**Working Title:** The genetic basis of two flowering time cues in switchgrass (*Panicum virgatum*)

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**Intended Audience:** *biologists interested in GxE, plant biologists interested in flowering*

**Target Journal:** *New Phytologist, TEJ suggests: PNAS, Nature Communications*

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**Abstract**

Switchgrass (*Panicum virgatum*) is a perennial, outcrossing species native to North America which has evolved into multiple divergent populations that vary in ploidy, morphology, and phenological timing. Its potential uses have expanded in the past few decades to include biofuels. Flowering time is a key life-history trait for biofuel production, as plants cease biomass accumulation upon completion of floral development. Here, we present evidence that photoperiodic sensitivity in switchgrass flowering time varies between genetic subpopulations using a diversity panel grown at eight field sites in the central United States spanning 17 degrees of latitude. We then map the genetic basis of flowering time in this population and in an independent four-way cross created from individuals from two highly divergent southern lowland and northern upland populations. We describe eight additive QTLs across these seven field sites with moderate effects on flowering, five of which had overlapping significant associations in the diversity panel. \_sentence about major gene candidates found – which genes are in Xiaoyu’s flowering homolog list\_\_.

**Keywords**: flowering time, GxE (genotype by environment interactions), switchgrass, Panicum virgatum, photoperiod

**Introduction**

The timing of floral development is a major component of plant fitness affected by multiple external environmental cues (e.g. temperature, daylength, and water availability) that signal existing or upcoming growing conditions. Genetic responses to these cues determine the speed, timing, and energy apportioned to reproductive growth and shape both the individual’s lifespan and its lifetime production of viable seed. Global climate forcing is increasing temperatures and causing more extreme weather events, such as droughts, heatwaves, and severe rain events (Ummenhofer and Meehl, 2017). These events will alter both the timing of reproductive development and the reliability of the environmental signals that plants use to cue flowering. It is thus of increasing importance that we understand the environmental cues driving floral development and the genetics underlying flowering responses to understand the genetic potential for adaptation to novel, changing environments.

Flowering at the right time of the year requires careful monitoring of environmental cues and correct integration of these cues with the endogenous molecular network. Day length (or photoperiod) is one of the most predictable cues in nature and plants sense day length to gauge both diurnal and seasonal time and to initiate reproductive development at the right time of the year. Photoperiod responses can be facultative or obligate, and these responses are typically classified into short day, long day, and day neutral types. Short day plant flowering is cued in day lengths below a critical maximum threshold, while long day plant flowering is cued in day lengths above a critical minimum threshold. In contrast, day neutral plants flower at the same time regardless of day length.

Extensive work on the molecular network underlying flowering has been conducted in the short day flowering model plants rice (*Oryza sativa*) and long day flowering model plants *Arabidopsis* *thaliana* (Wei et al., 2020; Cho et al., 2017; Shrestha et al., 2014; Brambilla and Fornara, 2013; Tsuji et al., 2013; Andres and Coupland, 2012; Tsuji et al., 2011; Wilczek et al., 2010). The genes that detect photoperiod signals and integrate this response into flowering pathways are largely conserved across species and have been well described (Amasino, 2010 ; Andres and Coupland, 2012; Kobayashi and Weigel, 2007). Photoperiod changes trigger expression of florigenic proteins – homologs of *FLOWERING LOCUS T* (*FT*) in *Arabidopsis*, and *Heading date 3a* (*Hd3a*) in rice - in leaves. These proteins move to the shoot apical meristem where they interact with additional genes to induce reproductive development. Though many of the same genes are involved in both long day and short day responses (Hayama et al., 2003), short day plants also possess unique genes and molecular pathways to regulate flowering (Wei et al., 2020; Brambilla and Fornara, 2013). Species with wide natural distributions can also segregate for multiple distinct photoperiod-related flowering responses: distinct populations of sunflower (*Helianthus annuus*) exhibit day-neutral, facultative short day, and facultative long-day responses, which vary with their environment (Blackman, 2013).

Distinct genetic responses that are detectable in different environments are known as genotype by environment interactions, or GxE. The methodology commonly employed to study natural variation in GxE include genetic studies using common gardens, reciprocal transplant experiments between contrasting environments, or environmental manipulations. These methods have been used in wild species to uncover widely varying genetic responsiveness to photoperiod-cued flowering (Brachi et al 2010; Blackman 2013; Dittmar et al., 2014; Henry et al., 2014; Agren et al 2016). In crop species, altering the timing of flowering has been a major crop improvement strategy to adapt crops for local or future environments (Jung & Müller, 2009). Changing flowering responsiveness to photoperiod cues has allowed geographic range expansion and increased yields in a number of cereal species (Turner et al., 2005; Faure et al., 2012; Hung et al., 2012; Zakhrabekova et al., 2012; Yang et al., 2013) and other crops (Pin et al., 2012; Weller et al., 2012). However, the majority of studies of flowering GxE have used inbreeding, short-lived species. It is not clear whether insights from these species can be extrapolated to species with different life histories. Plant life histories vary along two main axes: a fast-slow continuum and a reproductive strategy continuum (Salguero-Gomez et al., 2016). The positions of species along these axes are likely to affect their evolutionary dynamics, and thus far, GxE in flowering has been studied only in fast growing, semelparous species and not in outbred, perennial systems which may face a broader swath of environments over their lifetimes.

Switchgrass (*Panicum virgatum*) is a warm-season perennial with wide environmental adaptation across the eastern half of North America. The US Department of Energy named switchgrass a model herbaceous biofuel feedstock in 1992

14, and since then, cultivars have been bred that significantly outproduce ethanol relative to maize and other cellulosic feedstocks15. Over the course of one season, switchgrass behaves as a determinate plant. It typically produces a single flush of tillers, which all become reproductive after a period of vegetative (leaf) development, and – critically for a biofuel crop – cease biomass accumulation upon completion of floral development (Van Esbroeck et al 2003). Switchgrass has substantial untapped genetic and morphological diversity, with tetraploid and octoploid individuals (cite), phenotypically distinct ‘upland’ and ‘lowland’ ecotypes, and three geographically distinct, deeply diverged genetic subpopulations within tetraploid individuals (Lovell et al 202X). Upland individuals are smaller in stature than lowland individuals, and have divergent leaf and whole plant morphologies (Casler et al 2007; Lowry et al 2014; Casler et al 2004; Porter 1966, McMillan 1964; McMillan 1959). Breeding for plants with earlier green up and later flowering dates may allow growers in the northern United States to take advantage of longer growing seasons, as these plants will accumulate more biomass before flowering, contributing to higher biomass yields. Genetic differences in flowering date and photoperiod response across these genetic subpopulations will be an early point of exploitation in biomass crop breeding.

Here, we grow and phenotype a diversity panel of 978 distinct switchgrass genotypes, clonal replicates of which were planted at eight common garden sites across 17 degrees of latitude. We use this panel to interrogate environmental mechanisms controlling greenup and flowering. We then use an outbred F2 cross between individuals from the most distinctive subpopulations for flowering response to dissect the genetics of flowering in these groups. Finally, we combine the results from this cross with genome-wide association results from the diversity panel to narrow in on candidate genes affecting flowering. Taken together, our results allow us to describe the environmental cues, genes, and alleles affecting flowering across multiple distinct switchgrass populations.

**Results**

*Diversity panel captures genetic and genotype by environment interactions across the common gardens*

In 2019, we grew and phenotyped a switchgrass diversity panel at eight common garden sites (Figure 1A). The eight common gardens cover the majority of the latitudinal and climatic range of switchgrass and therefore capture the most comprehensive picture to date of genotype-specific environmental plasticity, or genotype-by-environment interactions, in this species. The diversity panel contained 134 sequenced, clonally propagated individuals from the Midwestern genetic subpopulation and 229 individuals from the Gulf subpopulation (Figure 1A; Lovell et al 202X). The four northernmost common gardens (hereafter ‘North’ gardens) were located within the natural range of the Midwestern genetic subpopulation, while the three Texas common gardens (hereafter ‘Texas’ gardens) were located within the natural range of the Gulf subpopulation, and the Oklahoma common garden was located near the natural range limits of both the Gulf and the Midwestern subpopulations. We scored plant greenup and flowering at these common gardens every two days. The Gulf and Midwest genetic subpopulations had the most distinct phenological responses across our common gardens and had distinct patterns of phenotypic correlations between common garden sites (Supplemental Figure X, Figure 1A, 1B). At the Texas gardens, Gulf genotypes typically greened up before and flowered after Midwestern genotypes, while at the North gardens, Gulf genotypes greened up and flowered after Midwestern genotypes (Figure 1A). At the Oklahoma common garden, Gulf and Midwestern individuals greened up over the same time period. These patterns led to strong negative phenotypic correlations for greenup between the North and Texas common gardens and contributed to positive phenotypic correlations for flowering time which increased at more northern gardens (Figure 1B).

*Figure 1. A) Map and trait histograms of greenup and flowering dates across two genetically distinct switchgrass subpopulations and eight common gardens. Purple represents individuals from the Midwest genetic subpopulation, and pink individuals from the Gulf subpopulation. Vertical dashed lines indicate the summer solstice. Common gardens are arranged in latitudinal order. B) Phenotypic correlations between clonal replicates planted at eight common gardens, within and between two genetic subpopulations. C) Narrow sense heritability of greenup and flowering within single common gardens (purple) and across all eight common gardens (green), within and between two genetic subpopulations. D) Variance components analysis of genetic (purple), genotype by environment (blue), environmental (green), and error (yellow) terms in models of flowering time as functions of Julian date or weather, for the four northern common gardens (North), the three Texas common gardens (Texas), and for all eight common gardens (All). Dashed lines indicate the cumulative contribution of G and GxE for flowering as a function of Julian date.*

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We determined narrow-sense heritabilities (h2) using a genomic relationship matrixfor greenup and flowering dates at single gardens and across all eight common gardens (Figure 1C). To allow for the possibility that different subpopulations had different strengths of connection between these phenotypes and the genotypes (Korte and Farlow 2013), we determined h2 both within and across the Midwestern and Gulf subpopulations. At individual gardens, h2 were typically quite high: 59% on average for greenup date, and 87% for flowering date. However, h2 were variable across common gardens, particularly for greenup at our OK and NE gardens. Greenup dates at these sites were uncorrelated or negatively correlated with greenup dates for clonal replicates at other sites (Figure 1B). These negative and small correlations undoubtedly contributed to the low h2 values for greenup and flowering date across all eight sites: h2 was 0.8% for greenup and 23.2% for flowering date in models including all sites. These data indicated the presence of numerous rank-changing genotype by environment interactions for these phenotypes across these common gardens.

Though we scored greenup and flowering as functions of Julian date, flowering is more likely cued by one or more environmental factors like temperature, rainfall, or daylength signals (Brachi et al., 2010, Casler 2012, Hartman et al 2012, Hartman & Nippert 2012). To evaluate these cues as genetic triggers of flowering, we defined greenup and flowering as functions of nine environmental cues, then determined the variance attributed to genetic effects (G), genotype by environment interactions (GxE), environmental effects (E), and error for these phenology-related traits across our eight common garden sites using linear mixed models. For example, to define flowering date as a function of day length, we replaced the phenotype of flowering Julian date with the daylength at that garden on that Julian date. We reasoned that if flowering as a function of a weather cue had higher heritability than flowering as a function of Julian date, then that environmental cue was both a better predictor of flowering and was more likely to have detectable genetic variation segregating within the tested population. To allow for the possibility that different subpopulations would have different cues within or outside of their native ranges, we also determined the variance explained by G, GxE, E, and error for each subpopulation at the North and Texas set of gardens.

Across all eight common gardens, greenup date had low G and low GxE (<10%), and the G and GxE values were not substantially improved by defining greenup as functions of weather-based cues (Supplementary Figure/Table). This result likely indicates that the weather functions we chose are not cuing greenup. Additional signals such as soil temperatures or chilling days may influence greenup for each subpopulation within its native range; however, we did not have good proxies for these values for this experiment. G and GxE estimates for greenup date were significantly higher when the sites were restricted to either the Texas or North set of gardens (Supplemental Figure). G and GxE estimates for greenup were higher for the Gulf subpopulation than for the Midwest, and higher outside of each subpopulation’s native range than within its native range (Supplementary Figure).

In contrast to greenup date, flowering date had moderate G and GxE, and these values were significantly increased by defining flowering as functions of weather based environmental cues (Figure 1D). In the Gulf subpopulation, a daylength cue explained more G and GxE than flowering date (G = 36.8% +/- 6.4; GxE = 34.4% +/- 6.0). In the Midwest subpopulation, a cumulative GDD cue explained more G than flowering date (5.8% +/- 2.8% vs 23.8% +/- 6.1%), while three additional cues, daylength, rainfall between greenup and flowering, and rainfall in the five days before flowering, explained more G and GxE than flowering date (Figure 1D). G and GxE estimates were also higher when the common gardens were restricted to either the Texas or the North gardens. For subpopulations growing outside of their native ranges, substantial G and GxE was seen for rainfall cues, particularly for rainfall on the day of flowering. Taken together, these data indicate substantial genetic variation for a GDD-based flowering cue in the Midwest subpopulation, and similar genetic variation for a daylength cue in the Gulf subpopulation. They also suggest the presence of GxE for rainfall, GDD, and photoperiod cues for flowering, with variation for these cues more visible outside of each subpopulations’ native range.

*Genetic effects of greenup and flowering as functions of environmental cues*

Across our eight common gardens, we observed heritable genetic variation for two distinct flowering time cues in our two genetic subpopulations, and little heritable genetic variation for greenup date. We therefore used genome-wide association on genetic BLUPs to evaluate consistent genetic associations for flowering as functions of two flowering time cues, daylength and cumulative GDD. We evaluated consistent genetic associations for flowering across all eight sites as well as at the Texas and North garden subsets, which correspond to the home ranges of the Gulf and Midwest subpopulations, respectively.

We first evaluated the suitability of GWAS on these environmental cues relative to flowering date by comparing the strength of associations on these cues and on flowering date. Weather-derived functions gave stronger statistical associations than flowering date. Across both subpopulations, the top 100 SNPs by the maximum -log10(*p*) for any phenotype (hereafter, “top 100 SNPs”) were more significant for cumulative GDD and for daylength than for flowering date (binomial test *p* = 5.6e-07 and 1.8e-07). Within both subpopulations, the top 100 SNPs were more significant for flowering as a function of daylength than for flowering date (Gulf binomial test *p* = 0.003; Midwest binomial test *p* = 2.2e-16), but were not significantly different for cumulative GDD and flowering date. Strikingly, across both subpopulations, flowering as a function of daylength had stronger associations in Texas, and flowering as a function of GDD had stronger associations in the North (Figure 2). Indeed, the Gulf subpopulation only had significant associations for daylength in the Texas gardens, not for GDD, while the Midwest subpopulation only had significant associations for cumulative GDD in the Texas gardens, not for daylength.

To make direct comparisons of genetic associations among subpopulations, which have different segregating SNPs, we summarized the 6,045 univariate GWAS associations into 3,123 20kb regions, for genetic effects at all gardens (nregions = 463), north gardens (nregions=2,551), and Texas gardens (nregions=420). 20kb represents the inflection point where linkage disequilibrium decay flattens in this species (Lovell et al., 202X). In nine single-subpopulation GWAS that had associations above a 10% FDR, 22 20kb regions had associations in three or more GWAS (Figure 2), and 369 had associations in two or more GWAS. These regions in particular may underlie consistent genetic effects detectable across the species’ natural range. In all but one comparison, 20kb windows did not overlap significantly for the same phenotypes and gardens across subpopulations (8 of 9 p-values > 0.085). The one exception was the daylength phenotype in the north, where 39 20kb regions were common associations across subpopulations (p = 0.00688). Within subpopulations but across the North and Texas garden subsets, only the Gulf subpopulation had significant overlap in 20kb windows, for daylength (*p =* 0.0078), though these overlaps were still quite rare (8 windows, <3.5% of 20kb windows). The remaining >96% of 20kb windows in the Gulf subpopulation and all 20kb windows in the Midwest subpopulation were exhibiting GxE at the continental scale, in that these windows significantly affected flowering only at one of two non-overlapping sets of gardens.

Our associations captured a considerable set of non-overlapping genomic regions. To validate these associations, we next determined if homologs from rice or *A. thaliana* with functionally validated roles in flowering (1599 genes, Supp. Table X; Bouche et al., 2015; Yao et al., 2017) were overrepresented in our genetic associations. Across all 3,123 20kb regions, at all gardens, and in the north gardens, flowering homologs were significantly enriched (OR 1.57, 1.80, 1.65; p-values 6.3e-05, 0.027, 4.8e-05). These enrichments increased as we increased the stringency of the FDR adjustment, providing further support for the likely functional roles of these genomic regions in flowering timing in this species.*Figure 2. Combined Manhattan plots displaying associations above a 10% FDR for 18 combinations of subpopulation, site subset, and weather-derived flowering phenotype. Black vertical lines represent 20kb regions with associations above a 10% FDR for five or more of these 18 GWAS. Colored boxes indicate significant QTL intervals in the fourway cross (with less than a 1.5 LOD drop,* A screenshot of a social media post

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*Confirmation of genetic effects using a four-way cross*

To confirm candidate genomic regions and allelic effects underlying the flowering responses in the Gulf and Midwest subpopulations, we analyzed flowering in an F2 cross between four individuals, two Midwest and two Gulf individuals. The parents of this cross were DAC, an early flowering Midwest individual, VS16, a late flowering Midwest individual, AP13, an early flowering Gulf individual, and WBC, a late flowering Gulf individual (Supplementary Figure X). We made F1 crosses of the two early flowering genotypes, AP13xDAC, and the two late flowering genotypes, WBCxVS16. We then clonally propagated and planted the four parents, the two F1 genotypes (AP13xDAC, and VS16xWBC), and 801 F2 genotypes at eight field sites, then recorded greenup and flowering date for the 2019 season.

To look for dominance in flowering cues, we compared the 2019 flowering dates of the two F1 genotypes to those of the fourway parent genotypes and to the diversity panel. The F1 genotypes differed in flowering date by 17 days on average; however, both F1 genotypes flowered at similar dates as their Midwest parent genotype each year, and as Midwest subpopulation individuals in general (Supplementary Figure X; early and late F1s flowered 8.1 +/- 8.1 and 7.9 +/- 13.3 days behind their F0 parents).

To confirm associations for flowering in our diversity GWAS panel, we conducted QTL mapping of greenup and flowering in 2019 for seven common gardens. We again analyzed flowering in three ways: as a function of Julian date (‘flowering date’), as a function of cumulative GDD between greenup and flowering (‘flowering GDD’), and as a function of daylength at flowering (‘flowering daylength’). There were no significant QTL for greenup. There were eight QTL for flowering date and five QTL for flowering GDD, three of which overlapped with QTL for flowering date. There were ten QTL for flowering daylength, five of which overlapped with QTL for flowering date, and two of which overlapped with flowering GDD. When multiple flowering phenotypes overlapped at a QTL, flowering daylength had the highest LOD scores in four of five cases. Both flowering GDD and flowering daylength had unique QTL, and daylength had higher explanatory power than flowering date for the majority of QTL. All QTL for flowering overlapped one or more homologs from rice or *A. thaliana* with functionally validated roles in flowering. The most significant QTL were on Chr02N, Chr04K, and two positions on Chr05N. Of these strongest four QTL, all but the QTL on Chr02N had consistent associations in five or more GWAS (Figure 2). The QTL on Chr02N overlapped Pavir.2NG474600, which is homologous to the rice gene SIP1. SIP1 participates in regulation of flowering time in rice by recruiting OsTrx1 to Ehd1, a major promoter of flowering. The QTL on Chr04K overlapped six genes with functional validated roles in flowering, including Pavir.4KG047800, which is homologous to the rice gene Hd3a. Hd3a promotes transition to flowering downstream of Hd1 under short-day conditions in rice. The QTL at 2Mb on Chr05N overlapped five genes with functional validated roles in flowering, including Pavir.4KG047800

Of the smaller QTL, the QTL for flowering daylength on Chr02K, and the QTL for flowering daylength on Chr09N overlapped with the 20kb interval with consistent associations in five or more GWAS. Thus, we confirmed that five genomic regions with consistent genetic associations also had effects on flowering in a four-way mapping population created from individuals from the same populations.

*Genotype-by-environment effects on greenup and flowering as functions of environmental cues*

At the continental scale we found no consistent associations for greenup, due to the presence of negative phenotypic and genetic correlations in greenup date between the Texas and North gardens. In contrast, there were some recurring associations for flowering at this scale. A variance components analysis of flowering G and GxE across the eight common gardens also suggested the presence of GxE for rainfall, GDD, and photoperiod cues for flowering, for which variation was more visible outside of each subpopulations’ native range. We explored GxE in greenup and flowering as it covaried with multiple environmental cues in additional analyses. We conducted univariate GWAS at each common garden for greenup and flowering, then analyzed the allelic effects of unlinked SNPs across common garden sites for the top 5000 or 19000 SNPs, respectively, using mash and specifying hypothesis-based covariance matrices based on covariation in environmental cues in the greenup or flowering date window for the Midwest, Gulf, and both subpopulations. When the same SNP set is used in multiple univariate GWAS, a subsequent mash analysis shares information on patterns of effect size and direction for SNPs across these GWAS, improving the power to detect significant, shared results. Importantly, mash allows – indeed, even generates – multiple covariance matrices that particular SNP effects can load on to. Mash then shrinks effect estimates for each SNP towards one of a set of covariance matrices. If a SNP affecting flowering then loads onto a covariance matrix created from the correlations between daylengths of flowering, we can infer that the linked effect of this SNP is responding to the environmental cue of flowering daylength. Just as different genetic subpopulations can have different strengths of connection between phenotypes and genotypes (Korte and Farlow 2013), and can have different genetic covariances between these phenotypes at different gardens (Figure 1B), distinct SNPs can have different patterns of effect, or covariances, on phenotypes at different gardens. These user-specified and data-driven covariance matrices are an important advantage mash offers for studying patterns of GxE.

We first used mash to examine GxE in greenup in the Gulf subpopulation across 16 conditions, representing combinations of garden (eight) and greenup function (two). 10581 of 21697 modeled SNPs had significant effects in one or more condition. The majority of these SNPs (89%) had significant effects in eight or fewer conditions (Figure 3a). Overall, SNP effects were much more varied across gardens than across greenup functions within each garden. SNP effects for greenup exhibited multiple complex types of GxE, demonstrating why attempts to find consistent genetic effects failed for this phenotype. Indeed, only 1.5% of significant SNPs loaded onto a covariance matrix which had equal effects for each condition. Four non-zero covariance matrices loaded 5% or more of total SNPs (Figure 3b). These covariance matrices represent the most common patterns of GxE in SNPs that have significant effects on greenup, and include both data-derived (ED) and canonical matrices. The first of these, ED\_tPCA, loaded 49% of the significant SNPs. 65.3% of the variation in this matrix was captured by two major patterns (eigenvector 1 and 2 from a singular value decomposition explained 65.3% of the total variation in this covariance matrix) (Figure 3c, 3d). The first eigenvector was similar to the first eigenvector of ED\_PCA\_1. ED\_PCA\_1 loaded 21.5% of the significant SNPs, and was characterized by large magnitude (>|0.5|) effects decreasing greenup date and greenup GDD at the Texas gardens and in Oklahoma, with moderate magnitude effects (|0.2| to |0.5|) increasing greenup date and greenup GDD in MO and MI (Figure 3e). The first eigenvectors of both ED\_PCA\_1 and ED\_tPCA have antagonistic pleiotropy for greenup between all four of the southern and two of the northern common gardens. The second eigenvector of ED\_tPCA, explaining 21.7% of the variation in this matrix, showed large magnitudes of effects at the MO and MI gardens, and moderate effects in the TX1, TX3, and SD gardens (Figure 3d). It also showed antagonistic pleiotropy between the SD gardens and the Texas, MO, and MI gardens. The third covariance matrix, ED\_PCA\_3, loaded 8.8% of the significant SNPs, and was characterized by large magnitude effects on both greenup functions at the NE garden, with differentially sensitive effects at the other northern gardens and antagonistically pleiotropic effects at the Texas and OK gardens (Figure 3f). The last, ED\_PCA\_4, loaded 5.4% of the significant SNPs, and was characterized by antagonistic pleiotropy between the gardens in the center of the latitudinal gradient, of large magnitude at OK, relative to gardens at the lowest and highest latitudes, of large magnitude at TX1 and SD (Figure 3g).

We characterized patterns of differential sensitivity and antagonistic pleiotropy between all SNPs and all pairs of gardens. Between pairs of gardens, the fraction of antagonistically pleiotropic loci varied between 0 and 53%, with the most antagonistic pleiotropy between the Texas and OK gardens and the MO and MI gardens (Figure 3h). The fraction of differentially sensitive loci varied between 0 and 44%, with the most differential sensitivity occurring between the NE garden and other gardens, particularly the Texas, OK, and SD gardens (Figure 3i).

We looked for enrichments of genes that could potentially affect greenup in the 20kb genomic windows surrounding the 10581 SNPs with significant effects on greenup. *Were these effects enriched for genes in rice with presumably similar functions? What were the GO enrichments for genes from regions that loaded onto each of these covariance matrices? Distinct GO enrichments in SNP sets that load onto different covariance matrices, maybe?*

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We then used mash to explore garden-specific patterns of GxE in flowering in both subpopulations across our eight gardens. We ran mash on the Gulf, Midwest, and both subpopulations using 30 ‘hypothesis’ covariance matrices that corresponded to correlations in ten environmental cues on the flowering dates in each of the three genetic subpopulations. We also included eight ‘data-driven’ covariance matrices and 14 ‘canonical’ covariance matrices. Including the hypothesized covariance matrices improved the log likelihood of the model fit relative to a model with just data-driven and canonical covariance matrices for all three subpopulation models. We first examined which covariance matrices SNP effects loaded on to.

In the Gulf subpopulation, most SNP effects (57.6%) loaded on to the data-driven covariance matrices, and 9.8% of effects loaded onto the canonical covariance matrices. However, 32.6% of SNP effects loaded on to the hypothesized covariance matrices, mostly onto daylength (13.0%) and Julian date (8.7%) for the Gulf and Midwest subpops, and also onto daylength change in the Midwest subpopulation (11.0%).

In the Midwest subpopulation, a similar proportion of SNP effects loaded onto the data-driven (45.1%) and canonical (9.4%) covariance matrices. 45.6% of SNP effects loaded on to the hypothesized covariance matrices. In contrast to the Gulf subpopulation, these effects almost entirely loaded on to matrices of covariation in cumulative GDD for the Gulf (18.2%), Midwest (18.4%), or both subpopulations (8.4%), with a small number of effects (0.6%) loaded onto cumulative rainfall in the Gulf subpopulation.

In mash on both subpopulations, similar proportions of SNP effects loaded onto the data-driven (51.2%) and canonical (2.9%) covariance matrices. Here, while a small number of effects continued to load on to cumulative GDD (3.8%), most SNPs loaded on to day length in the Gulf (11.5%) or Gulf & Midwest (6.2%) subpopulations, daylength change in the Midwest subpopulation (18.3%), and flowering in the Gulf & Midwest subpopulations (6.2%).

* Mashhattans colored by covariance matrices.
* What patterns do the data-driven matrices capture that our weather matrices don’t capture?
* We characterized patterns of differential sensitivity and antagonistic pleiotropy between all SNPs and all pairs of gardens.
* We looked for enrichments of genes that could potentially affect flowering in the 20kb genomic windows surrounding the SNPs with significant effects on flowering.
* Rerun greenup mash and rewrite greenup mash section.

*Confirmation of genotype-by environment effects using a fourway cross*

*How many mash regions with GxE colocalized with these 8 QTL?*

All eight QTL for flowering date exhibited significant GxE between common garden sites. Our cross design allowed us to estimate allelic effects of alleles from all four parents as contrasts of alleles in the F2 individuals. In the early flowering allele set, AxB, at TX2, five of eight lowland alleles delayed flowering date. In the CxD cross at TX2, four of eight lowland alleles delayed flowering date – four of the same five regions that delayed flowering date in the AxB cross. These effects were consistent with the observed order of flowering in F0 and F1 individuals. We therefore hypothesized that these regions: Chr02N, Chr04K, Chr05N, (Chr08N), and Chr09K were important regions affecting flowering in the Gulf subpopulation. At the northern sites, because of the dominance of Midwest phenotypes and alleles that we observed, we compared effects of the Midwest allele from the early and late F1 cross. For 29 site by QTL combinations for the AxB early flowering alleles, the Midwest allele was accelerating flowering by reducing flowering date. For 17 site by QTL combinations for the CxD late flowering alleles, the Midwest allele was accelerating flowering, and for 2 site by QTL the upland allele was delaying flowering. These effects, if additive, would be consistent with the observed order of flowering in the F0 and F1. All eight QTL affected flowering date for at least one northern site for both the early and late flowering allele sets. Thus, we hypothesized that all eight regions were important regions affecting flowering date in the Midwest subpopulation.

All five QTL for flowering GDD exhibited significant GxE between common garden sites. In the early flowering allele set, 27 AxB alleles accelerated flowering by decreasing the GDD required for flowering, while 20 CxD alleles accelerating flowering by decreasing GDD. No alleles delayed flowering at the northern five sites, but at TX2, four alleles accelerated flowering and two decelerated it in the AxB cross, while four accelerated it in the CxD cross. At TX1, two accelerated flowering by decreasing GDD requirements in the AxB cross, and two decelerated it, while only one allele accelerated flowering at TX1 in the CxD cross. In general, Kingsville (TX1) is a marked departure from the temperate climates of the other common gardens. Heritability for flowering was lowest at TX1 in the fourway cross (Supplementary Figure X), indicating a larger effect of environment on phenotypic variance at this site.

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**Discussion**

* *Possible reasons why we didn’t find candidates in GWAS for other three QTL intervals: false negatives, type of allele shifts we are testing here vs the fourway, allele frequency: Crosses are powerful for detecting effects between they equalize frequency of alternative alleles – the effect of a QTL depends both on the additive effect and it’s frequency, with a max when p=q. It could be that some of the effects we detect in 4-way are just rare in GWAS panel*
* *And what are other types of region we can detect with GWAS that we couldn’t detect in our fourway? Particularly differences between Gulf alleles… maybe offer up Gulf specific GWAS/mash here. Or GWAS on any individual that is tetraploid and looks like it’s photoperiod sensitive – including some admixed and Atlantic individuals. As an idea.*
* *Discussion of environmental cues and environmental covariation. There is some compelling evidence that the gulf pop has evolved different critical cues compared to Midwest.*
  + Strikingly, across both subpopulations, flowering as a function of daylength had stronger associations in Texas, and flowering as a function of GDD had stronger associations in the North (Figure 2). Indeed, the Gulf subpopulation only had significant associations for daylength in the Texas gardens, not for GDD, while the Midwest subpopulation only had significant associations for cumulative GDD in the Texas gardens, not for daylength.
  + Mash on Midwest subpopulation found SNP effects that covary as cumulative GDD covaries, while mash on the Gulf subpopulation found SNP effects that covary as daylength, or daylength change at an interval slightly before Gulf flowering, daylength change at Midwest flowering (Midwest almost always flowers a similar timespan before the Gulf flowerings).
  + These distinct cues led to somewhat confounding results in the Gulf/Midwest fourway cross and in the Gulf & Midwest diversity panel. In the diversity panel, daylength cues dominated, probably because of the large number of Gulf plants compared to Midwest plants (more than twice as many Gulf plants at most sites). In the fourway cross, F1s demonstrated that Midwest cues were dominant. However, the QTL with the highest LOD scores were for daylength. All QTL but one for cGDD colocalized with QTL for daylength change or daylength. In contrast, daylength had four QTL that did not colocalize with cGDD.
  + Our weather covariance matrices did explain some patterns of SNP effects, but there were major patterns of SNP effects not explained by weather data. Luckily, these patterns loaded onto data-driven covariance matrices, so we could understand these patterns and potential location-specific drivers, even if we could not map these to specific environmental cues at this time.

Spring growth is initiated by “adequate temperature” according to McMillan and Weiler (1959), with adequacy thought to be dependent on the cultivar (Parrish and Fike 2005). During vegetative growth, switchgrass phenology is closely correlated with growing degree days (GDD), the cumulative mean daily temperature less a base temperature (Madakadze et al 1998c; Sanderson and Wolf, 1995a, 1995b). A base temperature of 12 C for vegetative and reproductive development is commonly used for growth models (Kiniry et al 2005, Kinery et al 2008a, Berhman et al 2013). However, base temperatures vary by cultivar (Madakadze et al 2003) and there may be a photoperiod or vernalization mechanism rather than a temperature threshold *per se* that triggers spring growth (Parish and Fike 2005).

Switchgrass is considered a short-day plant that flowers when exposed to shortening days of a specific length (Benedict, 1940) and reproductive development is strongly linked to day-of-the year (Cornelius and Johnston, 1941; Eberhart and Newell, 1959; Hopkins et al., 1995a; Sanderson and Wolf, 1995a). However, the nature of switchgrass photoperiodicity may be genotype dependent – both northern and southern cultivars with distinctive upland and lowland ecotypes flowered under both 12 and 16 hour photoperiods, but flowering was delayed in the upland cultivar (Van Esbroeck et al 2003). Photoperiodicity likely differs with plant latitude of origin (Parish and Fike 2005). Moving plants from southern populations northward is thought to delay flowering, increasing leaf number and yields, while moving northern populations to southern latitudes is thought to hasten the transition to reproductive development, reducing vegetative growth and biomass yield (Sanderson et al 1996).

If flowering date frequently varies as a function of GDD in switchgrass, this explains observations that moving southern populations northwards delays flowering, and moving northern populations south hastens flowering (Sanderson et al 1996). Indeed, in our common gardens in 2019, the average number of days from greenup to flowering for the Midwest subpopulation was 27 days shorter at our southernmost site than our northernmost site (55d vs 82d), while this value was 26 days longer for the Gulf subpopulation at the southernmost site than at the northernmost site (130d vs 104d).

**Methods**

***Diversity panel and Single Nucleotide Polymorphism Dataset***

Overview of the diversity panel.

*Panel collection, propagation, cultivation, and phenotyping*

The formation of the diversity panel has been described previously (Lovell et al 202X). In brief, seeds, rhizomes, and clonal propagules from natural and common gardens were collected from 2015-2018, and propagated by clonal division from 2016 to 2018 with the aim of generating > 10 clones per unique accession. Plants were grown in 1 gallon pots in the final propagation before transplanting to the field. Planting at the ten field sites occurred in the spring of 2018 and followed the methods of Lowry et al 2019, with the exception that the Blackwell cultivar was used instead of the Alamo cultivar at edge positions of the plot to control for edge effects. Plant aboveground biomass was removed in the spring of 2019 before spring tiller emergence. Plants without new growth from the crown by June 1st, 2019 were removed from the experiment and replaced with Blackwell cultivar plants in July or September 2019.

<<Paragraph about how the phenotypes of greenup, emergence, and flowering were scored. Should talk with Jason for specifics after looking in his metadata about this.>>

*Panel sequencing and ecotype classification*

The resequencing of the diversity panel has been described previously (Lovell et al 202X). Briefly, 789 diversity panel samples were resequenced at a median depth of 59x (range 20 – 140x). 630 samples were used for this analysis, after filtering for missing sequence or phenotype data, outlier heterozygousity scores, and collection site discrepancies. The reads were mapped to the V5 assembly using bwa-memcite\_bwa, and SNPs were called using SAMtools mpileup84 and Varscan V2.4.085 with a minimum coverage of eight and a minimum alternate allele count of four. Only SNPs with ≤ 20% missing data and minor allele frequencies > 0.05 were retained, resulting in 8.8 to 12.3 million SNPs used for genome wide association, depending on the individuals retained.

*Environmental functions for greenup and flowering*

Given our large number of sites with genetically identical individuals, we looked for an environmental cue that maximized narrow-sense heritability within or across subpopulations and common garden sites. Our reasoning was that, if a flowering time phenotype, as defined by a particular environmental cue, had higher heritability, it is more likely that there is detectable genetic variation segregating for that cue’s role in flowering. Ideally, heritability when defining flowering using a specific cue should be higher than heritability using Julian date; otherwise, it makes little sense to define the phenotype using that cue. We looked at heritability for nine traits associated with 50% flowering: daylength (analogous for a critical daylength for flowering), Julian date, cumulative GDD between greenup and flowering, the change in daylength from the previous day at flowering, and five measures of cumulative rainfall: cumulative rainfall between greenup and flowering, and in the two days, three days, five days, and seven days before flowering.

Because switchgrass phenology is closely correlated with growing degree days (GDD) during vegetative growth, we tested for a temperature-based environmental cue for flowering. To do this, we evaluated flowering date as a function of cumulative GDD between plant green up and flowering, using a base temperature of 12 C (Kiniry et al 2005, Behrman 2013). We then modelled GDD as a function of subpopulation and the interaction between subpopulation and latitude of origin, both as random effects. To avoid confounding with a photoperiod-based environmental cue, we removed GDD response values for genotypes predicted to have a photoperiod cue at the four common gardens south of 38°N. Including these GDD response values substantially increased the residual variance for photoperiod sensitive individuals at these sites (data not shown//in supplement). Subpopulation explained most of the variation in flowering as a function of GDD (% Varsubpop = 85.9%). Predicted values of GDD necessary for flowering were largest for the Gulf subpopulation (1056; 95% CI 973-1099), smallest for the Midwest (667; 95% CI 517 – 814) and intermediate for the other three groups (741 (599-1019); 776 (483-1195); 747 (573 – 1043); Supplementary table of effects). The heritability for GDD at flowering for photoperiod insensitive, sequenced individuals was 30%, while the heritability for flowering as a Julian date for the same set of individuals was 10.8% (Supplementary Table: Variance Components analysis). Thus, more phenotypic variance was explained by GDD than by Julian date for these individuals.

*Genome-wide association mapping*

We used the switchgrassGWAS R package (https://github.com/Alice-MacQueen/switchgrassGWAS) to allow fast, less memory intensive GWAS on the diversity panel.

*Analysis of correlated SNP effects on phenotypes at multiple sites*

We used the switchgrassGWAS R package to estimate and test the significance of SNP effects on phenology phenotypes measured at our common garden sites.

***Four-way cross and Quantitative Trait Locus Dataset***

*Creation, propagation, cultivation, and phenotyping*

A four-way population (801 F2 individuals) with their grandparents and F1 hybrid parents was clonally propagated 10 times in 3.8L pots at the Brackenridge Field Laboratory, Austin, TX in 2014-2015 and transplanted to 10 common garden sites covering 17 degrees of latitude from South Texas to South Dakota in May-July of 2015. The formation of the four-way mapping population has been described previously (Milano et al 2016). Briefly, the population was developed by initial cross between AP13 (A) x DAC6 (B) and WBC3 (C) x VS16 (D), the F1 hybrids of each of those crosses were then intercrossed reciprocally to create the four-way outbred population, 801 F2 individuals. The four grandparents were derived from highly divergent southern lowland and northern upland ecotypes: AP13, an early flowering Gulf individual, and WBC, a late flowering Gulf individual, DAC, an early flowering Midwest individual, VS16, a late flowering Midwest individual. The details of the 10 common garden sites can be found in Lowry et al. (2019). Briefly, each field was covered with one layer of weed barrier cloth, and holes were cut into the weed cloth in a honeycomb design. Plants were randomized into the holes, with each plant having four nearest neighbors located 1.56m away from each other. A row of border plants which were derived from rhizome plugs of approximately 10-year-old Alamo switchgrass were planted at every edge position to prevent edge effects. Plants were well-watered in 2015 to facilitate establishment.

Phenology data, including greenup and flowering time, have been recorded from 2016 to 2019 for the four-way population at each site. Greenup (GR50) was recorded as the day of the year when 50% of the tillers from the crown on the plant turned green, flowering (FL50) was recorded as the day of the year when 50% of the plant tillers had panicles undergoing anthesis. Additionally, cumulative growing degree days (GDD) from GR50 to FL50 was calculated as GDD = , where Tmean is the daily average temperature, Tbase is the base temperature of 12 °C for switchgrass (Kiniry et al 2005, Behrman 2013). On a specific day, if Tmean is less than Tbase, the GDD for that day is 0; if Tmean is bigger than Tbase, the GDD for that day is the difference between Tmean and Tbase. To be comparable and consistent with the diversity panel, only 2019 phenology data of the four-way population from the same seven common garden sites were used in this study.

*Sequencing and QTL mapping*

Illumina fragment paired end libraries, representing each of the four grandparents (A: AP13, B: DAC; C: WBC; D: VS16) were aligned to the *Panicum virgatum* reference genome v5) with bwa *mem* (Li and Durbin 2009). Details on the genetic map construction, map polishing and fine-scale reordering can be accessed on <https://datadryad.org/stash/dataset/doi:10.5061/dryad.ghx3ffbjv>.

Narrow-sense heritability (h2) for each phenotype (GR50, FL50, GDD) at each field site was estimated using the additive kinship matrix based on marker genotypic information. The process was accomplished via the ‘sommer’ package (Covarrubias-Pazaran, 2020) in R (2020). Briefly, we used a multivariate mixed model (mmer) that takes the kinship matrix and other random incidence matrices to estimate the variance components for each phenotype at each field, and calculates h2 as the proportion of additive genetic variance to the total variance.

QTL mapping was conducted with R/qtl2 (Broman, 2020). Specifically, we performed a genome scan with a linear mixed model accounting for the relationships among individuals (i.e., kinship matrix) and the environmental covariates (i.e., field sites). The full model can be expressed as: phenotype = µ + QTL + E + QTL x E + kinship + e, where µ is the population mean, QTL is the marker genetic effect (i.e., kinship matrix), E is the environmental effects (i.e., field sites), QTL x E is the interaction between marker genetic and environmental effects, kinship corresponds to the background polygenic variation, and e is the error term. The genome scan was accomplished through ‘scan1’ function. The statistical significance of the genome scan was established by performing a stratified (i.e., stratifying on field sites) permutation test (n=1000) using ‘scan1perm’ function. The estimated QTL effect was obtained using ‘scan1coef’ function in R/qtl2.

***Data availability statement***

**References**

Parrish DJ, Fike JH. 2005. The Biology and Agronomy of Switchgrass for Biofuels. Critical Reviews in Plant Sciences 24:423-459.

Tables & Figures, Maybe Supplementary

**Supplementary Figure X.** Average flowering date from 2016 – 2019 for the four parents of the fourway cross, compared to the 2019 distribution of flowering date for the Gulf and Midwest subpopulations. The two Midwest parents are DAC and VS16, and the two Gulf parents are AP13 and WBC. Bold yellow line indicates the summer solstice.

A close up of a map

Description automatically generated