Received: 23 October 2023 | Accepted: 20 March 2024

DOI: 10.1002/ajb2.16349

RESEARCH ARTICLE

Botany

Genetically correlated leaf tensile and morphological traits are driven by growing season length in a widespread perennial grass

P. Camilla Durant[1] | Amit Bhasin[2] | Thomas E. Juenger[1] | Robert W. Heckman[1]

[1]Department of Integrated Biology, University of Texas at Austin, Austin, TX 78712, USA

[2]Department of Civil, Architectural and Environmental Engineering, University of Texas at Austin, Austin, TX 78712, USA

Correspondence

P. Camilla Durant and Robert W. Heckman, Department of Integrative Biology, University of Texas at Austin, Austin, TX 78712 USA.
Email: durantpa@msu.edu and robert.heckman@usda.gov

Present addresses

P. Camilla Durant, Department of Plant Biology, Michigan State University, East LansingMI 48824, USA.

Robert W. Heckman, U.S. Department of Agriculture Forest Service, Rocky Mountain Research Station, Cedar City, UT 84721, USA.

Abstract

Premise: Leaf tensile resistance, a leaf's ability to withstand pulling forces, is an important determinant of plant ecological strategies. One potential driver of leaf tensile resistance is growing season length. When growing seasons are long, strong leaves, which often require more time and resources to construct than weak leaves, may be more advantageous than when growing seasons are short. Growing season length and other ecological conditions may also impact the morphological traits that underlie leaf tensile resistance.

Methods: To understand variation in leaf tensile resistance, we measured size-dependent leaf strength and size-independent leaf toughness in diverse genotypes of the widespread perennial grass *Panicum virgatum* (switchgrass) in a common garden. We then used quantitative genetic approaches to estimate the heritability of leaf tensile resistance and whether there were genetic correlations between leaf tensile resistance and other morphological traits.

Results: Leaf tensile resistance was positively associated with aboveground biomass (a proxy for fitness). Moreover, both measures of leaf tensile resistance exhibited high heritability and were positively genetically correlated with leaf lamina thickness and leaf mass per area (LMA). Leaf tensile resistance also increased with the growing season length in the habitat of origin, and this effect was mediated by both LMA and leaf thickness.

Conclusions: Differences in growing season length may promote selection for different leaf lifespans and may explain existing variation in leaf tensile resistance in *P. virgatum*. In addition, the high heritability of leaf tensile resistance suggests that *P. virgatum* will be able to respond to climate change as growing seasons lengthen.

KEYWORDS

bioenergy crops, Cauchy combination, common garden, functional traits, genome-wide association, grass evolution, leaf economics spectrum, leaf longevity, Poaceae, structural equation modeling

Leaf structural resistance is a critical determinant of many ecologically important processes, including leaf longevity, decomposition, and nutrient turnover, resistance to herbivory, and plant growth rates (Reich et al., 1991; Wright and Cannon, 2001; Pérez-Harguindeguy et al., 2003; Kitajima and Poorter, 2010). Tough leaves often live longer than weak leaves, which may be especially advantageous when growing seasons are long (Kikuzawa, 1991; Kitajima and Poorter, 2010; Kikuzawa et al., 2013). In warm habitats with long growing seasons, leaves have longer to recoup their construction costs, which allows plants to exhibit a slow return on investment strategy (Kikuzawa, 1991; Kudo, 1996; Grady et al., 2013; Mason and Donovan, 2015). Despite the benefits of high leaf structural resistance, there are also costs—tougher leaves often require more resources, time, and energy to construct than weaker leaves—which can reduce growth rates and thereby decrease performance in resource-rich environments and environments with short growing seasons (Mooney, 1972; Coley et al., 1985; Ackerly, 1999; Kitajima and Poorter, 2010). Despite its role in many ecological functions, the potential basis of a trade-off between leaf structural resistance and growing season

length, including morphological traits and their underlying genetic basis, has been underexplored.

Leaf structural resistance is a key aspect of plant investment in growth and defense, which may depend on growing season length. Long growing seasons can promote greater leaf lifespans in deciduous species (Kikuzawa et al., 2013), potentially leading to the evolution of a conservative functional syndrome—long-lived, low-nutrient leaves with high construction costs, including high leaf mass per area (LMA) (Reich et al., 1991; Wright et al., 2004; Onoda et al., 2011; Wang et al., 2023). Similarly, at lower latitudes where growing seasons are typically longer, plants are more likely to experience strong selective pressure from pathogens and herbivores, which may promote increased allocation to defense (Coley and Barone, 1996; Schemske et al., 2009; Anstett et al., 2016). Plants allocating heavily to defensive traits, including chemical defenses, trichomes, silica content, and structural resistance, often grow slowly and better tolerate abiotic stresses (e.g., Wright and Cannon, 2001; Agrawal and Fishbein, 2006; Züst and Agrawal, 2017; Nardini, 2022). As climate change causes growing seasons in many regions to lengthen, altered selective pressure on leaf longevity may also promote changes in leaf structural resistance (Gray and Brady, 2016; Piao et al., 2019).

Leaves are exposed to many types of physical damage, which exert different stresses and require different mechanical properties to resist. One important mechanical property is leaf tensile resistance—the resistance of a leaf section to being torn or pulled apart, which is analogous to the stress of strong winds or large herbivores pulling on a leaf (Lucas et al., 2000). Measurements of leaf tensile resistance take two forms: size-dependent and size-independent. Size-dependent structural resistance (hereafter, leaf strength) tends to increase with leaf size (width, length, and area) for any given leaf composition—it takes more energy to break through more leaf material (Wright and Illius, 1995; Lucas et al., 2000). Size-independent structural resistance (hereafter, leaf toughness), on the other hand, is a material property related primarily to leaf composition, especially cell wall composition, leaf thickness, or leaf density (Lucas et al., 2000; Onoda et al., 2011; Westbrook et al., 2011; Balsamo et al., 2015; Alonso-Forn et al., 2023). Some commonly studied leaf functional traits such as leaf mass per area (LMA) and leaf thickness have also been shown to contribute to leaf tensile resistance in broad multispecies surveys (e.g., Onoda et al., 2011; Alonso-Forn et al., 2023). In addition to leaf tensile resistance, two other mechanical properties are commonly studied: resistance to shearing and resistance to punching (Lucas et al., 2000; Onoda et al., 2011). Shearing and punching are analogous to the stress caused by chewing insect herbivores (Lucas et al., 2000); resistances to shearing and punching were positively correlated with tensile resistance in a global meta-analysis (Onoda et al., 2011).

To date, most studies investigating the basis of leaf structural resistance have examined patterns across many species (Westbrook et al., 2011; Onoda et al., 2017). While this cross-species approach is valuable, it has limited ability to identify the drivers of current selection on leaf structural resistance, which is best accomplished within species (Agrawal, 2020). Moreover, working within a single species allows researchers to use the powerful suite of quantitative genetics tools. For instance, quantitative genetics methods can be used to examine the capacity of a trait such as leaf structural resistance to evolve predictably within a population or respond to artificial selection imposed by breeding programs. Predictable evolution requires two things: natural selection acting on the trait and standing genetic variation in the trait (Lynch and Walsh, 1998; Mackay, 2001). Traits that have little genetic variation within the population (i.e., low heritability) and/or traits under limited natural selection will evolve more slowly than traits with high heritability under strong natural selection (Lynch and Walsh, 1998). Quantitative genetics can also be used to examine the anatomical or compositional traits that best explain leaf structural resistance. Strong genetic correlations can result from genetic linkage (nearby loci are frequently inherited together), pleiotropy (a locus that affects multiple traits), or population structure (nonrandom mating within a population; Lynch and Walsh, 1998; Walsh and Blows, 2009). While heritability and genetic correlation describe the aggregate genetic contribution to traits or pairs of traits, genome-wide association (GWA) mapping can identify particular genomic regions associated with leaf structural resistance and whether any genomic regions are associated with both leaf structural resistance and leaf composition.

For the study of the genetic mechanisms underlying leaf structural resistance, it is advantageous to examine a species with a broad geographic distribution, large morphological differences, and adequate genetic resources. One such species is *Panicum virgatum* L. (switchgrass; Poaceae), a perennial grass whose distribution encompasses much of eastern and central North America, where it experiences considerable variation in growing season length, potentially leading to differing selective pressures on leaf strength (Casler, 2012; Lovell et al., 2021). We took advantage of this broad geographic extent to examine the geographic, anatomical, and genetic drivers of leaf tensile resistance in a diverse group of resequenced *P. virgatum* genotypes growing in a common location. We first assessed the links between growing season length and leaf structural resistance, then tested for genetic or phenotypic relationships between leaf structural resistance traits and leaf traits that characterize the size or construction of the leaf (LMA, leaf lamina thickness, and leaf density). In this study, we assessed four questions: (1) How much phenotypic and genetic variation in leaf tensile resistance is present within *P. virgatum*? (2) Is there evidence for selection for leaf tensile resistance in *P. virgatum*? (3) Which leaf morphological traits contribute to and share a genetic basis with leaf tensile resistance? (4) How do leaf morphology and its contribution to leaf tensile resistance change in response to growing season length?

MATERIALS AND METHODS

Study system, sequencing, and experimental design

Panicum virgatum is a morphologically diverse, widespread C_4 grass species whose range spans much of eastern and central North America, from southern Canada to Mexico (Casler et al., 2011; Lovell et al., 2021). The species has three genetically and geographically distinct subpopulations—Gulf, Midwest, and Atlantic (Lovell et al., 2021). The Gulf subpopulation occupies the southernmost portion of the United States range; it is adapted to long growing seasons and has large, long-lived leaves (Lovell et al., 2021). The Midwest subpopulation occurs in the north-central United States in upland environments; it is adapted to short growing seasons and has small, short-lived leaves (Lovell et al., 2021). The Atlantic subpopulation is found along the eastern seaboard; it has leaf attributes that are intermediate between the other subpopulations (Lovell et al., 2021).

The *P. virgatum* genotypes sampled in this study came from natural occurrences and cultivars collected from across the United States between 2010 and 2018 (Figure 1). The genotypes represented all three genetic subpopulations identified by Lovell et al. (2021), and their collection sites covered 18.5° of latitude. Before planting, all genotypes were propagated at the Brackenridge Field Laboratory, University of Texas at Austin (Austin, TX, USA). Each genotype was grown in an 18.9-L pot until the crown was large enough to be divided into multiple plants, each of which was then grown in a 3.78-L pot. One clone of each genotype was then transported to the J.J. Pickle Research Campus at the University of Texas at Austin in May 2018, where it was planted in a common garden covered in landscape fabric (Sunbelt 3.2 oz, DeWitt, Sikeston, MO, USA). Genotypes in the common garden were randomly arranged 1.56 m apart in a honeycomb pattern. The edges of the common garden were surrounded by a row of plants of Blackwell, a common upland *P. virgatum* cultivar. Of the 438 genotypes we surveyed, 161 were from the Atlantic subpopulation, 188 were from the Gulf subpopulation, and 89 were from the Midwest subpopulation.

The methods for sequencing and annotating the switchgrass genome and determining the genetic subpopulation for each genotype were discussed in detail by Lovell et al. (2021). Briefly, the sequenced *P. virgatum* genome came from a genotype of the Alamo cultivar (AP13; version 5.1, https://phytozome-next.jgi.doe.gov/info/Pvirgatum_v5_1). All genotypes were resequenced (Illumina HiSeq X10 and Illumina NovaSeq. 6000 paired-end sequencing, 2 × 150 bp) and mapped against the reference genome (bwa-mem; Li and Durbin 2009). 10.2 million single nucleotide polymorphisms

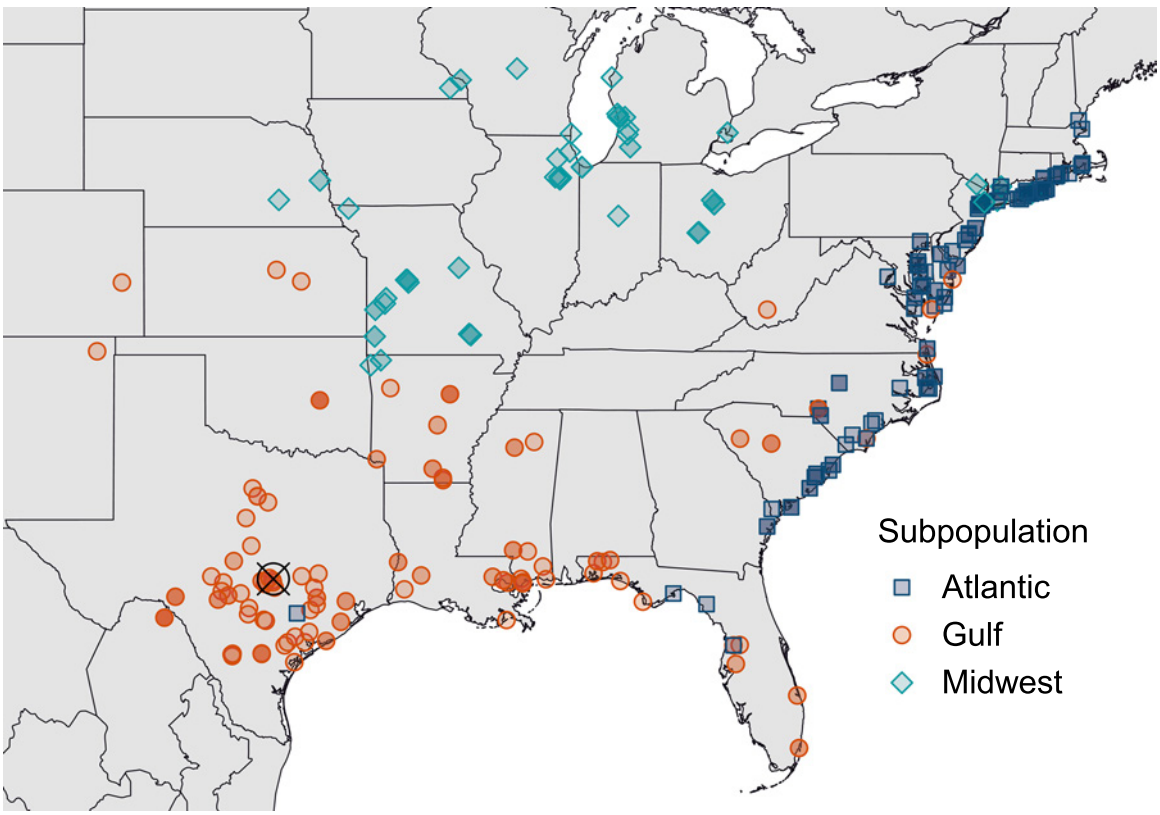

FIGURE 1 Origin of genotypes of *Panicum virgatum* used in this study. All genotypes were propagated at the University of Texas at Austin and then planted in a common garden in Austin, Texas, USA (denoted by a circle cross). *Panicum virgatum* has three genetic subpopulations, which are largely geographically separated.

(SNPs) remained after removing missing data and loci with minor allele frequencies <0.05. These SNPs were used in the analyses described below.

Phenotypic data collection

To evaluate leaf structural resistance in *P. virgatum*, we measured four leaf attributes—leaf strength, leaf width, leaf mass per area (LMA), and leaf thickness—which we then used to estimate two additional attributes—leaf toughness and leaf density. To accomplish this, we collected three leaves from each plant that were at a comparable developmental stage and were representative of the entire canopy. Each leaf chosen was young, fully expanded and the second leaf from the apical end of the tiller. Leaves were rehydrated by placing their cut end in 15-mL tubes filled with ~5 mL of water, then the leaves were stored in cool, dark conditions for up to 3 days. Maintaining consistent hydration across samples can be important because the tensile strength of plant tissue can change with moisture content (e.g., Hales and Miniat, 2017).

Leaf strength, the maximum tensile force that a leaf can withstand before failing (a size-dependent quantity), was estimated on rectangular leaf segments using a constant displacement rate tensile strength test with a tensiometer (Instron Model 1000 Universal Testing Machine, Instron, Norwood, Massachusetts, USA). Specifically, we first cut a 20 cm-long segment of each leaf; we avoided the ends of the leaf, where the leaf width tapers rapidly. To isolate the tensile properties of the leaf lamina from the considerably stronger midrib and to better control the site at which the lamina tore, we followed the method of Wright and Illius (1995) by weakening the midrib using a small incision ~5 cm from the basal end of the leaf segment. This approach is also consistent with studies of leaf toughness that measured the toughness of the lamina without the influence of the midrib or other major veins (e.g., Wright and Cannon, 2001). To further protect the tips of leaf segments from the tensiometer clamps, we covered them with ethylene-vinyl acetate craft foam. The arm of the tensiometer moved at a constant displacement rate of 0.01 mm s^{-1} until the leaf segment completely failed in tension. During the test, the tensile load and total displacement were collected using a 250-N load cell and a digital displacement sensor that is built in to the top of the actuator. When a leaf developed multiple fractures (e.g., when the leaf initially tore only partially), we still considered the maximum force applied to the sample as the measure of leaf strength. Leaf toughness is the ratio of the maximum load (leaf strength) to leaf width at the fracture point, which was 5 cm from the basal end of the leaf segment 72.7% of the time. Thus, leaf toughness normalizes the influence of varying leaf width but would still be influenced by leaf thickness and intrinsic strength.

We also calculated leaf mass per area (LMA, the ratio of dried leaf mass to fresh, one-sided leaf area) and measured leaf thickness. Leaf area was estimated using a leaf area meter (LI-3100C, LI-COR Biosciences, Lincoln, Nebraska, USA). Leaves were then dried at 50°C for 72 h and weighed. On these leaves, we also recorded the thickness of the leaf lamina at 5 cm from the leaf base and at the approximate midpoint along the leaf length using a digital thickness gauge (Mitutoyo 547-500 S, Mitutoyo America Corp, Aurora, IL, USA). The average of the thickness near the base and at the middle of the leaves was used in analyses. From these measurements, we then estimated leaf density (the ratio of LMA to the average leaf lamina thickness [base and midpoint]). Because leaf strength and LMA measurements are both destructive, we measured leaf strength on one leaf and LMA and leaf thickness on two other leaves from the same plant.

In 2019, 2020, and 2021, plants were harvested each fall at ~15 cm above ground level, and aboveground biomass was weighed to estimate biomass production. A representative subsample of vegetation from each plant was collected and weighed fresh, then dried and reweighed; the percentage moisture in this subsample was used to calculate the dry mass of the total plant. We then summed biomass for each plant across the 3 years. Biomass measured in 2019 was also reported by Lovell et al. (2021).

Analyses

We performed all analyses using R 4.2.2 (R Core Team, 2022). To address our first question—how much phenotypic and genetic variation in leaf tensile resistance is present within *P. virgatum*—we took two approaches. First, we examined phenotypic differences in leaf strength and leaf toughness among genetic subpopulations using an ANOVA and a post hoc Tukey's honestly significant difference test (R packages nlme and emmeans; Pinheiro and Bates, 2000; Lenth, 2023; Pinheiro et al., 2023). Second, we examined the genetic contributions to traits using a linear mixed model in which the random effect is an additive genetic relationship matrix (animal model, sommer package; Wilson et al., 2010; Covarrubias-Pazaran, 2016). The additive genetic relationship matrix was calculated using the vanRaden method—the similarity between each pair of plants was calculated at each locus (i.e., SNP) and weighted by the minor allele frequency of the locus, then summed across all loci (VanRaden, 2008; Lovell et al., 2021). We calculated narrow-sense heritability (h^2) in univariate-response models as the ratio of additive genetic variance (V_A) to total variance (V_A + residual variance, V_ε). This analysis was performed for all genotypes together as well as separately within each genetic subpopulation. Before the analysis, we mean-centered and variance-standardized (mean = 0, standard deviation = 1) responses.

To address our second question—is there evidence for selection for leaf tensile resistance in *P. virgatum*—we performed a phenotypic selection gradient analysis (Lande and Arnold, 1983). We first mean-standardized aboveground biomass for each genotype by dividing each genotype's biomass by the global mean aboveground biomass to obtain

a relativized score where average fitness = 1, above average fitness > 1, and below average fitness < 1 (Franklin and Morrissey, 2017). Previous work demonstrated that aboveground biomass is highly correlated with seed production in *P. virgatum* (Palik et al., 2016), making it a useful proxy for fitness (Lowry et al., 2019). We then examined how mean-centered and variance-standardized (mean = 0, standard deviation = 1) leaf toughness or leaf strength predicted mean-standardized biomass in a single generalized least squares model (gls() function in the R package nlme; Pinheiro and Bates, 2000; Pinheiro et al., 2023). The selection gradient model included linear and quadratic effects of leaf strength or leaf toughness and their interaction with subpopulation. All values were standardized separately by subpopulation. Quadratic effects were doubled before analysis (Stinchcombe et al., 2008). When a significant quadratic effect was detected, we then assessed whether the quadratic effect represented a humped relationship (i.e., stabilizing selection) or a nonlinear increase/decrease (i.e., directional selection; Mitchell-Olds and Shaw, 1987) (MOS test() function in the R package vegan; Oksanen et al., 2022). This approach tests the null hypothesis that the hump of a quadratic effect occurs at the minimum or maximum value.

To address our third question—which leaf morphological traits contribute to and share a genetic basis with leaf tensile resistance—we took two approaches. First, we calculated the phenotypic correlations [Pearson's correlation coefficient, r_P; cor() function] and genetic correlations (r_G) between leaf strength or leaf toughness and leaf thickness, LMA, and leaf density in bivariate-response linear mixed models with the additive genetic relationship matrix as a random effect (R package sommer; Covarrubias-Pazaran 2016). The correlation coefficient r_G is the ratio of the genetic covariance (Cov_A) between a pair of traits to the square root of the product of the additive genetic variances of the two traits —$r_G = Cov_A/\sqrt{V_{A1}V_{A2}}$ (Wilson et al., 2010). We quantified the significance of r_G using a likelihood ratio test to compare a model that freely estimated the genetic covariance between traits to a model that fixed their genetic covariance at 0. As with the univariate mixed model analyses described above, we analyzed all genotypes together, and we mean-centered and variance-standardized all responses.

Then, we performed genome-wide association (GWA) mapping for each leaf trait (leaf area, leaf thickness, LMA, leaf density, leaf strength, and leaf toughness) with linear models that included 10.2 million biallelic SNPs (R packages switchgrassGWAS and bigsnpr; Privé et al., 2018; Lovell et al., 2021). In each model, we accounted for population structure with principal components of the SNP matrix (Q, sensu Yu et al., 2006). Specifically, we included as predictors the number of principal components of the SNP matrix (1–15 principal components) that produced the best GWA model fit to the data for each leaf trait (λ_{GC} nearest 1 calculated from quantile-quantile plots). We used a 5% false discovery rate significance threshold (Storey and Tibshirani, 2003) to identify significant associations between leaf traits and SNPs. Quantile–quantile plots of expected and observed *P*-value distributions for each trait are available in the supplementary material (Appendix S1: Figure S1).

To identify significant associations between groups of leaf traits and SNPs, we used a data fusion method called the Cauchy combination test (CCT), which is a computationally efficient method with no assumption of independence between responses (ACAT package; Liu et al., 2019; Liu, 2020). Specifically, we examined associations between each SNP and (1) both tensile traits (leaf strength and leaf toughness), (2) pairs of leaf tensile and leaf morphological traits, and (3) all five leaf traits. A significant association would provide evidence that pleiotropic changes in leaf morphology can impact leaf tensile resistance. Despite the computational efficiency of the CCT, we still needed to reduce the number of SNPs tested to alleviate the computational burden (Kremling et al., 2019; Wu et al., 2022). To reduce the number, we first identified the minimum *P*-value for each SNP across all five traits in univariate GWAS, then selected the 10% of SNPs with the lowest minimum *P*-value. Because we performed only a portion of the total possible tests, we could not calculate the 5% false discovery rate significance threshold. Instead, we used the more stringent Bonferroni correction ($\alpha = 0.05$, $m = 10.2$ million biallelic SNPs) to reduce Type I errors.

To identify candidate genes associated with leaf traits for both CCT and single-trait GWA, we investigated the genes within 10 kbp of each SNP—the average decay of linkage disequilibrium across the *P. virgatum* genome (Lovell et al., 2021)—and their orthologous functions in the model plants *Arabidopsis thaliana* and *Oryza sativa*.

Finally, to address our fourth question—how do leaf morphology and its contribution to leaf tensile resistance change in response to growing season length—we performed a mediation analysis, which is a method for assessing the importance of indirect effects on a response (Zhao et al., 2010). Specifically, we examined whether the effect of growing season length on leaf tensile resistance was mediated by the effects of leaf morphology in a multivariate model (i.e., structural equation model, using R package lavaan; Rosseel, 2012). We estimated growing season length by calculating the growing degree days for the site-of-origin of each genotype—the sum of each day's mean temperature minus 12°C when mean daily temperature exceeds 12°C. We defined growing degree days using meteorological criteria to estimate the potential growing season length based on ecologically relevant temperature thresholds (Körner et al., 2023). Mean daily temperature was the average of daily maximum and minimum temperature at each site of origin for 2001–2019 (R package nasapower; Sparks, 2018, 2023). We excluded 25 genotypes from this analysis; 21 genotypes lacked precise sites of origin, and four other genotypes had sites of origin that were considerably outside the range of their genetic subpopulation. This model had two levels—the effect of growing degree days on LMA and leaf thickness and the effects of LMA and leaf thickness on leaf toughness or leaf strength [sem() function in R package lavaan]. Following Zhao et al. (2010), we estimated

the indirect effect of growing degree days on leaf strength or leaf toughness by multiplying two paths—the effect of growing degree days on LMA or leaf thickness and the effect of LMA or leaf thickness on leaf strength or leaf toughness—then estimated the significance of these effects with 10,000 bootstrapping iterations. A significant effect indicates that the relationship between growing season length and leaf tensile resistance is mediated by leaf morphology (Zhao et al., 2010). We then examined whether this mediation differed among genetic subpopulations using a multigroup model. Both the single group and multigroup models also included a correlation between LMA and leaf thickness. All single-group and multigroup models exceeded the minimum sample size ($N > 5$ per model parameter) recommended by Grace et al. (2015).

RESULTS

Leaf tensile resistance is variable and highly heritable

Leaf strength and leaf toughness both differed significantly among genetic subpopulations ($F = 208.33$, $P < 0.001$ and $F = 65.09$, $P < 0.001$, respectively; Table 1, Figure 2; Appendix S1: Table S1). Specifically, Gulf subpopulation leaves were more than twice as strong as Atlantic and Midwest leaves (108% and 197% stronger, respectively; $t = -16.41$, $P < 0.001$; $t = 17.36$, $P < 0.001$; Appendix S1: Table S2). Additionally, Atlantic leaves were 43% stronger, on average, than Midwest leaves ($t = 3.63$, $P < 0.001$; Appendix S1: Table S2). As with leaf strength, Gulf subpopulation leaves were significantly tougher than Atlantic and Midwest leaves; however, the differences in leaf toughness were of considerably smaller magnitude—Gulf leaves were 36% tougher than Atlantic leaves ($t = -10.33$, $P < 0.001$) and 35% tougher than Midwest leaves ($t = 8.32$, $P < 0.001$; Appendix S1: Table S2). The toughness of Atlantic and Midwest leaves did not differ ($t = -0.24$, $P = 0.97$; Appendix S1: Table S2). Both leaf strength and leaf toughness were highly heritable across all genotypes and in the Gulf and Atlantic subpopulations ($h^2 > 0.5$) but less heritable in the Midwest subpopulation ($h^2 < 0.3$; Table 1).

TABLE 1 Phenotypic mean and narrow-sense heritability of leaf traits across all *Panicum virgatum* subpopulations and within subpopulations of *Panicum virgatum*. Narrow-sense heritability (h^2) was calculated from a univariate model with an additive genetic relationship matrix as a random effect. Standard errors (SE) were calculated using the delta method.

Trait	Subpopulation	Mean ± SE	h^2 ± SE
Strength (N)	Overall	33.1 ± 0.8	0.88 ± 0.04
	Gulf	48.4 ± 1.2	0.90 ± 0.04
	Atlantic	23.3 ± 1.4	0.87 ± 0.09
	Midwest	16.3 ± 1.9	0.19 ± 0.25
Toughness (N cm^{-1})	Overall	24.0 ± 0.3	0.73 ± 0.08
	Gulf	28.1 ± 0.5	0.78 ± 0.08
	Atlantic	20.6 ± 0.6	0.54 ± 0.22
	Midwest	20.8 ± 0.8	0.27 ± 0.28
Leaf thickness (mm)	Overall	0.225 ± 0.002	0.76 ± 0.07
	Gulf	0.265 ± 0.003	0.87 ± 0.05
	Atlantic	0.197 ± 0.004	0.64 ± 0.19
	Midwest	0.185 ± 0.005	0.07 ± 0.20
LMA (g m^{-2})	Overall	87.6 ± 0.8	0.83 ± 0.05
	Gulf	100.9 ± 1.2	0.80 ± 0.07
	Atlantic	78.3 ± 1.3	0.74 ± 0.12
	Midwest	74.4 ± 1.7	—
Leaf density (g cm^{-3})	Overall	0.398 ± 0.003	0.55 ± 0.11
	Gulf	0.388 ± 0.005	0.65 ± 0.12
	Atlantic	0.405 ± 0.005	0.24 ± 0.21
	Midwest	0.408 ± 0.007	—

Notes: LMA, leaf mass to area ratio. A missing value indicates that the model estimate was unreliable; thus, h^2 is not reported.

Leaf tensile resistance is associated with biomass within and across subpopulations

In general, leaf tensile resistance was positively associated with plant biomass, a proxy for fitness (Appendix S1: Tables S3, S4; Figures S2, S3). Specifically, the linear effect of leaf strength on plant biomass was significantly greater than 0 in all three subpopulations ($P < 0.01$ for each subpopulation; Appendix S1: Table S5A). This effect was significantly larger in the Atlantic subpopulation than in the other two subpopulations (Atlantic–Gulf, $t = 6.84$, $P < 0.001$; Atlantic–Midwest, $t = 2.66$, $P = 0.022$) and marginally larger in the Midwest subpopulation than in the Gulf subpopulation ($t = -2.30$, $P = 0.056$; Appendix S1: Table S5A). There was also an overall significant quadratic effect of leaf strength on biomass that was concave down ($P < 0.001$; Appendix S1, Tables S3A, S4A), indicating that the overall relationship between biomass and leaf strength was nonlinear. Within subpopulations, this quadratic effect was significant in the Atlantic and Gulf subpopulations ($t = -5.44$, $P < 0.001$ and $t = -3.11$, $P = 0.002$, respectively), but not in the Midwest subpopulation ($t = -1.45$, $P = 0.146$; Appendix S1: Table S5A); none of the subpopulations differed significantly from one another (Appendix S1: Table S6A). In the Atlantic and Gulf subpopulations, we rejected the hypothesis that the hump of this quadratic relationship was at the maximum or minimum value (Atlantic, $P = 0.005$; Gulf, $P = 0.002$), providing evidence for stabilizing selection on leaf strength. In the Midwest subpopulation, the results suggest that directional selection prevails ($P = 0.815$). Finally, the leaf strength at which fitness was maximized—[−Linear selection coefficient/Quadratic selection coefficient] (Appendix S1: Table S5)—was slightly higher in the Atlantic and Gulf

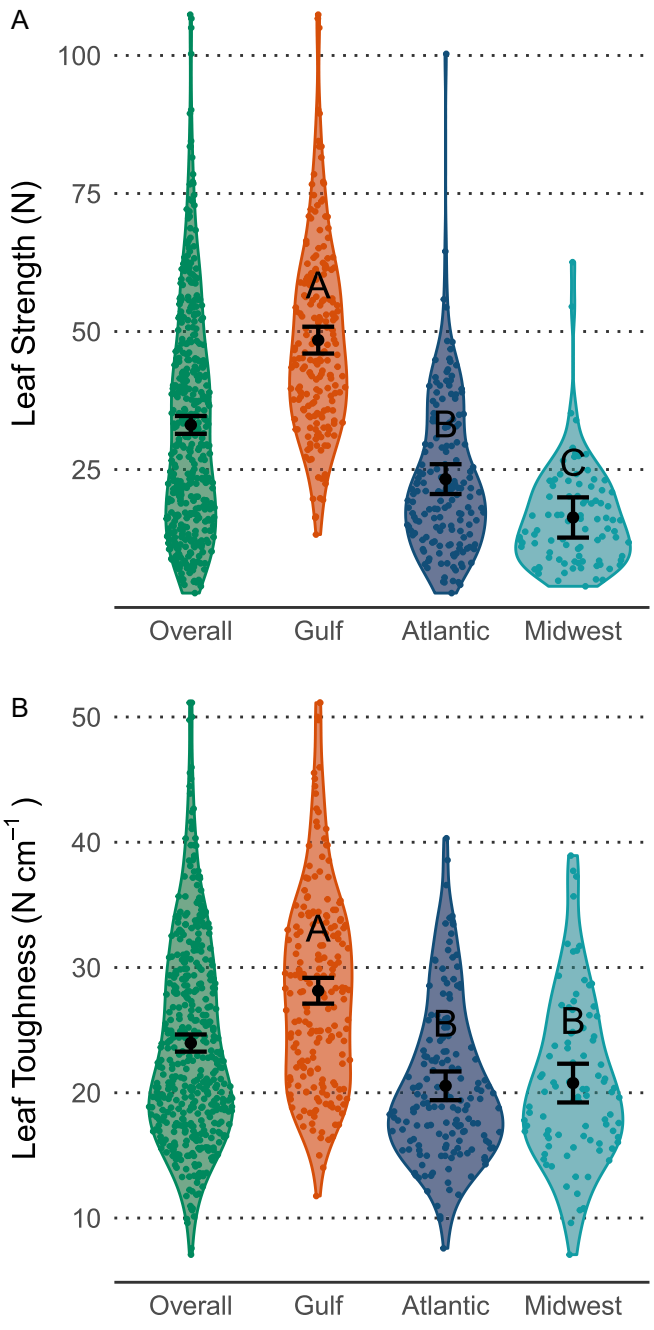

FIGURE 2 Differences in (A) leaf strength and (B) leaf toughness among subpopulations of *Panicum virgatum*. Means and 95% confidence intervals, shown by error bars, were model-derived. Points are raw values that were jittered horizontally to reduce overplotting.

subpopulations (61.15 N and 60.66 N, respectively) than in the Midwest subpopulation (57.02 N).

Like leaf strength, leaf toughness also had a positive association with plant biomass across all populations ($F = 12.21$, $P < 0.001$), and the effect differed among subpopulations ($F = 3.32$, $P = 0.037$; Appendix S1: Table S3B). Specifically, biomass increased with leaf toughness in the Atlantic ($t = 4.57$, $P < 0.001$) and Midwest subpopulations ($t = 2.75$, $P = 0.006$) but not in the Gulf subpopulation ($t = 1.20$, $P = 0.229$; Appendix S1: Table S5B). This relationship was significantly greater in the Atlantic subpopulation than in the Gulf subpopulation ($t = 2.76$, $P = 0.017$) but did not differ between other subpopulations (Atlantic–Midwest, $t = 0.67$, $P = 0.780$; Gulf–Midwest, $t = -1.65$, $P = 0.228$; Appendix S1: Table S6B). As with leaf strength, there was also an overall quadratic effect of leaf toughness on biomass that was concave down ($P < 0.001$; Appendix S1: Tables S3B, S4B), indicating a nonlinear effect of leaf toughness on biomass. Within subpopulations, this quadratic effect was significant in the Atlantic subpopulation ($t = -3.02$, $P = 0.003$) but not in the Gulf or Midwest subpopulations ($t = -1.60$, $P = 0.111$ and $t = -1.78$, $P = 0.076$, respectively; Appendix S1: Table S5B); none of the subpopulations differed significantly from one another (Appendix S1: Table S6B). In the Atlantic and Gulf subpopulations, there was marginally significant support for the hypothesis that the hump of this quadratic relationship was not at the maximum or minimum value (Atlantic, $P = 0.081$; Gulf, $P = 0.097$), which somewhat supports stabilizing selection on leaf toughness in these subpopulations. In the Midwest subpopulation, on the other hand, these results support directional selection ($P = 0.275$). Lastly, the Gulf subpopulation exhibited higher optimal leaf toughness (32.36 N cm^{-1}) than the Atlantic and Midwest subpopulations (27.51 N cm^{-1} and 28.38 N cm^{-1}, respectively). In total, although selection on leaf tensile resistance differed considerably among genetic subpopulations, selection in the Gulf region—where this study was performed—generally favored leaves that were stronger and tougher than average in each subpopulation.

Leaf size and LMA are correlated with leaf tensile resistance

Phenotypically, leaf strength was significantly and strongly associated with all three morphological traits. Leaf strength positively covaried with LMA ($r_P = 0.680$, $t = 19.8$, $P < 0.001$) and leaf thickness ($r_P = 0.737$, $t = 23.3$, $P < 0.001$) but negatively covaried with leaf density ($r_P = -0.205$, $t = -4.5$, $P < 0.001$). Leaf toughness was also significantly associated with these three morphological traits, but the associations were not as strong. As with leaf strength, leaf toughness positively covaried with LMA ($r_P = 0.488$, $t = 11.9$, $P < 0.001$) and leaf thickness ($r_P = 0.521$, $t = 13.0$, $P < 0.001$) and negatively covaried with leaf density ($r_P = -0.128$, $t = -2.8$, $P = 0.006$) (Figure 3).

The genetic correlations between these traits showed a similar pattern to the phenotypic correlations (Figure 4). There were positive genetic correlations between LMA and leaf strength ($r_G = 0.422 \pm 0.071$, $\chi^2 = 28.39$, $P < 0.001$) and LMA and leaf toughness ($r_G = 0.424 \pm 0.106$, $\chi^2 = 13.90$, $P < 0.001$). In addition, leaf thickness was positively genetically correlated with leaf strength ($r_G = 0.574 \pm 0.071$, $\chi^2 = 50.00$, $P < 0.001$) and leaf toughness ($r_G = 0.512 \pm 0.106$, $\chi^2 = 23.11$, $P < 0.001$). There was a marginally significant negative genetic correlation between leaf strength and leaf density ($r_G = -0.189 \pm 0.114$, $\chi^2 = 3.01$, $P = 0.083$) and a nonsignificant negative genetic correlation between leaf toughness and leaf density

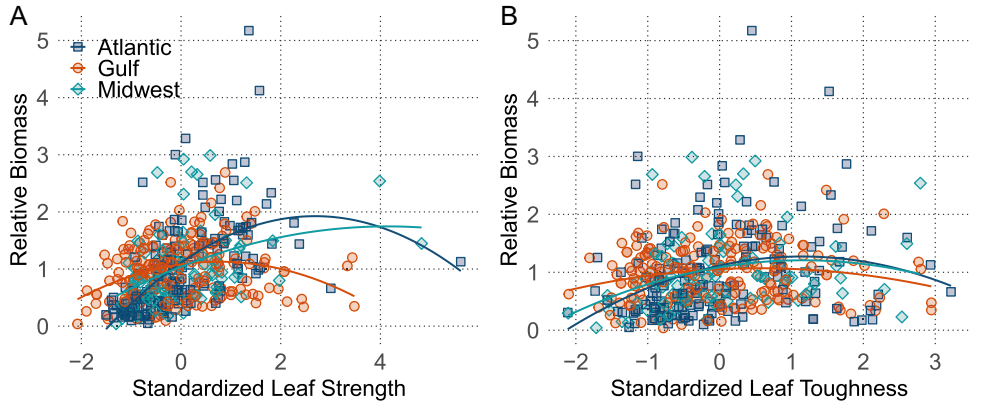

FIGURE 3 Effects of (A) standardized leaf strength and (B) standardized leaf toughness (transformed such that mean = 0 and standard deviation = 1 across all plants within a subpopulation) on relative aboveground biomass of *Panicum virgatum*. Model-derived quadratic parameter estimates are doubled. Colors and shapes are used to differentiate subpopulations.

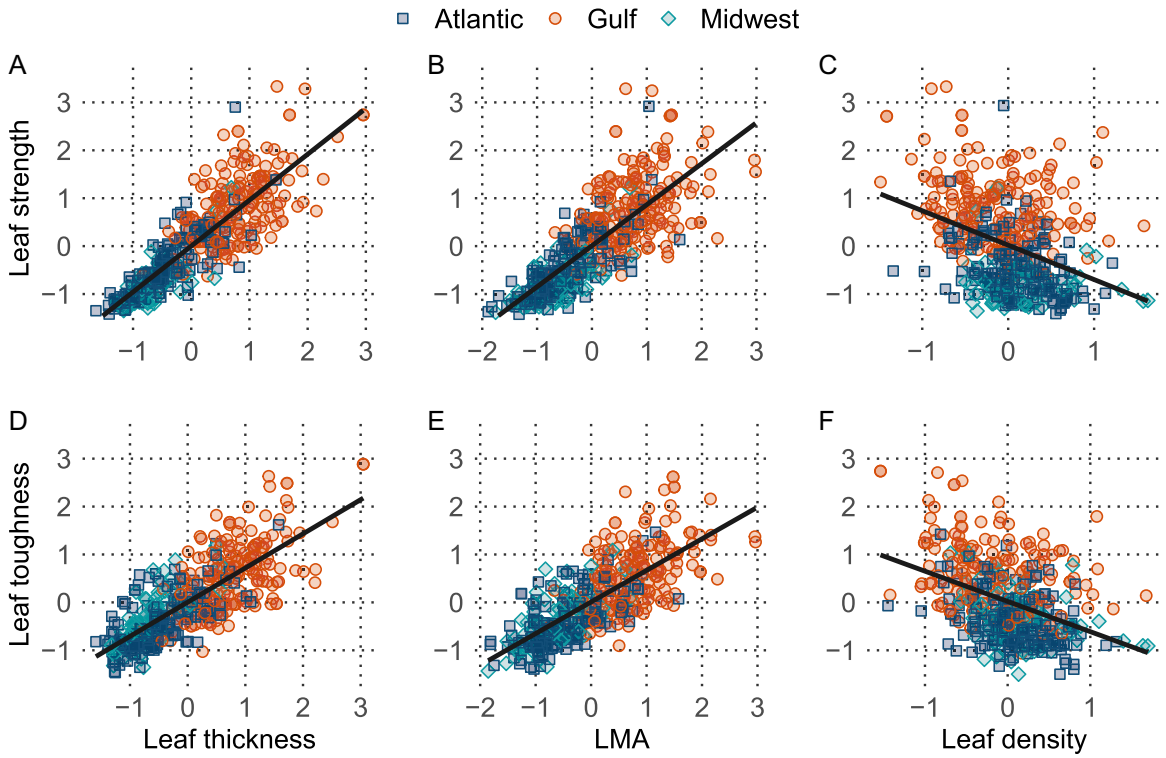

FIGURE 4 Panels A–F show genetic correlations between leaf structural resistance traits (leaf strength and leaf toughness) and leaf mass to area ratio (LMA), leaf thickness, and leaf density in subpopulations of *Panicum virgatum*. Models were fit using all genotypes; colors and shapes were added to differentiate subpopulations. Trend lines represent the fitted linear relationship between genetic best linear unbiased predictions (GBLUPs).

($r_G = -0.243 \pm 0.147$, $\chi^2 = 2.50$, $P = 0.11$). Together, these results indicate that leaf morphology and composition contribute to leaf tensile resistance.

Genome-wide association and candidate genes underlying leaf traits

Using GWA, we identified several variants associated with leaf tensile resistance and leaf morphological traits. Five significant single nucleotide polymorphisms (SNPs) were associated with leaf strength when using a Bonferroni-adjusted significance threshold ($\alpha = 0.05$). At the more relaxed 5% false discovery rate significance threshold, 33 SNPs were associated with leaf strength across 11 chromosomes (Figure 5; Appendix S2). There were 30 genes within 10 kbp of these SNPs (Appendix S3). Leaf toughness was not significantly associated with any variants at either the 5% false discovery rate threshold or the Bonferroni-adjusted threshold (Appendix S1: Figure S3A). Leaf morphological

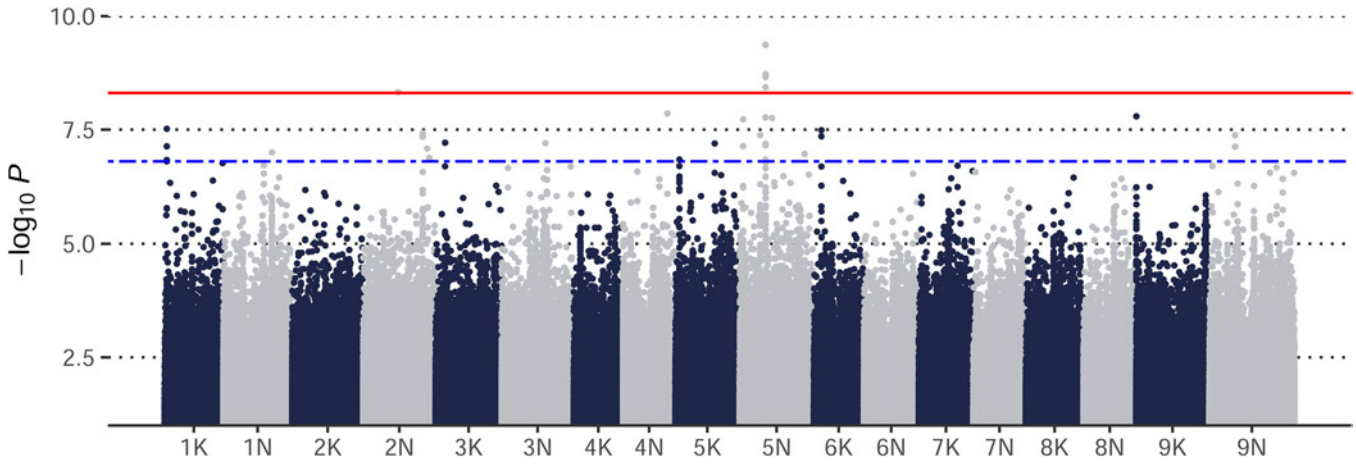

FIGURE 5 Manhattan plot showing the association between single nucleotide polymorphisms (SNPs) and leaf strength for *Panicum virgatum*. The solid red line represents a genome-wide Bonferroni-adjusted significance threshold, and the dashed blue line below represents a genome-wide 5% false discovery rate threshold; SNPs above these lines have a significant statistical association with leaf strength ($-\log_{10} P$). For plotting purposes, we removed all SNPs with $-\log_{10} P < 1$.

traits exhibited large differences in their degree of genetic association. Leaf area was associated with 292 variants across all 18 chromosomes at a 5% false discovery rate significance threshold (Appendix S1: Figure S3B; Appendix S2); 293 genes were located within 10 kbp of these SNPs (Appendix S3). Leaf thickness, on the other hand, was only associated with one variant (Appendix S1: Figure S3C; Appendix S2), which was located near two genes (Appendix S3). Leaf mass per area (LMA) was not significantly associated with any variants (Appendix S1: Figure S3D).

When we looked for overlap in SNPs associated with leaf tensile resistance and leaf morphology using Cauchy combination tests, SNPs fell into four categories: SNPs associated with leaf area and leaf tensile resistance ($N = 2$), SNPs associated with leaf tensile resistance and multiple leaf morphological traits ($N = 3$), SNPs only associated with leaf strength ($N = 30$), and SNPs only associated with leaf area ($N = 290$). There was also a single SNP that was associated with leaf tensile resistance and leaf thickness, and it was the same SNP that was significantly associated with leaf thickness.

Finally, many candidate genes were present within 10 kbp of significant SNPs in both univariate and Cauchy combination test GWA. Specifically, five candidate genes were exclusively associated with leaf strength. These included genes related to stress responses, such as an ortholog of the regulatory gene *GF14* (Chen et al., 2006), abscisic acid (*APA1*; Sebastián et al., 2020), and gibberellic acid (*SLY1*; Ariizumi et al., 2011). There were also genes associated with growth and development, including an ortholog of an auxin regulator *ABCB4* (Kubeš et al., 2012) and an ortholog of *Cyp40* (Pavir.4NG131800), which is necessary for vegetative phase change in *Arabidopsis thaliana* (Berardini et al., 2001). We detected one candidate pleiotropic gene (Pavir.7NG287700) associated with leaf area and leaf tensile resistance—an ortholog of *AGO1* which is involved in auxin regulation (Sorin et al., 2005), vegetative phase change (Yang et al., 2006), and normal leaf morphology (Bohmert et al., 1998; Jover-Gil et al., 2012). We also found a candidate pleiotropic gene (Pavir. 9KG134601) associated with leaf thickness and leaf tensile resistance, *NaPRT1*, which is involved in stress response (Hashida et al., 2009) and leaf senescence (Schippers et al., 2008; Hashida et al., 2009; Wu et al., 2016). These candidates suggest that genes related to stress response, auxin regulation within the cell, and development are critical in determining leaf tensile resistance and likely have pleiotropic effects on the leaf as a whole.

Leaf tensile resistance increases with growing season length

Growing season length may be an important determinant of leaf tensile resistance, possibly resulting from changes in leaf morphology. We evaluated this hypothesis using mediation analysis, an approach that assesses the importance of indirect effects on a response. Overall, the effect of habitat-of-origin growing degree days on leaf strength and leaf toughness was mediated by LMA (indirect effect on leaf strength = 0.168, $z = 4.813$, $P < 0.001$; indirect effect on leaf toughness = 0.145, $z = 3.506$, $P < 0.001$) and by leaf thickness (indirect effect on leaf strength = 0.302, $z = 6.377$, $P < 0.001$; indirect effect on leaf toughness = 0.228, $z = 4.185$, $P < 0.001$; Figure 6; Appendix S1: Tables S7, S8). Moreover, there was a significant direct effect of growing degree days on leaf strength (direct effect = 0.313, $z = 6.457$, $P < 0.001$) and on leaf toughness (direct effect = 0.148, $z = 2.290$, $P = 0.022$). These large direct effects indicate that another important mediator is probably absent from our analysis.

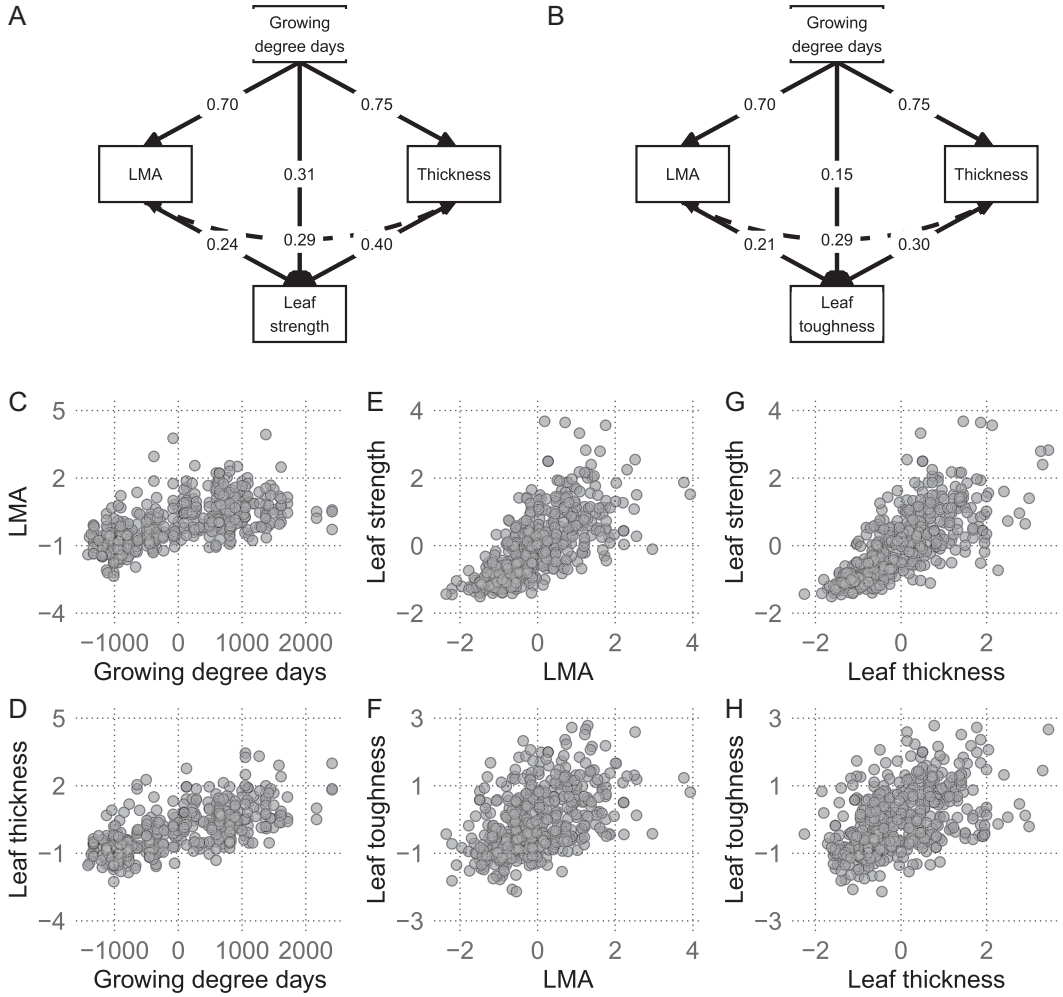

FIGURE 6 Direct and indirect effects of growing degree days, a measure of growing season length, on (A) leaf strength and (B) leaf toughness, showing the importance of two leaf morphological traits, leaf mass to area ratio (LMA) and leaf thickness. Black lines indicate that all model paths are significant. Panels C–H show bivariate relationships represented in panels A and B. Growing degree days was mean-centered. All other variables were standardized such that mean = 0 and standard deviation = 1 across all plants.

Another possible explanation is that the effects of habitat-of-origin growing degree days on leaf strength and leaf toughness were mediated to differing degrees by LMA and leaf thickness in each subpopulation (Appendix S1: Tables S9, S10; Figure S4). In the Atlantic subpopulation, leaf strength was mediated by both LMA and leaf thickness (LMA indirect effect = 0.121, $z = 2.140$, $P = 0.032$; thickness indirect effect = 0.181, $z = 4.016$, $P < 0.001$; Appendix S1, Table S9; Appendix S1: Figures S4A, S5). In the Gulf subpopulation, the leaf strength effect was significantly mediated by leaf thickness (indirect effect = 0.140, $z = 2.848$, $P = 0.004$) but not by LMA (indirect effect = −0.004, $z = -0.206$, $P = 0.837$). In the Midwest population, the leaf strength effect was mediated by LMA (indirect effect = 0.126, $z = 2.521$, $P = 0.012$) but not by leaf thickness (indirect effect = 0.055, $z = 1.370$, $P = 0.171$). Moreover, the direct effect of growing degree days on leaf strength was only significant in the Atlantic subpopulation (indirect effect = 0.365, $z = 5.659$, $P < 0.001$; Appendix S1: Table S9, Figure S4A).

The indirect effect of growing degree days on leaf toughness mediated by LMA and leaf thickness was also inconsistent within subpopulations. In the Atlantic and Midwest subpopulations, neither indirect effect was significant; in the Gulf subpopulation, the indirect leaf thickness effect was significant (Gulf indirect effect = 0.110, $z = 2.392$, $P = 0.017$), but the indirect LMA effect was not significant (Appendix S1: Table S10, Figure S4B). The direct effect of growing degree days on leaf toughness was not significant in any of the three subpopulations. Together, these results demonstrate that leaf morphology (leaf thickness and LMA) contributes substantially to the effect of growing season length on leaf tensile resistance (i.e., leaf morphology mediates this effect) across all genotypes of *P. virgatum*. Importantly, though, within genetic subpopulations, these relationships are less consistently mediated by leaf thickness and LMA.

DISCUSSION

In this study, we explored the intraspecific phenotypic and genetic variation in leaf tensile resistance in *P. virgatum*. We found that the three genetic subpopulations of *P. virgatum* exhibited large phenotypic differences in leaf tensile resistance, that leaf tensile resistance was highly heritable within and across subpopulations, and that high leaf tensile resistance was associated with high fitness as measured by biomass production. Moreover, leaf morphological traits, especially LMA and leaf thickness, were positively correlated —both phenotypically and genetically—with leaf tensile resistance, and these traits partially mediated the positive effect of growing season length on leaf tensile resistance. Additionally, multiple candidate genes for leaf tensile resistance were related to stress responses, auxin regulation within the cell, and leaf development and morphology; some of these candidate genes may have pleiotropic effects. Together, these results suggest that differences in climate across the broad range of *P. virgatum* contribute to the differences in leaf tensile resistance observed in this study—plants from the southernmost *P. virgatum* subpopulation, where growing seasons are longer, have evolved to produce stronger leaves with morphological traits (i.e., high LMA and leaf thickness) that may enhance leaf longevity. These results further suggest that leaf tensile resistance in *P. virgatum* may evolve in response to current and future climate change.

Much of the natural variation in leaf tensile resistance in *P. virgatum* can be attributed to the genetic variation present within the species. The high heritability (h^2) of leaf strength and leaf toughness in *P. virgatum* indicate that genetics is critical in determining leaf structural resistance, consistent with the significant genetic contribution of stem structural resistance to stem strength, stiffness, and rigidity in *Sorghum bicolor* (Gomez et al., 2020). Additionally, we identified several candidate genes associated with leaf strength, including genes functioning in leaf development, responses to abiotic stress, and hormones like abscisic acid, auxin, and gibberellic acid. Unlike a similar study in *A. thaliana*, which demonstrated an important role for cell wall composition in leaf strength (Balsamo et al., 2015), we did not detect any candidate genes associated with cell wall composition. Our lack of evidence here could have resulted from low statistical power—characteristics like leaf tensile resistance may be highly polygenic, and our sample size was relatively small, which would limit our ability to detect genes of moderate effect. In support of this statistical argument, we identified a candidate gene associated with cell wall composition at the less stringent 10% false discovery rate. It is likely that across tissues and systems, mechanical properties have a genetic basis. This underlying genetic basis implies that structurally robust plants can be bred in agricultural systems and that tissue toughness in many natural plant populations may adapt to environmental conditions. Specifically, in agricultural settings, stem lodging (i.e., stem bending near ground level) can reduce plant productivity and crop yields (Foulkes et al., 2011); in biofuel crops, stronger leaves are more likely to remain attached to the stem and contribute to biomass yield (Karp and Shield, 2008). In natural systems, this genetic basis means that in many species tissue toughness will be related to biotic pressures, such as herbivory, or abiotic pressures (Pérez-Harguindeguy et al., 2003; Moles et al., 2011). For instance, in *P. virgatum*, resistance to insect herbivory was positively genetically correlated with LMA (Headrick et al., 2024).

Although there are numerous studies on the drivers of leaf toughness across species (Wright and Cannon, 2001; Kitajima and Poorter, 2010; Onoda et al., 2017), less research has been done within individual species. Because interspecific and intraspecific patterns do not always concur (Agrawal, 2020), it is worthwhile to investigate the intraspecific relationship between leaf morphology and leaf structural resistance. We found that leaf strength and toughness were both positively phenotypically correlated with LMA and leaf thickness. This result is consistent with the findings of interspecific studies (Wright and Cannon, 2001; Onoda et al., 2011). On the other hand, leaf strength and toughness were marginally negatively correlated with leaf density, which is contrary to the positive interspecific relationship between leaf structural resistance and leaf density observed by Onoda et al. (2011). Perhaps this unexpected negative correlation was driven by differences in the abundance of major and minor veins in *P. virgatum* (Zhang et al., 2004), which might promote higher leaf tensile resistance without increasing leaf density. Thus, LMA and leaf thickness appear to be reliable predictors of leaf structural resistance across scales, while density may only be a useful predictor of leaf structural resistance at larger phylogenetic scales. In addition, this intraspecific study gave us the opportunity to explore genetic relationships between traits. We found a significant genetic relationship between leaf tensile resistance and both LMA and leaf thickness, including two candidate pleiotropic genes (orthologs of *AGO1* and *NaPRT1*; Pavir.7NG287700 and Pavir. 9KG134601, respectively) that may affect both leaf tensile resistance and morphology. More intraspecific studies on the effects of cell wall composition and leaf venation on leaf tensile resistance are needed to characterize the relationships between these traits within species and the capacity for these traits to evolve in response to a changing environment. Together, the results of these genetic analyses indicate that there is an underlying phenotypic and genetic relationship between leaf morphology and leaf tensile resistance.

In our mediation analysis, the large direct effect of growing season length on leaf toughness and leaf strength indicated that characteristics other than LMA and leaf thickness contributed substantially to leaf strength and leaf toughness. These characteristics could include many anatomical and compositional traits. For instance, plant tissues with thicker cell walls are typically tougher than those with thinner cell walls (Wright and Illius, 1995; Lucas

et al., 2000; Onoda et al., 2011; Alonso-Forn et al., 2023). Similarly, veins can be many times tougher than the rest of the lamina (Onoda et al., 2011; Sack and Scoffoni, 2013). Moreover, leaf tensile resistance often increases with fiber and lignin content (Wright and Illius, 1995; Onoda et al., 2011; Alonso-Forn et al., 2023).

Even though our findings show that genetics and leaf morphology strongly influence leaf tensile resistance, there were some limitations. First, population structure can be a confounding factor for quantitative genetic and genomic analyses (Sul et al., 2018). The high heritability of leaf strength and leaf toughness within the Atlantic and Gulf subpopulations, however, suggests that strong population structure was not the primary driver of these results. Second, only one study site was used, which prevents us from knowing how plasticity or genotype-by-environment interactions affect leaf tensile resistance in *P. virgatum* (Wilson et al., 2010). The conditions at this site differed from the climate of origin of some plants, particularly those from the Midwest and Atlantic subpopulations, which may result in changes to their observed phenotypes due to the hotter and more arid climate (Johnson et al., 2022; Napier et al., 2023). However, using this southern site meant that the plants originating from the southern United States did not experience the truncated growing season that would occur in a study site farther north. Third, we sampled considerably fewer Midwest genotypes (89) than Gulf and Atlantic plants (188 and 161, respectively). The combined effect of fewer Midwest plants being grown and measured in conditions vastly different from their natural habitat could have contributed to the low precision of heritability observed in the Midwest subpopulation. Despite these limitations, the sample of genotypes still had sufficient diversity and is likely representative of the natural genetic and phenotypic variation in *P. virgatum*.

In addition to having sufficient variation in leaf tensile resistance to adapt to different environments, there is evidence that *P. virgatum* has evolved tougher leaves in response to longer growing seasons across and within subpopulations. The ecological strategies of the Midwest and the Atlantic subpopulations may favor a faster growth rate to make the most of a shorter growing season, while the longer growing seasons of the Gulf subpopulation may promote the development of stronger but more resource-expensive leaves (Wright et al., 2004; Onoda et al., 2011; Lowry et al., 2014; Wang et al., 2023; Heckman et al., 2020, 2024). At this site, which is in the southern portion of the *P. virgatum* range and thus has a relatively long growing season, the individuals with tougher leaves had higher aboveground biomass, an indicator of higher fitness (Lowry et al., 2019), than those with weaker leaves. The high variation in leaf structural resistance present within *P. virgatum* and its strong links with the climate of genotype origin suggest that structural resistance may be an important contributor to the success of genotypes under climate change, potentially promoting local adaptation to changing growing season length.

CONCLUSIONS

Despite the potential costs associated with high leaf structural resistance, plants in this study with tougher and stronger leaves produced more biomass—a proxy for fitness—which may be associated with the long growing season at our common garden. Leaf tensile resistance was strongly associated with leaf morphological traits like leaf thickness and LMA, although these relationships often differed among genetic subpopulations, which suggests that leaves may be responding to different selective pressures across the range of *P. virgatum*. Together, the high heritability of leaf tensile resistance, the strong genetic links between tensile resistance and leaf morphology, and the impact of growing season length on tensile resistance suggest that leaves of *P. virgatum* will be able to respond to a warming climate in the coming decades.

AUTHOR CONTRIBUTIONS

T.E.J., A.B., and R.W.H. conceived the study; P.C.D. and R.W.H. implemented the experiment and analyzed the data; P.C.D. and R.W.H. wrote the manuscript with contributions from T.E.J. and A.B.

ACKNOWLEDGMENTS

The authors thank Angelo Filonzi for making the tensile resistance assays possible, and Jason Bonnette and Emily Breckenridge for help with data collection. The authors also thank David Lowry, Acer VanWallendael, Daniel Anstett, Joseph Edwards, Otto Kailing, Leslie Kollar, Jason Olsen, Madison Plunkert, and Lauren Stanley for comments and suggestions. Lastly, the authors thank two anonymous reviewers for helpful suggestions that improved the quality of this study. The findings and conclusions of this publication are those of the authors and should not be construed to represent any official USDA or U.S. Government determination or policy. The work was supported in part by the Great Lakes Bioenergy Research Center, U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research under Award Number DE-SC0018409. This research was also supported by the U.S. Department of Energy, Office of Science (BER), Grant no. DE-SC0014156; by the National Science Foundation, Plant Genome Research Program (IOS 1444533); and by the USDA Forest Service, Rocky Mountain Research Station.

DATA AVAILABILITY STATEMENT

All data that are not already available have been archived through Figshare: https://doi.org/10.6084/m9.figshare.25395514.v1.

ORCID

P. Camilla Durant http://orcid.org/0009-0006-6340-2113
Amit Bhasin http://orcid.org/0000-0001-8076-7719
Thomas E. Juenger http://orcid.org/0000-0001-9550-9288
Robert W. Heckman http://orcid.org/0000-0002-2281-3091

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Statistical tables and figures.

Appendix S2. List of single nucleotide polymorphisms (SNPs) significantly associated with one or more leaf morphological or tensile trait.

Appendix S3. List of candidate genes within 10 kbp of a significant SNP in genome-wide association mapping.

How to cite this article: Durant, P. C., A. Bhasin, T. E. Juenger, and R. W. Heckman. 2024. Genetically correlated leaf tensile and morphological traits are driven by growing season length in a widespread perennial grass. *American Journal of Botany* 111(5): e16349. https://doi.org/10.1002/ajb2.16349