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Evaluation of the over-expression of a *Miscanthus* x *giganteus* PPDK (C4ppdk1) in switchgrass (*Panicum virgatum*) for improved cold temperature C4 photosynthesis

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To the Graduate Council:

I am submitting herewith a thesis written by Mathew Christian Halter entitled "Evaluation of the over-expression of a *Miscanthus* x *giganteus* PPDK (C4ppdk1) in switchgrass (*Panicum virgatum*) for improved cold temperature C4 photosynthesis." 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.

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Evaluation of the over-expression of a *Miscanthus* x *giganteus* PPDK (C4ppdk1) in switchgrass (*Panicum virgatum*) for improved cold temperature C4 photosynthesis

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Abstract

Switchgrass (*Panicum virgatum*) has been proposed as a potential biofuel feedstock to aid in the displacement of petroleum-based combustible fuels over the course of the next several decades. Improving the yield potential of this perennial grass is therefore of economic interest. Increased net ethanol yields can be achieved in cellulosic feedstocks via two means: 1. Improved sugar release from digestible cell wall materials, 2. Increased overall biomass yield. The latter is the subject of this study.

As a C4 photosynthetic plant, switchgrass is highly productive in the hot, humid climate of the southeastern United States. By bypassing the photorespiratory pathway, C4 plants have the ability to avoid as much as 25% yield losses due to the oxygenase activity of Rubisco. The utilization of this alternate pathway exposes the plant to cold temperature growth inhibition. Early spring and higher latitudinal temperatures do not provide optimal growth conditions for most C4 plants.

One C4 plant, miscanthus (*Miscanthus* x *giganteus*), has been shown to avoid the inhibiting effects of cold temperatures through the upregulation of a particular C4 enzyme, *pyruvate phosphate dikinase* (PPDK). This enzyme, responsible for the rate limiting step in the C4 pathway, has been shown to increase cold temperature carbon fixation and growth in miscanthus when it is upregulated. For this reason, this enzyme is an interesting target for overexpression in transgenic switchgrass to increase cold temperature photosynthesis.

The miscanthus PPDK cDNA was synthesized and ultimately overexpressed in transgenic switchgrass, which were characterized for photosynthesis and growth under both cold and warm temperatures. There were no statistically significant increases in biomass yields or in

photosynthetic capacity between transformed plants and non-transformed control plants. These data suggest that the initial hypothesis was probably too simplistic; there is likely greater complexity to understand the relationship of cold temperature C4 photosynthesis and the role of PPDK. Future experiments are needed to evaluate the underlying regulation and complexities of the C4 pathway to further understand how to specifically target and influence gene expression to accomplish higher efficiencies.

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Chapter 1: Switchgrass as a lignocellulosic biofuels feedstock

1.1 Biofuels

1.1.1 Starch-based fuels

The growth of the United States and world economies has spurred the development of alternative combustible fuels to offset the consumption of finite stocks of combustible petroleum products, especially for transportation fuels. As of 2012, the Unites States consumed 133 billion gallons of gasoline (DOE). In an effort to displace the use and costs of this imported product, dilution with combustible ethanol has been utilized. Ethanol can be produced domestically from agricultural sugar sources, such as starchy crops like corn and sugar beet. Over the last decade, use of corn-based ethanol as a fuel has accounted for 40% of the total United States corn yield (Ranum *et al.*, 2014).

As with any crop product, the production of corn-based ethanol entails certain energy inputs. Fertilizer input, harvest, product transportation, and refinement all require an input of energy. The use of corn-derived starch for ethanol production has been shown to produce a net energy yield of 34% (Shapouri *et al.*, 2002). This number has steadily increased over the last several decades due to the increased yields involved in improved cultivation techniques, as well as improved starch to ethanol conversion technologies.

1.1.2 Lignocellulosic biofuels

While corn is a highly productive crop, it is, in the end, a crop that requires high inputs for high grain yields (Shapouri *et al.*, 2002). To maximize ethanol yields from domestic sources, and to achieve a 30% displacement of foreign based petroleum with domestically produced ethanol by 2030 (Perlack *et al.*, 2005), the use of low input crops that can be grown on marginal land has

been proposed (Lynd *et al.*, 1991, Tanaka *et al.*, 1985). There are a number of agricultural crop products that can be used to this end. Corn stover, which is the aboveground biomass that is left over after grain harvest, can be milled and used to produce ethanol in a process similar to the production of ethanol from the starchy grain (Tanaka *et al.*, 1985). Rather than using glucose derived from the starch in the grain for fermentation, glucose derived from the cellulose of the stalk is used. It is the same process with two different inputs. While this process does make use of leftover agricultural residues, it also increases the required fertilizer inputs for the crop the following year and the biomass-to-fuel conversion is also not as efficient as that using grain owing to the recalcitrance of cellulose hydrolysis. The removal of the crop residues that could otherwise be tilled into the soil removes a convenient source of fertilizer, which must then be replaced. This, in turn, decreases the net yield of the following year's crop.

There are, on the other hand, many low input non-traditional crops that have high net yields. Poplar (*Populus trichocarpa*) and switchgrass (*Panicum virgatum*) have been identified by the Department of Energy as potential crops for future cellulosic ethanol production. Both species are native to the United States, and therefore would not pose new ecological or invasive plant risks.

1.1.3 Switchgrass (*Panicum virgatum*)

Switchgrass (*Panicum virgatum*) is an herbaceous perennial prairie grass native to the United States that offers advantages over established crops in that it can be grown on marginal land and requires little agricultural input (Hill *et al.*, 2006). The use of marginal land is of importance to the agricultural industry in that it does not require the devotion of current agricultural land that is

currently used for food and feed commodities. Switchgrass has been used as a grazing crop on livestock farms for years, and high yielding cultivars have been selected and bred (Ward *et al.,* 1989).

As a C4 plant, switchgrass is highly adapted to the hot, humid conditions of the southern United States. In a recent study measuring net energy values, switchgrass was shown to yield 343% more energy than was needed as for input when used to produce ethanol (Schmer *et al.,* 2008). C4 plants are known to be highly efficient at water and nitrogen use (Wang *et al.,* 2012). As a perennial plant, switchgrass does not require replanting after harvest, and can produce multiple harvests per year.Since it is a perennial, switchgrass crops need not be established each year. Prior to senescence in late fall, switchgrass transports important, non-fermentable nutrients to the rhizomes (Sarath *et al.,* 2014). This increases plant productivity and minimizes the need for annual fertilizer inputs. These agronomic traits all contribute to the potential of switchgrass as a sustainable biofuels feedstock.

1.2 Switchgrass biotechnology

1.2.1 Genetic tools

The use of biotechnology to improve agronomic traits in the most widely grown crop commodities has been widely successful (Ji *et al.,* 2013). Next generation sequencing technologies, as well as high throughput bioinformatic capabilities have helped to bridge the gap between phenotype and genotype. Although traditional breeding also will certainly play a major role in improving switchgrass germplasm, biotechnology will allow for the incorporation of traits that may otherwise be unattainable using only breeding techniques. Genomic single nucleotide

polymorphism (SNP) maps have been developed (Lu *et al.,* 2013) along with extensive expressed sequence tag (EST) libraries (Ersoz *et al.,* 2012, Zhang *et al.,* 2013). These resources allow for the high throughput identification of candidate genes for both overexpression and knockdown analysis. Pathway regulation profiles can be produced through next-generation sequencing of transcriptomes, providing a detailed look relative gene expression, such as in the case of biotic and abiotic stress responses (Dong *et al.,* 2014). The use of these technologies, paired with efficient tissue culture and transformation techniques, allow for a quicker and more targeted realization of agronomic traits than can be accomplished through traditional breeding.

1.2.2 Tissue culture

In vitro callus production is a necessary step in the genetic modification of a whole plant. Recently, methods of inducing embryogenic callus production from immature inflorescence have been developed with increased efficiency (Burris *et al.,* 2009). This method is preferred over methods that involve the use of seed-derived callus (Gupta and Conger, 1998, Seo *et al.,* 2008), as it allows clonal propagation of plants, which constricts the factor of genetic variability in the analysis if transgenic traits. This system includes 5 mg/L of 2, 4-D (2,4-dichlorophenoxyacetic acid) and a decreased amount of proline (from 500 to 100 mg/L), resulting in soft, friable, easily transformed callus.

1.2.3 Genetic transformation

There are two dominant methods used to make transgenic plants. The first, known as biolistics or particle bombardment, involves the physical delivery of a nucleic acid of interest into the plant of interest using particle carriers (Sanford *et al.,* 1987). The plasmid DNA containing the

expression constructs of interest is bound to positively charged particles, typically gold or tungsten, which are then fired into the cells of the plant of interest. The Plasmid DNA, now floating freely inside of the cell's nucleus, is incorporated into the plant genome by native DNA repair mechanisms (Shrivastav *et al.,* 2008). Cells that successfully incorporate the introduced DNA into their genomes and survive the damage caused by the particle bombardment may then be selected for using appropriate selection agents coupled with the introduction of specific resistance genes. Transgenic cells are then ultimately regenerated into whole plants. The second method, known as *Agrobacterium* mediated transformation, utilizes the natural infectious ability of *Agrobacterium tumefaciens* to incorporate its tumor inducing (Ti) plasmid (Schell and Montagu, 1977) into a host plant's genome (Gelvin, 2012). The tumor inducing genes, which naturally cause crown-gall disease in plants and are located between T-DNA borders on the Ti plasmid, are replaced with genes of interest to be transformed into the plant. *Agrobacterium*, carrying the modified Ti plasmid, is then used to infect plant callus. The infection results in the incorporation of the genes of interest into the host genome. Then transgenic cells are selected for and plants regenerated as above.

Both methods of plant transformation have been successfully used to produce transgenic switchgrass plants (King *et al.,* 2014, Richards *et al.,* 2001), with *Agrobacterium* mediated transformation tending to be the more efficient DNA delivery method (Ramamoorthy and Kumar, 2012). As new genotypes are developed for increased efficiency in tissue culturability and genetic transformability, the gap between gene of interest cloning and transgenic plant regeneration will become increasingly smaller. This will allow for a more high throughput process, enabling quicker turnover of gene characterization and trait enhancement.

1.2.4 Switchgrass biotech success stories to date

The aforementioned tool box has allowed for significant steps in improving switchgrass, especially for improved cell walls for subsequent conversion to biofuels. Gene characterization in model grass species, such as rice, has resulted in a number of potential candidate genes for overexpression or knockdown analysis in switchgrass (Bartley *et al.*, 2013). Many of these candidate genes have resulted in an increase of ethanol yield per unit of dry-weight biomass in switchgrass, as well as an increase in total biomass (Bartley *et al.*, 2013).

As is the case in any lignocellulosic biomass feedstock, the cell wall of the plant must be degraded to release the sugars that make up many of the cell wall polymers (Sun and Cheng, 2002). Cellulose, as well as hemicellulose and pectin, are polymers of sugar residues that are fermentation targets in the process of ethanol production. The tight matrix of the cell wall is held together by another polymer known as lignin. This network of polymers in the cell wall provides structural support to the plant (Caffall and Mohnen, 2009, Himmel *et al.*, 2007, Gibeaut and Carpita, 1994). Lignin binds to the cellulose of the cell wall, making it less accessible to digestive enzymes that release its glucose monomers for fermentation in the ethanol production process (Bonawitz and Chapple, 2010), and has therefore been the proposed target to alter using breeding and biotechnology (Chen and Dixon, 2007). Reduction in lignin content by the down regulation of the lignin biosynthetic enzyme cinnamyl-alcohol dehydrogenase resulted in an increase of glucose residue release in cellulose treated biomass samples (Saathoff *et al.*, 2011). An increase in glucose release relative to controls will directly contribute to an increase in ethanol production per unit biomass. Another targeted knockdown of a lignin biosynthetic gene, caffeic acid *O*-methyltransferase, resulted in a 38% increase in ethanol yield relative to non-transgenic controls (Fu *et al.*, 2011). Transcription factors, which regulate gene expression on a

broad level, have also been successfully targeted to manipulate lignin production. Specifically, the switchgrass PvMYB4 transcription factor, when overexpressed, causes a reduction in lignin content as well as an increase in plant tillering (Shen *et al.,* 2012). Similar to transcription factors, micro RNA's also regulate gene expression and can be targeted for phenotypic changes. The overexpression of a specific micro RNA, miR156, in switchgrass resulted in increased soluble sugar yield and increased biomass (Fu *et al.,* 2012).

1.3 Photosynthesis

1.3.1 Light dependent reactions

Photosynthesis is the process by which plants produce sugars from atmospheric carbon dioxide using light energy from the sun. Unlike animals, fungi, and most single cell organisms, plants are unable to retrieve the carbon necessary for growth and anabolism via the consumption of other organisms. They are autotrophs, meaning they are self-feeding, or producers of fixed carbon. Rather than obtaining the carbon backbones for basic metabolism from another organism, plants build them from the single carbon molecule carbon dioxide. This process requires energy, and that energy is provided by sunlight. In a process relatable to aerobic respiration, plants use light radiation to produce ATP in their photosynthetic chloroplasts. The chloroplast, home to the light-dependent reactions of photosynthesis, is a plastid that can be found in all green plants. Inside the chloroplast, membranous sacks known as thylakoids are stacked. The membranes of the thylakoid are used to produce a proton gradient, with light photons driving the pumping of protons through the thylakoid membrane into the sack. Much like the electron transport chain in the mitochondria that is used to produce a proton gradient, the photosynthetic electron transport chain of the thylakoid membrane initiates a series of oxidation-reduction reactions using an

electron stripped from a single water molecule by a light capturing photosystem. There are two photosystems found in the thylakoid membrane, each a massive complex of proteins capable of absorbing visible light of certain wavelengths. At the core of each photosystem is a chlorophyll molecule, structurally optimized for light absorption. With each photon of light absorbed by a chlorophyll molecule within these photosystems, an electron is ejected from the molecule into the electron transport chain to be used to produce the proton gradient across the thylakoid membrane. The extremely electronegative chlorophyll ion left over then strips an electron from an available water molecule, releasing molecular oxygen in the process and recharging itself to absorb another photon. The proton gradient produced by this process then drives the production of ATP through a thylakoid membrane bound ATP synthase. The ATP herein produced is then used to drive the reactions involved in the capture of carbon dioxide from the atmosphere and its sequential incorporation into the carbon backbones used to produce necessary biomolecules.

1.3.2 Light independent reactions (Calvin-Benson cycle)

Atmospheric carbon dioxide gains access to photosynthetic cells within the plant via opening on the leaf surface known as stomata. Once inside the cell, the carbon dioxide is acted upon by the enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), thus initiating what is known as the Calvin-Benson cycle. Rubisco catalyzes the production of two molecules of 3-phosphoglycerate from carbon dioxide and ribulose 1,5-bisphosphate. One molecule of 3-phosphoglycerate produced by this reaction is then used in central metabolic pathways for production of more complex molecules, such as amino acids, while the other is used to regenerate ribulose 1,5-bisphosphate to be reused in the cycle.

1.3.3 Photorespiration

The major pitfall of the Calvin-Benson cycle is found in the oxygenase activity of Rubisco. This enzyme is capable of catalyzing two reactions, the first as described above, and the second being the incorporation of molecular oxygen, rather than carbon dioxide, into the ribuose 1,5-bisphosphate backbone. This second reaction product, 2-phosphoglycolate, is not metabolically useful and must be recycled to reproduce ribulose 1,5-bisphosphate. This process, known as photorespiration, is not energetically favored, and is costly to the photosynthetic cell (Ogren, 1984).

1.3.4 C_4 photosynthesis

Due to the detrimental effects of the photorespiratory pathway, an alternate pathway evolved in plants that limits the access of Rubisco to atmospheric molecular oxygen. This pathway, known as the C_4 photosynthetic pathway, achieves this by utilizing an alternate leaf anatomy. The Kranz anatomy of C_4 plants includes two different photosynthetic cell types: the mesophyll cell types, located at the surface of the leaf exposed to atmospheric gases, and the bundle sheath cell type, located underneath the mesophyll cells adjacent to the vascular bundles (Hattersley, 1984). Rubisco is specifically expressed in the bundle sheath cell type, where it is separated from the atmospheric oxygen that accompanies carbon dioxide into the mesophyll cells. Carbon dioxide is initially fixed into the four carbon compound oxaloacetate by the enzyme phospho*enol*pyruvate carboxylase in the mesophyll cell. The oxaloacetate is then converted to malate, another four carbon compound, and then actively transported into the bundle sheath cell, where carbon dioxide is enzymatically re-released from the malate. In this way, carbon dioxide is specifically concentrated in the bundle sheath cell, where Rubisco is exclusively expressed. Disallowing

Rubisco access to molecular oxygen renders it's oxygenase activity useless, and thereby avoids the photorespiratory pathway.

1.3.5 Switchgrass photosynthesis

Switchgrass is a C_4 photosynthetic plant. It is, therefore, highly productive in the southern and midwestern United States, but less so in cooler regions of the country. This presents an opportunity for regional or seasonal yield improvements through improving the capacity of switchgrass growth under cooler conditions. The ability of switchgrass to grow at higher latitudes and earlier/later in the season would improve net yields across the country, improving its potential as a biofuels feedstock.

Chapter 2: Cold tolerant biomass accumulation in switchgrass (*Panicum virgatum)* is not affected by stable overexpression of *Miscanthus* x *giganteus* PPDK (C4ppdk1)

2.1 Abstract

As the demand for biofuels in the United States and worldwide increases, so does the need for a reliable and high yielding feedstock. Switchgrass (*Panicum virgatum*), a cellulosic biomass crop native to North America, has been proposed as an alternative to starch-based feedstocks in the U.S., and is, therefore, the topic of much ongoing research targeting the improvement of net yield. As a plant with C_4 photosynthesis, switchgrass is prone to slow growth in cold temperatures. The growth of another grass, *Miscanthus* x *giganteus,* also a C_4 plant, does not have as slow growth in cold temperatures, putatively because of the up regulation of a particular C_4 pathway gene, *pyruvate orthophosphate dikinase (ppdk)*. PPDK is responsible for the rate limiting step of the C_4 pathway, and it has been suggested that its upregulation increases the flux of carbon through the pathway in cold temperatures, thus, increasing the plants ability to fix carbon under temperatures ranging from 12^o to 14^o celsius. Here, we have attempted to improve switchgrass' capacity for cold temperature growth by overexpression of the miscanthus *ppdk* cDNA in switchgrass. Stable transformants showed no improved growth under cold temperature conditions, as well as no improved photosynthetic capacity. Our data suggests that there is more to be understood about the underlying regulation of C_4 photosynthesis under stressful conditions.

2.2 Introduction

Lignocellulosic biofuels feedstocks have been promoted as a means to displace petroleum-based fuels in a rapidly expanding and volatile fuel-based economy (Kerr and Service, 2005). In the United States, switchgrass (*Panicum virgatum*) and poplar (*Populus trichocarpa*) are among the leading candidates to provide lignocellulosic biomass for biofuel production (McLaughlin and Kszos, 2005, Davison *et al.*, 2006). One major advantage in the use of these plants is the fact that both are capable of productivity on marginal land, rendering less new competition for current land under crop production. While this will allow for their cultivation with relatively low agricultural input, vast improvements to yield and productivity must be achieved to maximize the net energy yield of these crops. Plant breeding techniques, as well as biotechnology, can be employed to rapidly improve yield in both species, optimizing fuel outputs while minimizing monetary inputs.

The use of biotechnology to improve plant productivity has been proposed as a means to achieve three ends: 1) improved yield of devoted biomass energy feedstocks (Nageswara-Rao *et al.*, 2013), 2) improved yield of staple food crops (Ji *et al.*, 2013), and 3) increased underground carbon sequestration to offset ever-increasing carbon emissions (Groover, 2007). Productivity is a trait in plants and can be examined from many different angles depending on the crop species and desired end result, but increased photosynthetic capacity has broad implications and can be directly linked to improved productivity (Kebeish *et al.*, 2007). C_4 photosynthesis, in particular, presents an enormous opportunity for efficiency improvements owing to the complexity of the pathway. In addition, there is a wide array of important C_4 crop species (corn, sugarcane, sorghum, switchgrass, etc.) with variable efficiencies (Lawrence and Walbot, 2007). C_4 photosynthesis, unlike its C_3 counterpart, is capable of avoiding the detrimental effects of

RUBISCO oxygenation of Ru-1,5-BP and the downstream photorespiratory pathway, and is therefore highly favored in warm temperatures (Sage *et al.,* 2012). But, in cold temperatures, such as those ranging from 12^{o} to 14^{o} C, the C_4 pathway experiences chilling inhibition, slowing the cycle and decreasing the rate of carbon fixation (Bilska and Sowinski, 2010). Improving cold temperature/climate C_4 photosynthesis has the potential to result in earlier season growth, increased latitudinal growing regions, and increased yield.

Miscanthus x *giganteus* (miscanthus) is a C_4 grass capable of accumulating large amounts of aboveground biomass relative to other C_4 grasses over a single growing season (Dohleman and Long, 2009). Miscanthus, unlike most other C_4 species, has been shown to be resistant to the detrimental effects of chilling temperatures (Naidu and Long, 2004). The avoidance of the anabolic slow-down from chilling inhibition by miscanthus is attributed to the upregulation of a single C_4 pathway gene, one encoding pyruvate orthophosphate dikinase (PPDK) under cool conditions (Wang *et al.,* 2008). PPDK is responsible for the rate-limiting reaction of the C_4 pathway, the conversion of pyruvate to phospho*enol*pyruvate in the chloroplast of the mesophyll cell type (Wang *et al.,* 2008). Higher expression rates of PPDK under cold temperatures increases the otherwise waning flux of metabolites through the pathway, thereby increasing carbon fixation.

Miscanthus x *giganteus* is a sterile hybrid clone, and therefore, might not be a viable industrial scale biomass feedstock since genetic variation is zero and vegetative reproduction for propagules would be expensive. Switchgrass also has C_4 photosynthesis that is highly productive in the Midwestern and southern United States, but is susceptible to chilling inhibition. The use of genetic engineering has been successful in improving various traits in switchgrass, such as cell wall digestibility (Fu *et al.,* 2011, Shen *et al.,* 2009, Yee *et al.,* 2012). A wide array of

bioinformatic/technological tools is available for the improvement of switchgrass as a bioenergy feedstock (Mann *et al.,* 2012, Burris *et al.,* 2009), (www.phytozome.net). Further improvements could be achieved by utilizing the miscanthus cold temperature C_4 photosynthetic mechanism.

The goal of this study was to assess the overexpression of the *Miscanthus* x *giganteus* C4ppdk1 cDNA in switchgrass. Plants confirmed for transgene expression were analyzed for photosynthetic evaluation, as well as warm/cold temperature growth.

2.3 Materials and Methods

2.3.1 Plants

The ST1 clone of switchgrass was grown in the greenhouse to the E2 to E4 stage (Moore *et al.,* 1991), at which point un-emerged inflorescences were excised just below the fourth node, sterilized by shaking in 75% bleach solution, and rinsed thoroughly with sterile water. After surface sterilization, explants were subjected to callus induction on LP9 media, as described by Burris, et al. (Burris *et al.,* 2009). Friable, white callus was selected to be further subcultured and eventually used for *Agrobacterium*-mediated transformation (Seo *et al.,* 2008).

2.3.2 Vector construction

C4ppdk1 was synthesized to the exact sequence specifications from Genbank (Genbank accession: AY262272.1) by Blue Heron Biotech (Bothell, Washington). The synthesized cDNA was cloned into the pANIC-6A monocot transformation binary vector (Mann *et al.,* 2012), which contains a pporRFP orange fluorescent protein (OFP) visual selection cassette and a hygromycin resistance antibiotic selection cassette. The Gateway (Life Technologies, Carlsbad, California) entry site for the gene of interest is also fused in frame to an AcV5 epitope tag.

2.3.3 Agrobacterium mediated transformation and plant regeneration

The pANIC-6A/MgPPDK transformation vector was heat shock transformed into *Agrobacterium tumafaciens* strain EHA105. *Agrobacterium* was grown overnight in YEP media with 50 mg/L kanomycin at 27^{o}C and shaking at 150 RPM to an OD_{600} of 0.50. The culture was then induced with 180 µM acetosyringone for 1 h, before centrifuging to pellet. The pelleted *Agrobacterium* was then resuspended in LP9 liquid medium, again to OD_{600} = 0.50. Three month old callus was selected for transformation and added to the LP9/*Agrobacterium* suspension. The callus culture was allowed to shake at approximately 40 RPM in the solution under vacuum for 30 minutes and without vacuum for 30 minutes. After inoculation, the solution was poured off and the callus was thoroughly dabbed with sterile filter paper in order to remove excess *Agrobacterium* to prevent overgrowth. Callus was placed on sterile filter paper damped by LP9 + 180 µM acetosyringone in a sealed Petri dish and allowed to co-cultivate at 27^{o}C for 3 days. After cocultivation, the inoculated callus was placed on LP9 + 400 mg/L timentin for two weeks to recover before being placed on increasingly stringent hygromycin selection for six weeks (LP9 + 400 mg/L timentin + 20, 40, 60 mg/L hygromycin, moving callus every two weeks). Callus clearly surviving hygromycin selection and displaying OFP fluorescence were then moved to MSO + 5 µM BAP to induce shoot production. After several weeks, calli with green shoots were moved to MSO magenta boxes to induce rooting and complete full plant regeneration.

2.3.4 Putative confirmation of transgenic events

Fully regenerated plants were screened to avoid non-transgenic escapes before continuing to molecular characterization. Leaves were screened using an Olympus SZX12 epifluorescent microscope with appropriate OFP filters. Plants having fluorescent tillers with fluorescent roots

were allowed to acclimate to soil before genomic DNA was extracted (Stewart and Via, 1993) and used as a template for PCR detection of the C4ppdk1 gene of interest using forward primer 5'-ACCTCACTGCCGC TGACC-3' and reverse primer 5'-TGGACATTGCTATAGCAGC-3', the pporRFP using forward primer 5'-ATGGCTCTTTCAAAGCAAAG-3' and reverse primer 5'-TTAGTGATGGTGATGGTG-3', and the switchgrass ACC synthase gene as a positive control for genomic DNA using forward primer 5'-AAGCTGGAGTTGGGATCATGG-3' and reverse primer 5'-CAACAGTAACTGGGCCTTCCTC-3'. Leaf RNA was then isolated using TRIzol (Life Technologies), treated with DNAse (Life Technologies, Carlsbad, California) for one hour to remove genomic DNA, and used to synthesize cDNA (Applied Biosystems High Capacity cDNA Reverse Transcription Kit). The cDNA was then used as a PCR template to detect C4ppdk1 transcript presence in transgenic plants using gene specific forward primer 5'-AGGCTAGCTGCAGCTCAGGT-3' and AcV5 tag specific reverse primer 5'-CAGCCGCTCGCATCTTTC-3'. Switchgrass ACC synthase primers listed previously were again used as a positive control for cDNA presence.

2.3.5 Southern blot analysis

Genomic DNA was isolated from leaves (Stewart and Via, 1993), 5 µg of which was digested with NCOI. Digested DNA was blotted and hybridized with an *hpt* DIG (Roche, Nutley, New Jersey) labeled probe. The bioluminescent detection of hybridization was performed according to supplied manufacturer DIG-High Prime DNA Labeling and Detection Starter Kit (Roche).

2.3.6 Quantitative reverse transcriptase PCR

Plants were grown in triplicate at either 28^{o}C or 12^{o}C treatments. After two weeks of acclimation, RNA was isolated using TRIzol (Life Technologies) from leaf number one from each of the three plants for each event and control, treated with DNase (Life Technologies, Carlsbad, California), and used to synthesize cDNA (Applied Biosystems High Capacity cDNA Reverse Transcription Kit). Forward primer 5'-AGGCTAGCTGCAGCTCAGGT-3', along with an AcV5 specific primer 5'-CAGCCGCTCGCATCTTTC-3' to avoid amplification of native switchgrass PPDK, were used along with PvUBI1 forward 5'-TTGGTGCTCCGCCTGAGA-3' and reverse 5'-CCTGGATCTTGGCCTTCACA-3' primers as an internal reference.

2.3.7 Gas exchange and chlorophyll fluorescence

All measurements were made on the third or fourth mature leaf of newly formed shoots using an open path gas-exchange system (LI-6400; Li-Cor Inc., Lincoln, Nebraska) in which relative humidity was between 55-75% and leaf temperature was maintained at the respective growth temperature. Leaves were allowed to acclimate to cuvette conditions for 5 min prior to initiating photosynthetic response programs. Initially, the photosynthetic response to light (A/Q curve) at 400 μmol/ml CO_2 was measured on all individuals from each growth chamber to determine the light saturation points, calculated using photosynthetic response curve fitting software (Li-Cor, Inc.) for event and temperature treatments.

2.3.8 Biomass characterization

Plants were grown in triplicate in growth chambers at warm and cold temperatures (26/22^{o}C D/N and 14/12^{o}C D/N). Tiller height was estimated taking the average of the five tallest tillers on

each plant, tiller number tallied for each plant (in triplicate), and total aboveground biomass was tallied using the average of the dry weight biomass from the triplicates.

2.3.9 Statistical Analysis

Three technical replications were performed on each of three biological replications for each analysis described. Mean separation was performed by Tukey's HSD test using SPSS statistical software.

2.4 Results

2.4.1 Molecular characterization of switchgrass plants overexpressing C4ppdk1

Two transgenic events were recovered in which the miscanthus C4ppdk1 was stably integrated, each with 4 copies of the T-DNA **(Figure 1).** The miscanthus transgene was transcribed at drastically different levels in the two events. Event A1 highly expressed the transgene, with transcript abundance over twice that of the *PvUbi1* internal control gene. The second event, D1, had much lower transgene expression, with transcript abundance at about half that of *PvUbi1* (**Figure 2**).

2.4.2 Photosynthetic characterization of switchgrass events expressing C4ppdk1

Both high and low expressing events' photosynthesis was characterized using gas exchange methods on plants grown at 14^{o}C and 28^{o}C **(Figure 3)**. The light saturation point, or the point at which the maximum rate of photosystem activity in a chloroplast has been achieved and light intensity no longer increases photosynthesis, was measured relative to an untransformed control that had been regenerated from tissue culture in the same way as the transgenic plants. Unexpectedly, the light saturation point was lower for both transgenic events, with event D1

being fully inhibited at roughly half the light intensity of the ST1 non-transgenic control. The photosynthetic capacities, defined as the amount of carbon a plant is capable of fixing during a unit of time, were not different. The non-photorespiratory CO_2 evolution, or day respiration (R_d), rates were measured for each event and the non-transgenic control. The three lines were not significantly different, indicating that they were maintaining similar levels of metabolic activity, an observation consistent with the measured rates of photosynthetic capacity.

2.4.3 Biomass characterization of events A1 and D1grown under warm and cool conditions

Both events were significantly taller than the control (**Figure 4**). Event A(1), while significantly taller, produced fewer tillers than event D(1) and the ST1 non-transgenic control. The two transgenic events and control did not produce significantly different amounts of total biomass over this growth period at these temperatures.

The temperature was then adjusted to 14^oC days and 12^oC nights. Plants were grown for 6 weeks under these conditions to more closely simulate the length of the early growing season. After the 6 week period, it was evident that the same biomass characterization as was performed on the warm temperature grown plants could not be performed on the cold temperature grown plants **(Figure 5)**. Many of the nine total plants (3 lines, in triplicate) did not grow beyond initial leaf emergence, and there was extreme inconsistency among members of the same line in triplicate. Total above ground biomass was, although, measured and showed no significant difference between events and non-transgenic controls.

2.5 Discussion

Expression of C4ppdk1 did not result in an increase in cold temperature or warm temperature biomass accumulation in switchgrass. Of the two transgenic events produced, neither showed an increased rate of photosynthesis or growth. Because PPDK does not play a direct role in CO_2 uptake, as it is involved in the non-light dependent reactions of photosynthesis, it is unexpected that overexpression of this gene would result in decreasing the light saturation point, but rather, possibly antibiotic selection stress or even epigenetic variation might have caused the differences (Nakano and Mii, 1993, Miguel and Marum, 2011). Photosynthetic capacity of a C_4 plant would be directly affected by the activity of PPDK in the mesophyll cell chloroplasts. Phospho*enol*pyruvate availability for fixation with CO_2 by PEP carboxylase increases or decreases based on the rate of PPDK activity. Therefore, it is expected that the photosynthetic capacity of the transgenic events would be higher than that of the non-transgenic control. This, however, was also not observed.

Non-photorespiratory CO_2 evolution results from cellular respiration, a process necessary to supply the plant cell with the ATP required for sucrose synthesis and other metabolic processes (Amthor, 1984). This process provides a general indication of cellular and/or metabolic activity. ATP produced by cellular respiration is required for protein synthesis, as well as many other metabolic functions. Therefore, increased ATP production, and the associated increase in CO_2 release, serves as an indirect indication of increased productivity, growth, and metabolism. An increase in non-photorespiratory CO_2 evolution was not observed, which when taken along with other data discussed, is a clear indication that the hypothesized phenotype is not being produced. This could be due to the underlying complexity of the C_4 pathway, or due to something more straightforward. As C4ppdk1 cDNA was used in this study, the regulatory and/or functional

aspects of the genomic introns were not utilized. Introns play an important role in gene expression (Emami and Arumainayagam, 2008), and as C4ppdk1 is expressed tissue specifically, this could be a source of inconsistency between our switchgrass data and that of native miscanthus.

The major advantage of the use of biotechnology over plant breeding is the ability to fix a very specific phenotype without investing the time required by several generations of crossing and progeny evaluation. It has become increasingly routine to identify a single gene responsible for a desired trait, clone the gene, and express its associated trait in a plant of interest. Overexpression of single genes has resulted in insect resistant Bt corn (Koziel *et al.,* 1993), increased provitamin A in tomatoes (Romer *et al.,* 2000), and color change in petunia flowers (Napoli *et al.,* 1990). All of these traits are controlled by single genes, and are therefore easily manipulated. They are qualitative traits. Unfortunately, not all traits of interest fall into this relatively simple to engineer category. Traits such as biomass yield, for instance, are controlled by a wide array of factors, including multiple genes and the environmental conditions. Photosynthesis is a polygenic trait, and so it is reasonable that maintenance of photosynthesis is not controlled only by PPDK (Ji *et al.,* 2013).

It has been clearly shown that the expression of PPDK is upregulated in miscanthus under cold conditions (Wang *et al.,* 2008), a mechanism that surely contributes to the overall productivity of the plant under said conditions. That knowledge, combined with the data presented here, suggests that there are more factors to be considered in determining the underlying physiology behind miscanthus' increased productivity. A recent study in sorghum, for instance, identified 20 different quantitative trait loci (QTL) related to grain yield (Rajkumar *et al.,* 2013). Shoot production prior to photosynthesis, as well as nutrient mobilization, are two

key aspects that have been shown recently by transcriptome analysis to be involved in early spring rhizome/shoot growth in miscanthus (Barling *et al.*, 2013). While the expression patterns of PPDK surely play a role in the productivity of miscanthus, a deeper evaluation of genome wide expression is necessary to more thoroughly understand the ability of this feedstock to outgrow its competitors.

Chapter 3: Conclusion

Switchgrass, as a native perennial grass, is an ideal candidate to become a lignocellulosic biofuels feedstock. It is highly productive in the southern and midwestern United States, and has the potential to become even more so through traditional breeding practices and biotechnology. Targeting traits such as overall biomass production, as well as cell wall digestibility will help to improve the net energy yield per hectare. The use of next generation biotechnological techniques is helping to streamline the process of gene characterization and trait improvement.

This work has focused on the improvement of biomass yield in switchgrass. As a C_4 photosynthetic plant, switchgrass is highly productive in warm climates, but not so in colder climates. Another C_4 plant, miscanthus, has been shown to be highly productive under cold stress. This cold tolerance has been hypothesized to be attributed to the up regulation of a single C_4 pathway gene, PPDK. PPDK expression is increased under cold conditions, increasing the overall productivity of the C_4 pathway. Here, I described the first attempt to improve the cold tolerance of switchgrass through the stable overexpression of this C_4 enzyme.

Two events were produced which overexpressed, at different levels, the miscanthus PPDK enzyme. These plants were evaluated for photosynthetic efficiency and biomass production under cool conditions (12^oC). Neither transgenic events showed an increase in photosynthetic efficiency or biomass production. This unexpected result suggests that there is more to be understood about the overall regulation and activities of C_4 photosynthetic enzymes, especially under cool growing conditions. Quantitative traits such as biomass are typically regulated by the coordinated activity of many enzymes, and it is evident that cold tolerance in miscanthus is no exception. Future work is needed to track global gene expression changes in miscanthus or other

cold tolerant C_4 species to identify alternate routes to achieving increased cold temperature biomass yield.

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Appendix

Figures

Figure 2-1

Transgenic plant analysis. (a) Schematic representation of the T-DNA used to transform switchgrass. Orange fluorescent protein (pporRFP; OFP) and hygromycin resistance were used for selection. The gene of interest cassette is fused to an AcV5 epitope tag. (b) Plants regenerated from positive callus events A1 and D1 were positive for OFP fluorescence. Scale bars represent 5 mm. (c) PCR analysis for *C4ppdk1* and pporRFP in both transgenic events, with ACC synthase serving as a positive control for genomic DNA. (d) Reverse transcriptase PCR using *C4ppdk1* specific forward primers and AcV5 specific reverse primers and using ACC synthase primers used for positive control for RNA. (e) T-DNA insertion copy number analysis using Southern blots. Genomic DNA was hybridized with an *hpt* probe, as indicated above the schematic (a) with a red bar.

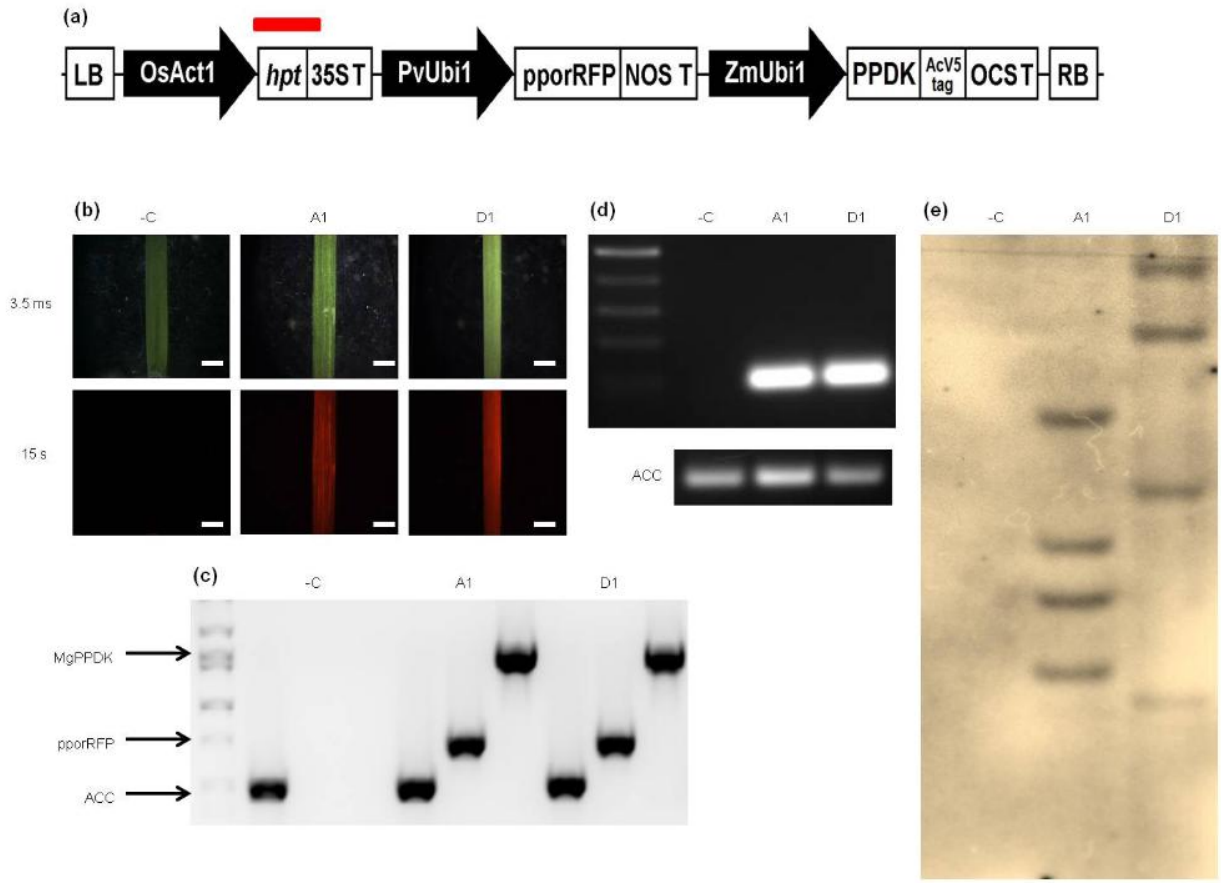

Figure 2-1 continued

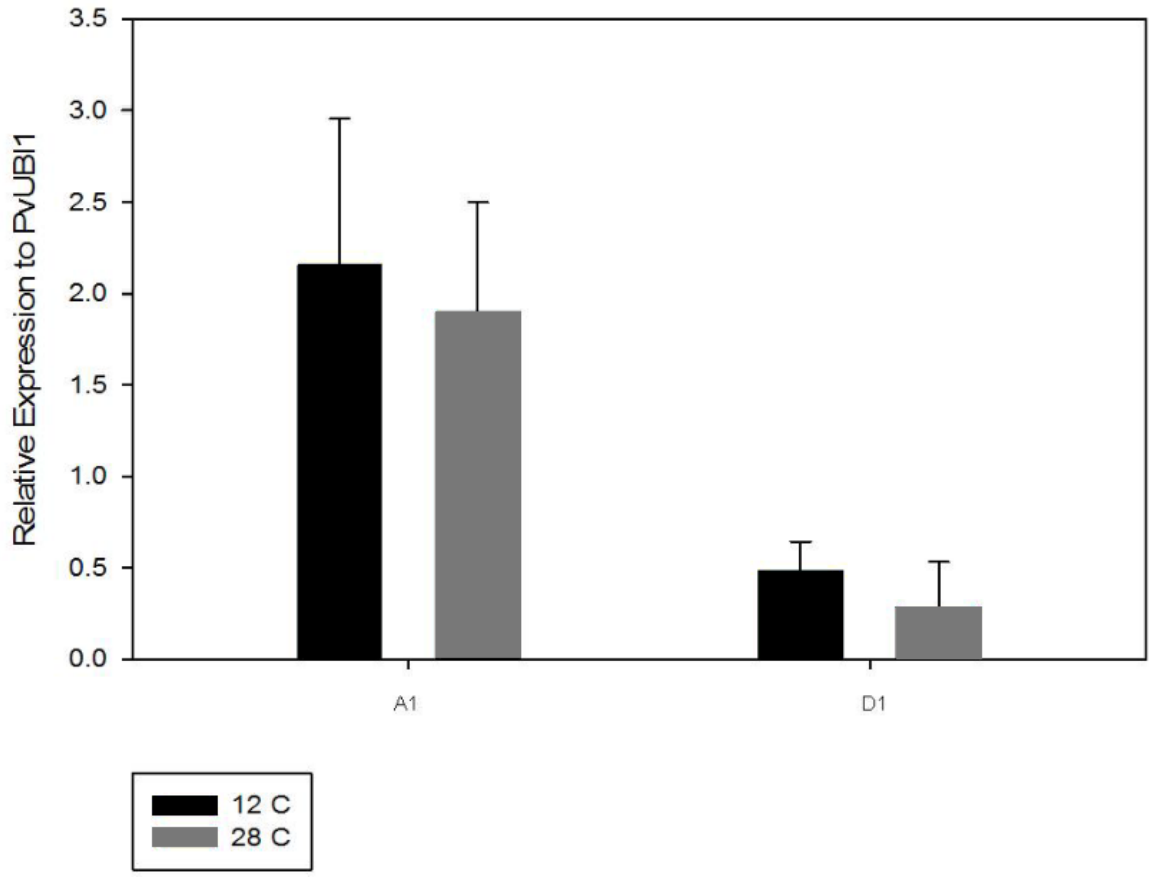

Figure 2-2

Relative transcript abundance of *C4ppdk1* to *PvUBI1* in warm (28^{o}C) and cool (12^{o}C) conditions.

(a) Light saturation point

	Event	14°C	28°C
Q_{sat} ($\mu mol\ m^{-2}s^{-1}$)	ST1	404.9 (60.39)	849.7 (347.46)
	A1	284.4 (106.62)	666.7 (150.79)
	D1	206.4 (67.84)	616.9 (217.48)

(b) Photosynthetic capacity

	Event	14°C	28°C
A_{max} ($\mu mol\ m^{-2}s^{-1}$)	ST1	4.7 (0.69)	10.9 (3.87)
	A1	4.1 (0.92)	9.0 (1.30)
	D1	3.5 (0.84)	7.6 (2.43)

(c) Non-photorespiratory CO_2 evolution

	Event	14°C	28°C
R_d ($\mu mol\ m^{-2}s^{-1}$)	ST1	-0.82 (0.335)	-0.75 (0.137)
	A1	-0.75 (0.249)	-0.90 (0.245)
	D1	-0.81 (0.584)	-0.82 (0.259)

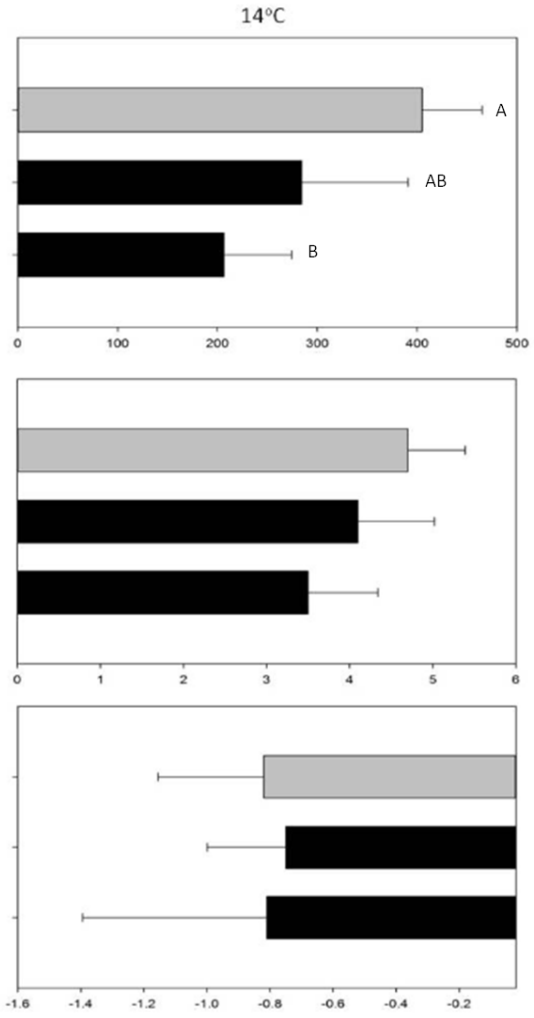

Figure 2-3

Photosynthetic characterization of events A1 and D1 expressing *C4ppdk1*. (a) Carbon dioxide fixation rates were measured at the light saturation point (Q_{sat}), (b) photosynthetic capacity was measured, and (c) non-photorespiratory CO_2 evolution was measured. All values in parentheses are standard errors. Letter groupings represent significantly different values at an alpha of 0.05 using Tukey's HSD method. A lack of letter groupings implies no significant difference.

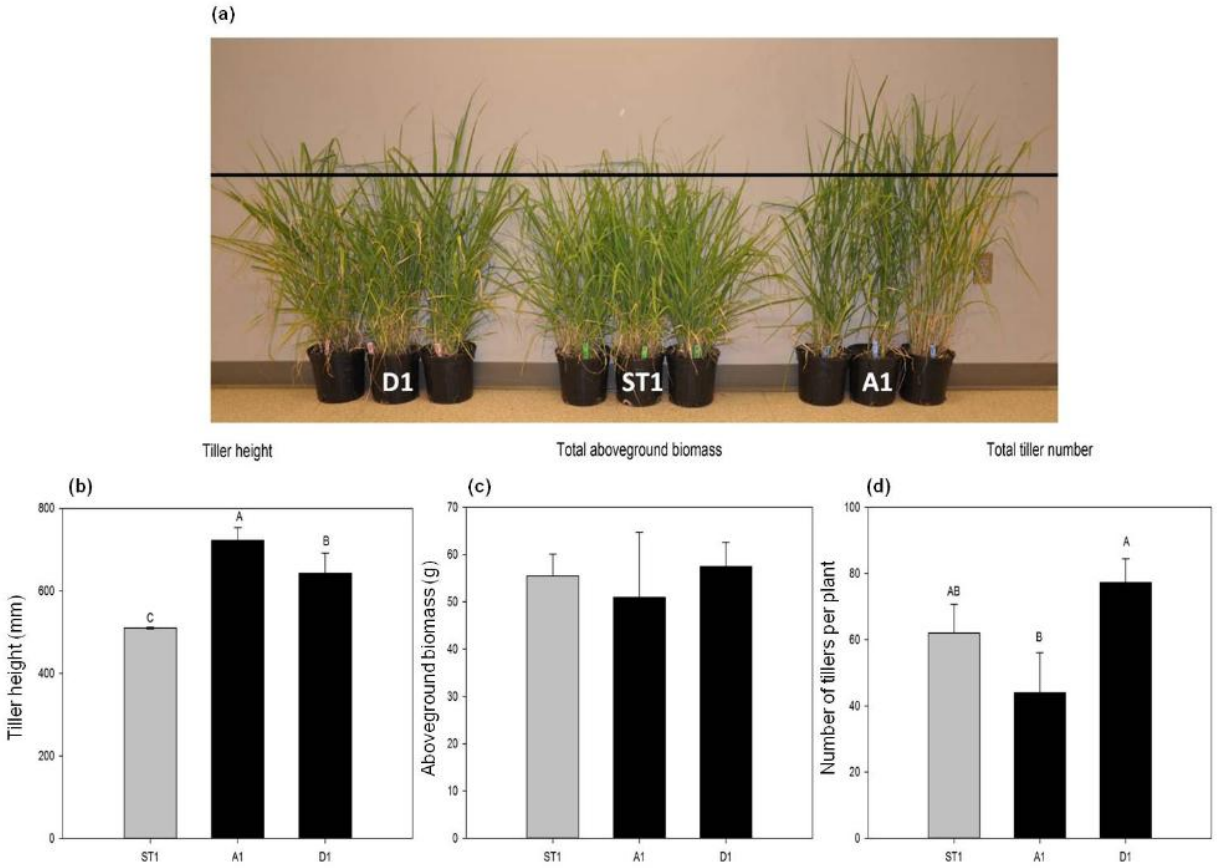

Figure 2-4

Biomass characterization of events A1 and D1 grown under warm conditions. Plants were grown in triplicate for 16 weeks at 26^{o}C days and 22^{o}C nights. (a) Phenotypic variation of the two events and non-transgenic control. (b) The five tallest tillers were collected from each plant, measured, and averaged for all three plants. (c) Total dry-weight aboveground biomass was measured for each of the three plants of each event. (d) The total number of tillers was counted for each plant of each event. Letter groupings represent significantly different values at an alpha of 0.05 using Tukey's HSD method. A lack of letter groupings implies no significant difference.

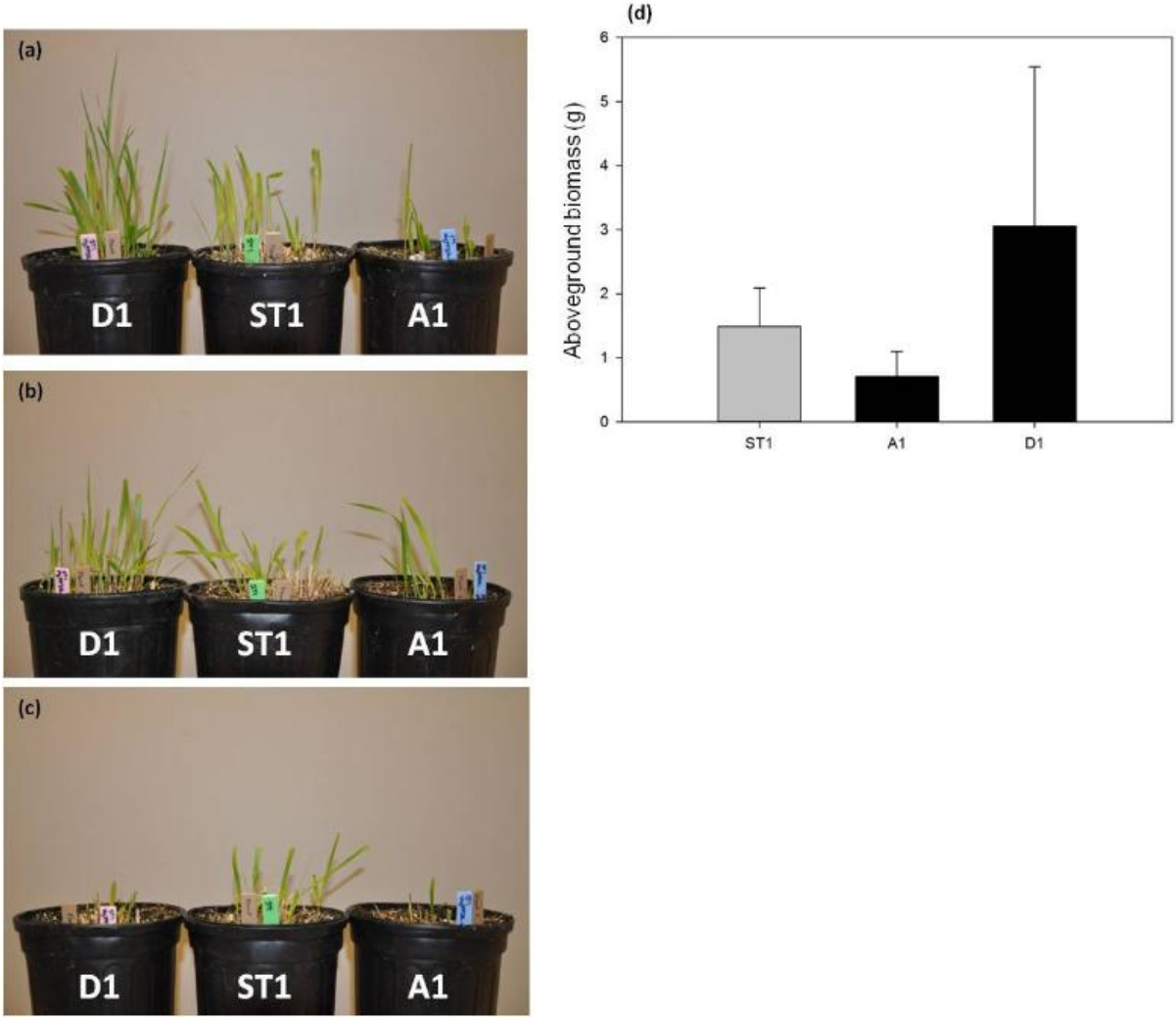

Figure 2-5

Biomass characterization of events A1 and D1 grown under cool conditions. Plants were grown in triplicate for 6 weeks at 14^{o}C days and 12^{o}C nights. Plants were arranged for imaging in grouping of largest triplicate to smallest (a, b, c). (d) Total dry-weight aboveground biomass was measured for each of the three plants for each event. Letter groupings represent significantly different values at an alpha of 0.05 using Tukey's HSD method. A lack of letter groupings implies no significant difference.

Vita

Mathew Christian Halter was born August 2, 1986 in Raleigh, North Carolina, to Michael and Erika Halter. Mathew attended Notre Dame High School in Chattanooga, Tennessee, and graduated in 2004. He then attended East Tennessee State University, graduating in 2009 with a Bachelor of Science degree in biochemistry.