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Photosynthetic parameters of switchgrass (*Panicum virgatum*) under low radiation: Influence of stable overexpression of *Miscanthus* × *giganteus* PPDK on responses to light and CO_2 under warm and cool growing conditions

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ABSTRACT

Background: Switchgrass (*Panicum virgatum*) is one of the leading candidates to provide lignocellulosic biomass for biofuel production. Switchgrass is capable of relatively high productivity on marginal land or when intercropped with trees. Production of switchgrass is dependent upon light use efficiency at the canopy level. Thus, maintenance of photosynthesis at light limiting and cool conditions ought to elongate the growing season and increase productivity of switchgrass. Photosynthesis under cool conditions and low light is maintained higher in giant miscanthus (*Miscanthus* × *giganteus*) than switchgrass by retaining relatively high expression of pyruvate orthophosphate dikinase (PPDK). Our main goal was to create lines of switchgrass with upregulated PPDK and to evaluate photosynthetic responses of those lines to growth temperature under low radiation conditions. Our approach was to grow replicate plants of each transgenic event with an untransformed control in low light environments at either warm (28 °C day/24 °C night) or cool (14 °C day/12 °C night) conditions. Photosynthesis parameters of all plants were assessed with fluorescence kinetics, light response curves and carbon dioxide response curves.

Results: We created several lines of transgenic switchgrass with documented upregulation of cDNA for the PPDK gene (C4ppdk1). Photoinhibition was higher in the transgenic lines, but electron transport rates (ETR) and quantum yield of photosystem II were not inhibited by cool conditions. The higher than expected ETR under cool conditions was associated with increased non-photochemical quenching, which indicated that enzymatic reactions of photosynthesis were inhibited more by cool conditions than photochemical processes. In all except one transgenic line, most metrics of biochemical processes decreased under cool growth conditions, which resulted in significantly lower productivity under cool conditions.

Conclusions: All transgenic lines were able to balance electron transport and biochemical process at low radiation keeping apparent quantum yield constant and the light saturation point relatively low. Thus, the photosynthetic changes associated with the transgenic events could make the transgenic lines appropriate for use in low light regions such as forest intercropping systems if productivity was increased. Although one transgenic line had weakly improved photosynthesis under cool conditions in this study, improving cold temperature photosynthesis in switchgrass will require more than manipulating the expression of a single gene.

Abbreviations: A/C_i curve, photosynthetic response to intercellular CO_2 concentration; A/Q curve, photosynthetic response to incident light curve; A_{sat}, light saturated photosynthetic rate; AQE, apparent quantum efficiency; A_{max}, CO_2 saturated photosynthetic rate; C4ppdk1, synthesized gene for PPDK; CE, carboxylation efficiency; ETR, electron transport rate; J_{max}, maximum electron flow; PPDK, pyruvate orthophosphate dikinase; PPFD, photosynthetic photon flux density; qN, non-photochemical quenching; Q_{comp}, light compensation point; Q_{sat}, light saturation point; R_d, dark respiration rate; TPU, triose phosphate use rate; V_{cmax}, maximum potential photosynthesis; Yield, quantum yield of photosystem II.

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1. Introduction

Lignocellulosic biofuel feedstocks have been promoted as a means to displace petroleum-based fuels in a rapidly expanding and volatile fuel-based economy [1]. In the United States, switchgrass (*Panicum virgatum*) and poplar (*Populus trichocarpa*) are among the leading candidates to provide lignocellulosic biomass for biofuel production [2,3]. 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 [4], (2) improved yield of staple food crops [5], and (3) increased underground carbon sequestration to offset ever-increasing carbon emissions [6]. 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 [7]. 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 [8], which are being targeted for bioenergy crop production. 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 [9] and high radiation environments. However, under low radiation, and in cold temperature conditions, such as those ranging from 12 °C to 14 °C, the C_4 pathway experiences inhibition, decreasing the rate of carbon fixation [10]. Improving low light and cold climate C_4 photosynthesis has the potential to result in earlier season growth, increased latitudinal growing regions, and increased yield.

Miscanthus × *giganteus* (miscanthus) is a C_4 grass capable of accumulating large amounts of aboveground biomass relative to other C_4 grasses in high light environments over a single growing season [11]. Miscanthus, unlike most other C_4 species, has been shown to be insensitive to the detrimental effects of chilling temperatures [12] and has the capability to acclimate to low light intensity [13]. Low light acclimation of miscanthus promotes a more efficient canopy and can allow for higher canopy photosynthesis early and late in the day, which in sum results in higher productivity [14].

The avoidance of anabolic slow-down from chilling inhibition by miscanthus has been attributed to the upregulation of a single C_4 pathway gene, one encoding pyruvate orthophosphate dikinase (PPDK) under cool conditions [15]. Significant reductions of 57% in the PPDK content of maize has been shown under 14/11 °C growing conditions [16] and nearly a 70% reduction following 14 days of 14 °C conditions, after being transferred from 25 °C treatment [17]. Miscanthus has displayed an increase of 2.1 times the PPDK content following transfer from warm conditions to 14 °C conditions after 14 days [17]. Also, miscanthus demonstrated no loss in the amount of Rubisco and a slight increase in PPDK under cool growing conditions (14/11 °C, compared with 25/20 °C), and showed no difference in photosynthetic rates between growth temperature treatments at the same measuring temperature [13,16]. Also, increases in the PPDK content has been shown in three sugarcane species (*Saccharum* spp.) under low temperature stress [18]. Sugarcane species, of different cold tolerances, have shown changes in the PPDK activity with cold stress; cold tolerant-sensitive hybrid species showed an increase in the PPDK content while cold sensitive species demonstrated a decrease in the PPDK activity, along with a decrease in malate dehydrogenase (NADP-MDH) [19]. These results suggest that PPDK content may be an underlying factor in the ability to tolerate cold temperatures in C_4 species.

Miscanthus × *giganteus* is a sterile hybrid clone, and therefore, might not be a viable industrial scale biomass feedstock since genetic variation is minimal 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 and is relatively tolerant of drought, but does not acclimate to low radiation and is susceptible to chilling inhibition [13]. The use of genetic engineering has been successful in improving various traits in switchgrass, such as cell wall digestibility [20–22]. A wide array of bioinformatics, technological tools now are available for the improvement of switchgrass as a bioenergy feedstock [23,24] (www.phytozome.net). Further improvements in photosynthetic acclimation to low radiation and cool temperature could be achieved by transferring the miscanthus cold temperature and low radiation attributes of the C_4 photosynthetic mechanism to switchgrass lines.

Given PPDK's role as the possible limiting factor in C_4 photosynthesis, overexpressing this gene may result in increased rates of assimilation and possibly in turn, productivity. Ohta et al. [25], using the *Flaveria brownii* A. Powell PPDK gene as a model, introduced point mutations into the maize PPDK gene to mimic that of the C-terminus of the *F. brownii* PPDK gene. The resulting transformed maize displayed cold tolerance of photosynthesis similar to *F. brownii* and increased photosynthetic rates of 23% over the nontransformed line [25].

The goal of this study was to assess the consequences of the overexpression of the *Miscanthus* × *giganteus* C4ppdk1 cDNA in Alamo switchgrass to photosynthesis under low light and cool temperature. Based on current knowledge of the relationship between PPDK and acclimation to cool growth temperatures under high light conditions we addressed the following hypotheses: (1) overexpressing C4ppdk1 in Alamo switchgrass will reduce the negative effect of cool temperatures on plant growth, (2) photosynthetic parameters of the transgenic events, particularly light related parameters, will be reduced for all plants in relation to that already found for high light adapted Alamo switchgrass, (3) overexpression of C4ppdk1 will cause less cold-induced reduction in photosynthetic parameters compared to a non-transformed control line. Photosynthetic parameters were obtained from fluorescence kinetics, light response curves and carbon dioxide response curves. Our approach was to characterize growth and photosynthetic parameters of stably transformed plants confirmed for transgene expression under low radiation conditions at warm and cool air temperatures.

2. Materials and methods

2.1. Plant callus source

The ST1 clone of switchgrass was grown in the greenhouse to the E2 to E4 stage [26] at which point non-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. [24] Friable, white callus was selected to be further subcultured and eventually used for *Agrobacterium*-mediated transformation [24].

2.2. 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 [23], 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. Agrobacterium *mediated transformation and plant regeneration*

The pANIC-6A/C4ppdk1 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 °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 were allowed to shake at approximately 40 rpm in the solution under vacuum for 30 min and without vacuum for 30 min. 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 °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 visibly 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.

2.4. 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 [28] 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 1 h 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.

2.5. Southern blot analysis

Genomic DNA was isolated from leaves [27], 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).

2.6. Quantitative real time PCR

Plants were grown in triplicate at either 28 °C or 12 °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.

2.7. Plants for gas exchange and biomass characterization

Four transgenic events plus a nontransgenic control, of 10 individuals each, were received by researchers at Virginia Tech for photosynthetic and productivity analysis. These plants were provided 3 g of four month slow-release fertilizer (Osmocote 19-6-12) and maintained in their original pots in the BIOL/VPI Plant Growth Facility at Virginia Tech. To achieve the desired sample size for each line, individuals were propagated by dividing and replanting in one gallon pots with Pro-Mix BX (Premier Tech Horticulture, Quebec, Canada) two weeks prior to being transferred into the growth chambers.

Four individuals from each of two transgenic events and two nontransgenic individuals (total of 10 plants) were transferred into each of 4 growth chambers (E8, CONVIRON, Winnipeg, Canada). Two of the growth chambers were programmed for warm conditions (28/24 °C; day/night) reflecting summer temperatures of the mid-Atlantic states and the other two growth chambers were programmed to reflect spring temperatures (14/12 °C; day/night), all with a 14/10 (light/dark) hour cycle and a photosynthetic photon flux density (PPFD) at flag leaf height of 200 $\mu mol\ m^{-2}\ s^{-1}$. The same photoperiod was used in both temperature treatments to isolate the effects of temperature from photoperiod. We selected a PPFD that was well above the compensation point yet below the anticipated light saturation point. The low radiation conditions we used simulate early morning or evening low radiation conditions, light intensity in intercropping systems between trees, and subcanopy locations with a thin tree canopy. Our results do not apply to plants grown in high light environments. Plants were watered as necessary to maintain a moist, yet not overly saturated rooting medium throughout the experimental period. Necessarily, the plants in the cooler chambers were watered less frequently because they utilized less water than those in the warm chambers. After equilibration in growth chambers, each individual plant was cut back to the ground. We left 5 tillers on each plant to insure that our cut-back treatment did not kill the plants. These preexisting tillers were marked so they would not be used for gas exchange or harvested during the experiment. Following establishment of at least 5 new tillers the preexisting tillers were removed. Once all gas exchange and chlorophyll fluorescence measurements were complete all the shoots produced in the growth chambers were harvested, dried to a consistent weight and summed to determine

total dry mass produced over the experimental period. The experiment was repeated in immediate succession for the two untested transgenic events.

2.8. Chlorophyll fluorescence and gas exchange

All fluorescence and gas exchange measurements were made on the most recently mature leaf of newly formed shoots (third or fourth leaf from the apex). To determine the response of photoinhibition (F_v/F_m), electron transport rate (ETR), non-photochemical quenching (qN), and quantum yield of photosystem II (Yield) to the ambient growth conditions, chlorophyll fluorescence was measured on three dark-adapted leaves from all individuals. Leaves were dark-adapted for 20 min prior to measuring fluorescence kinetics using a pulse-modulated fluorometer (OS-500, Opti-Sciences, New Hampshire, USA). The shorted dark-adaptation period was justified because of the low light intensity of the growth conditions prior to dark adaptation. A pulse of saturating radiation was used to measure F_v/F_m. The difference in the minimal (F_o) and the maximum (F_m) flourecsence yield divided by the maximum fluorescence yield for a dark-adapted leaf ($(F_m - F_o)/F_m$) was recorded as the F_v/F_m value. Following the pulse of saturating light an actinic light source was initiated. The yield (an indicator of photosystem II quantum yield) was determined from the steady state (F_s) and the maximal (F_{ms}) fluorescence during a saturating pulse for the leaf experiencing actinic light (Yield = $(F_{ms} - F_s)/F_{ms}$). Non-photochemical quenching (qN) was determined as $(F_s - F_{ms})/(F_m - F_o)$. The electron transport rate (ETR) was calculated as yield × PPFD × 0.5 × 0.84, where PPFD = flux density of incident photosynthetically active radiation ($\mu mol\ m^{-2}\ s^{-1}$); 0.5 refers to the need for two quanta for each electron transported; 0.84 refers to the average of 84% of incident quanta are absorbed by the leaf.

An open path gas-exchange system (LI-6400; Li-Cor Inc., Lincoln, Nebraska) in which relative humidity was between 55 and 75% and leaf temperature was maintained at the respective growth temperature (28 °C and 14 °C) was used to measure net photosynthetic parameters. Leaves were allowed to acclimate to cuvette conditions for 5 min prior to initiating photosynthetic response programs. The photosynthetic response to light was measured for one leaf from all plants in all chambers and followed the protocol established in the LI6400 software (A/Q curve). The protocol began with reaching a stable photosynthetic rate at 400 $\mu mol\ mol^{-1}$ CO_2 at PPFD = 2000 $\mu mol\ m^{-2}\ s^{-1}$, followed by decreasing PPFD values (2000, 1500, 1000, 800, 500, 300, 200, 100, 80, 50, 20, 10, 0 $\mu mol\ m^{-2}\ s^{-1}$). Transitions between PPFD values were made once photosynthesis was stable for at least 5 min at each level. Light response parameters (A_{sat} = light saturated rate of photosynthesis, Q_{comp} = light compensation point, Q_{sat} = light saturation point, AQE = apparent quantum efficiency, R_d = dark respiration rate) were calculated for each A/Q curve using photosynthetic response curve fitting software (Li-Cor Inc., Nebraska, USA). The photosynthetic response to CO_2 concentration (A/C_i curve) was measured for all plants in all chambers at saturating light, which varied depending on the species and growth temperature (PPFD was about 1000 $\mu mol\ m^{-2}\ s^{-1}$ for 14 °C and about 1500 $\mu mol\ m^{-2}\ s^{-1}$ for 28 °C conditions). The A/C_i protocol (as defined by the LI-Cor 6400 software) includes initial equilibration at 400 $\mu mol\ mol^{-1}$ CO_2 followed by decreasing and then increasing equilibration concentration steps for the sample CO_2 (400, 300, 200, 100, 50, 40, 40, 400, 400, 600, 800, 1000, 1200, 1400, 1600 $\mu mol\ mol^{-1}$ CO_2). Transitions between CO_2 concentrations levels were made after the photosynthetic rate remained constant for at least 5 min. Gas exchange parameters (A_{max} = CO_2 saturated photosynthetic rate, CE = carboxylation efficiency, V_{cmax} = maximum potential photosynthesis, J_{max} = maximum electron flow, TPU = triose phosphate use rate) were calculated using Photosynthesis Assistant (Dundee Scientific Ltd., Scotland, UK).

2.9. Statistical treatment

The results from the two experiments were lumped because the same growth chambers were used by both experiments, the same control line was used for both experiments and the experiments occurred in rapid succession. Two-way ANOVA was used to test for the effects of treatment (df = 1), line (df = 4), or their interaction (df = 4) on productivity, A/Q response curve parameters, fluorescence parameters, and A/C_i response curve parameters (n = 80 plants). Significant differences between lines or temperature treatments within line were performed with the Tukey HSD test ($p < 0.05$). All statistical analyses were performed with JMP (JMP®, Version 11 for Windows 7, SAS Institute Inc., Cary, NC).

3. Results

3.1. Molecular characterization of switchgrass plants overexpressing C4ppdk1

Two of the four transgenic events in which the C4ppdk1 was stably integrated, were characterized. Each characterized line had 4 copies of the T-DNA (Fig. 1). The transgene was transcribed at drastically different levels in the two events. Event A1 highly expressed the transgene, with transcript abundance more than twice that of the *PvUbi1* reference gene. The second event, D1, had much lower transgene expression, with transcript abundance at about half that of *PvUbi1*, and approximately 4× lower than transgenic event A1 (Fig. 2).

3.2. Aboveground biomass characterization

There was a significant effect of treatment (warm vs cold) and line (transgenic lines) on total above ground biomass accumulated by the end of the experimental period (Table 1). However, the interaction between treatment and line was not significant. All lines had significantly different aboveground biomass accumulation between growth temperature treatments (Table 2). Transgenic lines A1 and S1 produced significantly less aboveground biomass than the control group in both the warm and cool growth treatment (p values both <0.0001).

3.3. Fluorescence characterization of transgenic switchgrass events

There was no significant effect of treatment, line or their interaction on the indices of photoinhibition (F_v/F_m), electron transport rate (ETR) or quantum yield (Yield) under ambient growth conditions (Table 3). However, there was a highly significant effect of treatment on non-photochemical quenching (qN) under ambient growth conditions (Table 3).

There were no significant differences in the index of photoinhibition (F_v/F_m) among any pair of lines at either the 14 °C or 28 °C growth conditions (Table 4). However, each line and treatment group showed some degree of photoinhibition because the values of all treatments and lines were below 0.8. The value of F_v/F_m for plants in the cool growth temperature was significantly higher than in the warm treatment only for transgenic lines A1 and C. There were no significant differences in ETR or Yield among any plant lines at either the 14 °C or the 28 °C growth conditions (Table 4). There were no significant differences in qN among any plant lines at either the 14 °C or 28 °C growth conditions (Table 4). The qN was significantly lower in the higher temperature treatment for all lines except the control.

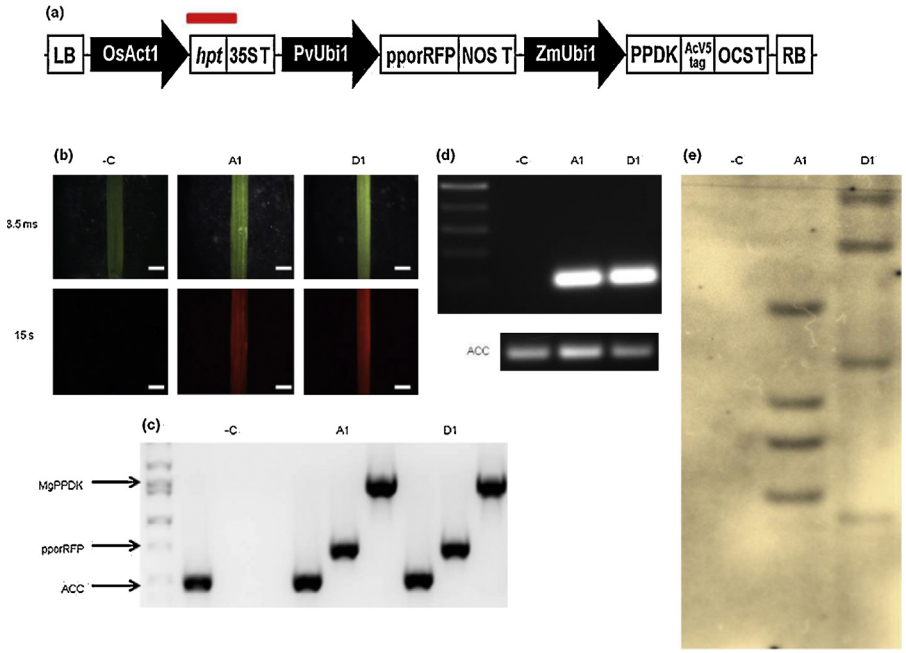

Fig. 1. Transgenic plant analysis. (a) Schematic representation of the T-DNA used to transform switchgrass. Orange fluorescent protein (pporRFP; OFP) and hygromycin resistance were used for selection. The gene of interest cassette is fused to an AcV5 epitope tag. (b) Plants regenerated from positive 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.

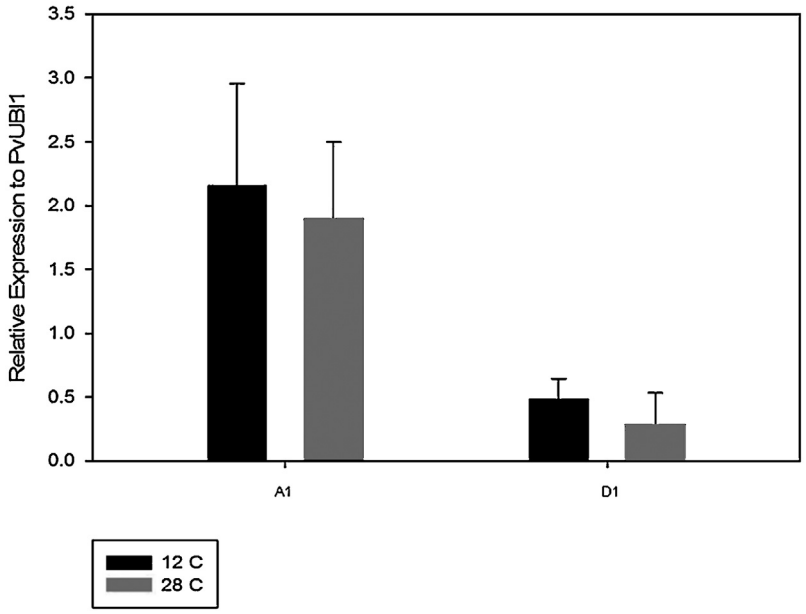

Fig. 2. Relative transcript abundance of *C4ppdk1* to *PvUBI1* in warm (28 °C) and cool (12 °C) conditions for two transgenic events (A1, D1) of Alamo Switchgrass.

Table 1
Results of two-way ANOVA for the effects of treatment (warm vs cold) and line (different transgenic lines) and their interaction on above ground biomass accumulation (final biomass). *p* values for significant effects are shown in bold.

Effect	DF	*F* ratio	*p* value
Final biomass			
Treatment	1	108.8	**<0.0001**
Line	4	18.3	**<0.0001**
Treatment * line	4	0.8	0.555

3.4. Gas exchange characterization of transgenic switchgrass plants

There was a significant effect (Table 5) of growth treatment, plant line and their interaction on Q_{sat}. There was a significant effect of plant line on Q_{conp} (Table 5). There were no significant effects of treatment, plant line or their interaction on AQE. Light saturated photosynthesis (A_{sat}) was highly significantly affected by both growth treatment and plant line. Yet the interaction term was not significant (Table 5). Treatment had a significant effect on R_d, but the effects of plant line and the interaction term were not significant.

Table 2
Means and standard deviation for biomass accumulated for lines of switchgrass transformed with C4ppdk1. C = non-transformed control; A1, D1, D2 and S1 are four transformation events. $N = 8$ treatment^{-1} line^{-1} for all parameters. Different small letters indicate significant differences at the alpha < 0.05 level among the transformed lines at each treatment temperature based on post hoc Tukey HSD. Significant p values for comparisons between treatments are shown in bold.

Parameter	C	A1	D1	D2	S1
Final weight 14 °C (g)	33.0 ± 8.0a	15.9 ± 3.6b	26.3 ± 6.1a	28.0 ± 5.0a	13.8 ± 7.3b
Final weight 28 °C (g)	57.9 ± 7.1a	31.7 ± 7.8b	50.6 ± 9.7a	53.2 ± 10.8a	33.5 ± 19.4b
p values between treatments	**0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.0170**

Table 3
Results of two-way ANOVA for the effects of treatment (warm vs cold) and line (different transgenic lines of switchgrass transformed with C4ppdk1) and their interaction on fluorescence parameters. p values for significant effects are shown in bold.

Effect	DF	F ratio	p value
Photoinhibition (F_v/F_m)			
Treatment	1	2.012	0.1605
Line	4	1.136	0.3469
Treatment * line	4	2.246	0.0728
Electron transport rate (ETR)			
Treatment	1	3.587	0.0624
Line	4	1.849	0.1293
Treatment * line	4	0.347	0.8451
Non photochemical quenching (qN)			
Treatment	1	31.32	**<0.0001**
Line	4	0.422	.7922
Treatment * line	4	1.803	.1379
Quantum yield (Y)			
Treatment	1	3.582	0.0625
Line	4	1.849	0.1293
Treatment * line	4	0.3471	0.8452

The Q_{sat} of all plant lines was significantly higher in the 28 °C growth condition than the 14 °C growth condition (Table 6). There were no significant differences in Q_{comp} among lines or treatments (Table 6). Two of the five plant lines had a significantly higher AQE under 28 °C growth conditions compared with 14 °C growth conditions (Table 6). The A_{sat} was significantly higher for all plant lines in the 28 °C growth conditions compared with the 14 °C growth conditions. There was no apparent difference in R_d among any of the lines (Table 6). Only line A1 had a significantly greater R_d in the 28 °C growth condition compared to the 14 °C.

There was a significant effect of treatment, line and the interaction on CE and on V_{cmax} (Table 5). Both the treatment and the interaction between treatment and line were significant for A_{max}. In contrast, only treatment had a significant effect on J_{max}, TPU, and A_{max}/J_{max}.

Lines C, A1 and D1 had a significantly higher CE under warm growth conditions (Table 6). All lines except S had a significantly higher A_{max} in the 28 °C growth conditions compared with the 14 °C. Both V_{cmax} and J_{max} of all lines were significantly higher in the 28 °C growth conditions compared with the 14 °C. Three of the lines had a significantly higher TPU under the 28 °C growth conditions compared with 14 °C. The TPU of lines D1, D2 and S were significantly lower than the control line at 28 °C ($p = 0.0391$, 0.0436, 0.0371 respectively). The A_{max}/J_{max} ratio was not different among lines at either the 14 °C or 28 °C growth conditions. Also, only the control and line A1 had a significantly higher A_{max}/J_{max} ratio under the 28 °C growth condition compared with the 14 °C growth conditions.

4. Discussion

4.1. Addressing hypothesis 1

Overall, the transgenic lines displayed shorter and more fragile appearing shoots than the non-transformed control line. The average productivity of the transgenic lines and the non-transformed control were about 60% lower under cool conditions, which is the same result as was found for non-genetically manipulated Alamo switchgrass in the same chambers and growth conditions [13]. The transgenic line with documented high C4ppdk1 expression (A1) had significantly lower productivity than the line with much lower expression of C4ppdk1 (D1). This result suggests that overexpressing C4ppdk1 caused a reduction in productivity. Perhaps that reduction was related to the energetic

Table 4
Means and standard deviation for mean fluorescence parameters for lines of switchgrass transformed with C4ppdk1. C = non-transformed control; A1, D1, D2, S1 are four transformation events. F_v/F_m = index of photoinhibition, ETR = electron transport rate, qN = non-photochemical quenching, Yield = quantum yield. $N = 8$ treatment^{-1} line^{-1} for all parameters. Different superscript letters indicate significant differences at the alpha < 0.05 level among the transformed lines based on post hoc Tukey HSD. Significant p values for comparisons between treatments are shown in bold.

Parameter	C	A1	D1	D2	S1
F_v/F_m 14 °C	0.729 ± 0.013a	0.723 ± 0.008a	0.711 ± 0.010a	0.710 ± 0.022a	0.714 ± 0.023a
F_v/F_m 28 °C	0.712 ± 0.009a	0.708 ± 0.007a	0.709 ± 0.040a	0.716 ± 0.010a	0.718 ± 0.070a
Treatments	**0.0426**	**0.0010**	0.7063	0.4964	0.6942
ETR 14 °C (μmol m^{-2} s^{-1})	186.7 ± 17.6a	204.6 ± 31.7a	185.5 ± 19.3a	185.6 ± 26.6a	178.2 ± 38.0a
ETR 28 °C (μmol m^{-2} s^{-1})	198.1 ± 16.2a	207.9 ± 18.0a	204.6 ± 20.2a	188.4 ± 26.8a	192.9 ± 18.1a
Treatments	0.2014	0.8024	0.0551	0.8368	0.3358
qN 14 °C	0.353 ± 0.099a	0.381 ± 0.089a	0.353 ± 0.102a	0.351 ± 0.126a	0.426 ± 0.083a
qN 28 °C	0.295 ± 0.054a	0.286 ± 0.085a	0.244 ± 0.030a	0.291 ± 0.061a	0.230 ± 0.055a
Treatments	0.1642	**0.0468**	**0.0118**	**0.02395**	<**0.0001**
Yield 14 °C	0.370 ± 0.035a	0.406 ± 0.063a	0.368 ± 0.038a	0.368 ± 0.053a	0.353 ± 0.075a
Yield 28 °C	0.393 ± 0.032a	0.412 ± 0.036a	0.406 ± 0.040a	0.373 ± 0.053a	0.383 ± 0.036a
Treatments	0.2017	0.8032	0.0552	0.8369	0.3359

Table 5
Results of two-way ANOVA for the effects of treatment (warm vs cold) and line (different transgenic lines of switchgrass transformed with C4ppdk1) and their interaction on parameters derived from light and carbon dioxide response curves. Q_{sat} = light saturation point, Q_{comp} = light compensation point, AQE = apparent quantum efficiency, A_{sat} = light saturated photosynthetic rate, R_d = dark respiration rate, CE = carboxylation efficiency, A_{max} = carbon dioxide saturated photosynthetic rate, V_{cmax} = maximum potential photosynthesis, J_{max} = maximum electron flow rate, TPU = triose phosphate use rate, A_{max}/J_{max} = saturated rate of photosynthesis relative to maximum electron flow rate. p values for significant effects are shown in bold.

Effect	DF	F ratio	p value
Q_{sat} (μmol m^{-2} s^{-1})			
Treatment	1	154.6	**<0.0001**
Line	4	4.0	**0.0056**
Treatment * line	4	9.7	**<0.0001**
Q_{comp} (μmol m^{-2} s^{-1})			
Treatment	1	2.1	0.1566
Line	4	2.9	**0.0300**
Treatment * line	4	1.3	0.3457
AQE			
Treatment	1	2.5	0.1188
Line	4	1.3	0.2927
Treatment * line	4	0.3	0.8575
A_{sat} (μmol m^{-2} s^{-1})			
Treatment	1	114.1	**<0.0001**
Line	4	9.9	**<0.0001**
Treatment * line	4	1.8	0.1305
R_d (μmol m^{-2} s^{-1})			
Treatment	1	9.6	**0.0028**
Line	4	1.4	0.2436
Treatment * line	4	0.7	0.6001
CE (μmol m^{-2} s^{-1} Pa^{-1})			
Treatment	1	22.28	**<0.0001**
Line	4	2.71	**0.0378**
Treatment * line	4	2.56	**0.0470**
A_{max} (μmol m^{-2} s^{-1})			
Treatment	1	92.69	**<0.0001**
Line	4	1.77	0.1441
Treatment * line	4	4.99	**0.0014**
V_{cmax} (μmol m^{-2} s^{-1})			
Treatment	1	110.44	**<0.0001**
Line	4	3.45	**0.0128**
Treatment * line	4	3.40	**0.0139**
J_{max} (μmol m^{-2} s^{-1})			
Treatment	1	120.5	**<0.0001**
Line	4	1.41	0.2419
Treatment * line	4	2.46	0.0542
TPU (μmol μmol^{-1})			
Treatment	1	12.22	**0.0009**
Line	4	2.16	0.0830
Treatment * line	4	3.04	**0.0232**
A_{max}/J_{max}			
Treatment	1	7.71	**0.0072**
Line	4	0.90	0.4672
Treatment * line	4	2.49	0.0519

costs of producing excess C4ppdk1. The higher R_d for transgenic event A1 supports this idea. Therefore, our first hypothesis was rejected because overexpressing C4ppdk1 did not improve cool condition productivity compared to the non-transformed control or non-GMO Alamo switchgrass.

4.2. Addressing hypothesis 2

All groups experienced higher levels of photoinhibition (F_v/F_m decreased) under the cooler temperatures, as would be expected with higher stress conditions. The mean F_v/F_m values were lower than those for non-GMO Alamo switchgrass [13], which indicated that the transformed lines and non-transformed control experienced slightly higher photoinhibition than non-GMO switchgrass. Although all lines had lower F_v/F_m under cool growing conditions, those differences were small and only significant in two cases (C, A1). There is minimal effect of cool growing conditions on photoinhibition. Because electron transport activity and biochemical reactions tend to be balanced, we expected that the ETR and Yield would be inhibited by the cool temperature conditions in cases where lower rates of assimilation were observed. The lack of inhibition of these parameters by cold, in cases when assimilation was inhibited, suggests that the limitation on photosynthesis under low temperatures is primarily in the CO_2 fixation pathway and not in the electron transport processes. The higher than expected rates of ETR and Yield under cool growth conditions that were not coordinated with CO_2 fixation results in an excess of free electrons, which must be dissipated via means such as non-photochemical quenching. The qNs of these transgenic lines were larger than those found for non-GMO Alamo switchgrass [13], which also supports the conclusion that the transformation process reduced the biochemical processes of photosynthesis more than the electron transport processes. As a result of this imbalance, qN was higher to help dissipate the excess radiation absorbed.

These low light saturation points of the transgenic lines found in this study are not a product of photoinhibition of PSII as indicated by the F_v/F_m values, which are relatively the same as the nontransgenic control. The low Q_{sat} values can be attributed to the low levels of PPFD in the growth chambers. These Q_{sat} values are considerably lower than those for non-GMO Alamo switchgrass under the same growing conditions [13]. The genetic manipulation used to generate these transgenic events caused a reduced ability to utilize high radiation, which has resulted in acclimation to the low radiation environment similar to that found for giant miscanthus under the same growing conditions [13]. The same significant differences between warm and cool A_{sat} rates in the transgenic groups as well as the control suggests that there is no effect of overexpression of the C4ppdk1 gene on light saturated photosynthetic rates in relation to the temperature treatments used here. Also, the A_{sat} rates for these transgenic lines are much lower than that for non-GMO switchgrass (10.3 μmol m^{-2} s^{-1} at 14 °C and 18.0 μmol m^{-2} s^{-1} at 28 °C) found in the earlier study [13]. These data contrast with those for giant miscanthus, which has been shown not to experience any significant decrease in A_{sat} in response to cool growth conditions at either high or low radiation conditions [13,16]. Similarly, there were no differences in the AQE response to temperature either. This lack of difference in AQE further suggests that the limitation to C_4 photosynthesis is not in the electron transport chain, but is located in the CO_2 fixation pathway.

The photosynthetic parameters measured in this study are considerably lower than those measured for varieties of switchgrass growing in high light environments [28,29]. Therefore, our second hypothesis was accepted. In fact, the A_{sat} values found in this study at low radiation are very similar to those found in other studies of non-GMO switchgrass varieties at low radiation [30].

4.3. Addressing hypothesis 3

PPDK does not play a direct role in O_2 evolution, because it is involved in the non-light dependent reactions of photosynthesis. Thus, it is unexpected that overexpression of this gene would result in decreasing the light saturation point. Rather, antibiotic selection stress or even epigenetic variation might have caused the differences [31,32]. Photosynthetic capacity of a C_4 plant would be directly affected by the activity of PPDK in the mesophyll cell chloroplasts. Phosphoenolpyruvate 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 CO_2 saturated photosynthetic capacity (A_{max}) of the transgenic events would be higher than that of the non-transformed control. In fact he A_{max} values found for transgenic lines in this study were all higher than the control at 14 °C. Yet, except for line S1, these differences were not significant.

Table 6
Means and standard deviation for mean photosynthetic parameters from light and carbon dioxide response curves for lines of switchgrass transformed with C4ppdk1. C = non-transformed control; A1, D1, D2, S1 are four transformation events. Q_{sat} = light saturation point, Q_{comp} = light compensation point, AQE = apparent quantum efficiency, A_{sat} = light saturated photosynthetic rate, R_d = dark respiration rate, CE = carboxylation efficiency, A_{max} = carbon dioxide saturated photosynthetic rate, V_{cmax} = maximum potential photosynthesis, J_{max} = maximum electron flow rate, TPU = triose phosphate use rate, A_{max}/J_{max} = saturated rate of photosynthesis relative to maximum electron flow rate. $N = 8$ treatment^{-1} line^{-1} for all parameters. Different superscript letters indicate significant differences at the alpha < 0.05 level among the transformed lines based on post hoc Tukey HSD. Significant p values for comparisons between treatments are shown in bold.

Parameter	C	A1	D1	D2	S1
Q_{sat} 14 °C (μmol m^{-2} s^{-1})	181.8 ± 78.64a	101.2 ± 16.9a	244.4 ± 110.7a	169.9 ± 69.19a	89.1 ± 16.3b
Q_{sat} 28 °C (μmol m^{-2} s^{-1})	700.7 ± 291.9a	666.8 ± 150.8a	361.4 ± 59.4b	355.4 ± 92.6b	616.9 ± 217.5a
Treatments	**0.0003**	**<0.0001**	**0.0206**	**0.0005**	**<0.0001**
Q_{comp} 14 °C (μmol m^{-2} s^{-1})	12.5 ± 3.7a	12.2 ± 3.5a	9.5 ± 2.0a	11.0 ± 2.8a	12.8 ± 1.3a
Q_{comp} 28 °C (μmol m^{-2} s^{-1})	12.5 ± 3.7a	14.5 ± 3.0 a	10.5 ± 2.1a	13.5 ± 3.0a	12.6 ± 3.6a
Treatments	0.5357	0.1545	0.5720	0.1066	0.8819
AQE 14 °C	0.047 ± 0.008a	0.046 ± 0.009a	0.078 ± 0.007a	0.064 ± 0.032a	0.063 ± 0.020a
AQE 28 °C	0.065 ± 0.007a	0.062 ± 0.010a	0.074 ± 0.027a	0.093 ± 0.009a	0.076 ± 0.029a
Treatments	**0.0007**	**0.0054**	0.8945	0.4258	0.3233
A_{sat} 14 °C (μmol m^{-2} s^{-1})	4.13 ± 1.46a	4.13 ± 1.17a	2.99 ± 0.98a	2.51 ± 0.50a	4.53 ± 0.74a
A_{sat} 28 °C (μmol m^{-2} s^{-1})	9.25 ± 3.22a	8.79 ± 1.04 a	5.81 ± 1.03b	5.52 ± 1.02b	7.67 ± 2.43a
Treatments	**0.0011**	**<0.0001**	**<0.0001**	**<0.0001**	**0.0041**
R_d 14 °C (μmol m^{-2} s^{-1})	−0.59 ± 0.19a	−0.55 ± 0.14a	−0.55 ± 0.22a	−0.62 ± 0.22a	−0.79 ± 0.22a
R_d 28 °C (μmol m^{-2} s^{-1})	−0.70 ± 0.16a	−0.81 ± 0.17a	−0.72 ± 0.22a	−0.91 ± 0.50a	−0.82 ± 0.26a
Treatments	0.2213	**0.0044**	0.2092	0.1583	0.7758
CE 14 °C (μmol m^{-2} s^{-1} Pa^{-1})	0.28 ± 0.28^a	0.15 ± 0.07^a	0.19 ± 0.22^a	0.16 ± 0.09^a	0.10 ± 0.07^a
CE 28 °C (μmol m^{-2} s^{-1} Pa^{-1})	5.57 ± 4.85^a	2.42 ± 1.44^a	1.26 ± 0.86^b	2.11 ± 2.64^a	1.70 ± 2.23^b
Treatments	**0.0136**	**0.0008**	**0.0057**	0.0549	0.0614
A_{max} 14 °C (μmol m^{-2} s^{-1})	4.84 ± 0.29^a	5.09 ± 0.70^a	5.60 ± 1.34^a	5.21 ± 0.71^a	7.10 ± 3.3^b
A_{max} 28 °C (μmol m^{-2} s^{-1})	17.78 ± 6.12^a	14.31 ± 4.71^a	11.21 ± 2.28^a	11.86 ± 3.80^a	9.82 ± 3.95^b
Treatments	**0.0001**	**<0.0001**	**0.0001**	**0.0003**	0.1574
V_{cmax} 14 °C (μmol m^{-2} s^{-1})	4.54 ± 1.30^a	4.14 ± 0.70^a	3.35 ± 1.10^b	4.06 ± 0.78^a	4.03 ± 0.59^a
V_{cmax} 28 °C (μmol m^{-2} s^{-1})	46.83 ± 23.1^a	44.60 ± 19.48^a	24.68 ± 4.15^a	27.11 ± 17.67^a	22.43 ± 12.0^b
Treatments	**0.0004**	**<0.0001**	**<0.0001**	**0.0024**	**0.0007**
J_{max} 14 °C (μmol m^{-2} s^{-1})	14.51 ± 2.21^a	14.95 ± 1.29^a	13.63 ± 3.44^a	15.81 ± 3.18^a	15.61 ± 2.26^a
J_{max} 28 °C (μmol m^{-2} s^{-1})	28.56 ± 5.11^a	29.76 ± 8.74^a	27.94 ± 4.73^a	27.20 ± 5.62^a	21.40 ± 5.51^a
Treatments	**<0.0001**	**0.0003**	**<0.0001**	**0.0002**	**0.0157**
TPU 14 °C (μmol μmol^{-1})	1.54 ± 0.62^a	1.87 ± 0.50^a	3.88 ± 0.73^a	1.35 ± 0.31^a	1.84 ± 0.93^a
TPU 28 °C (μmol μmol^{-1})	19.23 ± 2.31^a	9.56 ± 2.83^a	2.90 ± 1.08^a	5.05 ± 2.90^a	4.67 ± 2.06^a
Treatments	0.0657	**<0.0001**	0.7761	**0.0029**	**0.0033**
A_{max}/J_{max} 14 °C	0.341 ± 0.068^a	0.340 ± 0.040^a	0.438 ± 0.184^a	0.340 ± 0.080^a	0.466 ± 0.231^a
A_{sat}/J_{max} 28 °C	0.647 ± 0.269^a	0.480 ± 0.080^a	0.403 ± 0.069^a	0.455 ± 0.206^a	0.460 ± 0.157^a
Treatments	**0.0130**	**0.0008**	0.6948	0.1607	0.9574

The CO_2 saturated rates of photosynthesis (A_{max}) decreased substantially in cold temperatures for the control and three of the four transgenic lines. There was no significant decrease in A_{sat} at cold temperature for transgenic line S1, which had the lowest A_{max} among lines at 28 °C and the highest among lines at 14 °C. Although the similarity between 28 °C and 14 °C A_{max} values for line S1 suggests that overexpressing C4ppdk1 affected the cold response of photosynthesis, this similarity between temperatures was not observed in the rest of the A/C_i response variables for line S1.

Among the photosynthetic parameters derived from the A/C_i response curves, carboxylation efficiency (CE) is indirectly indicative of PPDK response to temperature, due to this region of the response curve being limited by PEPc activity [33]. CE, however, is unable to distinguish between limitations due to PEPc activity and Rubisco activity. Kubien and Sage [34] demonstrated Rubisco's limiting effect on CO_2 assimilation with C_4 *Flaveria bidentis*. Transformants of *F. bidens* with reduced Rubisco content had significantly reduced photosynthesis at cool temperature yet the in vitro k_{cat} of Rubisco matched the in vivo k_{cat}. Similar results were shown by Sage [35] where Rubisco activity in vivo matched the rate of CO_2 assimilation at temperatures below 20 °C. Therefore, cool temperature inhibition of C_4 is not due to an inactivation of Rubisco. In this study, all

transgenic lines and the control line had a significant decrease in CE in the cool growing conditions, indicating that the C4ppdk1 transformation did not enhance PPDK performance at 14 °C/12 °C relative to the control. Also, the CE of two transgenic lines (D1, S1) was substantially less than the control group suggesting that the transformation may have had negative effects on the CO_2 fixation apparatus.

The ratio of A_{max}/J_{max} gives the amount of CO_2 fixed per electron from PSII, giving a relative measure of electron use efficiency. The control line had values, in both growth temperature conditions, similar to those found for non-GMO Alamo switchgrass (switchgrass $A_{sat}/J_{max} = 0.37 \pm 0.155$ at 14 °C/12 °C and 0.84 ± 0.332 at 28 °C/25 °C) while the transgenic lines showed A_{sat}/J_{max} values substantially lower. However, line S1 showed very similar values as miscanthus [13] in a previous experiment (0.44 ± 0.187 at 14 °C/12 °C; 0.42 ± 0.172 at 28 °C/25 °C) also with no significant difference between temperatures. Transgenic line A1 showed a significantly lower A_{sat}/J_{max} in the cool conditions than in the warm. These results may indicate that the transformation event creating line S1) decreased the inhibition of assimilation by cool temperatures. Along with the lack of temperature effect on the A_{max}, the results for line S1 support our third hypothesis that overexpression of C4ppdk1 reduces cool temperature limitation of photosynthesis. The response of line S1 to cool growing conditions is a glimmer of hope that overexpression of C4ppdk1 in switchgrass could reduce low temperature inhibition of photosynthesis.

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 [36]. 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 (R_d) was not observed except for line A1, which when taken along with other data discussed, is a clear indication that the hypothesized phenotype of transgenic lines is not being produced. This could be due to the underlying complexity of the C_4 pathway, or due to something more straightforward. Because C4ppdk1 cDNA was used in this study, the regulatory and/or functional aspects of the genomic introns or other endogenous regulatory elements native to C4ppdk1 within the giant miscanthus genome were not utilized. Introns play an important role in gene expression [37], and as C4ppdk1 is expressed with tissue and cell-specificity, this could be a source of inconsistency between our switchgrass data and that of giant 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 [38], increased provitamin A in tomatoes [39], and color change in petunia flowers [40]. 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 and photosynthesis, 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 C_4 photosynthesis is not controlled by PPDK alone [11]. However, it is possible that transforming switchgrass with transcription factors that regulate multiple genes associated with cold tolerance may provide a higher possibility of increasing productivity under cool conditions.

It has been clearly shown that the expression of PPDK is up-regulated in giant miscanthus under cold conditions [15], a mechanism that surely contributes to the overall productivity of the plant under these conditions. Nonetheless, the transgenic switchgrass data presented here suggests that there are more factors to be considered in determining the underlying physiology behind giant miscanthus' increased productivity under cool conditions. A recent study in sorghum, for instance, identified 20 different quantitative trait loci (QTL) related to grain yield [41]. 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 giant miscanthus [42]. While the expression patterns of PPDK surely play a role in the productivity of giant miscanthus, a deeper evaluation of genome wide expression is necessary to more thoroughly understand the ability of this feedstock to outgrow its competitors.

5. Conclusions

Overexpression of C4ppdk1 in transgenic switchgrass was generally not sufficient to endow increased cold-temperature photosynthesis in all transgenic lines. However, one transgenic line (S1) had some photosynthetic traits that supported the notion that overexpressing C4ppdk1 in switchgrass could increase photosynthesis and productivity. In both the high and low expressing transgenic switchgrass events photosynthetic parameters were decreased under cold temperature, more so for the event with higher C4ppdk1 expression, but overall photosynthesis was not increased relative to the control plant. Therefore, improving cold temperature photosynthesis in switchgrass will require more than manipulating the expression of a single gene.

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References

[1] R.A. Kerr, R.F. Service, What can replace cheap oil – and when, Science 309 (2005) 101.
[2] S.B. McLaughlin, L.A. Kszos, Development of switchgrass (*Panicum virgatum*) as a bioenergy feedstock in the United States, Biomass Bioenergy 28 (2005) 515–535.
[3] B.H. Davison, S.R. Drescher, G.A. Tuskan, M.F. Davis, N.P. Nghiem, Variation of S/G ratio and lignin content in a *Populus* family influences the release of xylose by dilute acid hydrolysis, Appl. Biochem. Biotechnol. 129–132 (2006) 427–435.
[4] M. Nageswara-Rao, J.R. Soneji, C. Kwit, C.N. Stewart Jr., Advances in biotechnology and genomics of switchgrass, Biotechnol. Biofuels 6 (2013) 77.
[5] Q. Ji, X. Xu, K. Wang, Genetic transformation of major cereal crops, Int. J. Dev. Biol. 57 (2013) 495–508.
[6] A.T. Groover, Will genomics guide a greener forest biotech? Trends Plant Sci. 12 (2007) 234–238.
[7] R. Kebeish, M. Niessen, K. Thiruveedhi, R. Bari, H.J. Hirsch, R. Rosenkranz, N. Stäbler, B. Schönfeld, F. Kreuzaler, C. Peterhänsel, Chloroplastic photorespiratory bypass increases photosynthesis and biomass production in *Arabidopsis thaliana*, Nat. Biotechnol. 25 (2007) 593–599.
[8] C.J. Lawrence, V. Walbot, Translational genomics for bioenergy production from fuelstock grasses: maize as the model species, Plant Cell 19 (2007) 2091–2094.
[9] R.F. Sage, T.L. Sage, F. Kocacinar, Photorespiration and the evolution of C_4 photosynthesis, Ann. Rev. Plant Biol. 63 (2012) 19–47.
[10] A. Bilska, P. Sowinski, Closure of plasmodesmata in maize (*Zea mays*) at low temperature: a new mechanism for inhibition of photosynthesis, Ann. Bot. Lond. 106 (2010) 675–686.
[11] F.G. Dohleman, S.P. Long, More productive than maize in the Midwest: how does Miscanthus do it? Plant Physiol. 150 (2009) 2104–2115.
[12] S.L. Naidu, S.P. Long, Potential mechanisms of low-temperature tolerance of C_4 photosynthesis in *Miscanthus* × *giganteus*: an in vivo analysis, Planta 220 (2004) 145–155.
[13] J.L.B. Mitchell, M. Halter, C.N. Stewart Jr., E.T. Nilsen, Cool temperature effects on photosynthetic parameters of two biomass fuel feedstocks in a low light

intensity environment: low light intensity alters the significance of cold tolerance to productivity in cool climates, Biofuels 5 (2014) 533–544.
[14] E. Heaton, F.G. Dohleman, A. Fernando Miguez, J.A. Juvik, V. Lozowaya, J. Widholm, O.A. Zabotina, G.F. Mcisaac, M.B. David, T.B. Voigt, N.N. Boersma, S.P. Long, Miscanthus: a promising biomass crop, in: J.C. Kader, M. Delseny (Eds.), Advances in Botanical Research, vol. 56, Academic Press Ltd./Elsevier Science Ltd., London, 2010, pp. 75–137.
[15] D. Wang, A.R. Portis Jr., S.P. Moose, S.P. Long, Cool C_4 photosynthesis: pyruvate Pi dikinase expression and activity corresponds to the exceptional cold tolerance of carbon assimilation in *Miscanthus* × *giganteus*, Plant Physiol. 148 (2008) 557–567.
[16] S.L. Naidu, S.P. Moose, A.K. Al-Shoaibi, C.A. Raines, S.P. Long, Cold tolerance of C_4 photosynthesis in *Miscanthus* × *giganteus*: adaptation in amounts and sequence of C_4 photosynthetic enzymes, Plant Physiol. 132 (2003) 1688–1697.
[17] D. Wang, Can the cold tolerance of C_4 photosynthesis in *Miscanthus* × *giganteus* relative to *Zea mays* be explained by differences in activities and thermal properties of Rubisco? J. Exp. Bot. 59 (2008) 1779–1787.
[18] F.T.S. Nogueira, V.E. De Rosa Jr., M. Menossi, E.C. Ulian, P. Arruda, RNA expression profiles and data mining of sugarcane response to low temperature, Plant Physiol. 132 (2003) 1811–1824.
[19] Y.-C. Du, A. Nose, K. Wasano, Effects of chilling temperature on photosynthetic rates, photosynthetic enzyme activities and metabolite levels in leaves of three sugarcane species, Plant Cell Environ. 22 (1999) 317–324.
[20] C. Fu, J.R. Mielenz, X. Xiao, Y. Ge, C.Y. Hamilton, M. Rodriguez Jr., F.M. Chen, A. Ragauskas, J. Bouton, R.A. Dixon, Z.-Y. Wang, Genetic manipulation of lignin reduces recalcitrance and improves ethanol production from switchgrass, Proc. Natl. Acad. Sci. U.S.A. 108 (2011) 3803–3808.
[21] H. Shen, H. Shen, C.R. Poovaiah, A. Ziebell, T.J. Tschaplinski, S. Pattathil, E. Gjersing, N.L. Engle, R. Katahira, Y. Pu, R. Sykes, F. Chen, A.J. Ragauskas, J.R. Mielenz, M.G. Hahn, M. Davis, C.N. Stewart Jr., R.A. Dixon, Enhanced characteristics of genetically modified switchgrass (*Panicum virgatum* L.) for high biofuel production, Biotechnol. Biofuels 6 (2013) 71.
[22] K.L. Yee, M. Rodriguez Jr., T.J. Tschaplinski, N.L. Engle, M.Z. Martin, C. Fu, Z.-Y. Wang, S.D. Hamilton-Brehm, J.R. Mielenz, Evaluation of the bioconversion of genetically modified switchgrass using simultaneous saccharification and fermentation and a consolidated bioprocessing approach, Biotechnol. Biofuels 5 (2012) 81.
[23] D.G. Mann, P.R. Lafayette, L.L. Abercrombie, Z.R. King, M. Mazarei, M.C. Halter, C.R. Poovaiah, H. Baxter, H. Shen, R.A. Dixon, W.A. Parrott, C.N. Stewart Jr., Gateway-compatible vectors for high-throughput gene functional analysis in switchgrass (*Panicum virgatum* L.) and other monocot species, Plant Biotechnol. J. 10 (2012) 226–236.
[24] J.N. Burris, D.G.J. Mann, B.L. Joyce, C.N. Stewart Jr., An improved tissue culture system for embryogenic callus production and plant regeneration in switchgrass (*Panicum virgatum* L.), Bioenergy Res. 2 (2009) 267–274.
[25] S. Ohta, Y. Ishida, S. Usami, High-level expression of cold-tolerant pyruvate, orthophosphate dikinase from a genomic clone with site-directed mutations in transgenic maize, Mol. Breed. 18 (2006) 29–38.
[26] K.J. Moore, L.E. Moser, K.P. Vogel, S.S. Waller, B.E. Johnson, Describing and quantifying growth-stages of perennial forage grasses, Agron. J. 83 (1991) 1073–1077.
[27] C.N. Stewart Jr., L.E. Via, A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR applications, Biotechniques 14 (1993) 748–750.
[28] P.A. Fay, H.W. Polley, V.L. Jin, M.J. Aspinwall, Productivity of well-watered *Panicum virgatum* does not increase with CO_2 enrichment, J. Plant Ecol. 5 (2012) 366–375.
[29] R. Sripathi, V.G. Kakani, Y. Wu, Genotypic variation and trait relationships for morphological and physiological traits among new switchgrass populations, Euphytica 191 (2013) 437–453.
[30] J.M. Albaugh, T.J. Albaugh, R.R. Heiderman, Z. Leggett, J.L. Stape, K. King, K.P. O'Neill, J.S. King, Evaluating changes in switchgrass physiology, biomass, and light-use efficiency under artificial shade to estimate yields if intercropped with *Pinus taeda* L., Agrofor. Syst. 88 (2014) 489–503.
[31] M. Nakano, M. Mii, Antibiotics stimulate somatic embryogenesis without plant-growth regulators in several *Dianthus* cultivars, J. Plant Physiol. 141 (1992) 721–725.
[32] C. Miguel, L. Marum, An epigenetic view of plant cells cultured in vitro: somaclonal variation and beyond, J. Exp. Bot. 62 (2011) 3713–3725.
[33] R.F. Sage, D.S. Kubien, The temperature response of C_3 and C_4 photosynthesis, Plant Cell Environ. 30 (2007) 1086–1106.
[34] D.S. Kubien, R.F. Sage, C_4 grasses in boreal fens: their occurrence in relation to microsite characteristics, Oecologia 137 (2003) 330–337.
[35] R.F. Sage, Variation in the k_{cat} of Rubisco in C_3 and C_4 plants and some implications for photosynthetic performance at high and low temperature, J. Exp. Bot. 53 (2002) 609–620.
[36] J.S. Amthor, The effects of a stimulating intron on the expression of heterologous genes in *Arabidopsis thaliana*, Plant Biotechnol. J. 7 (1984) 561–569.
[37] S. Emami, D. Arumainayagam, The effects of a stimulating intron on the expression of heterologous genes in *Arabidopsis thaliana*, Plant Biotechnol. J. 11 (2008) 555–563.
[38] M.G. Koziel, G.L. Beland, C. Bowman, N.B. Carozzi, R. Crenshaw, L. Crossland, J. Dawson, N. Desal, M. Hill, S. Kadwell, K. Launis, K. Lewis, D. Maddox, K. McPherson, M.R. Meghji, E. Merlin, R. Rhodes, G.W. Warren, M. Wright, S.V. Evola, Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*, Biotechnology 11 (1993) 194–200.
[39] S. Römer, P.D. Fraser, J.W. Kiano, C.A. Shapton, N. Misawa, W. Schurch, P.M. Bramley, Elevation of the provitamin A content of transgenic tomato plants, Nat. Biotechnol. 18 (2000) 666–669.
[40] C. Napoli, C. Lemieux, R. Jorgensen, Introduction of a chimeric chalcone synthase gene into Petunia results in reversible co-suppression of homologous genes in trans, Plant Cell 2 (1990) 279–289.
[41] R.R. Nagaraja, R. Madhusudhana, S. Murali Mohan, D.V.N. Chakravarthi, S.P. Mehtre, N. Seetharama, J.V. Patil, Mapping QTL for grain yield and other agronomic traits in post-rainy sorghum *Sorghum bicolor* (L.) Moench, Theor. Angew. Genet. (Theor. Appl. Genet.), 126 (2013) 1921–1939.
[42] A. Barling, K. Swaminathan, T. Mitros, B.T. James, J. Morris, A. Ngamboma, M.C. Hall, J. Kirkpatrick, M. Alabady, A.K. Spence, M.E. Hudson, D.S. Rokhsar, S.P. Moose, A detailed gene expression study of the *Miscanthus* genus reveals changes in the transcriptome associated with the rejuvenation of spring rhizomes, BMC Genomics 14 (2013) 864.