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Reproduction and bioconfinement of miR156 transgenic switchgrass (*Panicum virgatum* L.)

Chelsea Renai Johnson
University of Tennessee, Knoxville, cjohn127@vols.utk.edu

To the Graduate Council:

I am submitting herewith a thesis written by Chelsea Renai Johnson entitled "Reproduction and bioconfinement of miR156 transgenic switchgrass (*Panicum virgatum* L.)." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Plant Sciences.

C. Neal Stewart Jr., Major Professor

We have read this thesis and recommend its acceptance:

Charles Kwit, Feng Chen

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

Reproduction and bioconfinement of miR156 transgenic switchgrass (*Panicum virgatum* L.)

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Abstract

Genetic engineering of switchgrass (*Panicum virgatum* L.), an emerging cellulosic bioenergy feedstock, has been performed to alter cell walls for improved biofuel conversion. However, gene flow from transgenic switchgrass presents regulatory issues that may prevent commercialization of the genetically engineered crop in the eastern United States. Depending on its expression level, microRNA156 (miR156) can reduce, delay or eliminate flowering, which may be useful to mitigate transgene flow. However, flowering transition is dependent upon both environmental and genetic cues. In this study of transgenic switchgrass, two low (T14 and T35) and two medium (T27 and T37) miR156 overexpressing 'Alamo' lines and nontransgenic control plants were used. A two-year field experiment was performed to compare flowering, reproduction, and biomass yield in eastern Tennessee, U.S.A. Growth chamber studies assessed temperature and photoperiod effects on flowering and reproduction across a simulated latitudinal cline.

In the field, medium miR156 overexpression line T37 resulted in the best overall combination of bioconfinement and biomass production. Though line T37 did flower, not all plants produced panicles, and panicle production was delayed in both years. Line T37 also produced fewer panicles, with a 65.9% reduction in year one and 23.8% reduction in year two over controls. T37 panicles produced 70.6% less flowers than control panicles during the second field year with commensurate decreased seed yield: 1205 seeds per plant vs. 18,539 produced by each control. These results are notable given that line T37 produced equivalent vegetative aboveground biomass as controls.

In latitudinal simulation growth chambers, elevated temperatures and decreased daylength promoted flowering of the miR156 transgenic switchgrass lines. As temperatures increased and day lengths decreased, more plants in lines T35, T37, and controls produced panicles. The simulated (Ecuador) tropical conditions were the only chambers in which three of the four transgenic lines flowered.

These results suggest that miR156 overexpression levels found in transgenic line T37 can be useful for bioconfinement, and the plants can significantly reproduce in tropical conditions, which would enable plant breeding for line improvement. Furthermore, the study suggests additional ways that miR156 can be manipulated to improve both biomass production and bioconfinement.

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Chapter 1 : Literature Review

Introduction

Pollen-mediated transgene flow from plants has been a topic of concern since the 1990s, the era of pre-and post-commercialization of transgenic crops. Pollen dispersal depends on multiple environmental factors such as wind speed and direction, temperature, humidity, and the location and density of related plant populations (Beckie and Hall 2008). The reproductive biology of both the crop and nearby congeners are also important and include features such as the timing and perpetuation of flower production, pollen viability and longevity, pollen size, and mode of pollen dispersal (Chandler and Dunwell 2008; Beckie and Hall 2008). Many cases of gene flow and hybridization between transgenic crops and sympatric weedy relatives, progenitors, or nontransgenic fields have been documented, such as with rice (*Oryza sativa*) (Chen et al. 2004; Messeguer et al. 2001; Song et al. 2003; Gealy et al. 2003), members of the *Brassica* genus or Brassicaceae family (Warwick and Martin 2013; Knispel et al. 2008; Hansen et al. 2001; Beckie et al. 2003; Warwick et al. 2008; Warwick et al. 2003; Hall et al. 2000; Rieger et al. 2002), lettuce (*Lactuca sativa*) (Giannino et al. 2008; Hooftman et al. 2005), and carrot (*Daucus carota* Apiaceae) (Mandel et al. 2016) to name a few. If the transgene provides fitness-enhancing benefits, hybrids could have negative ecological impacts on local plant communities by altered competitive plant interactions, possible extinction of species populations, or the development of weedy populations (Raybould and Gray 1994; Stewart et al. 2003; Mannasse 1992; Ellstrand et al. 1999; Kwit et al. 2011). Transgene introgression is also a risk. The occurrence of introgression depends on multiple factors such as fitness advantages from either intended or unintended effects of the transgene, strength of environmental selection pressures, pollinator overlap, the location of planting, and natural selective advantage traits associated with the crop

or its weedy relatives (Chandler and Dunwell 2008; Stewart et al. 2003; Chapman and Burke 2006; Kwit et al. 2011).

Examples of gene flow can be found in grass crop species. Grasses are notorious for their high amounts of gene flow because they commonly have a perennial life-cycle, are obligate outcrossers, and produce small pollen grains that can travel long distances via wind (Kausch et al. 2010). Many cases have been documented that describe the scope of gene flow in grasses. Studies on herbicide resistant creeping bentgrass (*Agrostis stolonifera*) have shown successful hybridization with related wild species such as *Agrostis capillaris*, *Agrostis castellana*, *Agrostis gigantea*, and *Agrostis exarata* at distances between 2 - 3.8 km, and creeping bentgrass pollen can travel up to 21 km (Belanger et al. 2003; Watrud et al. 2004; Reichman et al. 2006). Controlled field studies have also been conducted for both herbicide resistant annual ryegrass (*Lolium rigidum*) and herbicide resistant barnyardgrass (*Echinochloa crus-galii*). Ryegrass pollen can travel up to 3 km from an experimental source plot (Busi et al. 2008). Barnyardgrass gene flow decreased exponentially as distance increased, but pollen was still detected at 50 m (Bagavathiannan and Norsworthy 2014). These studies show that pollen mediated gene flow in grass species can occur at far distances.

While the grass species above are mainly grown for turf or forage, there are other grass species that are candidates for widespread cultivation as biofuel feedstocks. One such grass is switchgrass (*Panicum virgatum*). Switchgrass is a North American perennial grass that has been the subject of intensive research as a biofuel feedstock because of its large biomass and energy production, ability to be grown on marginal lands not conducive for growing row crops, conservative nutrient usage, and low input needs (Parrish and Fike 2005; McLaughlin and Kszos 2005; Sage et al. 2015). Under field conditions it has been observed that transgenic switchgrass

pollen can travel at least 100 m and is viable for pollination (Millwood et al. 2017). Models suggest switchgrass pollen can travel as far as 6.5 km under certain wind conditions (Ecker et al. 2013). Switchgrass pollen can also remain viable for 20 – 150 minutes depending on atmospheric conditions (Ge et al. 2011). Taken together, these studies suggest that switchgrass could be a significant vector for transgene spread under relevant agricultural environments.

Although many traits of switchgrass are favorable for bioenergy production, its cell walls contain high levels of lignin that interfere with cell wall degradation and subsequent biofuel production (Nigam and Singh 2011; Yang and Wyman 2008). It is becoming apparent that switchgrass will likely require altered cell wall chemistry, at least a decrease in lignin, to lower the economic barrier for effective processing of switchgrass biomass into biofuels. Moreover, genetic engineering is likely required to significantly change cell walls toward that end (Gressel 2008; Fu et al. 2011; Shen et al. 2013; Wuddineh et al. 2016). The likelihood of gene flow from transgenic switchgrass might trigger regulatory positions that could jeopardize commercialization of genetically engineered germplasm, at least in eastern North America where switchgrass is indigenous (Raghu et al. 2006; Stewart 2007; Kwit and Stewart 2012). Therefore, the elimination or mitigation of gene flow may be required for deregulation of transgenic switchgrass (Kausch et al. 2010; Sang et al. 2013).

Risk and deregulation of genetically engineered crops

Risk is generally defined as the probability of both hazard and exposure, where a hazard is any trait that may cause adverse effects, and exposure is the release of the hazard into the environment (Risk = Probability [Hazard x Exposure]) (Johnson et al. 2007; Andow and

Zwahlen 2006; Wilkinson et al. 2003). Both hazard and exposure must occur or be probable for risk to transpire. Risk is specifically determined for each transgenic plant on a case-by-case basis because the hazard and exposure depend on the transgene phenotype, transgene composition on a molecular level, target plant, and area of desired cultivation (Craig et al. 2008; Auer 2008).

Genetically engineered (GE) crops are put through an exhaustive and comprehensive safety assessment; they must pass an extensive multistep risk analysis to ensure consumer and environmental safety before they can be commercially released (Prado et al. 2014). First, to ensure all putative GE crop trait hazards have been tested, scientific experiments are carried out and generally follow a tiered framework (Figure 1) (Raybould and Cooper 2005; Wilkinson et al. 2003). This framework begins with hazard identification and small-scale lab experiments to identify and assess the probability of adverse effects occurring; it ends after large-scale greenhouse and field studies are conducted to quantify the amount of risk associated with the transgene(s) (Wilkinson et al. 2003; Andow and Zwahlen 2006). If the results from the tiered tests show a low probability of adverse effects, the GE crop begins the process of deregulation, which starts with an environmental risk assessment (ERA). The ERA uses information gathered during the research stages to determine whether introduction of the transgene into the environment can increase the risk of ecological harm, increase the plant susceptibility to pests, or enhance the weedy characteristics of the plant (Prado et al. 2014). ERA results are used to determine (1) if the plant is safe for release (no/low risk), (2) what kind of management practices should be applied (moderate risk), or (3) if approval of the crop should be withheld (high risk) (Craig et al. 2008). If any portion of the crop will be consumed by livestock or humans, a food and feed safety assessment is also be performed. This involves a comparison of the transgene,

both its amino acid sequence and the protein encoded, to human and animal allergens and toxins to screen for similarities (Prado et al. 2014).

Plant fitness could also be increased when the transgene is combined with certain inherent characteristics of the target plant, especially if those traits are associated with weediness. Weedy traits include 1) the ability to germinate in many environments, 2) the ability to reproduce by seed or vegetative tissues, 3) seed dormancy and longevity, 4) rapid growth/phase change and continuous seed production, 5) self-compatible reproduction, 6) multiple methods of pollination if cross-pollinated, 7) small pollen produced in large amounts, 8) a large, fibrous root system, and 9) a high photosynthetic rate (Baker 1965; Stewart 2004). These weedy traits have been removed from most food crops over years of selective breeding; however, plant species that have gained popularity for use as biofuels are often not highly domesticated and thus display many of these weedy traits (Raghu et al. 2006). In fact, the very traits that cause these plants to be considered weedy are the traits that make them useful as biofuel crops (Raghu et al. 2006). Switchgrass shows promise as a biofuel feedstock because of its ability to be vegetatively propagated, ability to grow on marginal land in various environments, rapid growth, and large root system; all of these traits are also considered to be weedy characteristics. These weedy traits, whether inherent or enhanced by the presence of a transgene, may be desired for a biofuel crop, but their dispersal into the environment is not because of hard to predict and potentially negative environmental effects.

Bioconfinement

Many methods have been proposed that could lower or eradicate gene flow in transgenic crops. Field isolation, fences, border plants, or flower removal could be used to reduce or eliminate pollen dispersion, but these methods are often labor and cost intensive (Moon et al. 2010). A biological mode of confinement (bioconfinement) is thought to be the most efficient technique. Many approaches have been tested (reviewed in Moon et al. 2010; Liu et al. 2013; Sang et al. 2013; Ding et al. 2014; Gressel 2015) and include methods such as pollen- and seed-specific sterility (Pedersen et al. 2003; Ishimaru et al. 2006; Kobayashi et al. 2006; Wang et al. 2012; Millwood et al. 2016), transgene excision (Zhang et al. 2003; Luo et al. 2007; Moon et al. 2011), and a delayed or non-flowering phenotype (Chuck et al. 2011; Fu et al. 2012; Jensen et al. 2013). A highly efficient engineering-based mechanism was described in Millwood et al. (2016). In this case, engineered tobacco (*Nicotiana tabacum*) expressed the restriction endonuclease *Eco*RI under a pollen specific promoter. Cells expressing *Eco*RI, resulted in over 99-100% transgene bioconfinement via selective plant male sterility (Millwood et al. 2016). Using tobacco as a model, transgene excision in pollen was tested using a CinH-*RS2* recombination system (Moon et al. 2011). Plants were engineered to produce a pollen-expressed green fluorescent protein (GFP), which the CinH-*RS2* recombination system was designed to excise (Moon et al. 2011). In three transgenic events, less than 1% of the pollen produced expressed GFP when CinH-*RS2* was present (Moon et al. 2011). While these two bioconfinement methods would be useful for crops where seed production is vital, biofuel feedstocks grown solely for their biomass would benefit more from a delayed or non-flowering phenotype because it has the potential to reduce gene flow while simultaneously boosting biomass production (Jakob et al. 2009). This idea is supported in a study focused on the biofuel grass *Miscaunthis sacchariflorus*, a parent of the hybrid

Miscanthus x *giganteus*, in which a delay of flowering for 61 days resulted 52% more biomass (Jensen et al. 2013). With this in mind, it is possible that a non-flowering phenotype could lead to increased biomass with the added benefit of no gene flow via pollen. Seed production would likely be reduced if flowering overlap and seed maturation time were decreased, and no seeds would be produced if flowering did not occur. For some crops a nonflowering phenotype would pose a problem because seeds would be required planting the next year, but because switchgrass can be vegetatively propagated, a switchgrass field could be transplanted. Switchgrass is also a perennial, so the field would not need to be re-planted the next season. If flowers were needed for breeding purposes, it is possible flowering and seed production might be performed in latitudes outside of the switchgrass production zone (Balasubramanian et al. 2006; Sherry et al. 2007; McClung et al. 2016) or under enclosed and controlled conditions.

miR156 switchgrass

A delayed or non-flowering phenotype can be achieved by overexpressing microRNAs (miRNAs) that are involved in regulating the vegetative-to-floral transition. Previous research has generated switchgrass lines that overexpress the maize (*Zea mays*) gene *Corngrass1* (*Cg1*), a member of the miR156 class of miRNAs (Chuck et al. 2011). During this study, no transgenic lines flowered during the two-year field study, and low *Cg1* overexpression resulted in higher biomass production and saccharification efficiency (Chuck et al. 2011). In a separate study, Fu et al. (2012) engineered switchgrass to overexpress, at various levels, a rice *pre-OsmiRNA156b* gene. Low and medium overexpression levels resulted in increased switchgrass biomass production, which was comprised, variably, of an increase in tiller number and similar plant

height compared to the control. High miR156 overexpression led to plants that were also heavily tillered, but no increase in biomass was observed due to severe dwarfism (Fu et al. 2012). Low miR156 overexpression plants flowered normally whereas medium and high overexpression plants had suppressed flowering (Fu et al. 2012).

The goal of my thesis research was to further characterize various Fu et al. (2012) miR156 overexpressing switchgrass lines with different transgene expression levels under several environmental conditions, with a special emphasis on flowering. I performed field experiments for two years, and three growth chamber experiments, representing one growing season each. Specific questions investigated included: 1) Does the overexpression of miR156 cause a significant delay in flowering or prevent flowering in the field? 2) If a change in flowering time occurs in the transgenic switchgrass, is it significant enough to reduce the amount of transgenic progeny via gene flow? 3) Does the amount of biomass produced by miR156 transgenic switchgrass exceed or match that of the wild-type switchgrass? 4) Do warmer temperatures, long days, or a combination cause normal flowering to occur in the miR156 transgenic switchgrass lines?

Chapter 2 : Field-grown miR156 transgenic switchgrass reproduction, yield, global gene expression analysis and bioconfinement

CRJ drafted the manuscript, carried out the field study, collected phenotypic measurement data, and performed quantitative RT-PCR. RJM assisted in experimental design, coordination of the study, and revisions to the manuscript. YT and JG performed the microarray. RWS, GBT, and MFD completed the lignin and sugar release assays. YS contributed to the development of the study and writing of the grant proposal. ZYW provided the original plant clones and helped develop the study. CNS conceived of the study and its design and coordination and revised the manuscript. All authors contributed to text and data analysis and interpretation. All authors read and approve the final version of the manuscript.

Field-grown miR156 transgenic switchgrass reproduction, yield, global gene expression analysis and bioconfinement

Chelsea R. Johnson[1], Reginald J. Millwood[1,2], Yuhong Tang[2,3], Jiqing Gou[2,3], Robert W. Sykes[2,4], Geoffrey B. Turner[2,4], Mark F. Davis[2,4], Yi Sang[1], Zeng-Yu Wang[2,3], C. Neal Stewart, Jr.[1,2]

[1]Department of Plant Sciences, University of Tennessee, Knoxville, Tennessee, USA

[2]BioEnergy Science Center, Oak Ridge National Laboratory, Oak Ridge, Tennessee, USA

[3]Noble Research Institute, Ardmore, Oklahoma, USA

[4]National Renewable Energy Laboratory, Golden, Colorado, USA

Abstract

Background

Genetic engineering has been effective in altering cell walls for biofuel production in the bioenergy crop, switchgrass (*Panicum virgatum*). However, regulatory issues arising from gene

flow may prevent commercialization of engineered switchgrass in the eastern United States where the species is native. Depending on its expression level, microRNA156 (miR156) can reduce, delay or eliminate flowering, which may serve to decrease transgene flow. In this unique field study of transgenic switchgrass that was permitted to flower, two low (T14 and T35) and two medium (T27 and T37) miR156 overexpressing 'Alamo' lines with the transgene under the control of the constitutive maize (*Zea mays*) ubiquitin promoter (Ubi1), along with nontransgenic control plants, were grown in eastern Tennessee over two seasons.

Results

miR156 expression was positively associated with decreased and delayed flowering in switchgrass. Line T27 did not flower during the two-year study. Line T37 did flower, but not all plants produced panicles. Flowering was delayed in T37 and resulted in 70.6% fewer flowers than controls during the second field year with commensurate decreased seed yield: 1205 seeds per plant vs. 18,539 produced by each control. These results are notable given that line T37 produced equivalent vegetative aboveground biomass to the controls. miR156 transcript abundance of field-grown plants was congruent with greenhouse results. The five miR156 SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (*SPL*) target genes had suppressed expression in one or more of the transgenic lines. Line T27, which had the highest miR156 overexpression, showed significant downregulation for all five *SPL* genes. On the contrary, line T35 had the lowest miR156 overexpression and had no significant change in any of the five *SPL* genes.

Conclusions

Because of the research field's geographical features, this study was the first instance of any genetically engineered trait in switchgrass, in which experimental plants were allowed to flower

in the field in the eastern U.S.; USDA-APHIS-BRS regulators allowed open-flowering. We found medium overexpression of miR156, e.g., line T37, resulted in delayed and reduced flowering accompanied by high biomass production. We propose induced miR156 expression could be further developed as a transgenic switchgrass bioconfinement tool to enable eventual commercialization.

Keywords: bioconfinement, floral transition, miR156, switchgrass, gene flow

Background

Switchgrass (*Panicum virgatum* L.) is a native North American perennial prairie grass mostly known for its use as a biofuel feedstock. The high biomass production, low input requirements, and its ability to be productive on marginal land are some features that make switchgrass an attractive cellulosic feedstock (Sage et al. 2015; McLaughlin and Kszos 2005). However, the significant lignification of secondary cell walls inhibits biomass conversion to fermentable sugars and biofuel in switchgrass, which, in turn, is an economic barrier to biofuel production (Parrish and Fike 2005; McLaughlin and Kszos 2005; Sage et al. 2015; Nigam and Singh 2011; Yang and Wyman 2008). Genetic engineering to reduce lignin levels in switchgrass cell walls appears to be essential for its optimal use as a biofuel crop (Stewart 2007; Gressel 2008; Jakob et al. 2009). Indeed, there are several success stories in producing transgenic switchgrass with altered lignification, which resulted in higher biofuel yield from field-grown biomass (e.g., Baxter et al. 2014; 2015), but the prospects of transgene flow from genetically engineered switchgrass is a regulatory concern. Transgene flow from switchgrass will likely need to be severely curtailed to facilitate the commercialization of transgenic varieties (Stewart 2007; Kausch et al. 2010). This situation is especially pertinent in the eastern United States where switchgrass is endemic and common (Kwit and Stewart 2012). Research has investigated several bioconfinement strategies, which include pollen ablation (Mariani et al. 1990; Luo et al. 2007; Millwood et al. 2016) and removal via site-specific recombinases (Moon et al. 2011; Somleva et al. 2014). In addition, the delay or elimination of flowering itself could promote simultaneous improvements for a transgenic biomass crop such as switchgrass: it could decrease or eliminate pollen while simultaneously potentially increasing vegetative biomass (Jakob et al. 2009).

MicroRNAs (miRNAs) are an extensive class of small (20-24 nucleotides), regulatory RNAs that could be useful in genetic engineering to improve biofuel feedstocks by targeting stress responses, biomass production, and lignin content (Reinhart et al. 2000; Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001; Bartel 2004; Zhang et al. 2006; Auer and Frederick 2009; Sun 2012; Sun et al. 2012; Zhou and Luo 2013; Cui et al. 2014; Trumbo et al. 2015; Zhang and Wang 2015; Djami-Tchatchou et al. 2017). Specifically, miR156 targets the SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (*SPL*) transcription factor family, which is involved in the transition from vegetative to reproductive phases (Rhoades et al. 2002; Poethig 2009; Wu et al. 2009; Matts et al. 2010). Overexpression of miR156 in switchgrass at low and moderate levels led to increased biomass and a non-flowering phenotype in the greenhouse (Fu et al. 2012). When two low and two moderate overexpressing lines were grown in the field, three of the lines flowered and one of these lines produced more biomass than the control (Baxter et al. 2017). These results indicate that growth environment and gene expression play significant roles in the phenology of switchgrass.

Our research objectives in this study were to deploy a range of miR156 overexpressing switchgrass in a relevant field situation to closely examine flowering, reproduction, and biomass. A field on the Cumberland Plateau in Tennessee that is surrounded by forest enabled a two-year study in which U.S. regulators allowed plants to reproduce. In assessing a delayed/decreased flowering strategy for bioconfinement of switchgrass, it was imperative to obtain two full flowering cycles in the field to gauge practical utility of this strategy. A transcriptomic study of the field-grown plants was performed to assess the influence of downstream genes impacted by miR156 expression, as well as any potential off-target effects, which are important for designing

next-generation transgenic plants to further fine-tune the spatio-temporal expression of miR156 in switchgrass.

Methods

Field design and plant materials

Plants were grown in a field site in Oliver Springs, Tennessee, USA for two years under USDA-APHIS-BRS release permits (13-046-104r-a1 and 16-056-103r). This highly secluded field on the hilly Cumberland Plateau is surrounded by a natural forest border (Figure 2), which allowed for open flowering and seed production of the transgenic switchgrass lines under permit conditions. The switchgrass plants were transplanted on June 05, 2015 into a twenty-plot complete randomized design (Figure 2 and 3). Four transgenic and two nontransgenic parent 'Alamo' switchgrass lines, all allotetraploids, were used to comparatively examine the phenotypic effects of miR156 overexpression (Figure 4). The four transgenic lines were engineered to overexpress the rice (*Oryza sativa*) *pre-miR156b* gene under the control of the maize (*Zea mays*) Ubi1 promoter as described in Fu et al. (2012) at relatively low- (lines T14 and T35) or medium- (lines T27 and T37) overexpression levels. All transgenic plant replicates were clones obtained through vegetative propagation of tillers from the respective transgenic event. Each of the deployed lines was clonally replicated in the greenhouse prior to field transplantation. Two replicates of a second nontransgenic clone (ST2) were included as pollen donors for the surrounding ten clones representing single lines per plot (Figure 3). Within plots, plants were spaced 0.76 m from each other, and each plot measured 2.29 m × 1.52 m. The entire field site was 21.59 m × 13.72 m. Plants were hand watered for four weeks after establishment.

No fertilizer or pesticide treatments were applied during the experiment. Weeds were manually removed.

Biomass and morphological characterization

Plants were checked weekly for the presence of panicles during both growing seasons, and first-date-to-flower was recorded. Aboveground biomass was harvested 10 cm above soil level after first frost (November) with plots pooled into a single harvest bag; the two ST2 plants from each plot were bagged separately from the surrounding plants per plot. All harvested biomass was oven-dried at 40°C for 168 h, then dry biomass was tallied on a per plot basis and data presented on a per plant basis. Panicles were removed prior to harvest due to permit restrictions and bagged separately. Bags were stored in a greenhouse and allowed to air dry. Total panicle weights were recorded, averaged, and added to the average vegetative biomass weight to give total aboveground biomass production.

Panicles were counted during the removal process, and the lengths were measured for two randomly chosen panicles from each of five randomly-selected plants per plot. A subsample of three panicles at the R4 stage of reproduction (Moore et al. 1991) was collected in September 2016 (year two) from each plot to tally flowers and spikelets per panicle.

The number of tillers per plant was tallied at each end-of-season harvest. Plant height (apex) was measured both before and after panicle removal. Leaf length, leaf width, stem diameter, and node number were taken at the end of the season on the two tallest tillers of each plant sampled. Leaf blade length and width was taken on the flag leaf or top-most mature leaf of each of the selected tillers. Tiller node number was counted from the soil line up, and representative internode diameter was taken using a Maxwell 150 mm digital caliper between the third and fourth nodes.

Seed collection and germination

After mature seeds were harvested from panicles, three subsamples per plant were tallied for 100-seed weight, then averaged. Seed number per plant was then derived by bulk seed weight and 100-seed weight. Seeds collected from transgenic lines or nontransgenic 'Alamo' controls were placed on solid MS-basal media (Murashige and Skoog 1962), and germination percentage was calculated at two weeks after plating.

Cell wall characterization

End-of-season vegetative dry biomass was chipped to approximately 10 cm segments using a CS-4325 chipper shredder (Troy-Bilt, Valley City, Ohio) and then milled with a Wiley mill (Thomas Scientific, Model 4, Swedesboro, N.J.) through a 1 mm screen. Milled material was used to analyze the lignin content, syringyl to guaiacyl (S/G) ratio, and sugar release of the cell walls of each line by the National Renewable Energy Laboratory standard protocols. Lignin content and the S/G ratio were determined by pyrolysis molecular beam mass spectrometry as described in Sykes et al. (2009) on an Extrel single quadrupole molecular beam mass spectrometer. The peak intensities of lignin precursors were summed and used to estimate total lignin content. The S/G ratio was calculated by dividing the intensity of the syringyl peaks by the intensity of the guaiacyl peaks.

Sugar release was determined using methods described in Selig et al. (2010). Hydrolysis took place using the Ctec2 enzyme cocktail (Novozymes North America, Franklinton, NC). Released glucose levels were measured using the D-Glucose Assay Kit (glucose oxidase/peroxidase; GOPOD), and released xylose levels were determined by the D-Xylose

Assay Kit (xylose dehydrogenase; XDH; Megasyme Intl., Bray, Ireland). Sugar release data was reported as grams of released sugar per gram of cell wall residue.

Transcriptomic analysis

Microarray analysis was performed to determine downstream gene expression effects of miR156 overexpression. Three tillers were collected from each plot, resulting in four biological replicates for each of the four transgenic and 'Alamo' nontransgenic control lines. Total RNA was extracted from the combined tissues of randomly selected V3 stage tillers, as defined in Hardin et al. 2013, from each line harvested on September 10, 2015 between 11:00 am and 1:00 pm. RNA was extracted using Tri-Reagent (Invitrogen, Carlsbad, Calif.) and subsequently cleaned and concentrated with the RNeasy® MinElute Cleanup Kit (Qiagen, Valencia, Calif.). Purified RNA (100 ng) was used for the expression analysis of each sample using a custom-designed switchgrass cDNA chip Pvi_cDNAa520831 (Affymetrix, Santa Clara, CA). Probe labeling, chip hybridization, and scanning were performed according to the manufacturer's instructions for 3' IVT PLUS Kit (Affymetrix). Data normalization among chips was conducted using the robust multichip average (RMA; Irizarry et al. 2003). Gene selections based on Associative T-test (Dozmorov and Centola 2003) were made using Matlab (MathWorks, Natick, MA). In this method, the background noise presented between replicates and technical noise during microarray experiments was measured by the residual presented among a group of genes whose residuals are homoscedastic. Genes whose residuals between the compared sample pairs that are significantly higher than the measured background noise level were considered to be differentially expressed. A selection threshold of 2 for transcript ratios and a Bonferroni-corrected P value threshold of 5.84201E-07 were used. The Bonferroni-corrected P value

threshold was derived from 0.05/N in these analyses, where N is the number of probes sets on the chip. Microarray data will be available in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress).

Quantitative RT-PCR (qRT-PCR) analysis was used to assess transcript abundance of miR156 and its known target *SPL* genes. Total RNA was extracted using Tri-Reagent (Invitrogen) from V3 stage tillers collected mid-day on July 26, 2016. RNA samples were cleaned with the RNeasy® Mini Kit (Qiagen). The mature miR156 levels was determined using a highly sensitive stem-loop pulsed reverse transcription procedure (Varkonyi-Gasic et al. 2007) using a miR156 specific stem-loop primer. RT-PCR for *SPL* expression was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, Calif.). SYBR Green (Applied Biosystems) was used as the reporter dye during qRT-PCR, and a QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems) was used. The miR156 target gene transcript abundance qRT-PCR analysis included *PvSPL1, PvSPL2, PvSPL3,* and *PvSPL6.* miR156 expression was normalized using miR390 expression, and switchgrass *PvUbq1* transcript abundance was used for normalization of data from each target gene with appropriate primers (Fu et al. 2012). Delta Cycle threshold (ΔCt) was calculated by subtracting the target gene Ct from Ct of the housekeeping gene (Housekeeping Ct – Target Ct = ΔCt).

Statistical analysis

SAS version 9.4 (SAS Institute Inc., Cary, NC) was used for all statistical analyses. A one-way ANOVA with Fisher's least significant difference was used to compare means among lines within each year. Differences were considered significant when *P*-values were less than or equal to 0.05.

Results

miR156 overexpression levels affect flowering timing and reproductive effort

The medium overexpression lines (T27 and T37) had notably decreased numbers of flowers that were also produced in a delayed floral transition phase (Figures 5 and 6). Line T27 never produced flowers in the field, but had attenuated biomass production. Only a subset of T37 plants flowered in the field in either growing season. The plants that did flower were delayed 12 weeks after the control in year one and two weeks in year two (Figure 5). T37 panicle number per plant was reduced 65.9% in year one and 23.8% in year two compared to the control, and the panicles were shorter (Table 1). The delayed and diminished flowering phenotype led to a commensurate and drastic reduction in both flower and seed production per plant in line T37 compared with the control (Figures 6 and 7). In year one, seed production was reduced 88.2% in T37 plants compared with the control, and in year two seed production was 93.5% less in T37 plants. There was a strong positive correlation between flower number and seed production when the two data sets were compared (Pearson correlation coefficient: $r = 0.77$, $P = <0.0001$).

All plants in the low overexpression lines flowered both years. T35 flowering phenology was delayed by six weeks relative to the control in year one, but was not delayed in year two (Figure 5). T35 produced 22.1% fewer panicles, but were no different in length than the control (Table 1). The opposite was found in year two; T35 and the control produced the same number of panicles, but T35 panicles were shorter. However, T35 plants produced fewer flowers and seeds than the control for both years (Figures 6 and 7). Line T14 flowered at the same time as the control in year one and two weeks before the control in year two (Figure 5). Although panicles emerged early in the season, they were fewer and smaller than control panicles (Table 1). T14 also produced fewer flowers and seeds than the control (Figures 6 and 7).

Seed germination

Seeds from the 'Alamo' non-transgenic control and transgenic lines were also collected and germinated. T35 was the only line to differ from the control in year one (18% higher germination), but there were no differences among transgenic lines in year 2, all of which had lower germination frequency than the control (Table 1).

Aboveground vegetative biomass production and plant morphology

Low expressing line T35 most closely resembled the control in the field: they had equivalent dry biomass production at the end of both seasons (Figure 8a), as well as other traits (Table 2; Figure 8). T35 did produce wider leaves and tillers with a greater stem diameter than the control in year two. Lines T14 and T27 produced less biomass, but line T27 produced the most tillers in year one and was matched only by T37 in year two. T27 plants were shorter (Figure 8c-d) and with diminutive stem diameters (Table 2), which resulted in very low biomass production (Figure 8a-b). The biomass of T27 plants was actually reduced by approximately 10 g in the second season (Figure 8a-b). T14 plants were shorter than the control, and they produced few, slender tillers. Line T37 plants and controls produced equivalent biomass in year one, but the control outperformed T37 in year two (Figure 8a). However, when panicles are removed from the biomass data, T37 and the control produced statistically equivalent biomass in both years, which is important from a commercialization perspective (Figure 8b). The difference in plant height is also less drastic when panicles were removed (Figure 8d). T37 plants had smaller diameter tillers with smaller leaves than the control (Table 2), but the increased tillering of T37 compensated for the stem and leaf traits, contributing to the high biomass production of T37.

Cell wall composition (lignin content, digestibility, and sugar release) of the transgenic switchgrass lines had a few notable changes compared with the control. In both seasons, line T14 plant cells contained more lignin than the control (Table 2). T14, along with line T35 (both low overexpression lines), had higher S/G ratios than the control, suggesting they are more easily digestible (Table 2). Both medium overexpression lines (T27 and T37) had lower S/G ratios than the control in both seasons. Transgenic lines did not differ from the control in sugar release (Table 2).

Transcriptomic analysis

The level of mature miR156 transcript was examined using quantitative RT-PCR, and results were congruent with results of the same clonal lines grown under greenhouse conditions (Fu et al. 2012) and in the field in which panicle removal was required (Baxter et al. 2017). Lines categorized as low overexpressors (T14 and T35) had two- and three-times increase respectively in miR156 levels compared to control plants in the field. Medium overexpression lines (T27 and T37) show eight- and 10-times increase respectively in mature miR156 levels compared to the control (Figure 9).

The expression level of four *SPL* genes (*PvSPL1, PvSPL2, PvSPL3,* and *PvSPL6*) were also examined using quantitative RT-PCR to determine the effects of miR156 overexpression on its target genes in field-grown plants. All expression levels were examined on V3 stage vegetative tillers collected in year two (2016). The high variation among biological replicates resulted in no statistically-significant differences for the expression levels of any *SPL* genes. Line T27 had the highest miR156 expression and showed the lowest *PvSPL* expression in general (Figure 10).

The V3 stage tillers collected from the field in year one (2015) represent relevant mid-year aboveground biomass for the global transcriptomic analysis (microarrays). Total RNA from all four transgenic lines and the 'Alamo' wild-type control was analyzed using Affymetrix microarray chips. Of the 85,587 probe sets examined, 14,507 were significantly up- or down-regulated for one or more of the transgenic lines. Genes related to the miR156 pathway and flowering were chosen for further examination. Of 49 probe sets annotated as *SPL* according to known *Arabidopsis thaliana* and rice *SPL* sequences, eight *SPL* probes were found to be downregulated in open flowering field conditions (Table 3). *SPL* down regulation was negatively correlated to mature miR156 overexpression (Figure 9 and 10). For the highest miR156 overexpression line T27, all eight *SPL* gene annotations were significantly downregulated (Table 3). Six *SPL* gene annotations were downregulated in T37, which had the second highest miR156 overexpression. The expression of *SPL* genes appeared to have similar patterns to nontransgenics in the low overexpression lines; only two *SPL* gene annotations were downregulated in T14, and none were downregulated in T35 (Table 3). Probes corresponding to other important genes involved in flowering pathways, such as Arabidopsis *AtFT* (*Flowering Locus T*)/ rice *OsFTL* (*Flowering Locus T-Like*) genes, were also significantly affected in miR156 overexpressing switchgrass (Table 3).

Discussion

Regulation of gene expression by miRNAs could be useful in the genetic engineering of biofuel feedstocks to enhance desired traits such as abiotic and biotic stress responses, biomass yield, and lignin content (Zhang et al. 2006; Auer and Frederick 2009; Sun 2012; Zhou and Luo 2013; Cui et al. 2014; Trumbo et al. 2015; Zhang and Wang 2015). miR156 targets the SQUAMOSA

PROMOTER BINDING PROTEIN-LIKE (*SPL*) transcription factor family which is involved in many plant developmental processes including the vegetative to reproductive phase developmental transition (Rhoades et al. 2002; Poethig 2009; Wu et al. 2009; Matts et al. 2010; Preston and Hileman 2013). The overexpression of miR156 has been shown to delay flowering and increase biomass yield in multiple plant species (Schwab et al. 2005; Wu and Poethig 2006; Chuck et al. 2011; Fu et al. 2012; Zheng et al. 2016). *Arabidopsis thaliana* plants engineered to overexpress miR156 had a moderate delay in flowering and an increase in total leaf number when grown under long days (Schwab et al. 2005). A similar phenotype was seen in red clover (*Trifolium pratense* L.) engineered to overexpress miR156; transgenic red clover plants had an increased number of shoots and delayed flowering (Zheng et al. 2016). Switchgrass engineered to overexpress maize *Corngrass1*, a gene in the miR156 class of miRNAs, did not flower in the field, and weak overexpression levels did not affect biomass production (Chuck et al. 2011). Transgenic switchgrass that overexpressed a rice miR156 precursor produced no flowering lines when grown in the greenhouse, and the low and medium overexpression lines produced more biomass than the control (Fu et al. 2012).

SPL downregulation causes delayed flowering in the field

Latitudinal origin and divergence of traits such as flowering time, growth and phenotype architecture, and disease susceptibility are used to classify switchgrass into either upland or lowland ecotypes (McMillan 1965; Porter 1966; Van Esbroeck et al. 1998; Casler et al. 2004, 2007; Casler 2005; Kiniry et al. 2013; Milano et al. 2016; Grabowski et al. 2017). Lowland switchgrass typically flowers later than varieties that originated in the north due to an elongated growth period (Grabowski et al. 2017). ‘Alamo,’ a lowland ecotype of switchgrass, typically

flowers in mid-late June when grown in the southern United States (Van Esbroeck et al. 1998). This study observed nontransgenic 'Alamo' switchgrass panicle production in mid- to late-June for both growing seasons. Because the 'Alamo' nontransgenic control flowered in the same period as past studies (Van Esbroeck et al. 1998; Grabowski et al. 2017), a delayed flowering phenotype observed in transgenic lines can be contributed to miR156 overexpression rather than environmental effects. Transgenic lines T14, T35, and T37 flowered in the field. While this phenotype was different than the previous greenhouse study (Fu et al. 2012), the same was reported in a field study in Knoxville, Tenn. using the same miR156 overexpressing plants (Baxter et al. 2017). Over the course of three years, T27 was the only line that did not produce panicles (Baxter et al. 2017). *SPL3* is an important upstream activator of floral meristem identity genes such as *LEAFY*, *FRUITFULL*, and *APETALA1* (Yamaguchi et al. 2009), and the microarray revealed significant down-regulation of *SPL3* and *APETALA1* in line T27 and T37 (Table 3). The medium overexpression lines were the only transgenic lines to have significant down-regulation in *SPL3, SPL4*, and *SPL5*, which have overlapping functions to promote floral induction and transform the vegetative meristem to an inflorescence meristem (Khan et al. 2014; Xu et al. 2016). This down-regulation of important *SPL* genes explains the delayed and non-flowering phenotypes of these two transgenic lines.

We observed all transgenic lines produced shorter panicles than the control in year two, and lines T14 and T37 were also shorter in year one (Table 1). Overexpression of miR156 in rice resulted in short panicles with reduced spikelet and grain number (Xie et al. 2006). Line T37 was the only transgenic line to consistently produce fewer panicles and seeds than the control. While Xie et al. (2006) found no difference in seed fertility, all miR156 switchgrass transgenic lines had lower seed germination than the control in year two (Table 1).

SPL downregulation results in altered plant phenotype

The trend in overexpression of miR156 in field grown plants was consistent with that of previous greenhouse and field studies, as was the inverse relationship between miR156 and *SPL* gene target abundance (Figure 9 and 10; Fu et al. 2012; Baxter et al. 2017). Medium overexpression lines (T27 and T37) produced a high number of tillers which is a common occurrence in plants overexpressing miR156 (Schwab et al. 2005; Chuck et al. 2011; Fu et al. 2012; Zheng et al. 2016; Baxter et al. 2017). The high tiller number and short stature of T27 is most likely caused by a reduction in *SPL1* and *SPL2* expression (Figure 10; Table 3) which are important for side tiller initiation and internode elongation (Wu et al. 2016). T27 and T37 had smaller tiller diameters compared to the control, and the leaves were smaller in both length and width for both lines (Table 2). When *Arabidopsis thaliana* was engineered to constitutively express miR156, plants produced leaves that were like young leaves in size, shape, and trichome production (Wu et al. 2009). miR156 promotes the expression of juvenile leaf traits by repressing *SPL* genes involved in plant maturation, such as *SPL2/10* and *SPL3/4/5*, all of which were reduced in T27 and T37 (Table 3; Wu and Poethig 2006; Shikata et al. 2009; Wu et al. 2009; Khan et al. 2014; Wu et al. 2016; Xu et al. 2016). The trend in vegetative biomass, height (without panicles), and tiller number were similar in ranking for year two data between this study and Baxter et al. (2017), even though the latter study required panicle removal as a federal regulatory requirement in the field release permit. The high tiller number of line T37 without a reduction in height 'rescued' its biomass production.

Conclusions

This two-year field study of miR156 overexpressing transgenic switchgrass is the first field experiment in the eastern U.S. in which USDA-APHIS-BRS regulators allowed open-flowering. Thus, the present study was the first opportunity to closely examine the dynamics of switchgrass reproduction in the field using transgenic lines with a range of a miR156 expression. We found medium overexpression levels of miR156 such as those in line T37 resulted in delayed and reduced flowering accompanied by high biomass production. Panicle size, seed production, and seed germination were also significantly reduced compared to the control. This outcome is the result of the down-regulation of important miR156 *SPL* gene targets including *SPL2/10* and *SPL3/4/5*. If miR156 overexpression were tied to developmental or environmental cues via conditional expression, then it could further optimize the use of miR156 overexpression as a bioconfinement tool.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

The datasets used and analyzed during the current study are available from the corresponding author upon request. Microarray data will be deposited in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress).

Competing interests

Authors report no competing interests.

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Chapter 3 : Light and temperature effects on miR156 transgenic switchgrass flowering: a simulated latitudinal study

CRJ performed the experiments, analyzed the data and drafted the manuscript. RJM assisted in experimental design and data analysis. ZYW produced the transgenic plants and conceived the experiments. CNS conceived the experiments and oversaw the project. All authors participated in manuscript preparation and agree to its content.

Light and temperature effects on miR156 transgenic switchgrass flowering: a simulated latitudinal study

Chelsea R. Johnson[1], Reginald J. Millwood[1,2], Zeng-Yu Wang[2,3], C. Neal Stewart, Jr.[1,2]

[1]Department of Plant Sciences, University of Tennessee, Knoxville, Tennessee, USA

[2]BioEnergy Science Center, Oak Ridge National Laboratory, Oak Ridge, Tennessee, USA

[3]Noble Research Institute, Ardmore, Oklahoma, USA

Abstract

The control of flowering in perennial grasses is an important trait, especially biofuel feedstocks. Lignocellulosic biomass may be increased commensurate with decreased or delayed flowering as the plant allocates energy for stems and leaves harvested for bioenergy at the end of the growing season. For transgenic feedstocks, such as switchgrass (*Panicum virgatum* L.) grown in its geographic center of distribution, it is foreseeable that regulators may require greatly decreased gene flow frequencies to enable commercialization. Transgenic switchgrass with various overexpression levels of a rice (*Oryza sativa*) microRNA gene, miR156, when grown in field conditions, holds promise for decreased flowering, yielding high biomass, and altered cell wall traits, which renders it as a potential crossing partner for further breeding with switchgrass lines

for decreased recalcitrance. In the current research, we simulated various climatic conditions—from northern temperate, to sub-tropical, to tropical to assess climate and photoperiod effects of flowering and reproduction among transgenic lines with low (T-14 and T-35) to moderate (T-27 and T-37) overexpression of miR156. Elevated temperatures and decreased daylength promoted flowering of the miR156 transgenic switchgrass lines. Tropical conditions rescued the flowering phenotype in all transgenic lines except T-27. Higher numbers of plants in lines T-35 and T-37 and controls produced panicles, which also occurred earlier in the study as temperatures increased and day length decreased. Line T-14 was the exception as more plant replicates flowered in cool-temperate conditions. Increased biomass was found in transgenic lines T-35 and T-37 in tropical conditions. No difference in biomass was found in sub-tropical chambers, and two lines (T-14 and T-35) produced less biomass than the control in cool-temperate conditions. Our findings suggest that warm temperatures and short days such as those found in tropical climates may be useful for breeding of switchgrass plants genetically engineered for decreased flowering phenotypes.

Keywords: Flower timing, latitudinal cline, bioenergy, switchgrass, perennial grasses

Introduction

Switchgrass (*Panicum virgatum* L.) cultivars can be divided into either lowland or upland ecotypes based on latitudinal origin (Porter 1966; Casler et al. 2004). Lowland ecotypes tend to mature later than upland ecotypes because of a longer growing season/later flowering date (Porter 1966; Casler et al. 2004; Casler 2012; Milano et al. 2016). Switchgrass populations within ecotypes can sufficiently perceive day-length and temperature, and growing them more than one USDA hardiness zone north or south of their adaptive zone can affect their flowering, vigor, and survival (Hopkins et al. 1995; Casler 2005; Wullschleger et al. 2010; Kiniry et al. 2013). With this in mind, it stands to reason that switchgrass plants genetically engineered for delayed flowering might have altered flowering phenology depending on latitude and temperature.

Plants heavily depend on endogenous cues, photoperiod, and temperature to correctly time their change from vegetative to reproductive state (Srikanth and Schmid 2011; Penfield 2008; Franklin 2009). There are numerous examples of temperature or photoperiod effects of flowering. Balasubramanian et al. (2006) showed that a 2-4 °C increase in growing temperature was just as effective at flower induction as a change in day length for *Arabidopsis thaliana*. Flowering in *Arabidopsis* is normally inhibited in a short-day cycle, but plants flowered at approximately the same rate in short day periods at 25 or 27 °C as *Arabidopsis* plants being grown in long-day cycles at 16 °C in growth chambers (Balasubramanian et al. 2006). A review by McClung et al. (2016) surveyed temperature effects on flower initiation; the effects can be mediated or confounded by temperature stress conditions. When various plant species were examined for environmental effects of reproductive timing, Sherry et al. (2007) found that field-grown switchgrass in Oklahoma had accelerated flowering under a 4 °C increase in growing

temperature, which was further exacerbated with increased water availability. Some switchgrass cultivars flower the same time each year regardless of temperature differences, suggesting that switchgrass may be more sensitive to photoperiod than some other environmental factors (Hopkins et al. 1995; Sanderson and Wolf 1995; Van Esbroeck et al. 2003). Studies have shown a change in flower initiation due to altered photoperiods in both upland (Castro et al. 2011) and lowland (Van Esbroeck et al. 2003; Alexander et al. 2014) switchgrass cultivars.

Besides exogenous cues, there are genetic determinants of flower timing. These have been studied recently using reverse genetics experiments. Switchgrass has been genetically engineered for altered flowering phenotypes. When miR156 was overexpressed in switchgrass (cv. 'Alamo') the level of expression appeared to convey several phenotypic effects, including altering flower timing (Chuck et al. 2011; Fu et al. 2012). Depending on the transgenic event the plant biomass, architecture, as well as flowering time ranged from undiscernible from the nontransgenic parent to extreme dwarf plants that never flowered when miR156 was highly overexpressed (Fu et al. 2012).

From a bioenergy feedstock perspective, the desirable phenotype is maximal biomass production with low inputs, low-to-decreased flowering, and cell walls that are readily converted to sugars. While delayed/non-flowering phenotype would be beneficial from a transgenic-regulatory standpoint in that gene flow would be decreased (Kausch et al. 2010; Sang et al. 2013), plant breeders would likely need some sexual reproductive capacity for conventional switchgrass improvement; i.e., seed production and the establishment of commercial fields (Wolfe and Fiske 1995; McLaughlin and Kszos 2005). Fu et al. (2012) performed a greenhouse experiment mimicking summertime cool-temperate conditions: (16 hr days, 26°C average

temperature), but it is possible that a change in temperature, day length, or a combination of the two could reinstate a flowering phenotype suitable for seed production.

To test the effect of temperature and photoperiod on switchgrass flowering, switchgrass plants genetically engineered to overexpress miR156 (Fu et al. 2012), a regulatory microRNA that is involved in the flower induction pathway, were grown in growth chambers that simulated the daylength, temperature, and season length of climates outside the adaptation zone of 'Alamo' switchgrass. The settings were based on the average weekly climate conditions of representative areas that included tropical (Guayaquil, Ecuador), sub-tropical (Laredo, Texas, USA), and cool-temperate (Brattleboro, Vermont, USA) along with their day lengths for each week of the year (Table 4). The high temperature and constant 12 hr (short, for switchgrass) day-length of the tropical growing conditions resulted in panicle production in the control and all but one of the transgenic lines. It is possible that switchgrass plants with delayed or non-flowering phenotypes could be grown in tropical climate conditions for seed production based on flower initiation in tropical growth chamber conditions.

Materials and methods

Plants, experimental design and growth conditions

miR156 low overexpression lines T-14 and T-35, medium overexpression lines T-27 and T-37, and one nontransgenic line from Fu et al. (2012) were used for each of the growth chamber experiments. All lines originated from the lowland switchgrass cultivar 'Alamo,' and transgenic lines have been characterized and described previously in the greenhouse (Fu et al. 2012) and a Knoxville, Tenn. USA field (Baxter et al. 2017). Plants were grown in Percival PCG-15 growth

chambers (Percival Scientific, Perry, IA) with temperature and photoperiod settings that corresponded to tropical, sub-tropical, or cool-temperate growing conditions from published day length and temperature highs and lows for each day of their respective growing seasons (Table 4 and 5). Typically, the lowland switchgrass growing season begins with vegetative flushes, which occur when weekly average temperatures are above 15/10˚C for day/night and ends when weekly minimum temperatures average below 15˚C (Sanderson and Wolf 1995; Gu et al. 2015). All experiments were started on the same day. Plants were culled to three tillers per pot, cut back to 20.32 cm, and grown in 12 L pots. Each growing condition was replicated in two growth chambers, and four clones of each line were randomly placed in each chamber (5 lines x 4 clones = 20 plants/chamber; Figure 11A). The pot was the experimental unit. Pot locations within the chamber were randomized again at mid-season to avoid any positional growing effects (Figure 11b). Plants were watered one to three times per week and fertilized with Peters 20-20-20 fertilizer (J.R. Peters Inc., Allentown, Penn. USAA) once every two weeks.

Plant characterization

The date for first flower emergence of each plant was recorded, and panicles were counted and removed throughout the growing season. Plant height was measured from the level of potting mix to the tallest point of the plant. The two tallest tillers were used to measure leaf length and width, node number, and internode diameter. The flag leaf or top-most mature leaf was used for length and width measurements. Internode diameter was measured using a Maxwell 150 mm digital caliper between the third and fourth nodes from the potting-mix level. All but 10 cm of aboveground biomass was harvested at the end of the experiment. The biomass by pot was

placed in a drying oven at 43°C for 300 hr prior to taking dry weight data. Tillers were tallied at harvest.

Statistical analysis

Statistical analysis was performed using SAS version 9.4 (SAS Institute Inc., Cary, NC). A one-way ANOVA with Fisher's least significant difference was used to compare means among lines within each treatment if the *P*-value was less than 0.05 for the ANOVA.

Results and discussion

Flowering phenotype

The nontransgenic control and low miR156 overexpression line T-35 were the only lines to flower under all three growing conditions. The medium overexpression line T-27 did not produce any panicles under any of the growth chamber conditions tested, as previously observed in greenhouse experiments (Fu et al. 2012) and during a three-year field experiment in Knoxville, Tenn. USA (Baxter et al. 2017). These findings suggest that the nonflowering phenotype of line T-27 is conferred by the relatively high miR156 overexpression and not growth conditions; i.e., genetics is more important than environment at a critical expression level (Baxter et al. 2017). The tropical experiment was the only one in which all lines, excluding T-27, produced panicles; line T-14 produced so few panicles that the average panicle number per plant was statistically zero (Table 6). Switchgrass flowering time appears to be more dependent on photoperiod than temperature (Sanderson and Wolf 1995), therefore the constant 12-hr day length in the tropical growth chambers may be responsible for more lines flowering compared to the sub-tropical and cool-temperate growth chambers, which had longer days during the growth season. The earliest

flowering time was observed in sub-tropical-condition growth chambers, which is most similar to where ‘Alamo’ would be cultivated; all flowering lines produced panicles by week five (Figure 12B). The high ambient temperature of the sub-tropical conditions most likely promoted flowering, especially during the short photoperiods in the beginning of the season (Table 5; Li et al. 2015). Short-day plants such as switchgrass have shown accelerated flowering when treated with warmer temperatures (Hartman and Nippert 2013; Cleland et al. 2006; Van Esbroeck et al. 2003; Sherry et al. 2007; Alexander et al. 2014).

The control was the only line to have all plants transition to the reproductive stage in sub-tropical conditions, and all replicates of the control and line T-35 began flowering by week 15 in the tropical chambers (Figure 12A and B). The control was also the only line in which all replicates flowered in the cool-temperate experiment (Figure 12C).

Although some data cannot be directly compared among experiments because of differences in season length, it is interesting to note the average number of panicles produced was higher in the tropical and sub-tropical experiments (short days) than the cool-temperate (long days) for all flowering lines except T-14 (Table 6). In general, switchgrass is thought to be a facultative short-day plant (Porter 1966; Van Esbroeck et al 2003; Alexander et al. 2014), but there is evidence suggesting upland cultivars may have a long-day flowering response (Casler 2012; Castro et al. 2011). The increase in panicle number, combined with other phenotypic traits of line T-14 grown under cool-temperate conditions, suggests that T-14 may behave more like an upland switchgrass ecotype.

Biomass and phenotypes

In general, the latitudinal cline simulation illuminated the integrated day length-seasonality-temperature effects of the transgene expressed in switchgrass. Biomass production followed tropical>sub-tropical>temperate, which corresponded to tillering and height (Table 6, Figure 13 and 14). When grown under tropical temperature and day length settings, transgenic lines T-35 and T-37 produced two- and three-fold more biomass than the control, respectively (Figure 13). The high biomass yield was most likely because of the increased tiller number of both T-35 and T-37 (Table 6), which could have been driven by high temperature (Hartman and Nippert 2013; Kandel et al. 2013). Increased tillering also appears to be a pleiotropic effect from overexpressing miR156 in switchgrass (Fu et al. 2012; Chuck et al. 2011; Baxter et al 2017) and red clover (Zheng et al. 2016) as well as other species (reviewed in Trumbo et al. 2014). No differences were observed in biomass production in the sub-tropical experiment compared to control plants (Figure 13). None of the four transgenic lines produced significantly more biomass than the control under cool-temperate conditions, but both low miR156 overexpression lines (T-14 and T-35) produced significantly less biomass (Figure 13).

While none of the transgenic lines was taller than controls, lines T-14 and T-27 were of significantly shorter in all growth conditions (Table 6). T-27 is commonly shorter than control lines in both sub-tropical and cool-temperate conditions as was observed in both greenhouse and field conditions (Baxter et al. 2017; Fu et al. 2012). Line T-35 was shorter in cool-temperate settings (Table 6). Leaf length differed significantly only in the cool-temperate experiment with all transgenic leaves being shorter than the control (Table 6), and this was the first time a difference in leaf length was reported for the transgenic lines (Fu et al. 2012). For leaf width, differences were found only under sub-tropical and cool-temperate conditions. Lines T-27 and T-

37 leaves had smaller widths than the control in both conditions, and T-14 leaf widths were smaller than the control only in the cool-temperate experiment (Table 6). These results suggest that perhaps the short day-length of the tropical conditions resulted in wide leaf production as Fu et al. (2012) also reported a decrease in leaf width for medium overexpression lines. Node number did not differ between lines when grown in cool-temperate settings, but T-37 and T-27 had significantly more nodes than the control in tropical and sub-tropical conditions, respectively (Table 6). The internode diameter of line T-35 did not differ from the control in any of the experimental settings, but medium overexpression lines T-27 and T-37 had tillers with decreased diameter than the control in all conditions. Line T-14 internode diameter was smaller than the control only in the tropical experiment (Table 6).

Conclusion

These experiments show that photoperiod and temperature can be used to alter the expected phenotype of switchgrass that has been genetically modified to flower late or not at all. If switchgrass plants overexpressing miR156 were grown in an area with short days and high temperatures, such as tropical Ecuador, flowering can occur and seed set may be possible.

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Chapter 4 : Conclusions

Switchgrass (*Panicum virgatum* L.) is an important biofuel feedstock candidate. The perennial nature of the plant, high biomass yield on marginal land not suitable for conventional crops, and low input need are all advantages of using switchgrass as a renewable energy source. However, its highly lignified cell walls decrease its biofuel conversion efficiency. Genetic modification techniques have been successful to alter the cell wall lignin content and composition, but the environmental release of transgenic switchgrass poses regulatory issues, especially if the transgenic switchgrass is grown in the eastern United States, where it is native. Gene flow from transgenic switchgrass to neighboring wild or nontransgenic switchgrass could cause negative ecological impacts. Gene flow of transgenic switchgrass must be addressed before commercial production can occur.

Transgene bioconfinement could allow for the mitigation of pollen mediated gene flow without the loss of desired traits in switchgrass. One such method would be to delay or inhibit flowering. This technique would be useful because it could simultaneously boost biomass production while reducing or eliminating pollen and seed production. The overexpression of microRNA-156 (miR156), which is a miRNA involved in the plant transition from a vegetative to reproductive phase, in switchgrass has been shown to delay flowering while producing sufficient amounts of biomass when grown in the greenhouse (Fu et al. 2012). Because environmental effects also play a key role in the induction of flowers in plants, plants engineered for delayed flowering were grown across multiple latitudinal climates through a field study and a simulated growth chamber studies. Studied were switchgrass lines overexpressing miR156 at low (T14 and T35) and moderate (T27 and T37) levels under the control of the maize (*Zea mays*) ubiquitin promotor (Ubi1), as well as an ‘Alamo’ nontransgenic control.

Medium overexpression of miR156 at levels such as those found in line T37 resulted in delayed flowering; reduced panicle, flower, and seed number per plant; and equal amounts of biomass compared to the control when grown in a field site in the normal climate of switchgrass production areas. It appears that environmental factors played an influential role in the differences found between our field study and the results of the greenhouse study conducted by Fu et al. (2012). Environmental factors could be used as cues for bioconfinement however, if they were connected to the engineered miR156 overexpression. For example, if an inducible promotor were used rather than the maize Ubi1 constitutive promotor, it may be possible to delay flowering further into the plant's lifecycle without affecting other traits such as biomass.

The purpose of the growth chamber studies was to simulate tropical, sub-tropical, and cool-temperate climate conditions via changing temperature and photoperiod settings throughout the experiment and observe the effects on flowering. The results suggest that warm temperatures and short days such as those found in tropical climates promoted flowering of the transgenic lines more than the other two climate conditions. While both low overexpression lines (T14 and T35) flowered in all three climate simulations, tropical growth chambers were the only ones in which the moderate miR156 overexpressing line T37 flowered. These results suggest that tropical climate areas may be useful for breeding of switchgrass plants genetically engineered for decreased flowering phenotypes. This study provides preliminary data needed to design large scale studies to fully characterize the effects of short day, high temperature conditions for miR156 switchgrass breeding purposes.

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Appendices

Appendix 1: Tables

Table 1. Flowering and reproduction of miR156-overexpressing switchgrass and the nontransgenic control in the field. Lines T14 and T35 have low overexpression of miR156 whereas lines T27 and T37 have moderate levels of overexpression of the transgene.

Year	Line	Panicle number per plant	Panicle length (cm)	Spikelets per panicle	Percent seed germination
2015	C	29.0 ± 1.6^{a}	54.33 ± 1.69^{a}	N/a	4.75 ± 3.47^{b}
	T14	22.2 ± 1.1^{b}	49.80 ± 1.29^{b}	N/a	5.50 ± 1.89^{b}
	T35	22.6 ± 1.7^{b}	51.55 ± 1.67^{ab}	N/a	22.75 ± 3.97^{a}
	T27	0.0 ± 0.0^{d}	N/a	N/a	N/a
	T37	9.9 ± 1.7^{c}	26.77 ± 2.07^{c}	N/a	0.25 ± 0.25^{b}
2016	C	103.5 ± 4.0^{a}	73.34 ± 0.66^{a}	27.5 ± 0.4^{a}	34.75 ± 6.30^{a}
	T14	60.6 ± 3.0^{c}	61.46 ± 0.91^{c}	24.6 ± 0.7^{c}	15.25 ± 1.93^{b}
	T35	98.8 ± 4.7^{a}	68.01 ± 0.78^{b}	25.8 ± 0.5^{bc}	19.25 ± 3.33^{b}
	T27	0.0 ± 0.0^{d}	N/a	N/a	N/a
	T37	78.9 ± 7.5^{b}	40.78 ± 1.26^{d}	26.4 ± 1.0^{ab}	18.25 ± 2.06^{b}

Values represent averages ± standard error. Letters indicate significant differences ($P < 0.05$) within year and trait using Fisher's LSD. Data sets were not compared between years. N/a, not applicable since there were no flowers produced.

Table 2. Year one (2015) and year two (2016) end-of-season vegetative morphological data and cell wall characterization of miR156-overexpressing switchgrass and the wild-type control in the field. CWR, cell wall residue; S/G, syringyl/guaiacyl.

Year	Line	Tiller number	Leaf length (cm)	Leaf width (cm)	Node number	Internode diameter (mm)	Lignin (% CWR)	S/G ratio	Sugar release (g/g CWR)
2015	C	47.5 ± 3.7^{c}	52.5 ± 1.1^{a}	1.47 ± 0.02^{a}	5.4 ± 0.1^{b}	4.73 ± 0.08^{a}	20.4 ± 0.4^{b}	0.66 ± 0.01^{b}	0.47 ± 0.00^{ab}
	T14	30.9 ± 2.5^{c}	36.6 ± 0.6^{c}	1.15 ± 0.02^{c}	4.9 ± 0.1^{c}	4.25 ± 0.09^{b}	21.3 ± 0.2^{a}	0.69 ± 0.01^{a}	0.44 ± 0.01^{b}
	T35	41.2 ± 3.6^{c}	48.2 ± 1.3^{b}	1.34 ± 0.02^{b}	5.1 ± 0.1^{bc}	4.95 ± 0.11^{a}	21.0 ± 0.1^{ab}	0.69 ± 0.01^{a}	0.49 ± 0.01^{a}
	T27	193.2 ± 11.7^{a}	15.3 ± 0.5^{e}	0.34 ± 0.01^{e}	5.5 ± 0.2^{b}	1.27 ± 0.03^{d}	20.5 ± 0.3^{b}	0.59 ± 0.01^{c}	0.44 ± 0.01^{b}
	T37	106.0 ± 5.5^{b}	27.3 ± 0.9^{d}	0.73 ± 0.02^{d}	7.8 ± 0.2^{a}	3.04 ± 0.06^{c}	20.5 ± 0.2^{b}	0.59 ± 0.01^{c}	0.49 ± 0.03^{a}
2016	C	112.2 ± 5.0^{b}	52.6 ± 1.1^{a}	1.17 ± 0.02^{b}	8.0 ± 0.2^{c}	5.36 ± 0.07^{b}	23.2 ± 0.1^{b}	0.66 ± 0.01^{ab}	N/a
	T14	66.1 ± 3.7^{c}	31.9 ± 1.1^{b}	0.86 ± 0.03^{c}	7.0 ± 0.2^{d}	4.18 ± 0.13^{c}	25.0 ± 0.2^{a}	0.70 ± 0.02^{a}	N/a
	T35	108.6 ± 6.8^{b}	48.5 ± 0.8^{a}	1.26 ± 0.03^{a}	7.7 ± 0.1^{cd}	5.79 ± 0.07^{a}	23.1 ± 0.1^{b}	0.64 ± 0.03^{b}	N/a
	T27	172.7 ± 16.0^{a}	9.6 ± 0.4^{c}	0.22 ± 0.01^{e}	8.8 ± 0.3^{b}	0.99 ± 0.03^{e}	21.4 ± 0.4^{c}	0.57 ± 0.00^{c}	N/a
	T37	182.2 ± 6.6^{a}	29.8 ± 1.1^{b}	0.58 ± 0.02^{d}	11.1 ± 0.2^{a}	3.37 ± 0.06^{d}	22.8 ± 0.2^{b}	0.58 ± 0.01^{c}	N/a

Values represent averages ± standard error. Letters indicate significant differences ($P < 0.05$) within year and trait using Fisher's LSD. Data sets were not compared between years.

Table 3. Summary of transcriptomic analysis of transgenic lines using gene-specific Affymetrix microarray genechips. Numbers represent the ratio of transgenic line expression divided by the control line expression for a single gene probe. SPL, Squamosa Promotor Binding Protein-Like; FT, Flowering Locus T; FTL, Flowering Locus T-Like; FPF, Flowering Promoting Factor; FPFL, Flowering Promoting Factor-Like; PFT, Phytochrome and Flowering Time regulatory protein; AP, Apetala; MADS, MADS-box genes; AGL, Agamous-Like; SEP, Sepallata; LEA, Late Embryogenesis Abundant; SAG, Senescence-Associated Genes; PI, Pistillata; AG, Agamous; STK, Seedstick; SVP, Short Vegetative Phase; DIA, AGL61/Diana; ERD, Early Responsive to Dehydration; SOC, Suppressor of Overexpression of Constans; COL, Constans-Like.

Probe set	Annotation	Short name	T-14/Ctrl	T-35/Ctrl	T-27/Ctrl	T-37/Ctrl
Pavir.2NG503700.1_at	Best-hit-arabi-name=AT3G15270.1 / Best-hit-rice-name=LOC_Os07g32170.1	AtSPL5 / OsSPL13	0.36	-	0.24	-
Pavir.2NG503500.1_at	Best-hit-arabi-name=AT1G53160.1 / Best-hit-rice-name=LOC_Os07g32170.1	AtSPL4 / OsSPL13	0.41	-	0.24	0.43
Pavir.2KG430000.1_at	Best-hit-arabi-name=AT2G33810.1 / Best-hit-rice-name=LOC_Os07g32170.1	AtSPL3 / OsSPL13	-	-	0.21	0.48
Pavir.2KG430400.1_at	Best-hit-arabi-name=AT3G15270.1 / Best-hit-rice-name=LOC_Os07g32170.1	AtSPL5 / OsSPL13	-	-	0.21	0.33
Pavir.1NG028400.1_x_at	Best-hit-arabi-name=AT1G27370.3 / Best-hit-rice-name=LOC_Os02g04680.2	AtSPL10 / OsSPL3	-	-	0.31	0.37
Pavir.1NG028400.2_x_at	Best-hit-arabi-name=AT1G27370.3 / Best-hit-rice-name=LOC_Os02g04680.2	AtSPL10 / OsSPL3	-	-	0.31	0.37
Pavir.1KG076500.1_at	Best-hit-arabi-name=AT5G43270.3 / Best-hit-rice-name=LOC_Os06g45310.1	AtSPL2 / OsSPL11	-	-	0.38	-
Pavir.1KG043600.1_at	Best-hit-arabi-name=AT1G27370.3 / Best-hit-rice-name=LOC_Os02g04680.2	AtSPL10 / OsSPL3	-	-	0.29	0.41
Pavir.5NG100600.1_at	Best-hit-arabi-name=AT1G65480.1 / Best-hit-rice-name=LOC_Os01g11940.1	AtFT / OsFTL1	0.45	-	0.14	-
Pavir.5NG198400.1_x_at	Best-hit-arabi-name=AT1G65480.1 / Best-hit-rice-name=LOC_Os05g44180.1	AtFT / OsFTL10	0.48	-	0.41	2.43

Table 3. Continued.

Probe set	Annotation	Short name	T-14/Ctrl	T-35/Ctrl	T-27/Ctrl	T-37/Ctrl
Pavir.3KG344200.1_x_at	Best-hit-arabi-name=AT1G65480.1 / Best-hit-rice-name=LOC_Os05g44180.1	AtFT / OsFTL10	-	-	-	4.70
Pavir.3KG349500.1_x_at	Best-hit-arabi-name=AT1G65480.1 / Best-hit-rice-name=LOC_Os05g44180.1	AtFT / OsFTL10	-	-	-	4.70
Pavir.5KG284600.1_s_at	Best-hit-arabi-name=AT1G65480.1 / Best-hit-rice-name=LOC_Os06g35940.1	AtFT / OsFTL12	-	-	0.44	-
Pavir.4KG264600.1_at	Best-hit-arabi-name=AT1G65480.1 / Best-hit-rice-name=LOC_Os06g35940.1	AtFT / OsFTL12	-	-	0.43	-
Pavir.5KG751900.1_at	Best-hit-arabi-name=AT5G24860.1 / Best-hit-rice-name=LOC_Os01g70730.1	AtFPF1 / OsFPFL1	-	0.50	-	-
Pavir.4KG047800.1_at	Best-hit-arabi-name=AT1G65480.1 / Best-hit-rice-name=LOC_Os06g06320.1	AtFT / OsFTL2	-	-	0.20	-
Pavir.J024900.1_at	Best-hit-arabi-name=AT5G10625.1 / Best-hit-rice-name=LOC_Os02g26210.1	AtFPF1 / OsFPFL1	-	-	0.46	-
Pavir.5KG166700.1_at	Best-hit-arabi-name=AT5G24860.1 / Best-hit-rice-name=LOC_Os01g15340.1	AtFPF1 / OsFPFL1	-	-	2.01	-
Pavir.5KG029000.1_at	Best-hit-arabi-name=AT1G65480.1 / Best-hit-rice-name=LOC_Os01g11940.1	AtFT / OsFTL1	-	-	0.36	-
Pavir.2KG594700.1_x_at	Best-hit-arabi-name=AT5G24860.1 / Best-hit-rice-name=LOC_Os07g47450.1	AtFPF1 / OsFPFL1	-	-	-	2.78
Pavir.2NG627900.1_at	Best-hit-arabi-name=AT5G24860.1 / Best-hit-rice-name=LOC_Os07g47450.1	AtFPF1 / OsFPFL1	-	2.52	-	3.60
Pavir.J167400.1_s_at	Best-hit-arabi-name=AT1G25540.2 / Best-hit-rice-name=LOC_Os09g13610.1	AtPFT1 / OsPFT1	2.15	-	-	-

Table 3. Continued.

Probe set	Annotation	Short name	T-14/Ctrl	T-35/Ctrl	T-27/Ctrl	T-37/Ctrl
Pavir.J167400.2_s_at	Best-hit-arabi-name=AT1G25540.2 / Best-hit-rice-name=LOC_Os09g13610.1	AtPFT1 / OsPFT1	2.15	-	-	-
Pavir.4NG331800.1_at	Best-hit-arabi-name=AT3G54340.1 / Best-hit-rice-name=LOC_Os06g49840.1	AtAP3 / OsMADS16	0.11	-	0.01	-
Pavir.6KG379800.1_x_at	Best-hit-arabi-name=AT1G24260.1 / Best-hit-rice-name=LOC_Os08g41950.2	AtAGL9, AtSEP3 / OsMADS7	0.14		0.06	
Pavir.6NG327400.1_at	Best-hit-arabi-name=AT1G24260.1 / Best-hit-rice-name=LOC_Os08g41950.2	AtAGL9, AtSEP3 / OsMADS7	0.19	-	0.04	-
Pavir.1KG449500.1_at	Best-hit-arabi-name=AT2G45650.1 / Best-hit-rice-name=LOC_Os02g45770.1	AtAGL6 / OsMADS6	0.19	-	0.05	0.43
Pavir.2NG422200.1_at	Best-hit-arabi-name=AT1G24260.1 / Best-hit-rice-name=LOC_Os09g32948.1	AtAGL9, AtSEP3 / OsMADS8	0.20	-	0.03	-
Pavir.2NG419400.2_s_at	Best-hit-arabi-name=AT1G24260.2 / Best-hit-rice-name=LOC_Os09g32948.1	AtAGL9, AtSEP3 / OsMADS8	0.22	-	0.02	-
Pavir.2NG419400.3_s_at	Best-hit-arabi-name=AT1G24260.2 / Best-hit-rice-name=LOC_Os09g32948.1	AtAGL9, AtSEP3 / OsMADS8	0.22	-	0.02	-
Pavir.5NG221200.1_at	Best-hit-arabi-name=AT4G02380.1 / Best-hit-rice-name=LOC_Os01g21250.1	AtLEA5, AtSAG21	0.23	0.26	-	-
Pavir.1NG424900.2_s_at	Best-hit-arabi-name=AT2G45650.1 / Best-hit-rice-name=LOC_Os02g45770.1	AtAGL6 / OsMADS6	0.24	-	0.10	-
Pavir.1NG424900.1_x_at	Best-hit-arabi-name=AT2G45650.1 / Best-hit-rice-name=LOC_Os02g45770.1	AtAGL6 / OsMADS6	0.27	-	0.12	-
Pavir.1NG424900.3_x_at	Best-hit-arabi-name=AT2G45650.1 / Best-hit-rice-name=LOC_Os02g45770.1	AtAGL6 / OsMADS6	0.27	-	0.12	-

Table 3. Continued.

Probe set	Annotation	Short name	T-14/Ctrl	T-35/Ctrl	T-27/Ctrl	T-37/Ctrl
Pavir.J149100.1_s_at	Best-hit-arabi-name=AT5G20240.1 / Best-hit-rice-name=LOC_Os05g34940.2	AtPI / OsMADS4	0.27	-	0.10	-
Pavir.9NG641900.1_at	Best-hit-arabi-name=AT3G02310.1 / Best-hit-rice-name=LOC_Os03g11614.1	AtAGL4, AtSEP2 / OsMADS1	0.28	-	0.20	-
Pavir.J149100.2_x_at	Best-hit-arabi-name=AT5G20240.1 / Best-hit-rice-name=LOC_Os05g34940.3	AtPI / OsMADS4	0.28	-	0.10	-
Pavir.J149100.3_x_at	Best-hit-arabi-name=AT5G20240.1 / Best-hit-rice-name=LOC_Os05g34940.2	AtPI / OsMADS4	0.28	-	0.10	-
Pavir.5NG045000.1_s_at	Best-hit-arabi-name=AT4G18960.1 / Best-hit-rice-name=LOC_Os01g10504.3	AtAG / OsMADS3	-	-	0.03	-
Pavir.5NG045000.2_s_at	Best-hit-arabi-name=AT4G18960.1 / Best-hit-rice-name=LOC_Os01g10504.2	AtAG / OsMADS3	-	-	0.03	-
Pavir.5NG045000.3_x_at	Best-hit-arabi-name=AT4G18960.1 / Best-hit-rice-name=LOC_Os01g10504.1	AtAG / OsMADS3	-	-	0.04	-
Pavir.5NG045000.4_x_at	Best-hit-arabi-name=AT4G18960.1 / Best-hit-rice-name=LOC_Os01g10504.1	AtAG / OsMADS3	-	-	0.04	-
Pavir.5NG045000.5_x_at	Best-hit-arabi-name=AT4G18960.1 / Best-hit-rice-name=LOC_Os01g10504.1	AtAG / OsMADS3	-	-	0.04	-
Pavir.5KG667500.1_s_at	Best-hit-arabi-name=AT5G20240.1 / Best-hit-rice-name=LOC_Os01g66030.1	AtPI / OsMADS2	-	-	0.09	-
Pavir.5KG676500.1_s_at	Best-hit-arabi-name=AT5G20240.1 / Best-hit-rice-name=LOC_Os01g66030.1	AtPI / OsMADS2	-	-	0.09	-
Pavir.4NG059400.1_s_at	Best-hit-arabi-name=AT1G24260.2 / Best-hit-rice-name=LOC_Os06g06750.1	AtAGL9, AtSEP3 / OsMADS5	0.36	-	0.17	0.44

Table 3. Continued.

Probe set	Annotation	Short name	T-14/Ctrl	T-35/Ctrl	T-27/Ctrl	T-37/Ctrl
Pavir.4NG059400.2_s_at	Best-hit-arabi-name=AT1G24260.1 / Best-hit-rice-name=LOC_Os06g06750.1	AtAGL9, AtSEP3 / OsMADS5	0.36	-	0.17	0.44
Pavir.9NG415300.1_at	Best-hit-arabi-name=AT3G54340.1 / Best-hit-rice-name=LOC_Os06g49840.2	AtAP3 / OsMADS16	0.37	-	0.04	-
Pavir.9KG622200.1_at	Best-hit-arabi-name=AT3G02310.1 / Best-hit-rice-name=LOC_Os03g11614.1	AtAGL4, AtSEP2 / OsMADS1	0.43	-	0.45	-
Pavir.4KG066600.1_s_at	Best-hit-arabi-name=AT1G24260.1 / Best-hit-rice-name=LOC_Os06g06750.1	AtAGL9, AtSEP3 / OsMADS5	0.43	-	0.17	0.38
Pavir.8KG297500.1_at	Best-hit-arabi-name=AT1G71190.1 / Best-hit-rice-name=LOC_Os01g73120.1	AtSAG18	0.44	0.13	0.22	0.35
Pavir.5NG475300.1_x_at	Best-hit-arabi-name=AT4G09960.2 / Best-hit-rice-name=LOC_Os01g52680.1	AtAGL11, AtSTK / OsMADS32	0.45	-	0.46	-
Pavir.3KG063200.1_at	Best-hit-arabi-name=AT4G18960.1 / Best-hit-rice-name=LOC_Os12g10540.1	AtAG / OsMADS13	-	-	0.32	-
Pavir.J610400.1_x_at	Best-hit-arabi-name=AT4G18960.1 / Best-hit-rice-name=LOC_Os05g11414.1	AtAG / OsMADS58	-	-	0.35	-
Pavir.3KG091900.1_x_at	Best-hit-arabi-name=AT4G18960.1 / Best-hit-rice-name=LOC_Os05g11414.1	AtAG / OsMADS58	-	-	0.42	-
Pavir.3KG091900.2_x_at	Best-hit-arabi-name=AT4G18960.1 / Best-hit-rice-name=LOC_Os05g11414.1	AtAG / OsMADS58	-	-	0.42	-
Pavir.3KG523200.1_s_at	Best-hit-arabi-name=AT3G02310.1 / Best-hit-rice-name=LOC_Os03g54170.1	AtAGL4, AtSEP2 / OsMADS34	-	-	0.34	0.43
Pavir.3KG523200.2_s_at	Best-hit-arabi-name=AT3G02310.1 / Best-hit-rice-name=LOC_Os03g54170.1	AtAGL4,SEP2 / OsMADS34	-	-	0.34	0.43

Table 3. Continued.

Probe set	Annotation	Short name	T-14/Ctrl	T-35/Ctrl	T-27/Ctrl	T-37/Ctrl
Pavir.5KG517700.1_x_at	Best-hit-arabi-name=AT4G09960.2 / Best-hit-rice-name=LOC_Os01g52680.1	AtAGL11, AtSTK / OsMADS32	-	2.54	-	-
Pavir.5KG517700.2_x_at	Best-hit-arabi-name=AT4G09960.2 / Best-hit-rice-name=LOC_Os01g52680.1	AtAGL11, AtSTK / OsMADS32	-	2.54	-	-
Pavir.5KG518600.1_s_at	Best-hit-rice-name=LOC_Os01g52680.1	OsMADS32	-	2.76	-	-
Pavir.2KG220600.2_at	Best-hit-arabi-name=AT5G45890.1 / Best-hit-rice-name=LOC_Os09g38920.1	AtSAG12	-	-	2.23	-
Pavir.5KG736600.3_at	Best-hit-arabi-name=AT4G11880.1 / Best-hit-rice-name=LOC_Os01g69850.1	AtAGL14 / OsMADS65	-	2.51	-	-
Pavir.4NG131700.1_at	Best-hit-arabi-name=AT2G22540.1 / Best-hit-rice-name=LOC_Os02g52340.1	AtAGL22, AtSVP / OsMADS22	-	-	0.39	-
Pavir.5KG518600.2_x_at	Best-hit-rice-name=LOC_Os01g52680.1	OsMADS32	-	3.35	-	-
Pavir.1NG073400.1_at	Best-hit-arabi-name=AT5G24930.1 / Best-hit-rice-name=LOC_Os02g08150.1	AtCOL4	-	-	0.18	-
Pavir.5KG325100.1_at	Best-hit-arabi-name=AT4G02380.1 / Best-hit-rice-name=LOC_Os05g29930.1	AtLEA5, AtSAG21	-	5.05	-	3.17
Pavir.2KG531900.1_at	Best-hit-arabi-name=AT1G69120.1 / Best-hit-rice-name=LOC_Os07g41370.1	AtAGL7, AtAP1 / OsMADS18	-	0.43	-	-
Pavir.3KG523400.1_x_at	Best-hit-arabi-name=AT1G69120.1 / Best-hit-rice-name=LOC_Os03g54160.2	AtAGL7, AtAP1 / OsMADS14	-	-	0.21	-
Pavir.3KG523400.2_x_at	Best-hit-arabi-name=AT1G69120.1 / Best-hit-rice-name=LOC_Os03g54160.2	AtAGL7, AtAP1 / OsMADS14	-	-	0.21	-

Table 3. Continued.

Probe set	Annotation	Short name	T-14/Ctrl	T-35/Ctrl	T-27/Ctrl	T-37/Ctrl
Pavir.J371200.1_x_at	Best-hit-arabi-name=AT1G69120.1 / Best-hit-rice-name=LOC_Os03g54160.2	AtAGL7, AtAP1 / OsMADS14	-	-	0.24	-
Pavir.9NG097300.1_x_at	Best-hit-arabi-name=AT1G69120.1 / Best-hit-rice-name=LOC_Os03g54160.2	AtAGL7, AtAP1 / OsMADS14	-	-	0.17	-
Pavir.9NG097300.2_x_at	Best-hit-arabi-name=AT1G69120.1 / Best-hit-rice-name=LOC_Os03g54160.2	AtAGL7, AtAP1 / OsMADS14	-	-	0.17	-
Pavir.2KG001200.1_s_at	Best-hit-arabi-name=AT1G69120.1 / Best-hit-rice-name=LOC_Os07g01820.3	AtAGL7, AtAP1 / OsMADS15	-	-	0.13	-
Pavir.2KG001200.2_s_at	Best-hit-arabi-name=AT1G69120.1 / Best-hit-rice-name=LOC_Os07g01820.3	AtAGL7, AtAP1 / OsMADS15	-	-	0.13	-
Pavir.2NG003000.1_at	Best-hit-arabi-name=AT1G69120.1 / Best-hit-rice-name=LOC_Os07g01820.3	AtAGL7, AtAP1 / OsMADS15	-	-	0.06	-
Pavir.1NG490300.1_x_at	Best-hit-arabi-name=AT2G22540.1 / Best-hit-rice-name=LOC_Os02g52340.1	AtAGL22, AtSVP / OsMADS22	-	-	2.21	-
Pavir.1NG470600.1_s_at	Best-hit-arabi-name=AT2G22540.1 / Best-hit-rice-name=LOC_Os02g52340.1	AtAGL22, AtSVP / OsMADS22	-	-	2.53	-
Pavir.1NG121800.1_x_at	Best-hit-arabi-name=AT5G45890.1 / Best-hit-rice-name=LOC_Os04g01710.1	AtSAG12	-	-	-	7.91
Pavir.4NG227400.1_x_at	Best-hit-arabi-name=AT2G24840.1 / Best-hit-rice-name=LOC_Os06g30810.1	AtAGL61, AtDIA / OsMADS75	-	2.59	2.81	-
Pavir.J027700.1_x_at	Best-hit-arabi-name=AT5G45890.1 / Best-hit-rice-name=LOC_Os09g21370.1	AtSAG12	-	2.04	-	7.11

Table 3. Continued.

Probe set	Annotation	Short name	T-14/Ctrl	T-35/Ctrl	T-27/Ctrl	T-37/Ctrl
Pavir.9NG775900.1_at	Best-hit-arabi-name=AT2G45660.1 / Best-hit-rice-name=LOC_Os10g39130.1	AtAGL20, AtSOC1 / OsMADS56	-	-	2.27	-
Pavir.9KG649500.1_s_at	Best-hit-arabi-name=AT2G22540.1 / Best-hit-rice-name=LOC_Os03g08754.1	AtAGL22, AtSVP / OsMADS47	-	-	4.12	-
Pavir.4NG227400.3_x_at	Best-hit-arabi-name=AT2G24840.1 / Best-hit-rice-name=LOC_Os06g30810.1	AtAGL61, AtDIA / OsMADS75	-	3.31	3.35	2.06
Pavir.1KG377300.1_at	Best-hit-arabi-name=AT5G57660.1 / Best-hit-rice-name=LOC_Os02g39710.1	AtCOL5	-	-	2.05	-
Pavir.1NG122000.1_x_at	Best-hit-arabi-name=AT5G45890.1 / Best-hit-rice-name=LOC_Os09g21370.1	AtSAG12	-	-	-	8.04
Pavir.4NG172100.4_x_at	Best-hit-rice-name=LOC_Os12g10540.4	OsMADS13	2.10	-	2.33	-
Pavir.1NG002100.1_s_at	Best-hit-arabi-name=AT2G45660.1 / Best-hit-rice-name=LOC_Os02g01355.1	AtAGL20, AtSOC1	2.17	-	-	-
Pavir.1NG002100.2_s_at	Best-hit-arabi-name=AT2G45660.1 / Best-hit-rice-name=LOC_Os02g01355.1	AtAGL20, AtSOC1	2.17	-	-	-
Pavir.3KG523600.1_at	Best-hit-arabi-name=AT5G45890.1 / Best-hit-rice-name=LOC_Os03g54130.1	AtSAG12	-	3.90	-	-
Pavir.4NG227400.2_x_at	Best-hit-arabi-name=AT2G24840.1 / Best-hit-rice-name=LOC_Os06g30810.1	AtAGL61, AtDIA / OsMADS75	2.17	3.04	2.95	2.04
Pavir.4NG227400.4_x_at	Best-hit-arabi-name=AT2G24840.1 / Best-hit-rice-name=LOC_Os06g30810.1	AtAGL61, AtDIA / OsMADS75	2.17	3.04	2.95	2.04
Pavir.4NG327000.1_at	Best-hit-arabi-name=AT2G17840.1 / Best-hit-rice-name=LOC_Os06g50330.1	AtERD7	2.26	-	-	-

Table 3. Continued.

Probe set	Annotation	Short name	T-14/Ctrl	T-35/Ctrl	T-27/Ctrl	T-37/Ctrl
Pavir.4NG172100.1_x_at	Best-hit-rice-name=LOC_Os12g10540.1	OsMADS13	2.31	-	3.14	2.43
Pavir.6NG327900.1_s_at	Best-hit-arabi-name=AT4G11880.1 / Best-hit-rice-name=LOC_Os08g41960.1	AtAGL14 / OsMADS37	2.35	-	-	-
Pavir.6NG327900.2_s_at	Best-hit-arabi-name=AT4G11880.1 / Best-hit-rice-name=LOC_Os08g41960.1	AtAGL14 / OsMADS37	2.35	-	-	-
Pavir.3NG137200.1_at	Best-hit-arabi-name=AT4G02380.1 / Best-hit-rice-name=LOC_Os05g29930.1	AtLEA5, AtSAG21	-	9.04	0.33	6.18
Pavir.9KG649500.2_at	Best-hit-arabi-name=AT2G22540.1 / Best-hit-rice-name=LOC_Os03g08754.2	AtAGL22, AtSVP / OsMADS47	2.43	-	3.80	-
Pavir.4NG172100.6_x_at	Best-hit-rice-name=LOC_Os12g10540.2	OsMADS13	2.71	-	3.69	2.74
Pavir.4KG233600.1_at	Best-hit-arabi-name=AT2G24840.1 / Best-hit-rice-name=LOC_Os06g30810.1	AtAGL61, AtDIA / OsMADS75	3.44	-	-	-
Pavir.6NG327900.3_at	Best-hit-arabi-name=AT4G11880.1 / Best-hit-rice-name=LOC_Os08g41960.1	AtAGL14 / OsMADS37	3.64	-	-	-

Table 4. The minimum, maximum, and average season length, temperature, and photoperiod settings for each of the three growth chamber experiments. Temperature and day length settings were changed weekly to mimic season fluctuation.

	Tropical (Guayaquil, Ecuador)	Sub-Tropical (Laredo, TX)	Cool-Temperate (Brattleboro, VT)
Growth Season Length (Weeks)			
	52	41	23
Temperature (Day/Night ˚C)			
Minimum	33/25	20/14	16/14
Maximum	33/25	40/26	29/17
Average	33/25	33/21	24/14
Photoperiod (Hour:Minute)			
Minimum	12:00	10:37	11:47
Maximum	12:00	13:52	15:20
Average	12:00	12:34	14:11

Table 5. Growth chamber regimes simulated sub-tropical and cool-temperate conditions. Tropical settings were static the entire 52-week period (35/25°C day/night temperature, 12:00 hr day length). Some night temperatures reached below 14°C (highlighted), but no settings below 14°C were used due to growth chamber setting restrictions.

Sub-tropical (Laredo, TX, USA)			**Cool-temperate (Brattleboro, VT, USA)**		
Week	Avg. Temp. (D/N) ˚C	Day length	Week	Avg. Temp. (D/N) ˚C	Day length
1	21/9	11:18	1	16/3	14:00
2	26/14	11:28	2	20/6	14:18
3	25/13	11:40	3	21/8	14:34
4	24/11	11:52	4	21/12	14:48
5	27/14	12:03	5	25/13	15:00
6	31/17	12:15	6	22/10	15:09
7	28/17	12:26	7	23/11	15:16
8	32/19	12:37	8	26/12	15:20
9	33/18	12:49	9	27/14	15:20
10	31/18	12:59	10	29/17	15:17
11	34/19	13:09	11	29/16	15:11
12	32/20	13:19	12	29/17	15:01
13	34/20	13:28	13	27/15	14:50
14	32/19	13:35	14	27/15	14:36
15	35/23	13:42	15	27/14	14:20
16	37/24	13:47	16	25/13	14:04
14	37/24	13:50	14	25/12	13:46
18	38/26	13:52	18	27/13	13:27
19	38/26	13:52	19	26/15	13:07
20	36/26	13:51	20	23/9	12:48
21	38/25	13:47	21	20/6	12:28
22	36/25	13:42	22	19/7	12:07
23	37/25	13:36	23	20/10	11:47
24	39/26	13:29			
25	40/26	13:20			
26	40/26	13:11			
27	39/26	13:01			
28	38/26	12:51			
29	39/26	12:40			
30	37/25	12:29			
31	38/25	12:17			
32	35/23	12:06			
33	33/23	11:55			
34	34/22	11:44			
35	33/22	11:32			
36	33/21	11:22			
37	31/20	11:11			
38	30/19	11:01			
39	30/18	10:52			
40	27/17	10:44			
41	20/12	10:37			

Table 6. Phenotypic characterization of miR156 transgenic switchgrass plants under tropical, sub-tropical, and cool-temperate growth chamber settings.

		Panicle number	Tiller number	Plant height (cm)	Leaf length (cm)	Leaf width (cm)	Node number	Internode diameter (mm)
Tropical	Control	25 ± 3.1^{a}	31 ± 2.5^{b}	169.3 ± 4.5^{a}	28.1 ± 4.3^{ab}	0.6 ± 0.1^{ab}	10 ± 0.8^{b}	2.35 ± 0.15^{a}
	T-14	0 ± 0.2^{b}	6 ± 1.0^{b}	101.4 ± 14.8^{b}	26.0 ± 5.9^{ab}	0.7 ± 0.1^{a}	7 ± 1.6^{b}	1.66 ± 0.20^{b}
	T-35	18 ± 2.6^{a}	68 ± 6.3^{b}	157.1 ± 3.7^{a}	39.5 ± 2.4^{a}	0.8 ± 0.1^{a}	7 ± 0.4^{b}	2.74 ± 0.14^{a}
	T-27	0^{b}	194 ± 41.2^{a}	113.1 ± 8.7^{b}	24.9 ± 1.5^{b}	0.3 ± 0.04^{b}	8 ± 0.3^{b}	0.81 ± 0.14^{b}
	T-37	3 ± 2.0^{b}	226 ± 24.2^{a}	146.3 ± 5.9^{a}	18.7 ± 1.4^{b}	0.3 ± 0.04^{b}	13 ± 0.8^{a}	0.81 ± 0.15^{b}
Sub-tropical	Control	13 ± 2.0^{a}	30 ± 2.9^{b}	163.4 ± 6.4^{a}	28.9 ± 3.1^{a}	0.8 ± 0.1^{a}	11 ± 0.7^{a}	3.61 ± 0.16^{ab}
	T-14	2 ± 1.0^{b}	16 ± 3.2^{b}	118.7 ± 13.0^{cd}	30.8 ± 2.9^{a}	0.8 ± 0.1^{a}	8 ± 0.7^{ab}	2.53 ± 0.15^{bc}
	T-35	3 ± 1.1^{b}	48 ± 7.9^{b}	154.9 ± 3.9^{ab}	32.1 ± 3.3^{a}	0.8 ± 0.1^{a}	8 ± 0.5^{ab}	3.91 ± 0.14^{a}
	T-27	0^{b}	168 ± 34.6^{a}	97.2 ± 5.8^{d}	27.1 ± 1.3^{a}	0.2 ± 0.02^{b}	7 ± 0.3^{b}	0.66 ± 0.09^{d}
	T-37	0^{b}	161 ±16.8^{a}	128.8 ± 6.9^{bc}	26.4 ± 3.3^{a}	0.5 ± 0.04^{b}	9 ± 0.6^{ab}	1.42 ± 0.04^{cd}
Cool-temperate	Control	2 ± 0.6^{ab}	24 ± 2.2^{c}	160.9 ± 3.9^{a}	65.2 ± 1.8^{a}	1.3 ± 0.04^{a}	5 ± 0.3^{ab}	4.62 ± 0.21^{a}
	T-14	3 ± 0.7^{a}	18 ± 2.2^{c}	128.8 ± 4.7^{b}	42.5 ± 1.4^{c}	1.1 ± 0.1^{b}	5 ± 0.2^{a}	3.96 ± 0.17^{ab}
	T-35	1 ± 0.2^{bc}	17 ± 2.2^{c}	139.2 ± 5.5^{b}	56.6 ± 1.3^{b}	1.2 ± 0.04^{ab}	4 ± 0.2^{b}	4.07 ± 0.23^{a}
	T-27	0^{c}	196 ± 8.2^{a}	101.8 ± 3.2^{c}	26.6 ± 1.0^{d}	0.3 ± 0.02^{d}	5 ± 0.2^{a}	1.20 ± 0.07^{c}
	T-37	0^{c}	197 ± 8.2^{b}	137.0 ± 3.4^{b}	45.2 ± 1.8^{c}	0.8 ± 0.03^{c}	5 ± 0.2^{a}	2.97 ± 0.10^{b}

All data was taken at the end of the respective season. The topmost leaf was used to measure leaf blade length and width, and internode three was used for internode diameter. Two tillers were measured for each replicate. Values are mean +/- SE (n = 8). Letters indicate significant difference at $P < 0.05$, Fisher's LSD

Appendix 2: Figures

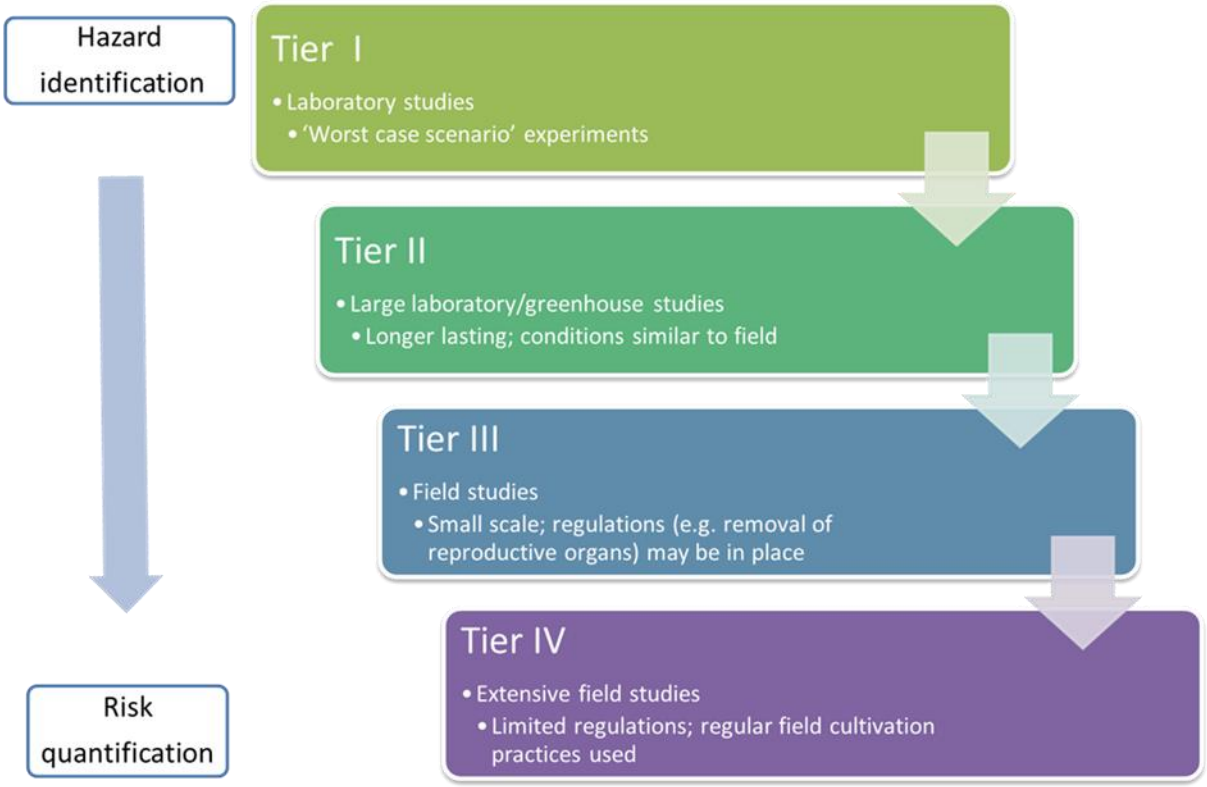

Figure 1. Explanation of each step in the tiered test process. Studies usually begin small scale with high exposure and hazard factors (Tier I). As the scale increases, the exposure and hazard levels adjust closer to more natural, expected levels (Tier IV). Adapted from Wilkinson et al. (2003).

Figure 2. Images of the field site located in Oliver Springs, Tennessee, USA. A) Google Earth image showing the heavily forested border of the field. Satellite image was taken on March 07, 2017. The red line represents a 20 m length. B) View of the experimental plots from the NE corner facing SW during year two on June 14, 2016.

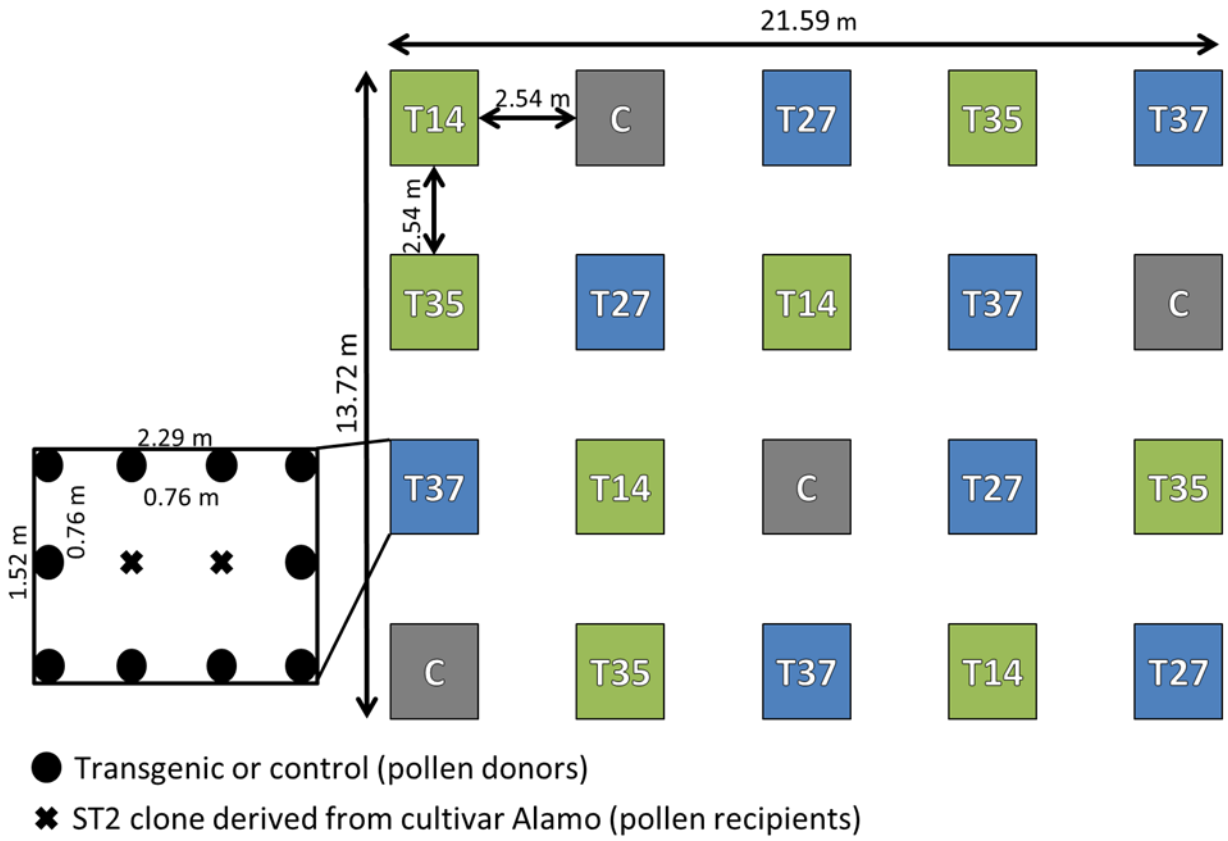

Figure 3. Complete randomized field design for open-flowering miR156-overexpressing transgenic switchgrass in Oliver Springs, TN, USA. In each of the 20 plots, two 'Alamo' ST2 clones (X's) act as pollen recipient plants and are surrounded by 10 pollen donor plants (filled black circles). Donor plants are either one of the four transgenic lines (T14, T35, T27, or T37) or the 'Alamo' control (C). Low overexpression lines are labeled in green, and medium overexpression plots are in blue.

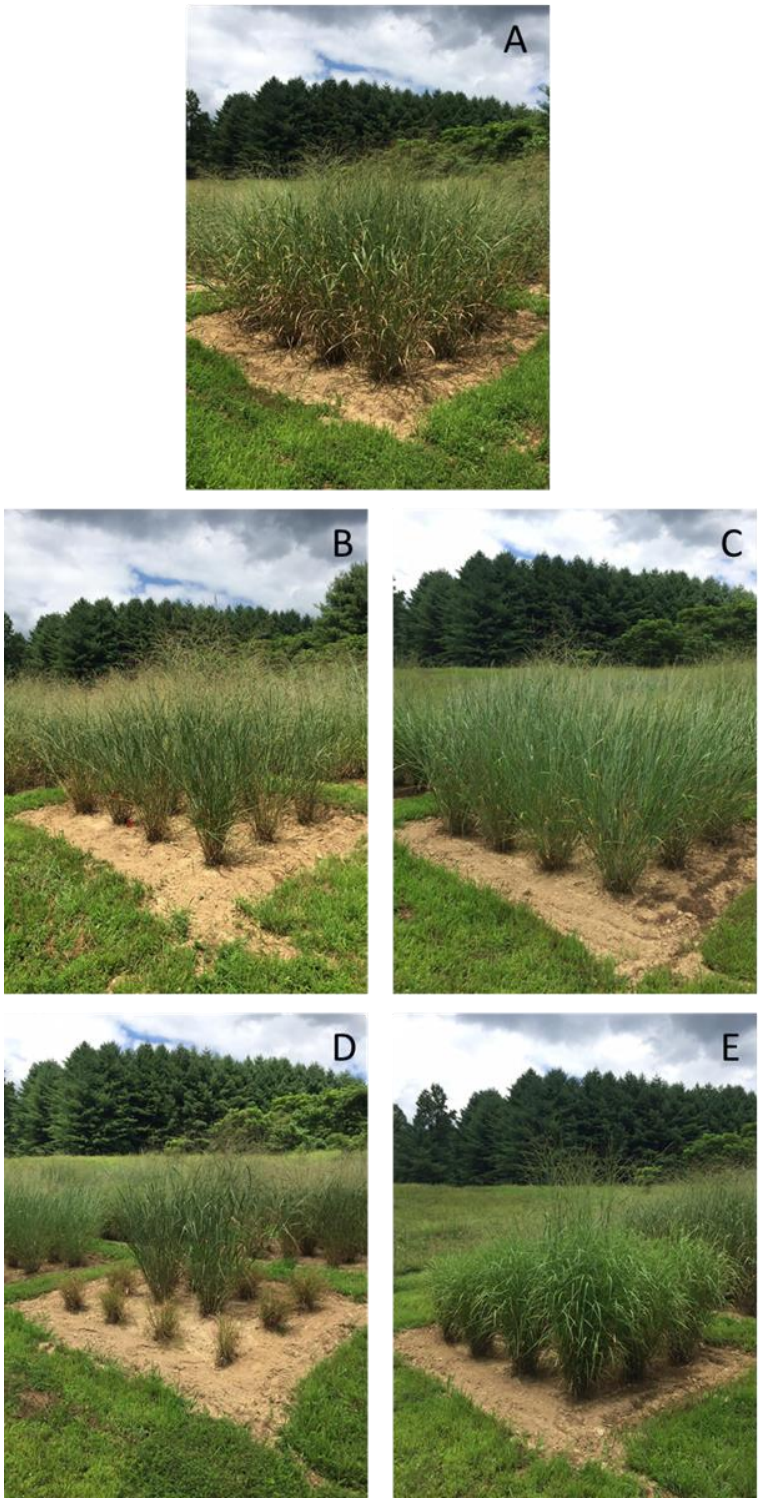

Figure 4. Representative images of each line in the field. Pictures were taken in year two on August 11, 2016. A) Nontransgenic ‘Alamo’. B) Low overexpression line T14. C) Low overexpression line T35. D) Medium overexpression line T27 surrounding easily visible ST2 nontransgenic plants in the center of the plot. E) Medium overexpression line T37.

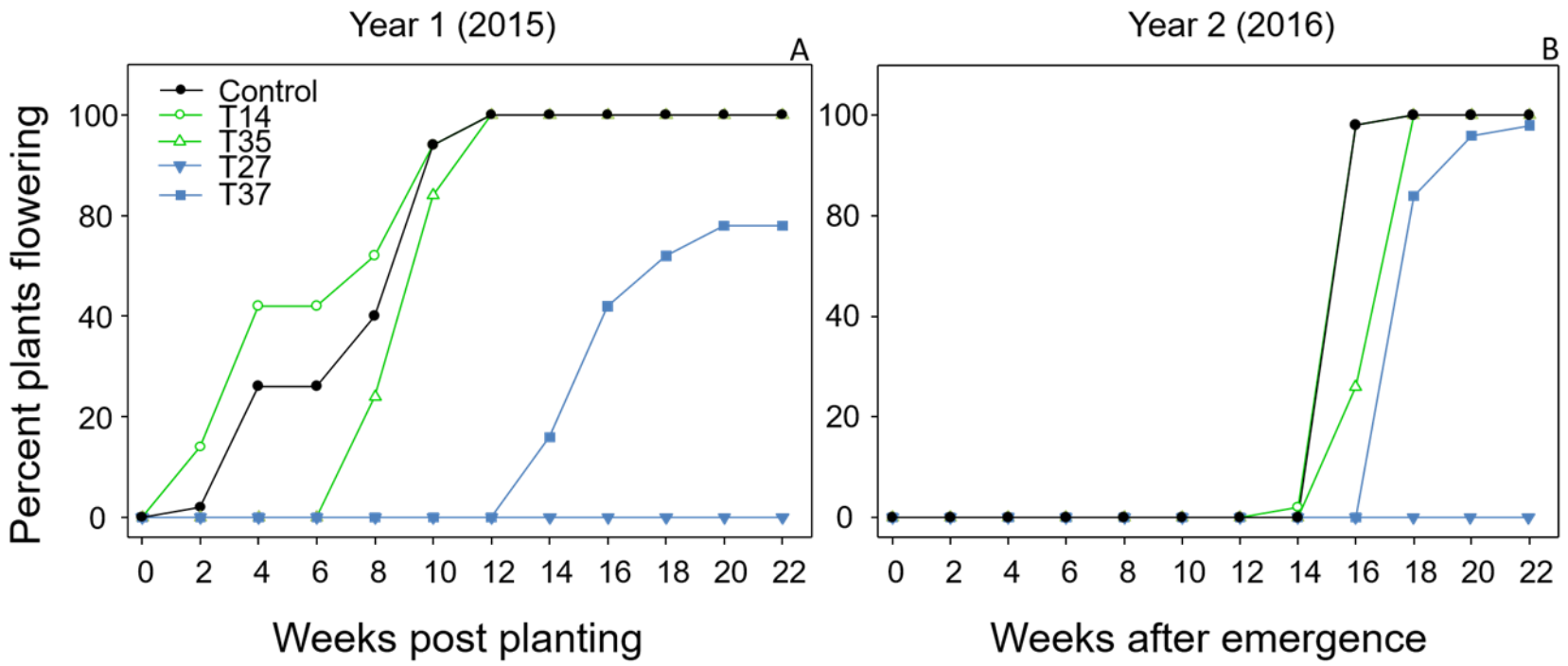

Figure 5. Time to first flower in the field for miR156 transgenic switchgrass lines and wild-type control. A) Year one (2015) weeks to first panicle emergence for each line after planting on June 05, 2015 (Wk 0). B) Year two (2016) weeks to first flower for each line after plant vegetative growth began on March 30, 2016 (Wk 0).

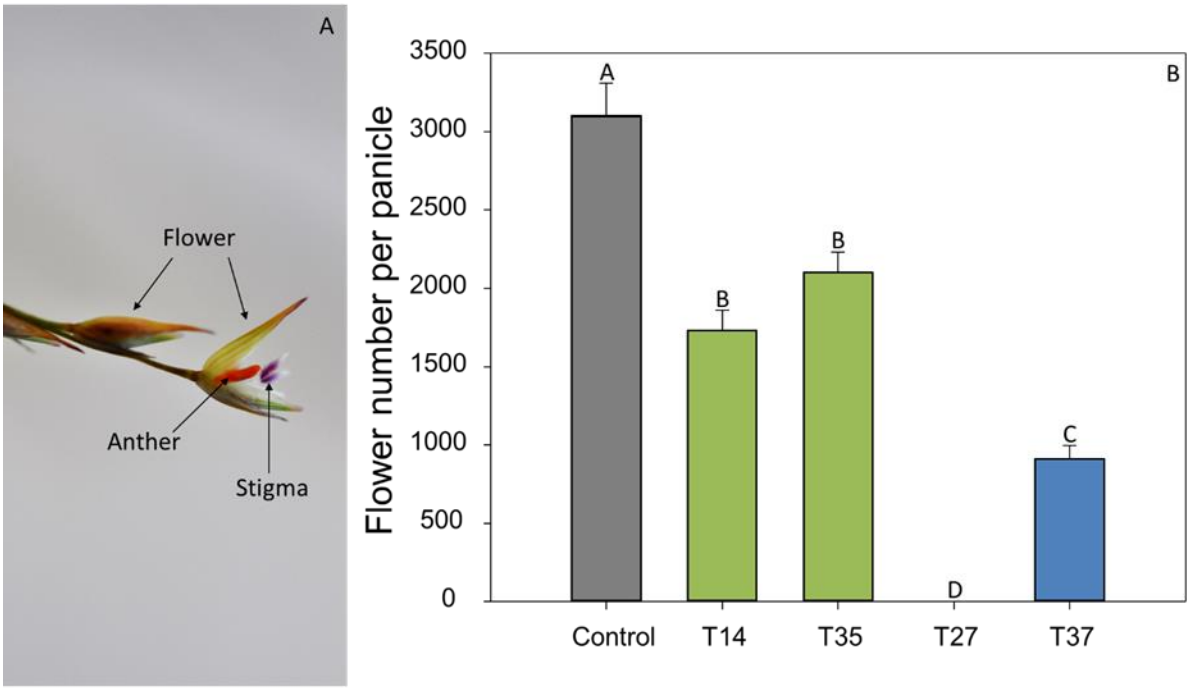

Figure 6. Flower number per panicle in year two (2016). A) Image of closed and open switchgrass flowers. Taken with a Nikon D90, 60 mm micro lens (Nikon USA, Melville, N.Y.). B) Letters represent significant differences between means (Fisher's LSD, $P < 0.05$). Error bars represent standard error of the means. $P = < 0.0001$.

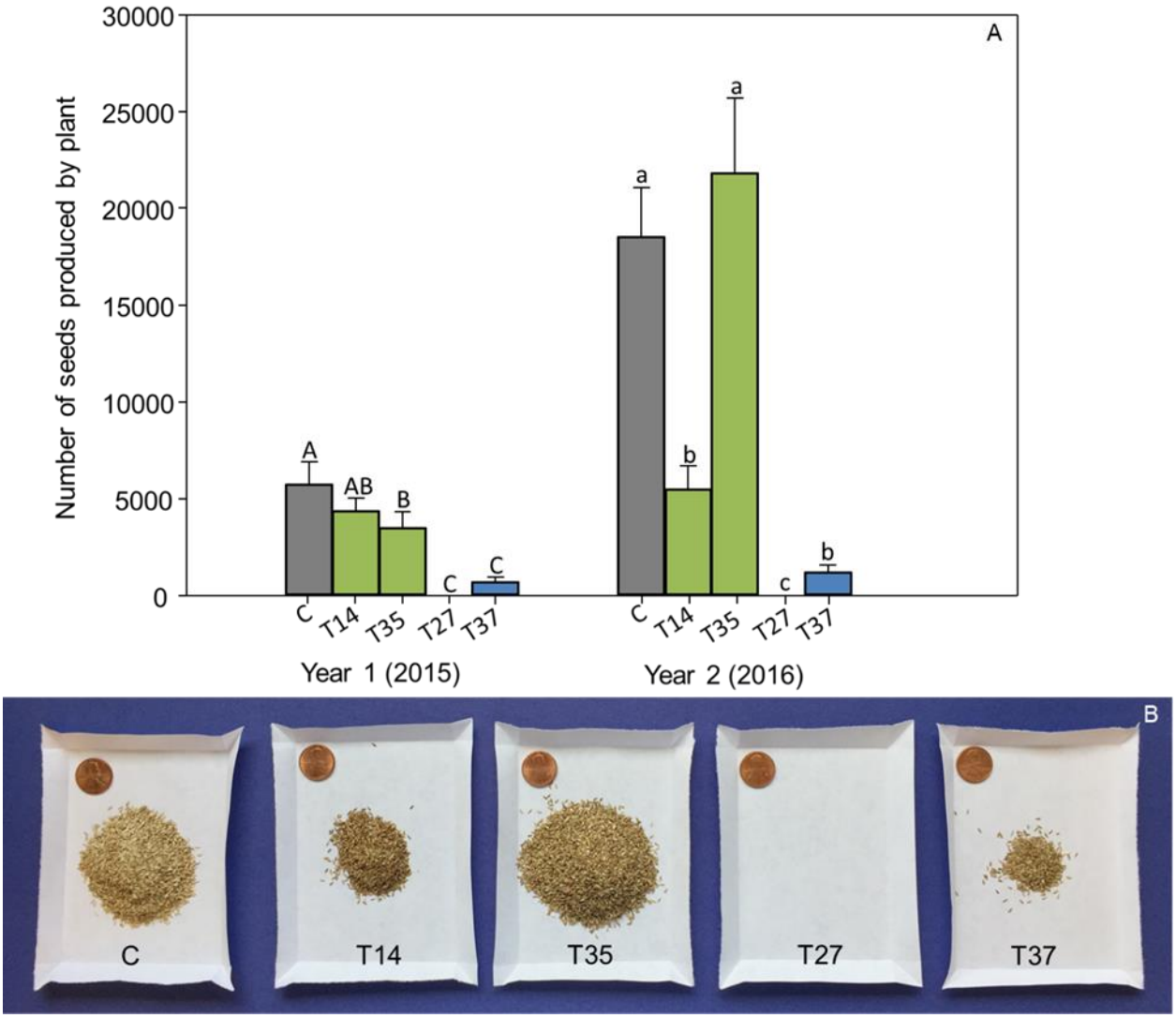

Figure 7. Number of seeds produced by plant for each transgenic line. Lines include the control (C), low miR156 overexpression lines (T14 and T35), and medium miR156 overexpression lines (T27 and T37). A) Capital letters represent significant differences between means in year one (2015) (P = <0.0001), and lowercase letters represent significant differences between means in year two (2016) (P = <0.0001; Fisher's LSD, P < 0.05). Error bars represent standard error of the means. B) Visual representation of the average number of seeds produced per plant in year two (2016). Penny used for scale.

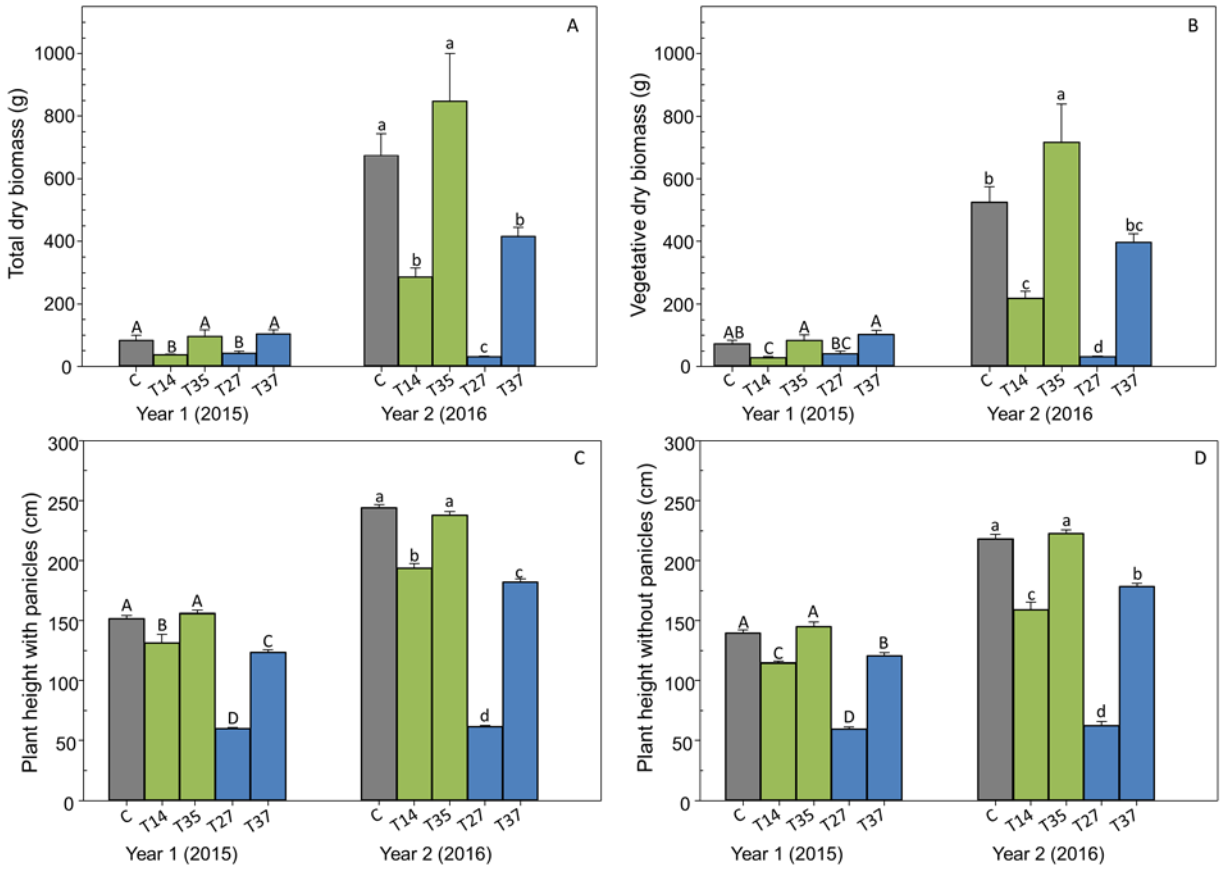

Figure 8. End-of-season dry biomass and height of miR156 transgenic switchgrass and control field grown in East Tennessee for two years. Capital letters represent significant differences between means in year one (2015), and lowercase letters represent significant differences between means in year two (2016) (Fisher's LSD, $P < 0.05$). Error bars represent standard error of the means. Year one growing season took place from June 05 – November 24, 2015. Year two growing season took place from March 30 – November 18, 2016. A) Dry biomass of both vegetative and reproductive tissues. Year one P = 0.0066; year two P = <0.0001. B) Dry biomass without panicles. Year one P = 0.002; year two P = <0.0001. C) Tallest part of the plant before panicle removal. P = <0.0001 for both years. D) Plant height after panicle removal. P = <0.0001 for both years.

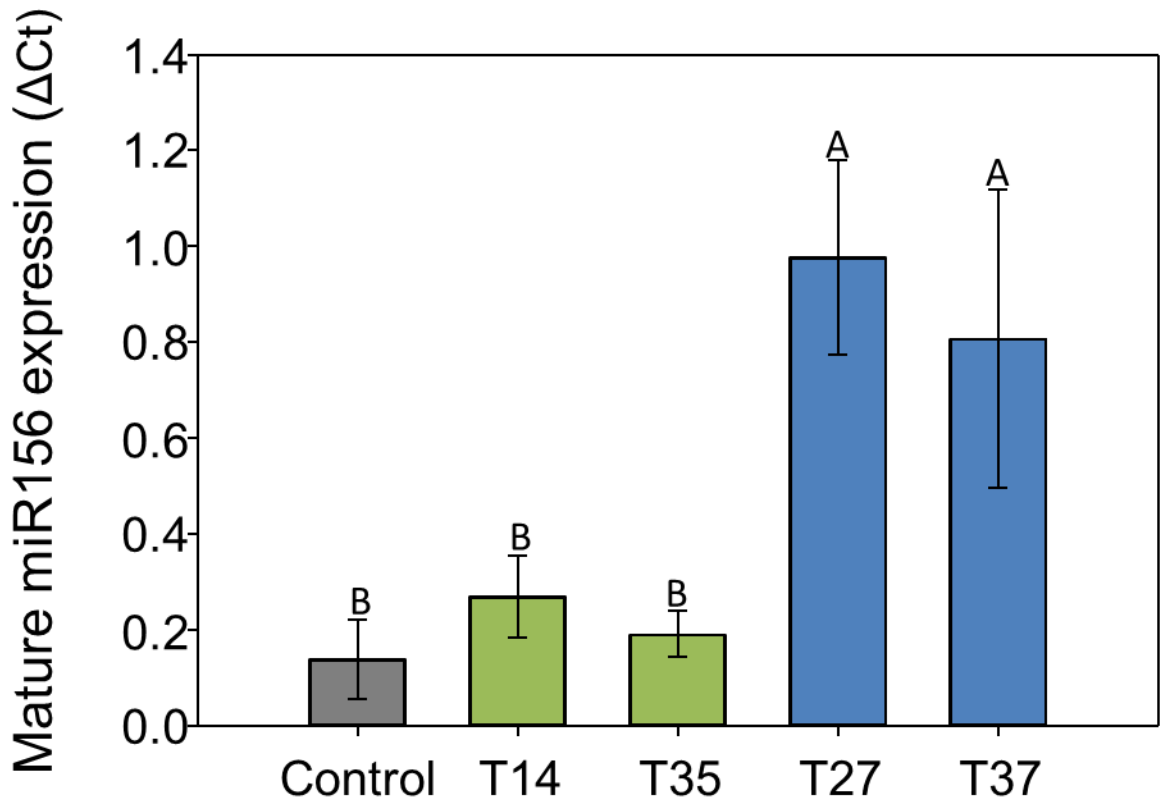

Figure 9. Relative mature miR156 expression results from qRT-PCR. The expression level of miR156 was normalized using miR390 expression. Combined leaf and tiller tissue from V3 stage tillers were used for mRNA extraction. Letters represent significant differences between means (Fisher's LSD, $P < 0.05$). Error bars represent standard error of the means. $P = 0.0103$.

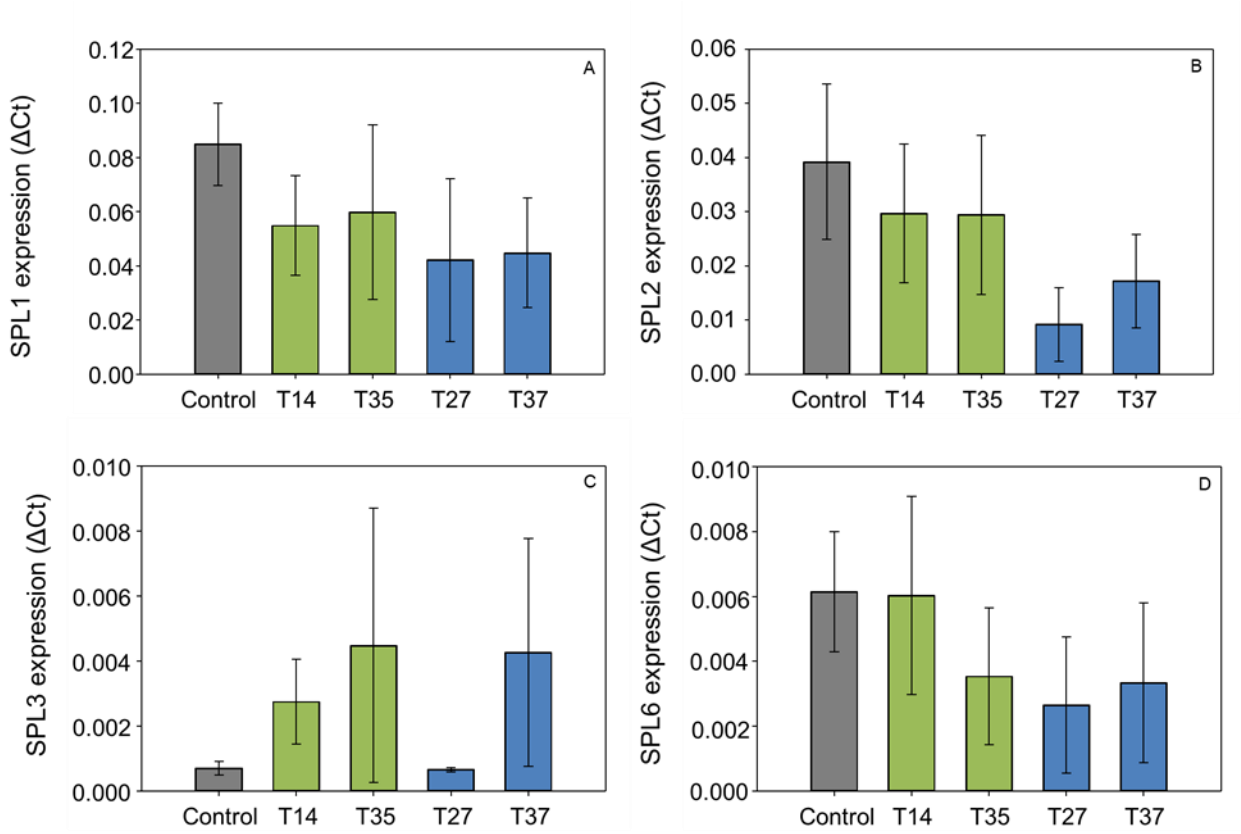

Figure 10. qRT-PCR results of combined V3 tiller and leaf tissue using the *PvSPL* primers. Error bars represent ± standard error of the means. No significant differences were found between means of any of the four target *SPL* genes. A) *SPL1* expression (P = 0.7374; ANOVA). B) *SPL2* expression (P = 0.4402; ANOVA). C) *SPL3* expression (P = 0.8544). D) *SPL6* expression (P = 0.7508; ANOVA).

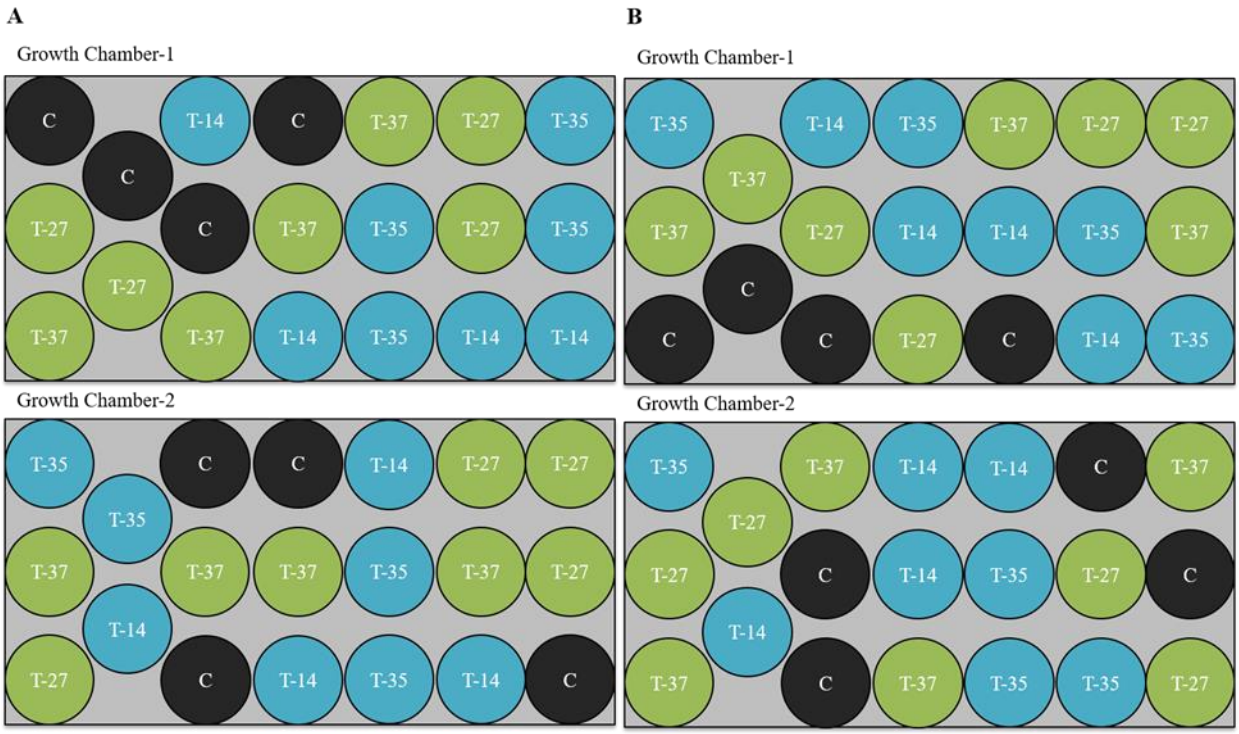

Figure 11. Growth chamber study with a randomized complete block design. Each experiment was replicated in two growth chambers, and each contained four replicates of each line. Lines are color-coded, which include a nontransgenic control (C) shown in black, low overexpression lines T-14 and T-35 (blue), and two medium overexpression lines T-27 and T-37 (green). A) Arrangement of pots from beginning of the season to mid-season. B) Pots were re-arranged in a different randomized design from mid-season to end of season.

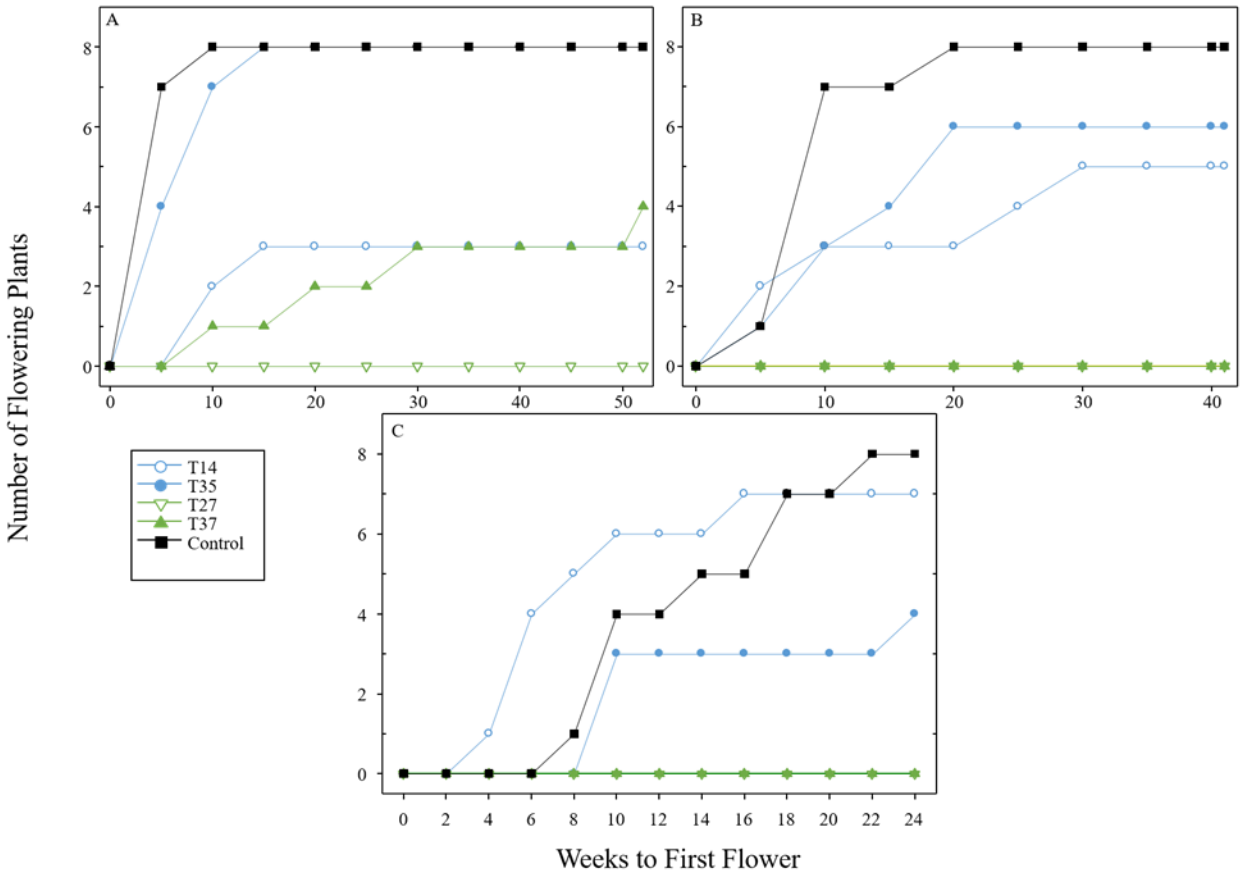

Figure 12. Time to first flower and number of plants flowering throughout the A) tropical, B) sub-tropical, and C) cool-temperate growing seasons. Lines labeled in blue (T-14 and T-35) represent low miR156 overexpression, and lines labeled in green (T-27 and T-37) represent medium miR156 overexpression.

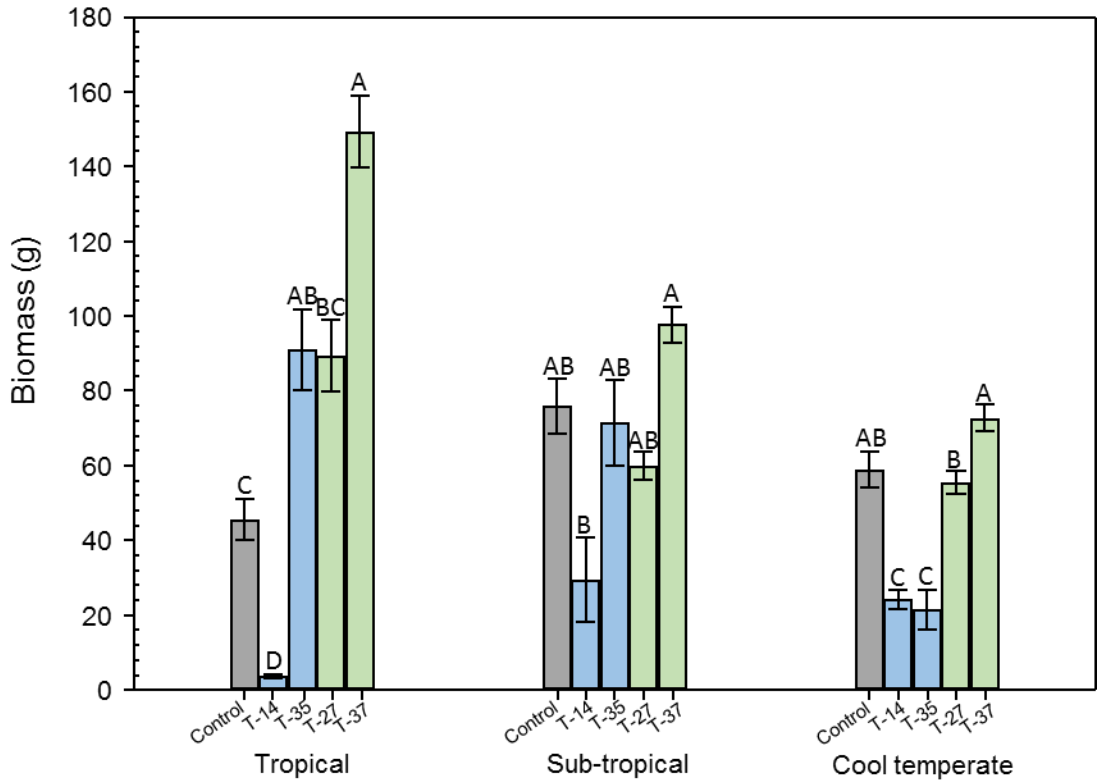

Figure 13. Biomass production per pot of miR156 transgenic switchgrass and control grown in tropical, sub-tropical, or cool-temperate conditions. Error bars represent SE. Letters denote statistical differences within each growth condition at P = 0.05, Fisher's LSD.

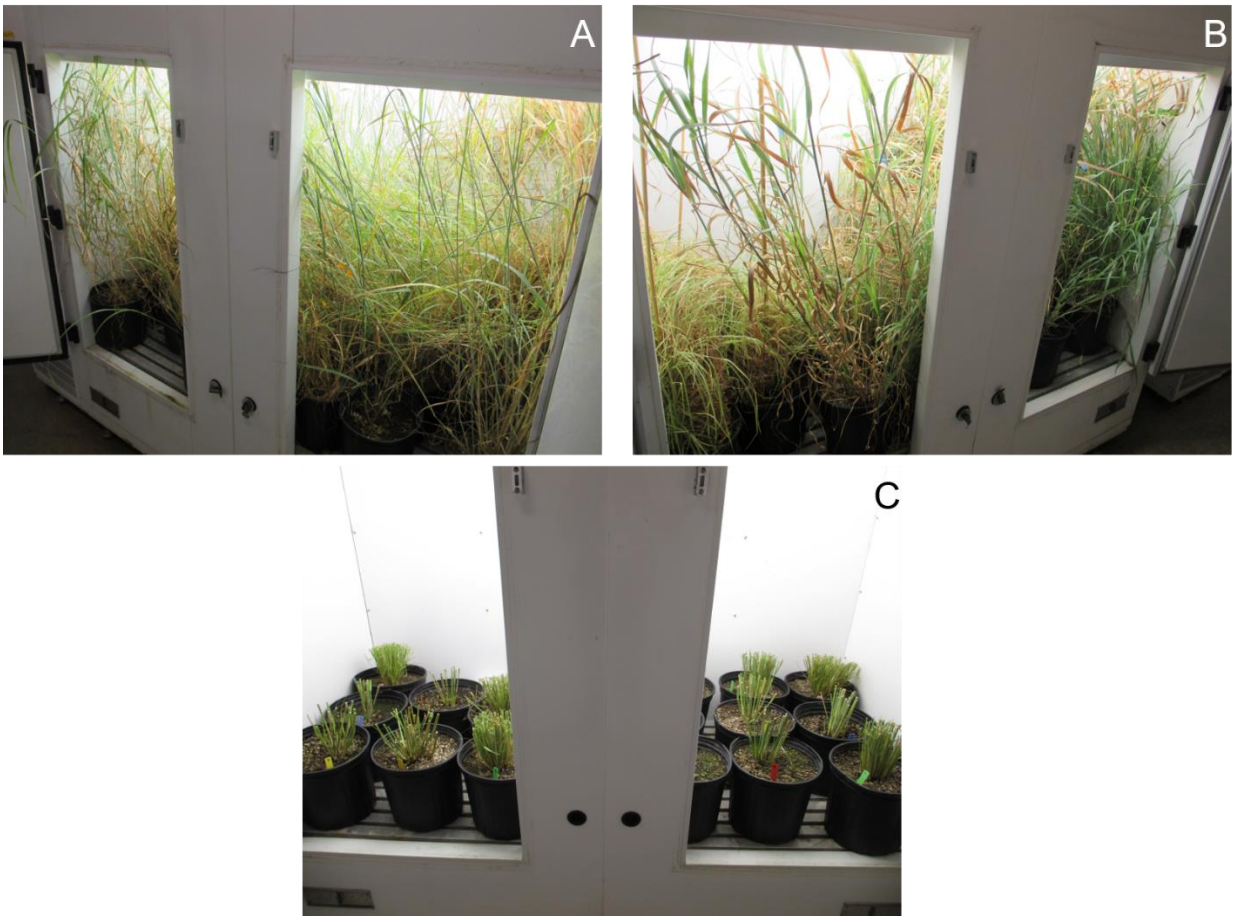

Figure 14. Switchgrass lines overexpressing miR156 at Low (T-14 and T-35) and medium (T-27 and T-37) levels and nontransgenic ‘Alamo’ control plants grown in three different climate simulations.

Vita

Chelsea Renai Johnson was born May 18, 1992 to Bill and Renai Johnson and grew up in Flintville, TN. She graduated from Lincoln County High School with Honors in 2010 and began her undergraduate education at the University of Tennessee that fall. She earned her Bachelor of Science in Plant Sciences with a concentration in Biotechnology in the fall of 2014. Wasting no time, she began work on her Master of Science in Plant Science, Plant Molecular Genetics in January 2015. After completing her master's, she plans to move back home with her fiancé, Eric, to begin their life together.