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Transcriptome Profiling Reveals Differentially Expressed Genes Associated with Flowering Time in Contrasting Switchgrass Genotypes

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Abbreviations: cDNA, complementary DNA; DAVID, the database for annotation, visualization, and integrated discovery; DEG, differentially expressed gene; FPKM, fragments per kilobase pair of exon model per million fragments mapped; GA, gibberellin; KEGG, Kyoto Encyclopedia of Genes and Genomes; PCA, principal component analysis; qRT-PCR, quantitative reverse transcription polymerase chain reaction; TF, transcription factor.

ABSTRACT

Switchgrass (*Panicum virgatum* L.) has become an important biofuel crop. The experiment was designed to identify differentially expressed genes (DEGs) for flowering time in switchgrass genotypes contrasting in heading and anthesis dates. Phytomers of early- (S041) and late-flowering (B901) parents and early- (7071) and late-flowering (7055) F₂ genotypes were collected on two sampling dates of vegetative stages, and transcriptome profiling was completed to identify DEGs involved in flowering time using RNA sequencing. Across two sampling dates, the comparison between S041 and B901 identified six upregulated (*TOC1*, *FKF1*, two *PHYA*, and two *PFT1*) and five downregulated flowering DEGs (*FT*, *GASA*, *COPI*, and two *CHS*) in S041, but one upregulated *FT* and one downregulated *GASA* in B901. When comparing two F₂ genotypes, three DEGs (*PFT1* and two *FT*) were upregulated in 7071, and six DEGs (*PRR7*, *PRR5*, *PHYB*, *TOC1*, and two *CKB4*) were upregulated and one DEG (*COPI*) was downregulated in 7055 genotype. The upregulation of *PRR5* and *FKF1* and downregulation of *GASA* and *COPI* were found in both early-flowering genotypes, whereas *FKF1*, *PRR5*, *PRR7*, and *PHYB* were upregulated and two *GASA* were downregulated in both late-flowering genotypes. Across both early-flowering genotypes, seven upregulated and 12 downregulated transcription factors (TFs) from nine families related to flowering were differentially expressed, whereas eight upregulated and 30 downregulated flowering related TFs from 16 families were differentially expressed across both late-flowering genotypes. The identification of key genes

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involved in the flowering pathways could facilitate the development of desirable flowering phenotypes of switchgrass.

Switchgrass (*Panicum virgatum* L.) has emerged as the leading North American native grass species that has high biomass yield for potential biofuel production. Adapted to a wide geographic range of habitats, switchgrass has evolved into two cytotypes: lowland and upland (Hultquist et al., 1996). Upland cytotypes are commonly tetraploid but can be octoploid or hexaploid, whereas lowland cytotypes are tetraploids (Narasimhamoorthy et al., 2008). The upland cytotypes are short, rhizomatous, and thin-stemmed, flower early, are more adapted to northern latitudes, and generally have lower biomass yield than the lowland cytotypes. There is genetic variability for morphological and adaptive traits within each lowland and upland cytotype, both of which have genotypes adapted to northern or southern geographic ranges (Casler et al., 2004). This variation provides an important basis for selecting genotypes with high biomass yield and flexibility of climatic adaptation.

There are many factors that influence the biomass yield of switchgrass. One key factor is the timing of phase change from juvenile (vegetative) to adult with reproductive competence. This switch to the flowering stage creates a sink that draws photosynthetic resources to reproductive tissue, a process that is a fundamental characteristic of this wild and undomesticated plant. As a result, a decline in biomass yield is typically observed in switchgrass immediately after completion of flowering. At northern latitudes, this often occurs up to 8 wk prior to killing frost, effectively reducing the length of the growing season; however, if flowering time is delayed by 4 to 6 wk, biomass yield can potentially increase by 30–50% (Casler, 2012). To develop a rational strategy for creating improved switchgrass with late flowering, it would be beneficial to have a better understanding of the genes that control flowering time in switchgrass.

The flowering pathways have been well characterized in *Arabidopsis thaliana* (L.) Heynh. (*Arabidopsis* thereafter) and rice (*Oryza sativa* L.) (Higgins et al., 2010). The flowering time genes are involved in multiple pathways including photoperiod, circadian clock, gibberellic acid, vernalization, aging, and autonomous (Distelfeld et al., 2009; Greenup et al., 2009; Khan et al., 2014). Genes involved in photoperiod and autonomous pathways are well conserved between *Arabidopsis* and rice (Colasanti and Coneva, 2009; Distelfeld et al., 2009; Greenup et al., 2009; Jung and Muller, 2009; Higgins et al., 2010). The wild grass *Brachypodium distachyon* (L.) P. Beauv. (*Brachypodium* thereafter) Bd21 has well-conserved homologues of circadian clock, photoperiod pathway, and autonomous pathway genes defined in *Arabidopsis* (Higgins et al., 2010). The photoperiod pathway of *Arabidopsis* consists of various light photoreceptors and proteins (e.g., PHYA–PHYE), interacting with the circadian clock and enabling plants to anticipate and adapt to daily and seasonal changes in the environment. Flowering regulation through this pathway is ultimately controlled by transcript levels of a floral integrator named *FLOWERING LOCUS T* (*FT*) (Song et al., 2015). *FLOWERING LOCUS T* proteins named florigen are synthesized in leaves and move to the shoot apical meristem, activating multiple floral identity genes such as *LEAFY* (*LFY*) and *APETALA1* (*API*) (Golembeski and Imaizumi, 2015). Many plants possess *FT* and *FT*-like genes that promote flowering (Andrés and Coupland, 2012), including rice (Komiya et

al., 2008), maize (*Zea mays* L.) (Meng et al., 2011), and *Brachypodium* (Higgins et al., 2010), whereas other *FT*-like genes act as a floral repressor that delays flowering time in some dicot species (Pin et al., 2010; Harig et al., 2012). The activation of *FT* gene could be regulated by CONSTANS (CO), a B-box zinc finger protein, by integrating various signals into the photoperiodic flowering pathway (Shim et al., 2017). Although *FT* genes are found in flowering time pathway of both monocots and dicots, not all *FT* gene orthologs function in the same capacity across plant species.

Environmental cues dictate the initiation of flowering, specifically influencing the circadian clock-controlled mechanisms that integrate light signals to begin developmental changes and induce genes that trigger flowering (Michael et al., 2008). The circadian clock primarily relies on feedback loops that are rhythmically and transcriptionally regulated. Most genes involved in the feedback loops act as repressors but a few are positive regulators for flowering (Nohales and Kay, 2016; Wu et al., 2016). The simplified model of the *Arabidopsis* circadian clock has been reviewed by Shim et al. (2017). The morning loop is composed of the *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL 1* (*LHY*), two MYB transcription factors (TFs) that can repress expression of the clock genes from midday to evening such as *PSUEDO RESPONSE REGULATOR 9* (*PRR9*), *PRR7*, *PRR5*, *TIMING OF CAB 1* (*TOC1*), *LUX ARRHYTHMO* (*LUX*), and *EARLY FLOWERING 3* (*ELF3*) and *ELF4*. The evening loop consists of *ELF3*, *ELF4*, and *LUX*, named as the evening complex, that represses *PRR9* and *LUX*. This loop interconnects with the morning loop and is suppressed by *TOC1* expression (Nusinow et al., 2011). Moreover, *CCA1* can directly repress the expression of both *PRR9* and *PRR7* (Kamioka et al., 2016). Other floral transition promoters, such as *CAULIFLOWER* (*CAL*), *SEPALLATA* (*SEP*), *FRUITFUL* (*FUL*), and *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOCI*), can also induce floral transition in the photoperiod and circadian pathways (Khan et al., 2014).

The flowering pathways revealed in model plant species provide valuable references for identifying flowering orthologs in perennial grass species. Transcriptomic analysis of lowland and upland genotypes of switchgrass identified seven putative floral promoters involved in photoperiod–clock, autonomous, and vernalization pathways, and five floral repressors in the photoperiod–clock and autonomous pathways and in the category of *FPI* or *FMI* genes (Tornqvist et al., 2017). An example of a putative floral promoter, an ortholog of the *Arabidopsis* gene, *LUX ARRHYTHMO* (*LUX*), had little to no expression in the late flowering lowland genotypes, but the expression level spiked in early-flowering, upland genotypes, whereas a putative floral repressor, an ortholog of the *Arabidopsis* gene, *BRI1-ESSENTIAL 1* (*BES1*), had little to no expression in leaves of the upland, early-flowering genotypes but the expression level gradually increased in the late-flowering, lowland genotype (Tornqvist et al., 2017). Several other flowering orthologs such as *FT*, *BES1*, *PRR5*, *SUPPRESSOR OF FRIGIDA 4* (*SUF4*), and *API* involved in the circadian clock, vernalization, and floral meristem identity have been identified through genome-wide association analysis in natural populations (Grabowski et al., 2017) and an F₂ population (Taylor et al., 2018), and quantitative trait locus mapping (Tornqvist et al., 2018). Functional studies verified the role of *PvFT1* in promoting early flowering in switchgrass (Niu et al., 2016) and demonstrated that the *Arabidopsis* NAC transcriptional factor gene *LONG VEGETATIVE PHASE ONE*

(*AtLOV1*) caused delayed flowering time of switchgrass (Xu et al., 2012). However, the previously identified flowering genes and their expression patterns in switchgrass were not consistent due to variations of genotypes, growing conditions, tissue types, and sampling procedure across experiments (Niu et al., 2016; Grabowski et al., 2017; Tornqvist et al., 2017; Taylor et al., 2018). For example, *PvFT1* expression increased in leaves, especially at the time of floral transition in switchgrass (Niu et al., 2016); however, the transcriptomic analysis indicated very low expression levels of *FT* genes in switchgrass genotypes (Tornqvist et al., 2017).

The identification of differentially expressed flowering orthologs and TFs related to flowering time has not been performed using the contrasting parents and their progeny of switchgrass. We developed an F₂ population derived from an early-flowering cultivar and a late-flowering cultivar, and the population varied largely in heading and anthesis dates (Tornqvist et al., 2018). The contrasting parents and F₂ genotypes enabled us to compare flowering genes between parents, between F₂ genotypes, as well as between the parents and F₂ genotypes with early (or late) flowering, respectively. We attempted to identify novel flowering genes (including TFs) or validate previously identified flowering genes in contrasting switchgrass genotypes through transcriptome profiling by RNA sequencing. Such efforts were made towards developing a more comprehensive understanding of genes that control flowering time in switchgrass. The identified key candidate genes would lay a foundation for the development of potential markers for creating desirable switchgrass cultivars with late flowering and high biomass yield.

MATERIALS AND METHODS

Plant Materials and Sample Collection

The early-flowering genotype S041 from the upland 'Summer' cultivar, late-flowering genotype B901 from the lowland 'Ellsworth' cultivar, and two of their F₂ progeny (early-flowering Genotype 7071 and late-flowering Genotype 7055) were used in this experiment. All four were tetraploid genotypes. Briefly, S041 and B901 were crossed initially and F₁ seeds were harvested from the B901 parent as the female and germinated. Two random F₁ plants were selected to create a pseudo-F₂ population (Tornqvist et al., 2018). The S041, B901, and F₂ genotypes were propagated by tillers and established at Lafayette, IN, in July 2014. Detailed information about experimental design and plant maintenance was previously given (Taylor et al., 2018).

Heading and anthesis dates for the population were recorded in 2015 and 2016. Heading date was scored when 50% of the tillers had a visible panicle, whereas anthesis date was scored when 50% of the panicles reached the fully expanded stage. For plant sampling for RNA sequencing, phytomer tissues consisting of internode, node sheath and leaf blade were collected on 24 April (A) and 8 May (B) of 2017 in Lafayette, representing V1 to V2 and V2 to V3 of vegetative growth stages, respectively. The phytomer tissue was cut at ~5 cm under the node from the well-grown tiller in the middle of the crown of the plant, as illustrated by Supplemental Figure S1. At the first (A) and second harvest (B), each genotype sample

consisted of three phytomers from three tillers per plant, and each sample had three independent field replicates (nine phytomers). The samples were frozen in liquid nitrogen immediately on collection and stored in a freezer at -80°C for RNA extraction.

RNA Extraction and Sequencing Analysis

The RNA was extracted using 1 ml of TRIzol (Life Technologies) and purified using a DirectZol RNA-mini prep kit (Zymo Research Corporation). The transcriptome sequencing library was constructed and sequenced in paired end-mode on an Illumina HiSeq 2500 at the Purdue University Genomic Center. Prior to assembly, clean reads were obtained after filtering out adapters or low-quality reads using Trimmomatic version 0.36 (Bolger et al., 2014). The quality of clean reads was then assessed using FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), including total raw reads, total clean reads, Q20 (sequencing error rate = 1%), Q30 (sequencing error rate = 0.1%), and GC content. The clean reads were aligned to the switchgrass reference genome sequence (*Panicum virgatum* version 4.1, <https://phytozome.jgi.doe.gov/>) using Bowtie version 2.3.3 (Langmead and Salzberg, 2012) and TopHat version 2.1.1 (Kim et al., 2013) with a minimum intron size of 5 bp, a maximum intron size of 6000 bp, and a maximum of two mismatches. Fragments per kilobase pair of exon model per million fragments mapped (FPKM) was used to normalize gene expression values and determined using Cufflinks 2.2.1 (Trapnell et al., 2010). The Cuffdiff program within Cufflinks was used to detect differentially expressed genes (DEGs) between different sampling times of the same sample and then between different samples after comparisons of sampling times. A combined criterion of *t* test at *p* value $< .05$ and false discovery rate $< .05$ was adopted to identify statistically significant DEGs. We used the CummeRbund package in R to manage, visualize, and integrate the results of the RNA sequencing analysis.

KEGG Pathway Enrichment, Flowering Orthologs, and Transcription Factors

Mean FPKM values for the genes with FPKM > 0 in each of the eight samples (four genotypes with two sampling time points) were used for principal component analysis (PCA) using the “Bprcomp” function in the R programming language (R Core Team, 2014). Differentially expressed genes identified in all genotypes across their respective sampling times were annotated based on orthologues of *Arabidopsis*, given that *Arabidopsis* provides more information on GeneFarm, structural, and functional annotation. A Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was conducted using DAVID (the database for annotation, visualization, and integrated discovery) Bioinformatics Resources 6.8 (<https://david.ncifcrf.gov/>) software. To perform KEGG pathway enrichment analysis, all protein sequences of DEGs in switchgrass were subjected to a basic local alignment search tool (BLAST) search against the *Arabidopsis* sequences downloaded from the KEGG Orthology-Based Annotation System (KOBAS, <http://kobas.cbi.pku.edu.cn/>). A local BLASTX search was performed with an expected threshold of 1×10^{-10} , and the search results were filtered by keeping BLASTX hits showing at least 50% identity and a minimum aligned length of 200 amino acids. The homologous genes from *Arabidopsis* were submitted

to DAVID Bioinformatics software for functional annotation of DEGs. The hypergeometric test was subsequently performed to identify the differentially regulated pathways with a threshold P value $\leq .05$ as the significance level.

Differentially expressed genes mainly involved in circadian rhythm (from KEGG analysis) were extracted for identification of flowering orthologs for all samples in this study. To maximize the identification of flowering genes in switchgrass, we used these switchgrass flowering time orthologs along with flowering genes in *Arabidopsis* (Chen et al., 2012; Park et al., 2016) to search for the homologous genes involved in flowering pathways other than circadian clock. In addition, the flowering time orthologs identified previously in switchgrass, through searching protein sequences of known flowering time genes against *Arabidopsis*, maize, and/or rice (Tornqvist et al., 2017), were used for gene identification in this study.

We obtained the global TFs of switchgrass from Plant Transcription Factor Database (<http://planttfdb.cbi.pku.edu.cn/prediction.php>). In addition, the known TFs involved in regulation of flowering time from *Brachypodium* (Higgins et al., 2010; Chen et al., 2017), *Arabidopsis* (Brachi et al., 2010; Chen et al., 2012; Zhu et al., 2015; Shim et al., 2017; Zhang et al., 2018), rice (Zhang et al., 2015), and soybean [*Glycine max* (L.) Merr.] (Yin et al., 2013) were used for searching against the protein orthologs of switchgrass for flowering time.

Comparison of Differentially Expressed Genes

The initial comparisons of gene expressions were made between the two sampling points (A and B) in the early-flowering parent S041, the late-flowering parent B901, the early-flowering F₂ Genotype 7071, the late-flowering F₂ Genotype 7055, respectively. To identify significant DEGs between the early and late flowering genotypes, comparisons were made between the two parents (S041 vs. B901) and between two F₂ genotypes (7071 vs. 7055). To identify significant DEGs only in early- or late-flowering genotypes, comparisons were made between S041 and 7071 and between B901 and 7055. We also identified significant DEGs commonly observed in S041, B901, 7071, and 7055 by comparing four genotypes across A and B sampling points. Expression clusters were constructed using the Heatmap function in R language (R Core Team, 2014).

Quantitative Real-Time Reverse Transcription PCR

The quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis was performed using the same tissues for the RNA sequencing. The qRT-PCR was conducted for the selected DEGs in all early- and late-flowering genotypes at two sampling dates (A and B). Briefly, total RNA was isolated using a Direct-zol RNA MiniPrep Kit (Zymo Research Corporation), and 500 ng RNA was used for the reverse transcription performed with an iScript complementary DNA (cDNA) Synthesis Kit (Bio-Rad). A 10- μ L mixture was used for all qPCR reactions containing 1 μ L of cDNA, the relevant primers, and iTaq Universal SYBR Green (Bio-Rad) in a Mx3000P qPCR system (Agilent Technologies), with reaction for 10 min at 95°C followed by 40 amplification cycles of 10 s at 95°C, 30 s at 55°C, and 30 s at

72°C. Primer sequences for target genes and for switchgrass housekeeping gene of elongation factor 1- α (*eEF-1 α*) (Gimeno et al., 2014). The method of $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001) was used to calculate the gene expression level between sampling times. The analysis included three biological replicates and three technical replicates for each sampling time.

RESULTS

Genotype Heading and Anthesis Dates

Genotypes significantly varied in heading and anthesis dates across 2015 and 2016 (Figure 1). The late-flowering parent B901 had significantly later heading and anthesis dates than the early-flowering parent S041 and the F₂ Genotype 7071, whereas the late-flowering F₂ Genotype 7055 showed later heading and anthesis dates than 7071. No differences in heading and anthesis dates were observed between two early- or two late-flowering genotypes. On average, the early-flowering genotypes reached heading and anthesis dates 17 and 19 d before the late-flowering genotypes, respectively (Figure 1).

Characterization of the Sequenced Libraries

Transcriptome sequencing of the libraries produced 27.9–46.9 million clean reads after quality checking and filtering (Supplemental Table S1). Over 96.1% of the clean reads had quality scores at the Q20 level, and >87.2% had quality scores at the Q30 level (Supplemental Table S1). Approximately 89.0–92.2% of the reads from each library were matched to the reference genes, and >82.6% of the reads in the libraries were mapped to single locations (Supplemental Table S2). In addition, ~40% of these uniquely mapped reads in each library were mapped to the sense strand and the antisense strand, respectively (Supplemental Table S2). All raw sequence reads are available from the National Center for Biotechnology Information under Project PRJNA560156.

Differentially Expressed Genes

Based on the switchgrass reference genome sequence (*Panicum virgatum* version 4.1), a total of 89,336 genes were identified across all four genotypes at the first sampling time (A) and the second sampling time (B) (Supplemental Table S3). Genotypes S041, B901, 7071, and 7055 had 63,041, 63,050, 63,150, and 62,488 genes, respectively. Out of 89,336 genes in the FPKM matrix, 71,799 genes with FPKM > 0 were used for PCA. Combined, Principal Components 1 and 2 accounted for ~48.6% of the total variation within the FPKM matrix (Supplemental Figure S2). Principal Component 1 accounted for genotype and/or flowering time variation, and the parent S041 and B901 separated better than F₂ 7071 and 7055. Principal Component 2 clearly accounted for sampling time variation (A and B).

Comparing A and B sampling times, more DEGs were upregulated in S041, B901, and 7055, whereas slightly more DGEs were downregulated in 7071 (Supplemental Figures S3 and S4). The number of DGEs unique to each genotype or shared between two genotypes was shown in Figure 2 and Supplemental Table S4. Specifically, comparing the two parents, S041

contained 4780 unique DEGs and B901 contained 1125 DEGs, and both shared 2033 common DEGs. Similarly, F₂ 7071 had 1098 DEGs and F₂ 7055 consisted of 3976 DEGs, and both shared 830 DEGs. Both early-flowering genotypes shared 858 DEGs and both late-flowering genotypes shared 1633 DEGs. A total of 399 common DEGs were identified in all genotypes across the two sampling time points (Figure 2).

Functional Enrichment Analysis

Enrichment analysis identified significant DEGs, which indicated enriched metabolic pathways or signal transduction pathways in comparison with the whole genome background. Twenty-five significantly enriched pathways were identified in one or more genotypes (Figure 3). Biosynthesis of secondary metabolites, flavonoid biosynthesis, and metabolic pathways were enriched in all four genotypes, and the photosynthesis pathway was enriched in three genotypes, excluding F₂ 7071. Differentially expressed genes belonging to carbon and carbohydrate metabolism pathways were mainly identified in F₂ 7071 and 7055. Circadian rhythm pathways were enriched in S041 and 7071, but not in B901 and 7055. Differentially expressed genes involved in ribosome biogenesis, oxidative phosphorylation, and DNA replication pathways were predominantly shown in S041 and 7055, whereas DEGs involved in glycerolipid metabolism and amino acid metabolism were mainly detected in B091 and 7071. Fatty acid elongation was enriched only in S041, whereas vitamin B6, sulfur, and phenylalanine metabolism and steroid biosynthesis were enriched only in B901. Pathways such as phenylpropanoid biosynthesis, ascorbate, and some amino acid metabolism were significantly enriched in 7071, but galactose metabolism and carbon fixation were enriched only in 7055.

Flowering Orthologs

To identify DEGs between early- and late-flowering genotypes, we compared two parents and two F₂ genotypes across two sampling time points (A and B) (Figure 4I–4IV, Supplemental Table S5). When comparing two parents, 11 significant flowering DEGs were detected in the early-flowering S041, including *PHYTOCHROME A (PHYA)*, *CHALCONE SYNTHASE (CHS)*, *FLAVIN-BINDING KELCH REPEAT F-BOX (FLK1)*, *PHYTOCHROME AND FLOWERING TIME REGULATORY PROTEIN (PFT1)*, *CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COPI)*, *TOC1*, *GIBBERELLIN-REGULATED PROTIEN (GASA)*, and *FT* orthologs (Figure 4I). Specifically, *TOC1*, *FKF1*, two *PHYA*, and two *PFT1* were upregulated, and *GASA*, *COPI*, *FT*, and two *CHS* were downregulated in S041. However, only one upregulated *FT* and one downregulated *GASA* were identified in B901 across two sampling time points (Figure 4I).

When comparing two F₂ genotypes, two *FT* genes and one *PFT1* gene were detected in early-flowering F₂ 7071, and all three were upregulated across two sampling time points (Figure 4II, Supplemental Table S5). There were seven significant DEGs identified in late-flowering F₂ 7055, including upregulated *PSUEDO RESPONSE REGULATOR (PRR7)* and *PRR5 (Pavir.8NG050900)*, *PHYTOCHROME B (PHYB)*, *TOC1*, and two *CKB4*, and downregulated *COPI* across two sampling time points (Figure 4II).

To identify significant DEGs only in the two early- or two late-flowering genotypes, we compared S041 with 7071 and B901 with 7055, respectively. Expressions of *PRR5* (*Pavir.2NG448600*) and *FKF1* were upregulated and expressions of *GASA* and *COP1* were downregulated in both early-flowering S041 and 7071, whereas upregulation of *FKF1*, *PRR5* (*Pavir.2NG448600*), *PRR7*, and *PHYB* and downregulation of two *GASA* genes were observed in two late-flowering genotypes B901 and 7055 across two sampling time points (Figure 4III, Supplemental Table S5). Three DEGs—*Pavir.2NG448600* (*PRR5*), *Pavir.8NG130300* (*FKF1*), and *Parvi.9NG045700* (*GASA*)—were upregulated in all four genotypes across two sampling time points (Figure 4IV, Supplemental Table S5).

Differentially Expressed Transcription Factors for Flowering Time

Similarly, the homologs of differentially expressed TFs for flowering time were identified between two parents, two F₂ genotypes, between the two early- or two late-flowering genotypes, and among all four genotypes across their respective sampling times A and B. A total of 539 TFs from 44 families were differentially expressed between the early- and late-flowering genotypes across their respective two sampling times A and B (Supplemental Table S6). Of them, 129 differentially expressed TFs were related to flowering time (Supplemental Table S7), including homologs of 18 MYB or MYB-related and 14 NAC involved in circadian control in *Brachypodium* (Zhu et al., 2015; Chen et al., 2017) and homologs of 13 *WRKY* involved in circadian control in soybean (Li et al., 2016). Comparing two parents, 17 TFs in nine families were upregulated and 24 TFs in 12 families were downregulated in S041, whereas upregulations of five TFs in four families and downregulations of 11 TFs in eight families were found for B901 (Supplemental Table S7, Supplemental Figures S5 and S6). When comparing two F₂ genotypes, 10 TFs in five families were upregulated and four TFs in four families were downregulated in 7071, whereas upregulations of 10 TFs in eight families and downregulations of 45 TFs in 14 families were detected in 7055 (Supplemental Table S7, Supplemental Figures S5 and S6).

Across both early-flowering genotypes of S041 and 7071, one *GRAS*, three *WRKY*, and three *NAC* were upregulated, and two *bHLH*, three *MYB*, three *CO-like*, two *bZIP*, one *DOF*, and one *G2-like* were downregulated across two sampling time points (Figure 5I, Supplemental Table S7). For both late-flowering genotypes of B901 and 7055, upregulations of one *bHLH*, one *AP2*, two *NAC*, two *WRKY*, and two *GRAS* and downregulation of eight *CO-like*, one *C3H*, five *bZIP*, one *WRKY*, one *G2-like*, one *NF-YA*, one *NF-YB*, four *MYB*, one *SBP*, one *MADS*, one *bHLH*, one *DBB*, two *NAC*, and two *DOF* were identified across two sampling time points (Figure 5II, Supplemental Table S7). Twelve TFs were commonly expressed in all genotypes, including upregulation of one *RAS*, one *NAC*, and one *WRKY*, and downregulation of one *bZIP*, three *CO-like*, one *G2-like*, two *MYB*, and one *NAC* (Figure 5).

The qRT-PCR Analysis of Flowering Gene Expression

We performed qRT-PCR analysis to confirm the reliability of expression of DEGs in the samples from the first sampling time (A) compared with the second sampling time (B) (Figure 6). Primers for each gene are listed in Supplemental Table S8. Fourteen DEGs related

to flowering time were selected in the early- and late-flowering genotypes, and qRT-PCR results were consistent with expression analysis of DGEs through RNA sequencing (Figures 4–6, Supplemental Figures S5 and S6). There was a strong correlation between qRT-PCR results and RNA sequence values (Figure 7). Specifically, expressions of *PRR5* (*Pavir.2NG448600*) and *WRKY72* (*Pavir.8KG145400*) significantly increased at the second sampling time in all four genotypes. Expression of *PFT1* (*Pavir.2NG195200*) was upregulated in S041, whereas expressions of *PHYA* (*Pavir.5KG291600*) and *NAC* (*Pavir.3KG002200*) were upregulated in B901 at the second sampling time. The expressions levels of *FT* (*Pavir.5KG044000*) and *NAC* (*Pavir.9NG282000*) increased in F₂ 7071, and expression levels of *PRR7* (*Pavir.2NG126000*), *PFT4* (*Pavir.9NG081800*), and *TOC1* (*Pavir.1KG3853000*) increased in F₂ 7055 at the second sampling time. Expressions of *NAM* (*Pavir.9NG437100*) and *GA3* (*Pavir.4NG214600*) were upregulated in both early-flowering genotypes, whereas expressions of *GRAS* (*Pavir.2KG467700*) and *AP2* (*Pavir.3KG118100*) were upregulated in both late-flowering genotypes across sampling times.

DISCUSSION

Floral transition can be considered as a reprogramming of the meristem to form flowers and reproductive organs (Howell, 1998). This transition is initiated by both endogenous (e.g., age and developmental phase) and environmental signals. From a plant developmental standpoint, the key stage in determining flowering is the transition of the shoot apical meristem from the vegetative to reproductive phase. This transition in switchgrass may occur several weeks before heading is observed (Tornqvist et al., 2017). Therefore, we made sampling at vegetative stages, which is crucial for identifying flowering genes that are expressed for regulation of physiological changes from a vegetative to a reproductive mode.

Switchgrass requires no vernalization for flowering. This may be related to flowering time being in the summer and autumn, not in spring (Michaels and Amasino, 2000). Switchgrass is photoperiodic. Phenotypic variation of flowering time in switchgrass is largely driven by the latitude of genotype origin (McMillan, 1965; Casler and Boe, 2003). In this study, the parent S041 from northern latitude reached heading and anthesis dates before the parent B901 from southern latitude (Figure 1). Similarly, F₂ Genotype 7071 flowered earlier than F₂ Genotype 7055 (Figure 1). These phenotypes allowed the flowering mechanism (e.g., gene expression) to be identified and compared between the early- and late-flowering genotypes or between two early- or two late-flowering genotypes. In this study, the early-flowering parent S041 had more upregulated and downregulated flowering orthologs than late-flowering parent B901, whereas late-flowering F₂ 7055 had more upregulated flowering orthologs than the early-flowering F₂ 7071 after pair comparisons across their respective two sampling times (Figures 4I and 4II). In addition, early- and late-flowering genotypes also largely varied in the number of upregulated or downregulated TFs related to flowering (Figure 5, Supplemental Table S6, Supplemental Figures S5 and S6). The results indicated that these differentially expressed flowering genes or TFs likely influenced flowering initiation during vegetative growth to promote or suppress flowering time of switchgrass.

Most flowering genes identified in this study were involved in the photoperiod pathway–circadian clock, including *PHYA*, *PHYB*, *TOC1*, *PRR5*, *PRR7*, *CO-like*, and *FT*. A previous transcriptomic analysis in switchgrass revealed a few genes involved in photoperiod–circadian clock including five flowering promoters (*PHYA*, *PHYB*, *CO*, *CCA1-Like*, *LUX*) and one repressor (*CCA1-like*) (Tornqvist et al., 2017). Phytochromes (*PHYA*–*PHYE*) are light photoreceptors that play fundamental roles in plant growth and development (Li et al., 2011). We found that two *PHYA* genes (*Pavir.9NG128200* and *Pavir.5KG291600*) were upregulated in the early-flowering parent S041, but not in the late-flowering parent B901 across two sampling time points (Figure 4I). However, these differentially expressed *PHYA* genes were not shown in the early F₂ Genotype 7071 compared with late-flowering F₂ Genotype 7055. The *PHYA* gene (*Pavir.5KG291600*, previously known as *Pavir.J04655*), a homolog of *AT1G09570* and *Os03g51030* (Supplemental Table S5), acted as a flowering promoter expressed in early-flowering groups in switchgrass (Tornqvist et al., 2017). In *Arabidopsis*, a *PHYA* mutant flowered later than wild-type plants (Johnson et al., 1994). The results suggested a role of *PHYA* in promoting early flowering. By contrast, *PHYB* can play an inhibitory role in floral initiation, and *PHYB* mutants flowered earlier than the wild type with decreased photoperiodic sensitivities in several plant species (Weller and Reid, 1993; Childs et al., 1997; Mockler et al., 1999). A sorghum [*Sorghum bicolor* (L.) Moench] photoperiod sensitivity gene, *Ma3*, encodes a *PHYB*, acting as a floral repressor (Childs et al., 1997). The effects of *PHYB* on late flowering could be due to its interpreting thermal signals to regulate anthesis and using gibberellic acid to control flowering time by repressing anthesis (Blázquez and Weigel, 1999). Our results also demonstrated *PHYB* (*Pavir.9KG418200*), a homolog of *AT2G18790* and *Os03g19590* (Supplemental Table S5), was upregulated in both late-flowering parent B901 and F₂ 7055, indicating its possible role in regulating late flowering in switchgrass. However, expression of the gene (previously known as *Pavir.Ib01239*) was not consistent with that found in lowland groups of switchgrass (Tornqvist et al., 2017). It appeared that expression of *PHYB* could be genotypic specific and influenced by environmental conditions.

Many circadian clock-associated genes have been identified in plants, including *TOC1*, *CCA1*, *LHY*, *HLI*, and *PRR* families (Nakamichi et al., 2005a, 2005b). However, the expression profiles of these genes in regulating flowering time were not always consistent. For example, *PRR5* mutant or overexpression of *PRR5* led to early or late flowering time in *Arabidopsis*, depending on environmental conditions (Yamamoto et al., 2003; Niinuma et al., 2008). In barley (*Hordeum vulgare* L.), expression of *PRR5* or *PRR9* was higher in late-flowering genotypes under long day conditions (Campoli et al., 2012). Not all *PRR* genes act similarly in switchgrass, and their expression patterns varied with genotypes. Previously, it was reported that *PRR5* expression was either up- or downregulated or remained unchanged in early- or late-flowering genotypes at different growth stages of switchgrass (Tornqvist et al., 2017). In this study, differentially expressed *PRR5* (*Pavir.2NG448600*), a homolog of *AT5G24470* and *Os09g36220* (Supplemental Table S5), was upregulated in all genotypes across two sampling time points. For regulating early flowering, *PRR* proteins could stabilize CO and increase binding of CO to the promoter of *FT*, leading to early flowering under long day conditions (Hayama et al., 2017). It illustrated a role of *PRR* proteins by acting on the

CO protein to promote flowering. We also found that another *PRR5* gene (*Pavir.8NG050900*), a homolog of *AT5G24470* and *Os11g05930* (Supplemental Table S5), was only upregulated in the late-flowering F₂ 7055 compared with the early-flowering F₂ 7071 (Figure 4II). In addition, upregulation of *PRR7* (*Pavir.2NG126000*), a homolog of *AT5G02810* and *Os07g49460* (Supplemental Table S5), was only observed in both late-flowering parent B901 and F₂ 7055 (Figure 4IV). In sorghum, three maturity loci (Ma1, Ma3, and Ma6) act as floral repressors that are involved in photoperiod response (Wolabu and Tadege, 2016). Of them, Ma1, corresponding to *SbPRR37*, is the ortholog of *Arabidopsis PRR7* (Murphy et al., 2011). Our results supported that *PRR5* (*Pavir.8NG050900*) and *PRR7* might act as floral repressors and delay flowering time of switchgrass. However, the mechanisms of *PRR* on regulating switchgrass flowering time deserve further investigation.

FLOWERING LOCUS T is a floral integrator of the photoperiodic pathway. Our results showed that three *FT* genes (*Pavir.5KG044000*, *Pavir.7NG223500*, and *Pavir.6KG345800*), homologs of *OsFT-like 8*, *OsFT-like6*, and *OsFT-like4*, respectively (Supplemental Table S5), were upregulated in the early-flowering genotypes, and one of them (*Pavir.7NG223500*) was downregulated in the late-flowering genotype, demonstrating the role of *FT* genes in promoting flowering in switchgrass (Figures 4I and 4II). Also in switchgrass, *PvFT1* expression increased in leaves especially at the time of floral transition, and ectopic expression of *PvFT1* in *Arabidopsis*, *Brachypodium*, and switchgrass led to extremely early flowering and activation of *FT* downstream target genes (Niu et al., 2016). However, the transcriptomic analysis indicated very low expression levels of *FT* genes in early- and low-flowering switchgrass genotypes (Tornqvist et al., 2017). The differences in tissue sampling and genotypes could cause the variations of *FT* expression in switchgrass. At the population level, genome-wide association studies identified a homolog of *FT* related to heading date in F₂ mapping populations (Taylor et al., 2018), whereas a different *FT* gene was associated with early heading in natural populations (Grabowski et al., 2017). There are at least 47 *FT* genes in the switchgrass genome (Taylor et al., 2018), and they could play different roles in regulating flowering time in switchgrass. In other plant species, expressions of *FT* genes are also complex. Some *FT* and *FT*-like genes promote flowering (Andrés and Coupland, 2012), including genes found in rice (Komiya et al., 2008), maize (Meng et al., 2011), and *Brachypodium* (Higgins et al., 2010), whereas other *FT*-like genes may act as floral repressors that delay flowering in sugar beet (*Beta vulgaris* L.) (Pin et al., 2010) and tobacco (*Nicotiana tabacum* L.) (Harig et al., 2012). In rice, one *FT* gene promoted flowering in short days only, and another *FT* gene responded to long days only (Tsuji et al., 2011). Collectively, *FT* genes act differently in regulating flowering, and their functions may vary with plant developmental stages and specific genotypes.

Several other flowering-related DEGs were upregulated or downregulated in early- or late-flowering genotypes of switchgrass, including *PFT1*, *CKB4*, *COP1*, *FKF1*, *GASA*, *GA3*, and *CHS* (Figure 4). For example, upregulation of three *PFT1* genes (*Pavir.2NG195200*, *Pavir.2KG188500*, and *Pavir.6KG047300*), homologs of *AT1G25540* and *Os09g13610* (Supplemental Table S5), were identified in early-flowering genotypes, indicating a possible role of *PFT1* in contributing to early flowering in switchgrass. In *Arabidopsis*, *PFT1* was found to promote flowering independently by regulating *FT* and *CONSTANS* (Iñigo et al.,

2012). The *FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1)* gene is activated by blue light and promotes flowering of *Arabidopsis* through regulation of *CONSTANS* under long-day conditions (Imaizumi et al., 2005). The *FKF1* mutants had delayed flowering in rice under both short- and long-day conditions (Han et al., 2015). Along with research findings in other plant species, upregulation of two *FKF1* genes (*Pavir.8NG141400* and *Pavir.8NG130300*), homologs of *OsFBO10* (Supplemental Table S5), was also detected in early-flowering switchgrass genotypes in this study, suggesting a role of *FKF1* genes in promoting early flowering. In addition, the gibberellin (GA) class of plant hormones regulates plant development including flowering. Gibberellin requiring 3 (*GA3*), a multifunctional cytochrome P450 in GA biosynthesis, interacts with *FLC* to influence flowering (Deng et al., 2011). In *Arabidopsis*, GA signaling is required for floral induction through the activation of genes encoding the floral integrators *SOC1*, *LFY*, and *FT* in the inflorescence, floral meristems, and leaves, respectively (Mutasa-Göttgens and Hedden, 2009). Upregulation of *GA3* (*Pavir.4NG214600*), a homolog of *AT5G25900* and *Os06g37330* (Supplemental Table S5), in both early-flowering genotypes indicated that *GA3* could induce flowering in switchgrass.

Transcription factors play an important role in regulating flowering time by interacting with the flowering pathways and environmental cues (Park et al., 2016). Key regulators of flowering time such as *CO*, *FT*, *FLC* and *SOC1* are often the primary targets for transcriptional control of flowering (Liu et al., 2013; Weng et al., 2016). Generally, for TF related to flowering time, the number of downregulated TFs was almost double compared with the upregulated TFs when combined across all genotypes (Figure 5, Supplemental Table S6, Supplemental Figures S5 and S6). The early-flowering genotypes, especially the parent S041, had more upregulated TFs, whereas the late-flowering genotypes, especially the F₂ 7055, had more downregulated TFs. The results showed complex patterns of TFs in regulating genes to influence flowering time of switchgrass. Specifically, *CONSTANS* is an important floral regulator in the photoperiod pathway, integrating the circadian clock and light signal into a control for flowering time. In this study, three *CO-like* genes (*Pavir.1KG080800*, *Pavir.9KG330800*, and *Pavir.9NG456000*) were downregulated in early- and late-flowering genotypes, whereas an additional five *CO-like* genes were downregulated only in late-flowering genotypes (Figure 5). The results in these five *CO-like* genes supported the previous discoveries that expression of *CO* (*Pavir.Db01632*) in leaves decreased with increasing plant growth in the late flowering groups of switchgrass (Tornqvist et al., 2017). In *Arabidopsis*, a *CONSTANS* gene promotes flowering and encodes a protein with similarities to zinc finger TFs (Putterill et al., 1995). Expression of *CO* promoted flowering under long days in *Arabidopsis* and induced transcription of *FT*, leading to anthesis (Cockram et al., 2012). However, overexpression of *COL9*, a *CONSTANS*-like gene, delayed flowering in *Arabidopsis* by reducing expression of *CO* and *FT*, whereas a gene knockout line exhibited earlier flowering under long-day conditions (Cheng and Wang, 2005). Moreover, overexpression of this gene induced flowering in *Arabidopsis* under short-day conditions with a concomitant change in *FT* and *SOC1* expression, but plants with loss of *COL5* function did not have altered flowering time (Hassidim et al., 2009). Taken together,

regulation of *CO-like* genes on flowering time is complex and could be through different regulatory mechanisms.

NAC proteins are a family of TFs that have a variety of regulatory roles in plants including flowering time (Ning et al., 2015). Through analysis of 77 NAC members involved in circadian control in *Brachypodium* (Zhu et al., 2015), we identified 14 differentially expressed NAC homologs in switchgrass (Figure 5, Supplemental Table S6, Supplemental Figure S6). Of them, both early-flowering S041 and 7071 had three upregulated NAC genes, whereas both late-flowering B901 and 7055 had two upregulated and two downregulated NAC genes (Figure 5). These NAC genes may contribute to either early or late flowering in switchgrass, indicating their different roles in regulating flowering time. In particular, one NAC gene (*Pavir.8NG018500*), a homolog of *AT1G69490* and *Os11g03300*, Supplemental Table S7), was upregulated in the early-flowering and downregulated in the late-flowering genotypes, indicating its role in contributing to early flowering. This NAC gene has multiple biological functions in *Arabidopsis*, including early flower development, leaf senescence, and fruit ripening, and is expressed in floral primordia and upregulated by AP3 and PI (Wellmer et al., 2006).

The WRKY TFs are key regulators of many plant processes including flowering time (Li et al., 2016). Through analysis of 99 WRKY members involved in circadian regulation in soybean (Yin et al., 2013), we identified 13 WRKY homologs in switchgrass that were differentially expressed between early- and late-flowering genotypes (Figure 5, Supplemental Table S6, Supplemental Figure S6). Except for upregulation of one WRKY gene (*Pavir.8KG145400*) shown in all genotypes, two additional WRKY genes were upregulated in both early-flowering S041 and 7071, and one additional WRKY gene was upregulated and one was downregulated in both late-flowering B091 and 7055. The results supported a diverse function of WRKY genes in regulating flowering time in plant species (Li et al., 2016). In particular, a WRKY gene (*Pavir.8NG230700*), a homolog of *AT5G13080* (WRKY75) and *Os11g29870* (WRKY72) (Supplemental Table S7), was upregulated in both early-flowering genotypes. Previous research showed that a mutation of this gene WRKY75 resulted in delayed flowering, whereas overexpression of WRKY75 accelerated flowering in *Arabidopsis* (Zhang et al., 2018). These authors further demonstrated that WRKY75 positively regulated flowering in a FT-dependent manner and may be involved in the GA-mediated signaling pathway to regulate flowering in *Arabidopsis* (Zhang et al., 2018). Thus, we speculated that upregulation of *Pavir.8NG230700* might induce flowering of switchgrass.

Through analysis of 83 MYB members involved in circadian control in *Brachypodium* (Chen et al., 2017), we identified 18 differentially expressed MYB or MYB-related homologs in switchgrass (Figure 5, Supplemental Table S6, Supplemental Figure S6). Three MYB genes were downregulated in both early-flowering genotypes, and four were downregulated in both late-flowering genotypes (Figure 5). The results suggested their specific role in regulating early or late flowering time in switchgrass. Collectively, the regulations of differentially expressed NAC, WRKY, and MYB genes on flowering time are complex, and their role in flowering time deserves further characterization in switchgrass.

CONCLUSIONS

Flowering pathways are complex and many genes are involved in regulation of flowering time. This study identified differentially expressed flowering genes and TFs related to flowering time between the parents and F₂ individuals contrasting in flowering time. Most flowering genes identified in this study were involved in the photoperiod pathway–circadian clock. The late-flowering genotypes had fewer differentially expressed flowering genes but more differentially expressed TFs related to flowering than the early-flowering genotypes. Our results supported that various TFs could be involved in the regulation of early or late flowering time in switchgrass. Further research could be conducted to verify the functional role of key genes in regulating flowering time of switchgrass, especially for those genes that may contribute to late flowering. Such research efforts could facilitate breeding programs aimed at improvement of switchgrass with late flowering time and high biomass yield.

Supplemental Material

Supplemental material is available online for this article.

Conflict of Interest

The authors declare that there is no conflict of interest.

Author Contributions

M. Taylor collected phenotypic data. X. Zhao led analysis of RNA sequencing data. M. Casler participated in interpreting results and writing the manuscript. Y. Jiang and M. Taylor designed the experiments and led writing of the manuscript.

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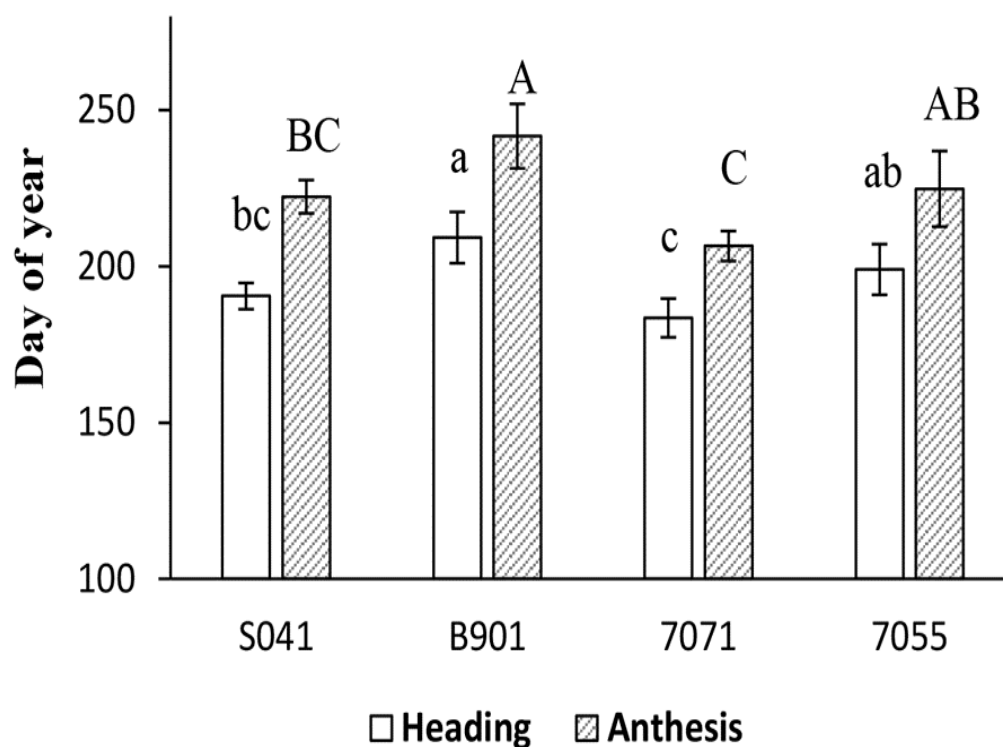


FIGURE 1. Heading and anthesis dates of the early-flowering parent S041, late-flowering parent B901, early-flowering F₂ Genotype 7071, and late-flowering F₂ Genotype 7055 across 2 yr. Comparisons were made for heading (lowercase letters) and anthesis (uppercase letters) among four genotypes. Means followed by a common letter are not significantly different at $P < .05$. Bars indicate standard deviations.

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FIGURE 2. Venn diagrams of the numbers of significant differentially expressed genes between four pairwise comparisons including S041A vs. S041B, B901A vs. B901B, 7071A vs. 7071B, and 7055A vs. 7055B. A, first time of sampling; B, second time of sampling. S041, early-flowering parent; 7071, early-flowering F₂ individual; B901, late-flowering parent; 7055, late-flowering F₂ genotype.

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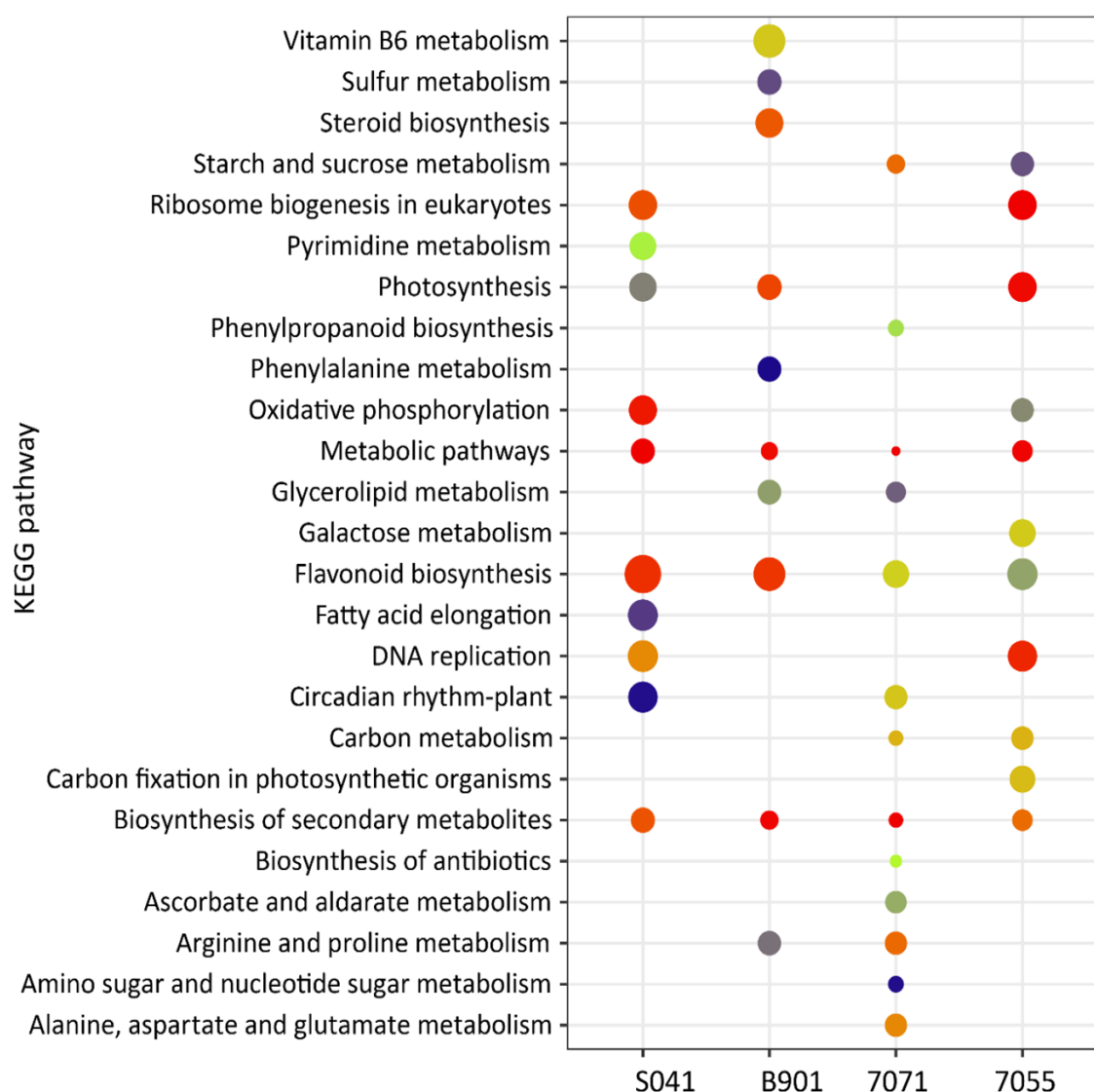


FIGURE 3. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis for differentially expressed genes (DEGs) that showed some statistical significance in early-flowering parent S041, late-flowering parent B901, early-flowering F₂ individual 7071, or late-flowering F₂ individual 7055. The y axis corresponds to the KEGG pathway, and the x axis indicates the four genotypes. The hypergeometric test was performed to identify the differentially regulated pathways with a threshold of $P \leq .05$ as the significance level. Rich factor is the ratio of the number of DEGs for a particular KEGG pathway to the number of all annotated genes in the background for that pathway.

AOC

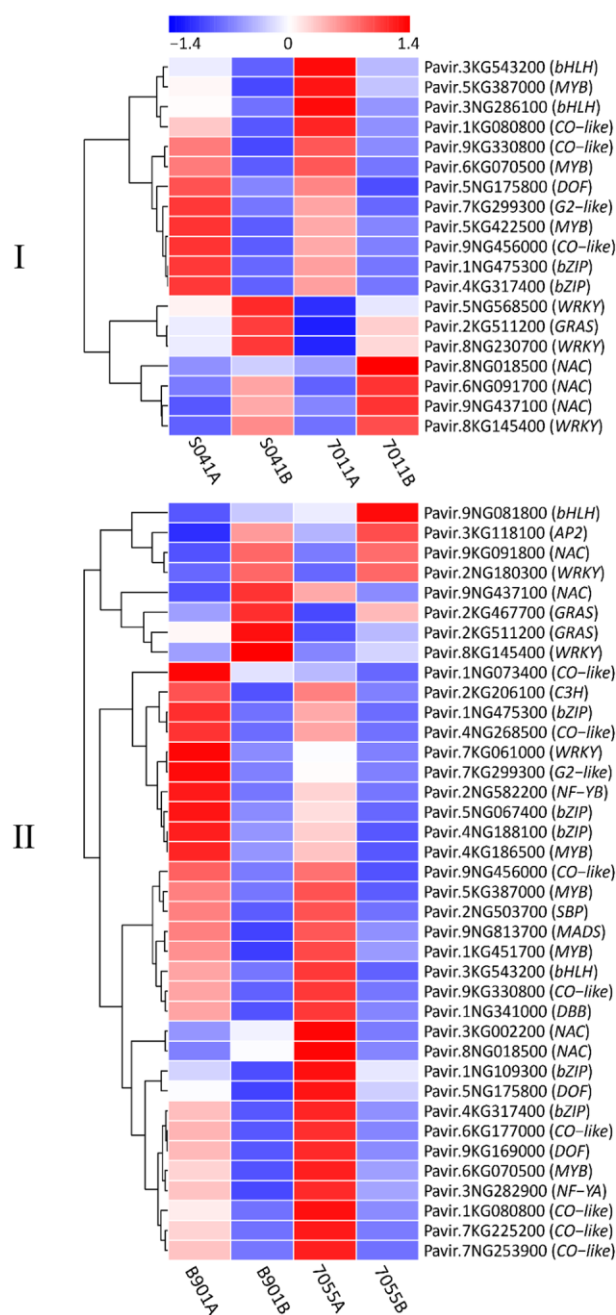


FIGURE 5. Hierarchical clustering of common differentially expressed transcription factors for flowering in early- or late-flowering switchgrass genotypes. Two pairwise comparisons were made: (I) early-flowering parents S041A and S041B vs. early-flowering F₂ Genotypes 7071A and 7071B, and (II) late-flowering parent B901 and late-flowering F₂ Genotypes 7075A and 7075B (A, first time of sampling; B, second time of sampling). The color scale of blue (low), white (medium), and red (high) represents the normalized expression level.

A

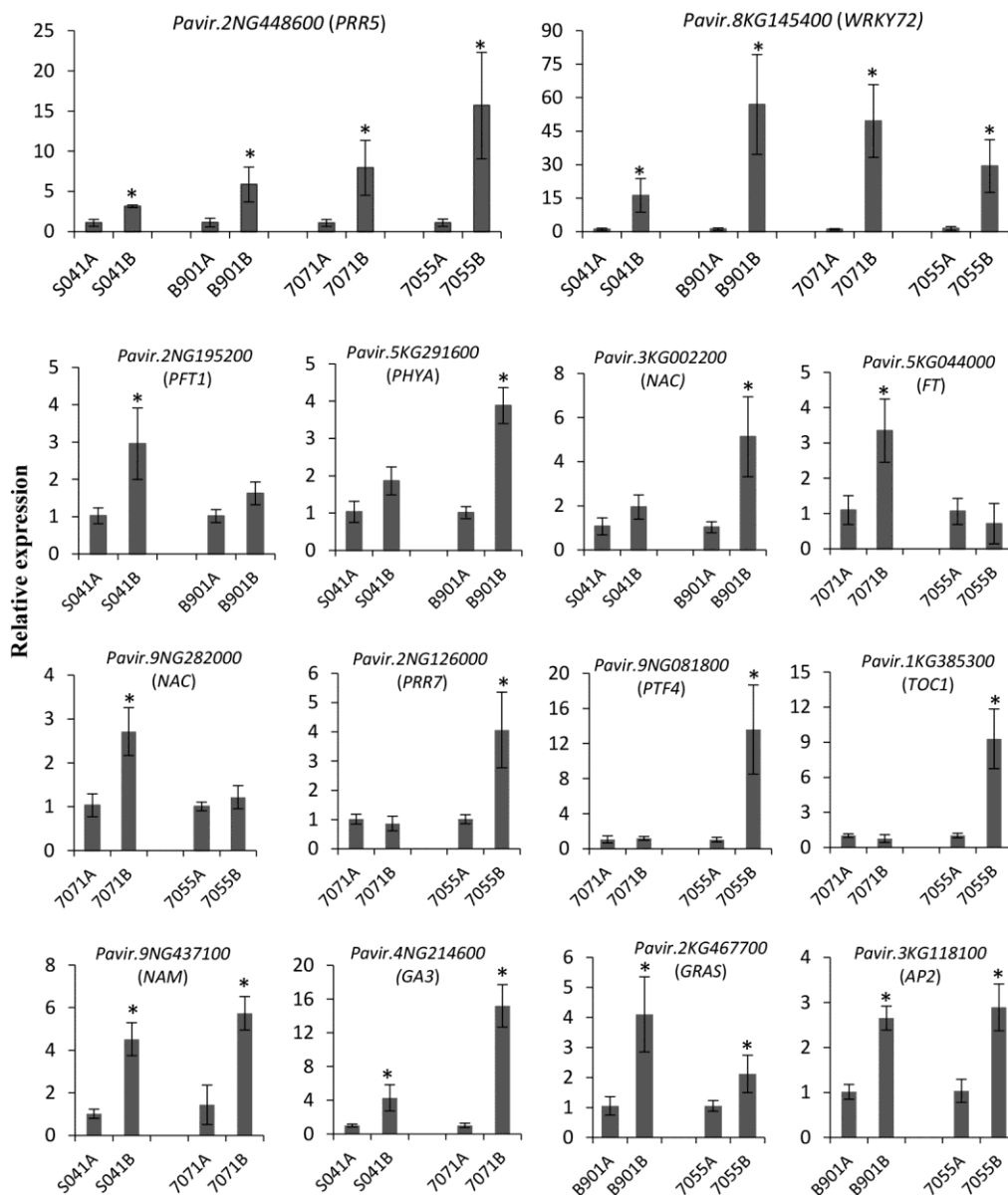


FIGURE 6. The quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of 14 selected flowering genes in switchgrass: S041, early-flowering parent; 7071, early-flowering F₂ individual; B901, late-flowering parent; 7055, late-flowering F₂ genotype; A, first time of sampling; B, second time of sampling. Comparison was made between A and B for a given genotype. * Significance at $P < .05$. Bars indicate standard deviations.

AC

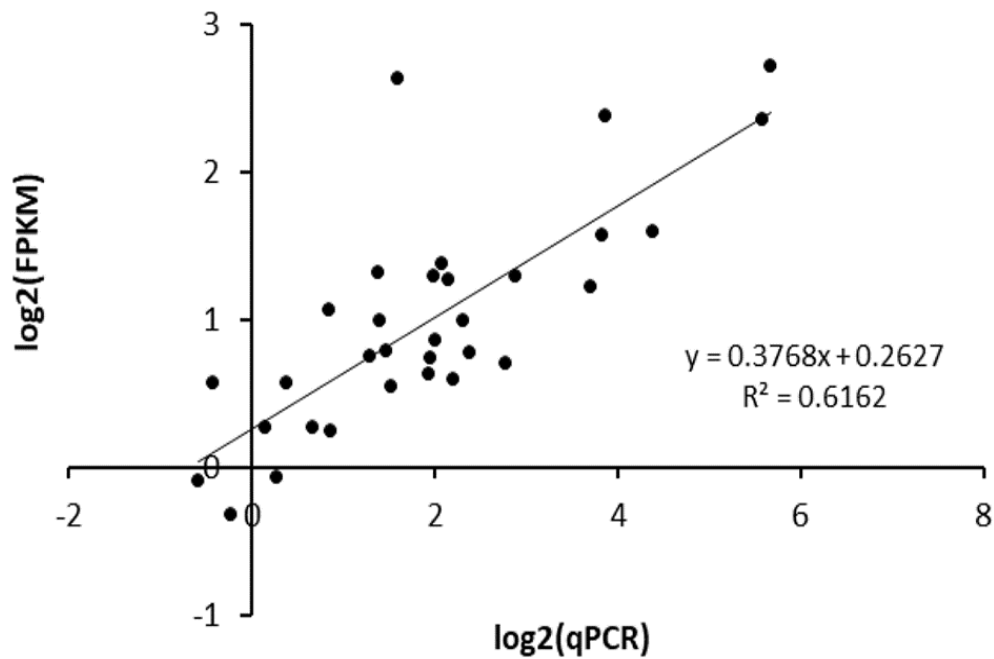


FIGURE 7. Correlations between quantitative reverse transcription polymerase chain reaction (qRT-PCR) and reads per kilobase of transcript per million (FPKM) from RNA sequence of 14 selected flowering genes in four contrasting switchgrass genotypes.

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