.jgi.doe.gov/>) using TopHat v1.4.1 with a minimum intron size of 5 bp, a maximum intron size of 5000 bp, and segment length set to 25; all other parameters were set to default. Expression abundances (fragments per kilobase of gene model per million mapped reads (FPKM)) were generated using Cufflinks v1.3.0 [14] using both bias correction (–b) and quantification against the reference transcript annotation (–G), to generate an expression

matrix of 98,005 genes across 83 samples (Online Resource S1). If the FPKM status for the gene was not “OK,” the FPKM value was replaced with “NA” in the matrix. Raw sequence reads are available from the National Center for Biotechnology Information under project PRJNA361131.

Expression Analysis

Pearson’s correlations between replicates indicated high reproducibility, with the lowest correlation of 0.9 for a single sample and most correlations in the 0.96–0.99 range (Online Resource S2). Of the 98,005 genes in the switchgrass reference genome (version 1.1), 81,430 had an FPKM >0 among the 83 samples sequenced. From the FPKM matrix, a mean expression matrix was calculated by averaging the FPKM values of the replicates (2 or 3) for each sample. Mean FPKM values for the genes with FPKM >0 in each of the 34 samples were used for principal component analysis (PCA) using the “prcomp” function in the R programming language [15].

For data exploration and differential expression analysis, the FPKM matrix was imported into ArrayStar software version 12.1 (DNASTAR, Madison, WI). For differential expression analysis, genotypes were grouped by ecotype: upland (WS08-R3 and WS08-U1) or lowland (5305-7 and 5300-8), and by flowering time: early- (WS08-R3, WS08-U1, and 5305-7) or late-flowering (5300-8). These groups are hereafter referred to as “comparison groups.” Mean FPKM values for each tissue type of each comparison group were used for differential expression analyses.

Identification of Flowering Time Orthologs

Protein sequences of 93 known flowering time genes from *Arabidopsis*, maize, and/or rice were used to search for putative flowering time gene orthologs. OrthoMCL (v1.4) analysis was performed using default parameters [16] with proteomes of six grass species including switchgrass (v1.1; phytozome.jgi.doe.gov), rice (v7; <http://rice.plantbiology.msu.edu/>), sorghum (v2.1; phytozome.jgi.doe.gov), *B. distachyon* (v1.2; phytozome.jgi.doe.gov), maize (v5b; maizesequence.org), and *Setaria italica* (v2.1; phytozome.jgi.doe.gov) with protein sequences of the 93 known flowering time genes; 448 gene orthologs from the six species including 101 from switchgrass were identified (Online Resource S3).

Differential Expression Analysis of Flowering Time Orthologs

For each tissue and developmental stage, the mean expression values (FPKM) of 101 switchgrass flowering time gene orthologs were compared between upland and lowland groups

and between early- and late-flowering groups. Genes upregulated or downregulated by at least 1.7-fold (T test p value <0.05) between the early- and late-flowering and/or upland and lowland comparison groups for the same tissue and stage and with a mean FPKM >2 (arbitrary FPKM threshold) in at least one of the comparison groups were deemed differentially expressed. The expression values of these differentially expressed genes were subsequently plotted for each comparison group and tissue type, along the entire developmental series, to compare expression patterns and classify genes as putative promoters or repressors of flowering.

Results and Discussion

Dates of Flowering for Each Genotype

In this study, flowering date was defined as the day of the year on which at least half of the tillers on a plant had reached R3, the stage at which panicles are fully expanded. The two upland genotypes, WS08-R3 and WS08-U1, flowered on August 8, or day 220 of the year. The early-flowering lowland genotype, 5305-7, flowered on August 15, or day 227 of the year. The late-flowering lowland genotype, 5300-8, flowered on September 5, or day 248 of the year (Table 1).

Morphological Characterization of the SAM

For the uplands, WS08-R3 and WS08-U1, which both flowered on the same date, the rachis, or first branch, of the reproductive meristem was detected in samples collected at the early V3 stage (V3.1), day 158 of the year. For the early-flowering lowland genotype, 5305-7, which flowered a week after the uplands, the rachis meristem was detected at the late V3 stage (V3.2), or day 171 of the year. For the late-flowering lowland, 5300-8, which flowered 3 weeks after the uplands, the rachis meristem was detected in samples from the early V4 stage (V4.1), or day 179 of the year. Since the signal to transition from vegetative to reproductive meristem occurs before the change in SAM morphology is observed, the stage prior to the one in which the SAM transition was detected was used as the pre-transition, or vegetative SAM, sample for transcriptional analysis.

Principal Component Analysis

Out of 98,005 genes in the FPKM matrix, 16,575 had a FPKM value of zero in all samples analyzed. Therefore, 81,430 genes with FPKM >0 were used for PCA. Mean FPKM values for each of 34 samples were used for PCA. Combined, principal components 1 and 2 (PC1 and PC2) accounted for approximately 42% of the total variation within the FPKM matrix.

Fig. 2 Principal component analysis (PCA) of FPKM expression values for 34 sample types sequenced from four genotypes. The entire FPKM matrix contains expression data for 98,005 genes, of which 16,575 had an FPKM value of zero in all samples sequenced. Therefore, 81,430 genes had FPKM values >0 within the sample set and were used for the PCA. Principal components 1 and 2 (PC1 and PC2) account for ~42% of the total variation within the FPKM matrix. PC1 clearly accounts for tissue variation and PC2 accounts for genotype, ecotype, or flowering time variation. Each point is the eigenvalue calculated for each sample, from mean global expression values from three or two replicates for upland and lowland genotypes, respectively

PC1 clearly accounted for tissue variation and PC2 accounted for genotype, ecotype, and/or flowering time variation (Fig. 2), indicating differential expression between these categories.

Differential Expression of Known Flowering Time Gene Orthologs

For 23 of the 93 known flowering-associated genes that had been identified in *Arabidopsis*, rice, and/or maize, no orthologs were identified in the switchgrass reference genome version 1.1; however, this could be due to an incomplete version of the genome. For the 70 known flowering-associated genes with identified switchgrass orthologs, 101, in total, were found. Of the 101 switchgrass orthologs, 23

had mean FPKM values of less than two (FPKM threshold) in all tissues across the four comparison groups (Online Resource S4). Therefore, 78 switchgrass orthologs met the FPKM threshold for at least one comparison group and were used for differential expression analyses. The arbitrary cutoff of FPKM >2 removed some known key players in the flowering pathway that may be expressed at low levels (e.g., FT/TSF); however, we were most interested in genes that were expressed early on, before meristem transition, that play roles upstream of FPI/FMI components.

Hierarchical clustering analysis of the 78 switchgrass orthologs with mean FPKM values greater than two for at least one comparison group revealed coexpressed genes and, for the most part, identical expression patterns between groups

Fig. 3 Heat map of expression of 78 switchgrass flowering time candidate gene orthologs in leaves, after hierarchical clustering. Expression values are the $\log_2$ -transformed mean FPKM for each comparison group, upland (WS08-R3 and WS08-U1) and lowland (5305-7 and 5300-8). Headings indicate comparison group and sample. The gene tree is shown on the left and gene IDs on the right. Arrowheads mark genes that were classified as promoters or repressors of flowering. The color-scale legend at the bottom shows the range of expression as $\log_2$ -transformed FPKM values

with differing flowering times. However, for a number of orthologs, differential expression was detected between comparison groups (Figs. 3, 4, 5, and 6).

The developmental stage of SAM transition, as determined by microscopy, informed the selection of tissue stages to sequence. The stages analyzed included the stage of SAM transition and stages flanking the transition, with the expectation that genes involved in flowering time would have differential expression from one transition flanking stage to the other. In addition, genes necessary for flowering time differences between comparison groups were expected to have differential expression between those groups. Based on the stage or stages at which genes had differential expression between comparison groups, the genes could be classified as putative promoters or repressors of flowering or having an unknown role.

Putative promoters of flowering had higher expression in early stages (V2, V3) in upland or early-flowering groups than in lowland or late-flowering groups, respectively. Conversely, putative repressors of flowering had higher expression in early stages (V2, V3) in lowland or late-flowering, compared to upland or early-flowering groups, respectively. Genes that fit the criteria for being a putative promoter or repressor are presented here.

Promoters of Flowering

Switchgrass orthologs of seven known flowering time genes were classified as putative floral promoters (Table 2). Five of these genes fall into the photoperiod/circadian clock pathway, one in the autonomous, and one in the vernalization pathway.

Fig. 4 Heat map of expression of 78 switchgrass flowering time candidate gene orthologs in SAM-enriched tissue, after hierarchical clustering. Expression values are the $\log_2$ -transformed mean FPKM for each comparison group, upland (WS08-R3 and WS08-U1) and lowland (5305-7 and 5300-8). Headings indicate comparison group and sample. The gene tree is shown on the *left* and gene IDs on the *right*. Arrowheads mark genes that were classified as promoters or repressors of flowering. The color-scale legend at the *bottom* shows the range of expression as $\log_2$ -transformed FPKM values

Fig. 5 Heat map of expression of 78 switchgrass flowering time candidate gene orthologs in leaves, after hierarchical clustering. Expression values are the $\log_2$ -transformed mean FPKM for each comparison group, early (WS08-R3, WS08-U1, and 5305-7) and late (5300-8). Headings indicate comparison group and sample. The gene tree is shown on the left and gene IDs on the right. Arrowheads mark genes that were classified as promoters or repressors of flowering. The color-scale legend at the bottom shows the range of expression as $\log_2$ -transformed FPKM values

One striking example of a putative floral promoter is the gene Pavir.Ea04181, an ortholog of the *Arabidopsis* gene, LUX ARR1TMO (LUX) [17]. In leaves of lowland and late-flowering genotypes (5305-7 and 5300-8), Pavir.Ea04181 had little to no expression throughout the three developmental stages analyzed, while the expression level in early-flowering, upland genotypes (WS08-R3 and WS08-U1) spiked at the stage of SAM transition as determined by microscopy (Fig. 7a). LUX, a Myb domain transcription factor, has been shown in *Arabidopsis* to be essential for producing circadian rhythms. Specifically, the proposed function of LUX is upregulation of the photoperiod floral activation pathway and acts indirectly to upregulate the photoperiod/circadian clock gene *CONSTANS* (CO) via *GIGANTEA* (GI) [18], thereby activating FPIs and FMIs in the subsequent SAM

transition [17]. In the SAM of upland genotypes (WS08-R3 and WS08-U1), Pavir.Ea04181 expression increased from V2 stage to V3.1, where it plateaued. In lowland genotypes (5305-7 and 5300-8), Pavir.Ea04181 expression began at the V3.1 stage, increasing sharply to the level of uplands at stage V4.2 (Fig. 7a). Of note, in Fig. 7a, are the overlapping expression patterns of the lowland (5305-7 and 5300-8) and late-flowering (5300-8) groups, suggesting an ecotype-specific expression pattern.

Repressors of Flowering

Switchgrass orthologs of five known flowering time genes were classified as putative floral repressors (Table 3). Two of

Fig. 6 Heat map of expression of 78 switchgrass flowering time candidate gene orthologs in SAM-enriched tissue, after hierarchical clustering. Expression values are the $\log_2$ -transformed mean FPKM for each comparison group, early (WS08-R3, WS08-U1, and 5305-7) and late (5300-8). Headings indicate comparison group and sample. The gene tree is shown on the *left* and gene IDs on the *right*. Arrowheads mark genes that were classified as promoters or repressors of flowering. The color-scale legend at the *bottom* shows the range of expression as $\log_2$ -transformed FPKM values

these genes fall into the category of FPI or FMI genes, two in the autonomous, and one in the photoperiod/clock pathway.

An example of a putative floral repressor that shows dramatic differential expression between flowering time groups is Pavir.Bb03208, an ortholog of the *Arabidopsis* gene, BRI1-ESSENTIAL 1 (BES1) [19]. In leaves of the upland and early-flowering genotypes (WS08-R3, WS08-U1, and 5305-7), Pavir.Bb03208 had little to no expression (FPKM <1) over the three developmental stages analyzed, while in the late-flowering, lowland genotype (5300-8), Pavir.Bb03208 transcript was present in gradually increasing levels through the series of the three leaf stages (Fig. 7b). Since BES1 in *Arabidopsis* represses FPI gene expression [20, 21], the lack of BES1 expression in the early-flowering genotypes suggests

a de-repression of floral activators, indicative of the transition to reproductive meristem growth.

In the SAM of the late-flowering, lowland genotype (5300-8), Pavir.Bb03208 expression is more than 6-fold higher than in the early-flowering genotypes (WS08-R3, WS08-U1, and 5305-7), suggesting a role in suppression of floral activator expression in the SAM. Note that the expression patterns of Pavir.Bb03208 in the upland (WS08-R3 and WS08-U1) and early-flowering (WS08-R3, WS08-U1, and 5305-7) comparison groups are nearly identical, while the expression pattern of the late-flowering genotype (5300-8) differs from the lowland group (5305-7 and 5300-8) pattern. This suggests that the expression of Pavir.Bb03208 in the SAM is flowering time-specific.

Table 2 Putative floral promoters identified through differential expression analysis of switchgrass flowering time orthologs

Locus	Gene ID	Late leaf ^a	Late SAM ^b	Low leaf ^c	Low SAM ^d	Pathway
Pavir.J34844	MSI4/FVE			V3 (0.5)		Autonomous
Pavir.Db01632	CONSTANS	V2 (3); V3 (0.1); V4 (0.4)		V3 (0.1); V4 (0.3)		Photoperiod/clock
Pavir.J04655	PHYA	V3 (0.5)		V3 (0.5); V4 (1.6)	V2, V3.1 (1.5); V4.1, V4.2 (1.9)	Photoperiod/clock
Pavir.Fa01905	CCA1-like		V3.2 (2.8); V4.2 (3)	V2 (0.4)	V3.1 (2.4); V3.2 (5.1); V4.1 (1.9); V4.2 (3.9)	Photoperiod/clock
Pavir.Ib01239	PHYB			V2, V3 (0.6)		Photoperiod/clock
Pavir.Ea04181	LUX			V3, V4 (0.1)	V3.1 (0.2); V3.2 (0.04); V4.1 (0.4); V4.2 (0.6)	Photoperiod/clock
Pavir.J37137	EFS			V3 (0.5)		Vernalization

^a Relative expression in leaf in late- versus early-flowering groups. Leaf stage indicated. Fold change in parentheses

^b Relative expression in SAM in late- versus early-flowering groups. SAM stage indicated. Fold change in parentheses

^c Relative expression in leaf in lowland versus upland groups. Leaf stage indicated. Fold change in parentheses

^d Relative expression in SAM in lowland versus upland groups. SAM stage indicated. Fold change in parentheses

Conclusions

In this study, flowering time differences, measured as heading date, among upland and lowland genotypes were mirrored in the SAM transition dates, suggesting that the date of transition from vegetative to reproductive meristem defines the time of flowering. Orthologs of known flowering time genes had either ecotype- or flowering time-specific patterns, allowing for the possible separation of flowering time traits from determinants of other ecotype-specific traits in breeding or transgenic manipulation efforts. Lastly, known flowering time gene orthologs from multiple pathways, but mainly the photoperiod/clock and autonomous pathways, were differentially expressed between genotypes with differing flowering times, indicating conservation of these pathways, at least in part, across species.

At the convergence of the flowering time pathway, found in both monocots and dicots, is the FPI and mobile signal called “florigen,” which is translocated from the leaf through the phloem to the SAM [22]. The specific gene that encodes the florigen protein was discovered in *Arabidopsis* and named FLOWERING LOCUS T (FT) [23–25]. Genes with FT-like functions have been identified in rice [26], maize [7, 27], and *Brachypodium* [5]. Even with conserved function, not all flowering time gene orthologs function in the same capacity across plant species. In rice, for example, there are two florigens, one that promotes flowering in short days only and the other acting in long days only [28]. For switchgrass, investigations into the flowering time pathway have also revealed complexities. A recent report on control of floral transition in switchgrass analyzed the expression of a switchgrass FT ortholog [29]. The authors found that, in greenhouse conditions, FT transcript increased from the V3 to E3 stage and suggested that the floral transition occurred between stages

E1 to E3. Interestingly, both of the switchgrass FT orthologs identified here were not expressed, with mean comparison group FPKM values below 2 across all tissues, despite having observed morphological changes in the SAM that indicated the occurrence of the transition from vegetative to reproductive growth. The possible reasons for the discrepancy in FT expression between the Niu et al. [29] study and this are 3-fold: (1) there may be other FT-like genes acting as FPIs that were not identified in the ortholog search, (2) the expression of the switchgrass FT ortholog may have been spatially restricted to the middle or top section of the leaf blade or the leaf sheath tissues which were not included in this study, and (3) there may be differences in FT expression among different genotypes, as the ones surveyed here are different from the single lowland variety used in the Niu et al. [29] study. Not only could FT expression patterns differ between genotypes, but the numerous FT-like genes in switchgrass may work in concert, each having partial roles in control of flowering. In addition, in a recent switchgrass flowering time association study, a single nucleotide polymorphism (SNP) in an FT-like gene was found to be associated with early flowering [30]. However, the early flowering allele was evident in only northern populations, leading the authors to suggest possible subfunctionalization of this FT homolog to play a role in cold hardiness [30].

The gene candidates evaluated in this study provide insight into the flowering time pathway in switchgrass. By surveying multiple genotypes from two ecotypes with different flowering times, gene candidates could be classified as either putative promoters or repressors of flowering. In addition, due to one lowland genotype having early flowering, expression patterns could be parsed out as ecotype- or flowering time-specific, thereby being able to dissect pathways shared between ecotypes. Orthologs of known floral genes, particularly

Fig. 7 Switchgrass orthologs of two known flowering time genes are differentially expressed between genotypes from two ecotypes. **a** Expression levels in FPKM of Pavir.Ea04181 in the leaf (left) and SAM-enriched tissue (right) along a developmental series from the second through the fourth leaf stage (V2–V4). Pavir.Ea04181 is an ortholog of the *Arabidopsis* gene, LUX ARRHYTHMO (LUX), a promoter of flowering. **b** Expression patterns, as in **a**, of Pavir.Bb03208 in the leaf (left) and SAM-enriched tissue (right). Pavir.Bb03208 is an ortholog of the *Arabidopsis* gene, BRI1-EMS-SUPPRESSOR 1 (BES1), a repressor of

flowering. Each data point is the mean FPKM value of the respective grouping. Groupings are: *UP*, both upland genotypes (WS08-R3 and WS08-U1); *LOW*, both lowland genotypes (5305-7 and 5300-8); *Early*, both uplands and the early-flowering lowland genotype (WS08-R3, WS08-U1, and 5305-7); and *Late*, the single late-flowering lowland genotype (5300-8). Significant differences between early-/late-flowering or upland/lowland, as determined by a *T* test with *p* value <0.05, are denoted by an asterisk. Error bars are $\pm$ standard error

from the photoperiod/circadian clock pathway, were expressed differentially in ecotype- and flowering time-specific manners, suggesting that parts of these pathways may have different expression patterns between ecotypes, but play no role in determining flowering time. Several novel genes that encode protein domains like those found in known flowering time genes were identified, such as Myb and zinc-finger transcription factors. These novel gene candidates all showed flowering time-specific expression patterns, indicating a shared pathway across ecotypes. It remains to be seen whether these conserved genes also share conserved functions or have evolved divergent functions as with the rice FT orthologs.

Switchgrass is photoperiodic, but the day length requirements are variable between cultivars and have been shown to correlate with latitude of origin, with southern-derived cultivars having a short-day requirement and northern cultivars responding to long days or being photoperiod-insensitive [31]. High night temperatures have been suggested to restrict flowering in some cultivars of switchgrass [32]; therefore, cool nights may possibly induce flowering, or, at least, permit it. Flowering in switchgrass is influenced by many factors,

both endogenous and exogenous. Some components of the photoperiod and autonomous pathways have been identified in switchgrass [33]; however, investigations in sorghum, another high biomass crop, have yielded more pieces of the flowering pathway in grasses. Three well-characterized maturity loci in sorghum, Ma1, Ma3, and Ma6, harbor floral repressors that confer photoperiod sensitivity [34]. Ma1 corresponds to SbPRR37, the ortholog of *Arabidopsis* PRR7 [35]; PHYB is the gene at the Ma3 locus [36]; and SbGhd7, the ortholog of rice Ghd7, was identified as Ma6 [37]. Work by Wolabu et al. [38] has shown that sorghum has 19 genes that encode a phosphatidylethanolamine-binding protein (PEBP) domain—the characteristic domain of FT and TERMINAL FLOWER LIKE 1 (TFL1), florigen and anti-florigen, respectively. Three of the 19 PEBP-containing genes have been shown to act as functional florigens, responding to different cues, and two are TFL1-like [38]. The model of multiple florigens is supported by previous work in rice, with its two florigens activated by different photoperiods. Switchgrass, with over 40 FT-like genes [30], could possibly also have multiple florigens that are activated by different photoperiods or environmental conditions.

Table 3 Putative floral repressors identified through differential expression analysis of switchgrass flowering time orthologs

Locus	Gene ID	Late leaf ^a	Late SAM ^b	Low leaf ^c	Low SAM ^d	Pathway
Pavir.Bb01323	FCA-like	V2 (2)		V2 (1.8); V3 (1.7)		Autonomous
Pavir.J11433	RAP2-7	V2 (2.4)	V4.2 (2.3)			FPI/FMI
Pavir.Ba00457	AGL8-like	V2 (2.3)				FPI/FMI
Pavir.Bb03208	BES1	V2 (20); V3 (21); V4 (26)	V2 (5.6); V3.1 (6.2); V3.2 (7.7); V4.1 (5.4); V4.2 (6.9)	V2 (20); V4 (26)	V2 (2); V3.1 (3.1); V3.2 (2); V4.1 (2.5); V4.2 (4)	Autonomous
Pavir.Fb00402	CCA1-like			V3 (4.4)	V3.2 (2.8)	Photoperiod/clock

^a Relative expression in leaf in late- versus early-flowering groups. Leaf stage indicated. Fold change in parentheses

^b Relative expression in SAM in late- versus early-flowering groups. SAM stage indicated. Fold change in parentheses

^c Relative expression in leaf in lowland versus upland groups. Leaf stage indicated. Fold change in parentheses

^d Relative expression in SAM in lowland versus upland groups. SAM stage indicated. Fold change in parentheses

Subsequent steps to confirm and analyze function of these candidate genes could be carried out initially in the model grass *Brachypodium*, by using readily available mutant knockout lines and/or through genetic transformations. However, as *Brachypodium* is a C3 annual grass, it may not serve as the best system for functional analyses of switchgrass genes. *Setaria viridis*, a C4 grass closely related to switchgrass has been developed for floral dip transformation [39] and, therefore, may be a promising system for determining the functions of switchgrass candidate genes. Recently, a virus-based technique for delivering florigens to various plant systems has been described [40]. This technique allows for transient expression of florigens in species that are difficult to stably transform. The main benefit of virus-induced flowering is the ability to stage plants with highly divergent flowering times for crossing; however, this could be an efficient way to assess the function of florigens (or anti-florigens) directly in switchgrass. In addition, concurrent flowering time QTL mapping studies using populations generated from upland/lowland switchgrass hybrids are expected to support the findings in this study. The identification of components of the switchgrass flowering time pathway that are potential promoters or repressors will enable future breeding and/or transgenic work to improve switchgrass biomass yield through modification of flowering time. For example, CONSTANS, the central component of the photoperiod response pathway in *Arabidopsis*, is upregulated in early-flowering genotypes, compared to late-flowering genotypes. One could possibly alter the promoter region of CONSTANS, through gene editing, to suppress its expression in upland varieties to delay flowering. In addition, upstream of CONSTANS, in the photoperiod/circadian clock-coupled pathway, a number of genes in this study showed differential expression between genotypes of differing flowering times. One example detailed earlier is LUX, but the phytochromes, PHYA and PHYB,

that entrain the circadian clock [10] also showed differential expression. It could be that the late-flowering switchgrass genotypes in this study may have variation in the clock-entraining mechanisms that result in differential photoperiod sensitivity. Functional analyses of the differentially expressed orthologs presented here are, therefore, warranted.

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Compliance With Ethical Standards

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Conflict of Interest The authors declare that they have no conflict of interest.

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