

Cool C₄ Photosynthesis: Pyruvate P_i Dikinase Expression and Activity Corresponds to the Exceptional Cold Tolerance of Carbon Assimilation in *Miscanthus × giganteus*^{1,2[W][OA]}

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The bioenergy feedstock grass *Miscanthus × giganteus* is exceptional among C₄ species for its high productivity in cold climates. It can maintain photosynthetically active leaves at temperatures 6°C below the minimum for maize (*Zea mays*), which allows it a longer growing season in cool climates. Understanding the basis for this difference between these two closely related plants may be critical in adapting maize to colder weather. When *M. × giganteus* and maize grown at 25°C were transferred to 14°C, light-saturated CO₂ assimilation and quantum yield of photosystem II declined by 30% and 40%, respectively, in the first 48 h in these two species. The decline continued in maize but arrested and then recovered partially in *M. × giganteus*. Within 24 h of the temperature transition, the pyruvate phosphate dikinase (PPDK) protein content per leaf area transiently declined in *M. × giganteus* but then steadily increased, such that after 7 d the enzyme content was significantly higher than in leaves growing in 25°C. By contrast it declined throughout the chilling period in maize leaves. Rubisco levels remained constant in *M. × giganteus* but declined in maize. Consistent with increased PPDK protein content, the extractable PPDK activity per unit leaf area ($V_{\max,ppdk}$) in cold-grown *M. × giganteus* leaves was higher than in warm-grown leaves, while $V_{\max,ppdk}$ was lower in cold-grown than in warm-grown maize. The rate of light activation of PPDK was also slower in cold-grown maize than *M. × giganteus*. The energy of activation (E_a) of extracted PPDK was lower in cold-grown than warm-grown *M. × giganteus* but not in maize. The specific activities and E_a of purified recombinant PPDK from *M. × giganteus* and maize cloned into *Escherichia coli* were similar. The increase in PPDK protein in the *M. × giganteus* leaves corresponded to an increase in PPDK mRNA level. These results indicate that of the two enzymes known to limit C₄ photosynthesis, increase of PPDK, not Rubisco content, corresponds to the recovery and maintenance of photosynthetic capacity. Functionally, increased enzyme concentration is shown to increase stability of *M. × giganteus* PPDK at low temperature. The results suggest that increases in either PPDK RNA transcription and/or the stability of this RNA are important for the increase in PPDK protein content and activity in *M. × giganteus* under chilling conditions relative to maize.

In C₄ grasses, the mesophyll cells fix atmospheric CO₂ via cytosolic phosphoenolpyruvate carboxylase (PEPc; E.C.4.1.1.31) using PEP regenerated via chloroplastic pyruvate phosphate dikinase (PPDK; E.C.2.7.9.1). The C₄ product is transported to bundle sheath cells, where CO₂ is released by decarboxylation of this product

and refixed via Rubisco (E.C.4.1.1.39). The physiological significance of this process is to increase CO₂ concentration relative to O₂ around Rubisco such that the oxygenase activity of Rubisco and in turn photorespiration are in practice eliminated. As a result, C₄ photosynthesis has a higher potential efficiency of light, water, and nitrogen use than C₃ photosynthesis (Long, 1983, 1999; Sage, 1999). Although the process has a theoretical advantage, even in cool climates (Long, 1983, 1999), this is rarely realized. This is because few C₄ plants can maintain photosynthetically competent leaves at chilling temperatures (<15°C). Although many C₄ species can survive in cold conditions, *Miscanthus × giganteus* appears exceptional in being able to maintain high photosynthetic rates when transferred to chilling conditions (Beale et al., 1996). Even the productive cool-temperate grass *Spartina anglica* shows a loss of assimilatory capacity when transferred to cool conditions (Dunn et al., 1987), a loss that is not observed for *M. × giganteus* (Beale et al., 1996; Farage et al., 2006). Loss of photosynthetic capacity in chilling conditions, rather than chilling effects on the multitude of

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other physiological processes that could limit growth, has been shown to be the key limitation to extending the range of maize (*Zea mays*), the most cold-tolerant of the major C₄ crops, into colder climates and to extending its growing season in the temperate zone (Miedema, 1982; Long, 1983; Long et al., 1994).

An exception among C₄ grasses is the bioenergy crop *Miscanthus* × *giganteus*, a rhizomatous perennial of the Andropogoneae, which is highly productive in cool climates (Beale and Long, 1995; Bullard et al., 1995; Beale et al., 1996; Heaton et al., 2004). *M. × giganteus* is closely related to sugar cane (*Saccharum officinarum*), sorghum (*Sorghum bicolor*), and maize. Like these crop species, *M. × giganteus* uses the NADP-malic enzyme pathway of C₄ photosynthesis, while sequences of its photosynthetic genes share >99% sequence identity with sugar cane (Naidu et al., 2003; Wang et al., 2008). When grown in the cool temperate climate of southern England (52°N), *M. × giganteus* produced 30 tons of dry matter per ha per year, one of the highest dry matter yields recorded for the United Kingdom. In marked contrast to maize grown at the same location, it showed no loss of maximum photosynthetic efficiency during cold spring weather (Baker et al., 1989; Beale and Long, 1995; Beale et al., 1996). Growing maize at 14°C results in a more than 90% reduction of maximum photosynthetic rate relative to leaves grown at 25°C, whereas *M. × giganteus* grown at 14°C or 10°C shows little loss of photosynthetic capacity (Nie et al., 1992; Naidu and Long, 2004; Farage et al., 2006). Understanding how this is achieved may be critical to adapting maize and other C₄ crops to colder climates or in extending the growing season into cooler periods of the year so allowing greater use of the available solar radiation.

The molecular mechanism by which *M. × giganteus* is able to maintain high photosynthetic rates at low temperatures remains unclear. In saturating light and chilling temperatures (<15°C), CO₂ assimilation for chilling-intolerant C₄ species is severely reduced, and in turn utilization of observed excitation energy leads to photoinhibition and photooxidation (Long, 1983; Baker et al., 1989; Long et al., 1994). *M. × giganteus* avoids this damage both by maintaining a high rate of CO₂ uptake, to increase utilization of absorbed light (Beale et al., 1996), and by increased nonphotochemical quenching, which correlates with a large increase in xanthophyll content and its de-epoxidation (Farage et al., 2006). Chilling-induced decreases in the leaf photosynthetic rate of CO₂ uptake of C₄ species have been correlated with decreases in: carboxylation efficiency via PEPc (Kingston-Smith et al., 1997; Chinthapalli et al., 2003), capacity for PEP regeneration via PPDK (Du et al., 1999a), Rubisco activity (Kingston-Smith et al., 1997; Du et al., 1999a; Pittermann and Sage, 2000; Pittermann and Sage, 2001; Chinthapalli et al., 2003), or by a combination of these. Analyses of flux control coefficients in transgenic plants of the C₄ dicot *Flaveria bidentis* suggest that Rubisco and PPDK share control and limit light-saturated C₄ photosynthesis (Furbank et al., 1997). This applies at saturating intercellular

mesophyll CO₂ concentration, a condition that is met in the current atmosphere in both cold- and warm-grown *M. × giganteus* (Naidu and Long, 2004).

In warm-grown *F. bidentis*, transformed with antisense Rubisco small subunit gene constructs, in vitro activities of Rubisco matched the in vivo photosynthetic rates at low temperature, which suggested that Rubisco content controlled the C₄ photosynthetic rate at low temperature (Kubien et al., 2003). In addition, for each of the chilling-tolerant C₄ species *Bouteloua gracilis*, *Muhlenbergia glomerata*, and *Amaranthus retroflexus*, in vivo photosynthetic rates below 20°C showed the same pattern of decline as maximum activities of Rubisco in crude extracts (Pittermann and Sage, 2000; Sage, 2002; Kubien and Sage, 2004). These findings led Sage and McKown (2006) to propose that because Kranz anatomy limits the enzyme to the bundle sheath, C₄ plants are inherently more prone to Rubisco limitation as the activity of the enzyme declines with temperature. When grown at 14°C and measured at 10°C, *M. × giganteus* achieves about twice the photosynthetic rate of the cold-tolerant C₄ species *M. glomerata* (Kubien and Sage, 2004; Naidu and Long, 2004). Yet in cold-grown *M. × giganteus*, Rubisco content remained unchanged relative to warm-grown plants, while it decreased in cold-grown maize (Naidu et al., 2003). Furthermore, the cold tolerance of C₄ photosynthesis in *M. × giganteus* could not be attributed to the catalytic properties of Rubisco, which showed no differences between cold- and warm-grown *M. × giganteus* nor with warm-grown maize (Wang et al., 2008).

Other studies have inferred that PPDK is the key limiting factor for C₄ photosynthesis at low temperature. Low extractable activities, often only just sufficient to support in vivo rates of photosynthesis, have been reported frequently (Long, 1983; Edwards et al., 1985). PPDK catalyzes the regeneration of PEP as the primary acceptor of atmospheric CO₂ from pyruvate, ATP, and phosphate in C₄ photosynthesis. PPDK undergoes diurnal dark/light regulation of activity, which is controlled by a bifunctional regulatory protein (Burnell and Hatch, 1984; Ashton et al., 1990; Chastain et al., 2008). The fully functional C₄ isoform of PPDK is a tetramer. The subunits dissociate between 10°C and 15°C, causing an increase in the energy of activation (E_a) and a loss of catalytic rate with further decline in temperature (Shirahashi et al., 1978; Ohta et al., 1996; Du et al., 1999b). In addition, the maximum extractable PPDK activity correlated well with cold tolerance in three *Saccharum* species (Du et al., 1999a) and across maize cultivars (Sugiyama and Boku, 1976). In contrast to the effects of temperature on the PPDK enzyme, there appears to be no correlation between thermal properties of other C₄ photosynthetic enzymes and differences in cold sensitivity of plant ecotypes (Du et al., 1999b; Hamel and Simon, 2000). Furthermore, an analysis of transgenic *F. bidentis* with antisense PPDK gene constructs showed that PPDK shares control of C₄ photosynthetic rate with Rubisco under ambient CO₂ and high light (Furbank et al., 1997). A key role for

PPDK in low temperature tolerance is also inferred in two chilling-tolerant PEP carboxykinase type C₄ species, which apparently maintain photosynthetic rates at low temperature by bypassing PPDK in PEP synthesis, via PEP carboxykinase (Smith and Woolhouse, 1983; Matsuba et al., 1997). Although analysis of the cDNA sequences for PPDK in *M. × giganteus* and maize have failed to reveal any differences in putative amino acids sequences that could confer cold tolerance, the amount of PPDK was reported to increase significantly in *M. × giganteus* when grown at low temperature. This was in sharp contrast to the PPDK decreases observed in maize (Naidu et al., 2003). If PPDK is a limitation at low temperature, then low temperature-adapted species might be expected either to up-regulate expression of the enzyme in response to chilling or possess an isoform with a higher specific activity at low temperatures.

Here, the hypothesis that an increase in PPDK content, but not kinetics properties of PPDK, corresponds to the ability of *M. × giganteus* to maintain photosynthetic capacity when transferred to low temperature is examined. CO₂ assimilation rate and quantum yield of PSII (Φ_{PSII}) were measured in parallel with protein and transcript levels of PPDK in the leaves of *M. × giganteus* and maize during a transition of growth conditions from warm to cold temperature. The catalytic rates, E_a , and light activation kinetics of PPDK in both crude leaf extracts and in purified recombinant PPDK were compared between *M. × giganteus* and maize, as was the effect of enzyme concentration on the cold stability of PPDK.

RESULTS

Influence of Chilling Temperature on Chlorophyll Content, CO₂ Assimilation Rate, and Φ_{PSII}

Following transition from a 25°C (warm) to a 14°C (chilling) growth temperature, CO₂ assimilation rate (A) and Φ_{PSII} measured on existing fully expanded maize leaves declined progressively over 9 d to about 38% and 30%, respectively, of prechilling levels (Fig. 1). A similar decline occurred in fully expanded *M. × giganteus* leaves for the first 2 d, but was followed by a recovery stabilizing at about 88% of the preexposure level for A and 73% for Φ_{PSII} by day 9 (Fig. 1).

Chlorophyll contents of the existing leaves of both species were lower after being transferred to 14°C growth condition. Before transition to 14°C, the chlorophyll contents for fully expanded *M. × giganteus* and maize leaves were 562 ± 22 and $585 \pm 26 \mu\text{mol}/\text{m}^2$, respectively, while the chlorophyll contents declined to 69% in *M. × giganteus* and to 35% in maize after 14 d at 14°C (not shown).

Changes in Protein and Transcript Contents of PPDK in *M. × giganteus* and Maize

M. × giganteus leaves developed at 25°C and then transferred to 14°C after completion of expansion

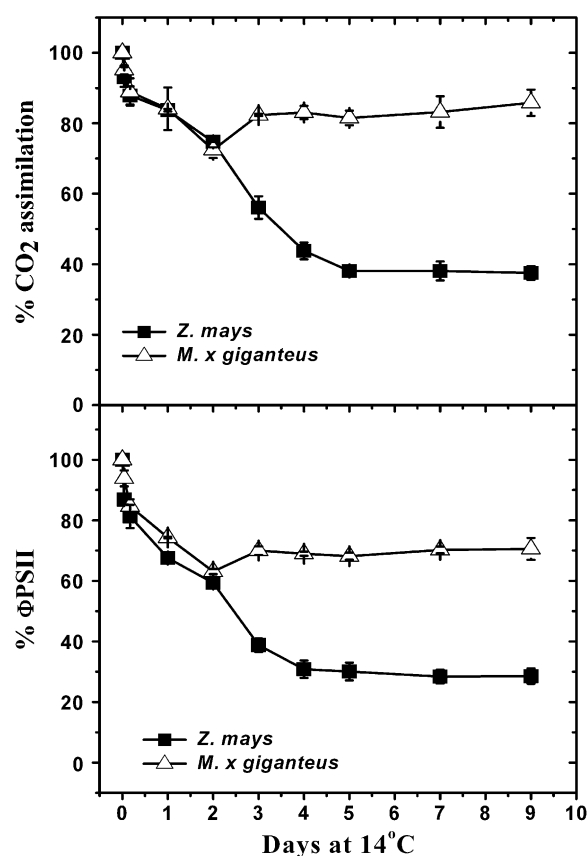


Figure 1. Change in leaf CO₂ uptake rate and Φ_{PSII} following transfer of plants from 25°C to 14°C. Means (± 1 se) for five plants are expressed as a percentage of rate observed immediately on transfer to 14°C, which were $14.8 \pm 0.6 \mu\text{mol m}^{-2} \text{s}^{-1}$ and 0.12 ± 0.02 for maize and $16.2 \pm 0.9 \mu\text{mol m}^{-2} \text{s}^{-1}$ and 0.14 ± 0.03 for *M. × giganteus*.

accumulated PPDK protein, while maize mature leaves given the same treatment lost PPDK (Fig. 2A). In parallel with the kinetics of the response of A to transfer to 14°C, PPDK protein declined slightly in *M. × giganteus* on the first day at low temperature, but then accumulated above the original level by day 3 and the amount was nearly doubled (calculated from band density) by day 7 (Fig. 2A). When quantified in absolute terms, PPDK content in 25°C grown *M. × giganteus* leaves was $0.30 \text{ g}/\text{m}^2$ and increased to $0.56 \text{ g}/\text{m}^2$ after 14 d at 14°C (Supplemental Fig. S1). However, for Rubisco, *M. × giganteus* mature leaves developed at 25°C and then transferred to 14°C showed no significant loss of Rubisco, in contrast to a marked decline in maize leaves (Fig. 2B).

Quantitative real-time reverse transcription (RT)-PCR showed that the changes in PPDK protein upon transfer of plants to 14°C were paralleled by changes in the amounts of the mRNA coding for PPDK (Fig. 3). An obvious increase in *M. × giganteus* PPDK mRNA was observed after day 3 following the exposure to 14°C (Fig. 3). In contrast, maize PPDK mRNA declined continuously following transfer to 14°C (Fig. 3). By the end of 14 d of exposure to 14°C, the *M. × giganteus*

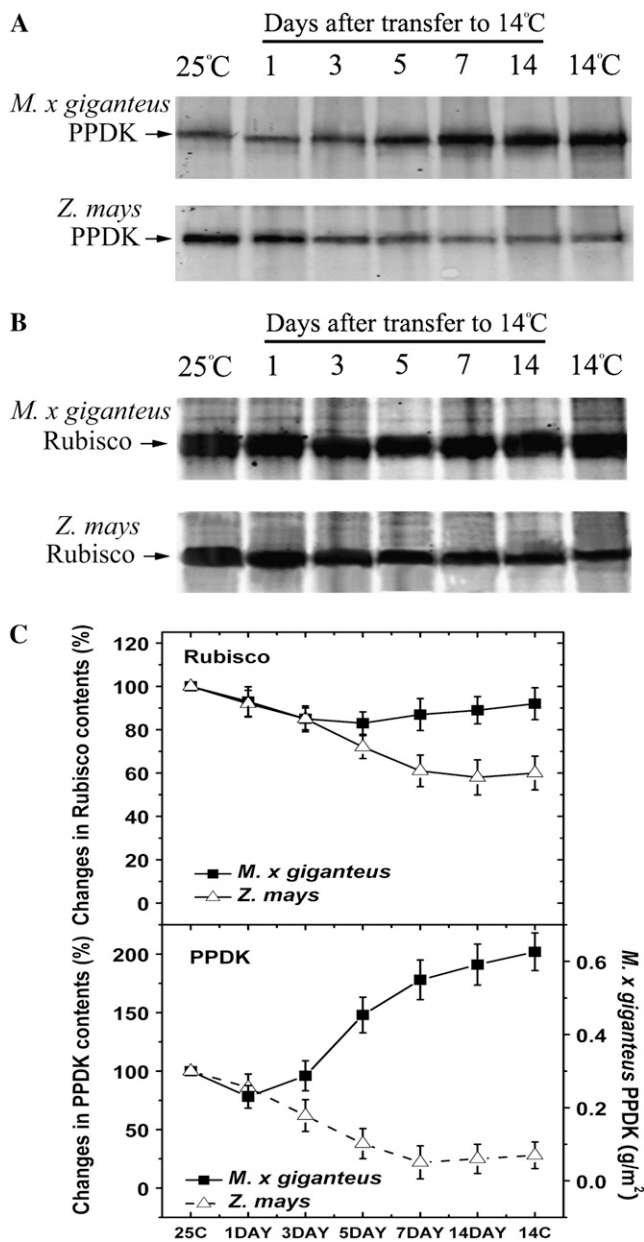


Figure 2. PPDK (A) and Rubisco (B) protein contents in *M. × giganteus* and maize for the leaves of Figure 1 following transfer from 25°C/20°C to 14°C/12°C (day/night). Prior to transfer, these plants had been grown continuously at 25°C/20°C. In addition, the lane marked “25C” shows protein contents immediately prior to the transfer. The lane marked “14C” is for leaves of the same developmental stage of plants that were grown and developed entirely at 14°C/12°C (day/night), this is included for comparison. For each lane, protein was extracted from 1.9 cm² of leaf area into 500 μL of extraction buffer, of which 20 μL was loaded and separated by 10% SDS-PAGE. Western blots used polyclonal primary antibodies against maize PPDK and spinach Rubisco, respectively. The bands on Figure 2, A and B, were quantified in relative terms by densitometry (Odyssey V1.2 application software; C). Means (± 1 SE) for three plants’ Rubisco and PPDK protein contents, expressed as a percentage of the amount present immediately prior to the transfer to 14°C/12°C; i.e. the first lane of A and B. The amounts of *M. × giganteus* PPDK were also quantified from a standard curve, generated from known quantities of recombinant PPDK (Supplemental Fig. S1) and shown via the right-hand axis of the lower segment.

PPDK mRNA was 2.1-fold higher than that grown continuously at 25°C, while for maize PPDK mRNA was 45% lower. For both species, the PPDK mRNA levels after 14-d chilling exposure were similar to those in leaves grown continuously at 14°C (Fig. 3).

Temperature Dependency and E_a of PPDK Activity in Crude Leaf Extracts

The extractable activity of PPDK 14 d after transfer to 14°C corresponded closely to *A* in these leaves, 5.3 versus 5.6 μmol m⁻² s⁻¹, respectively, for maize, and 18.3 versus 14.1 μmol m⁻² s⁻¹ for *M. × giganteus* (Table I; Fig. 1). The apparent E_a was calculated separately for a low (6°C–18°C; $E_{a,6-18}$) and a high (18°C–28°C; $E_{a,18-28}$) temperature range (Fig. 4, A and B). For the control plants grown at 25°C, the $E_{a,6-18}$ was about 1.5-fold of $E_{a,18-28}$ for PPDK from *M. × giganteus*, and was slightly and significantly greater at 1.8-fold for PPDK from maize (Table I). For plants exposed to 14°C for 14 d, the $E_{a,6-18}$ was about 1.4-fold of $E_{a,18-28}$ for PPDK from *M. × giganteus* and again higher at 2.0-fold for PPDK from maize (Table I). For *M. × giganteus*, the $E_{a,6-18}$ of PPDK extracted from leaves exposed to 14°C was 15% lower than that of the control plant, indicating some acclamatory effect, while no significant difference was observed for $E_{a,18-28}$ (Table I). In sharp contrast to *M. × giganteus*, the $E_{a,6-18}$ and $E_{a,18-28}$ of PPDK activity from maize leaves exposed to 14°C was 37% and 24% higher than that of the control plant, respectively (Table I).

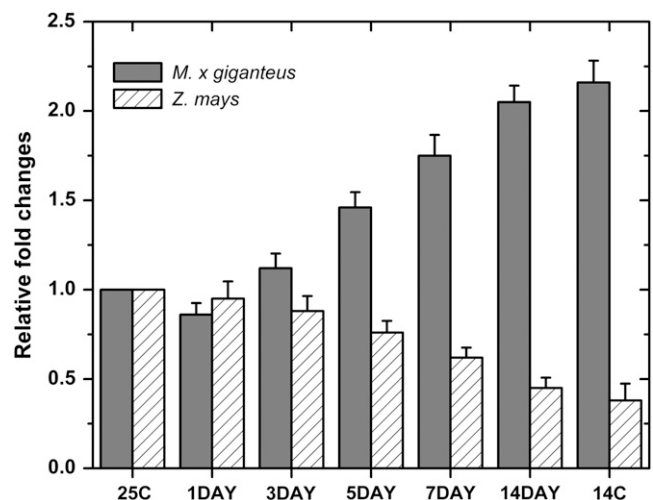


Figure 3. Quantitative real-time RT-PCR analysis of PPDK expression in leaves of *M. × giganteus* and maize. The leaf samples were collected 1, 3, 5, 7, and 14 d after transfer from 25°C to 14°C or collected from plants grown continuously at 25°C (25C) and 14°C (14C), respectively. The total RNAs were isolated and quantitative real-time RT-PCR in triplicate was conducted using a pair of primers specific for PPDK genes. The expression of PPDK from plants continuously grown at 25°C (25C) was used as control and set as 1.0. The changes in expression of PPDK were determined relative to the control. Bars are the means (± 1 SE) of three replicate plants, relative to the level immediately prior to transfer to 14°C.

Table 1. V_{\max} and E_a of PPDK *M. × giganteus* and maize from crude leaf extracts ($n = 5$ replicate plants) and from *E. coli* ($n = 3$ independent transformations) into which the genes from each species had been cloned

Values given are means (and ses) and their units are given at the end of the table. Leaf and recombinant protein extracts were made as described in "Materials and Methods."

| Species | Growth Temperature | $V_{\max,ppdk}^a$ | | E_a (kJ mol ⁻¹) ^b | |
|--|--------------------|-------------------|------------|--|--------------------------|
| | | 25°C | 15°C | 6°C–18°C (6°C–15°C) | 18°C–28°C (18°C–28°C) |
| <i>M. × giganteus</i> crude leaf extract | 25°C | 39.2 (1.5) | 14.2 (0.9) | 93.5 (6.4) | 63.2 (3.8) |
| | 14°C | 43.5 (1.3) | 18.3 (0.7) | 79.1 (5.2) | 57.3 (3.2) |
| Maize crude leaf extract | 25°C | 46.1 (0.9) | 17.1 (1.6) | 106.2 (8.9) | 58.1 (2.9) |
| | 14°C | 18.0 (1.2) | 5.3 (0.9) | 145.8 (9.5) | 71.9 (4.2) |
| <i>M. × giganteus</i> recombinant PPDK | – | 7.3 (0.5) | 3.3 (0.4) | 115.7 (6.2) | 51.6 (2.9) |
| Maize recombinant PPDK | – | 7.6 (0.4) | 3.1 (0.3) | 114.3 (5.8) | 52.1 (3.6) |

^aThe units for $V_{\max,ppdk}$ of PPDK in crude leaf extract are $\mu\text{mol s}^{-1} \text{m}^{-2}$ and for recombinant PPDK $\mu\text{mol min}^{-1} \text{mg}^{-1}$. ^b E_a for PPDK in crude leaf extract was calculated from slopes of Arrhenius plots at low (6°C–18°C) and high (18°C–28°C) temperature ranges; for recombinant PPDK at low (6°C–15°C) and high (18°C–28°C) temperature ranges.

The Maximum Enzyme Rates and E_a of Purified Recombinant PPDK

To test if the lower E_a of PPDK in crude extracts from *M. × giganteus* at low temperature is due to any inherent difference in the protein and related gene sequence differences, *C*₄ PPDK genes from both species were cloned and expressed in *Escherichia coli* so that the two enzymes could be synthesized and extracted from a common background. The specific activities of purified His-tagged recombinant PPDK of *M. × giganteus* and maize increased with temperature from 6°C to 28°C but showed no significant difference across this range (Fig. 5A). For example, the specific activities of PPDK of *M. × giganteus* and maize at 25°C were 7.3 ± 0.5 and $7.6 \pm 0.4 \mu\text{mol min}^{-1} \text{mg}^{-1}$ respectively, and 3.3 ± 0.4 and $3.1 \pm 0.3 \mu\text{mol min}^{-1}$

mg^{-1} , respectively, at 15°C. In contrast to crude extracts, the Arrhenius plots of $V_{\max,ppdk}$ showed no significant difference between *M. × giganteus* and maize across all measuring temperatures (Fig. 5B; Table I). An apparent break-point in the Arrhenius plot was observed at 15°C for both *M. × giganteus* and maize PPDK (Fig. 5B). A shift in the slopes of the fitted lines at high (18°C–28°C) and low (6°C–15°C) temperature was observed (Fig. 5B). Therefore, the apparent E_a of the PPDK was calculated separately for these two regions of the plot. The E_a at low measuring temperatures was 2.2-fold of that above 15°C for PPDK activity from both *M. × giganteus* and maize (Table I). No significant difference in the E_a in either temperature range was observed between recombinant PPDK from two species. The lack of difference between the

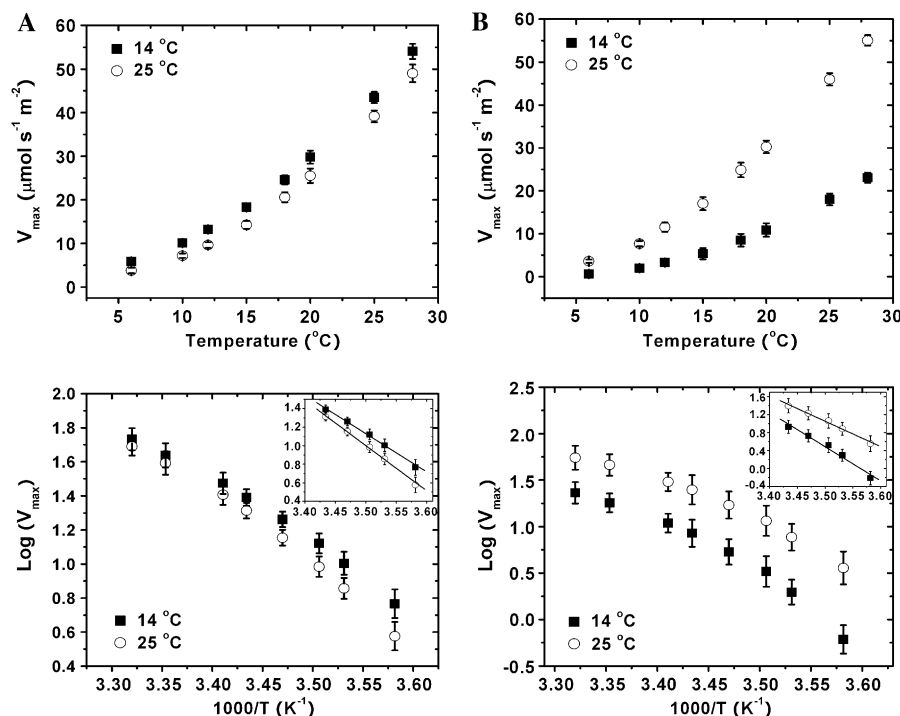


Figure 4. Maximum rates of PPDK-catalyzed PEP synthesis expressed on a leaf area basis versus assay temperature, transformed to provide Arrhenius plots for *M. × giganteus* (A) and maize (B) grown at 25°C (○) and transferred to 14°C (■) for 14 d. PPDK was extracted from leaves of *M. × giganteus* and maize. Values are the means (± 1 SE) of five plants. The inset shows the linear regression fitted to the Arrhenius plots for low (6°C–18°C) measuring temperatures.

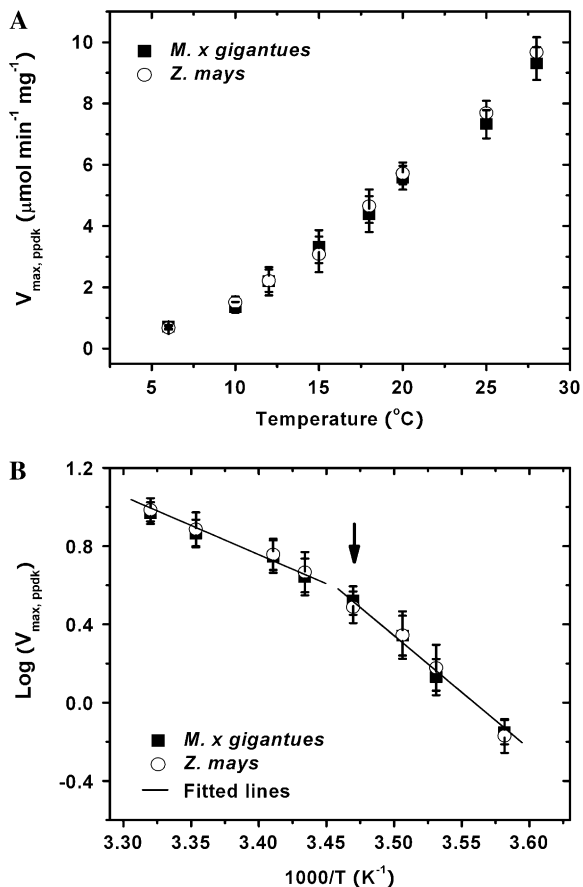


Figure 5. Maximum rates of PPDK-catalyzed PEP synthesis per unit protein versus assay temperatures (A) and transformed to provide Arrhenius plots (B) for purified recombinant His-tagged PPDK of *M. x giganteus* (■) and maize (○) cloned in *E. coli*. The linear regressions in the Arrhenius plot are for low (6°C–15°C) and high (18°C–28°C) measuring temperatures. Values are the means (± 1 SE) of three different transformation events. The arrow indicates the apparent break point in Arrhenius plot for both *M. x giganteus* and maize PPDKs at 15°C.

two recombinant PPDKs suggests that differences observed in leaf extracts are a result of posttranslational differences or differences within the stromal environments and not differences between the gene sequences coding for the two polypeptides.

Cold Inactivation of PPDK at Varying Protein Concentrations

To examine the relationship between cold inactivation of PPDK activity and enzyme concentration, the specific PPDK activity at varying protein concentrations (0.1–2 mg/mL) was measured following 10, 20, 40, and 60 min of incubation at 0°C (Fig. 6). The initial PPDK activities immediately before 0°C incubation (at time 0) were very similar and about $6.9 \mu\text{mol mg}^{-1} \text{min}^{-1}$ at all enzyme concentrations. The PPDK activities decreased during the cold treatment at all PPDK concentrations, but the decreases were much slower at higher enzyme concentration. The half-time when

PPDK activity falls to 50% of the initial value ($t_{1/2}$) at a protein concentration of 0.1 mg/mL was 9.2 min, while $t_{1/2}$ at a concentration of 2 mg/mL was 22.6 min (Fig. 6). By the end of 10 min incubation at 0°C, the PPDK activity at the concentration of 0.1 mg/mL was only 36% of the control, while the PPDK activity at the concentration of 2 mg/mL was 73% of the control (Fig. 6).

Influence of Chilling Temperature on Light Activation of PPDK

Activity of the C_4 PPDK is known to be tightly regulated by the light/dark cycle. To examine the influence of chilling, the activation kinetics of PPDK from warm-grown and chilling-exposed *M. x giganteus* and maize were compared by measuring maximum activities of PPDK during the dark to light transition. The PPDK polypeptide contents showed no significant changes within 1 h following the dark to light transition in either species or in either temperature (Supplemental Fig. S2). However, acclimation to 14°C reduced the rate of activation of PPDK in both species, but to a much greater extent in maize (Fig. 7; Table II). The time required to reach 50% of the maximum PPDK activity from chilling-acclimated plants increased to 2.6-fold (*M. x giganteus*) and 4.7-fold (maize) in comparison to that for warm-grown plants (Fig. 7; Table II). However, on completion of activation, the activity in cold-grown leaves of *M. x giganteus* was about 20% higher than in warm-grown leaves, in sharp contrast to maize (Table II).

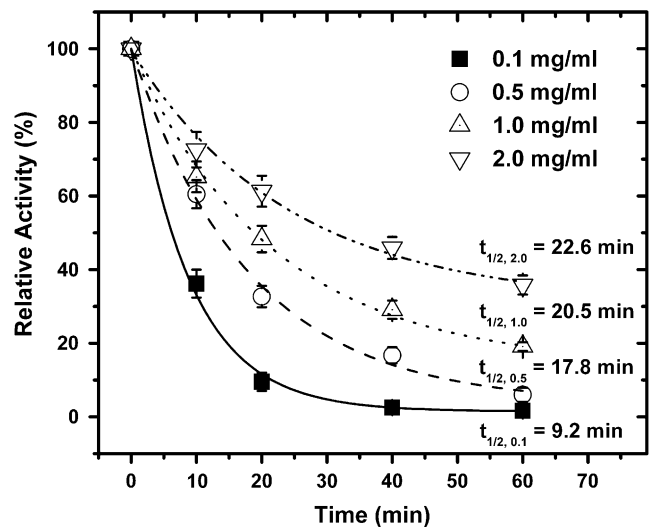


Figure 6. Cold inactivation of *M. x giganteus* recombinant PPDK at varying enzyme concentrations. The purified recombinant *M. x giganteus* PPDKs were diluted to varying protein concentrations and incubated at 0°C for the time indicated. The specific PPDK activities immediately before the cold treatment at time 0 were assigned as 100%. The data were fitted to a first-order exponential decay (Origin-Pro 7.5, OriginLab). The $t_{1/2}$ (the time points at which PPDK activity fell to 50% of the initial value) at the different protein concentrations are shown.

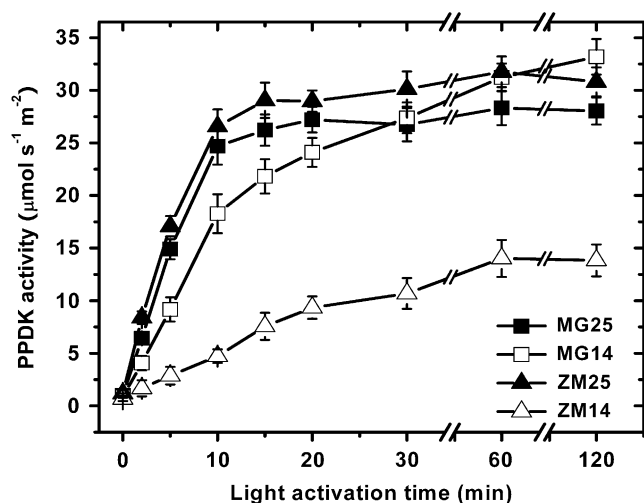


Figure 7. Activation kinetics of PPDK in leaf crude extract from *M. × giganteus* (MG) and maize (ZM) grown continuously at 25°C (25) or grown at 25°C and then at 14°C (14) for 14 d. The leaves were dark adapted for 10 h in their respective growth environments. The leaf discs were collected into liquid N₂ immediately before illumination and then at time points following illumination time points. The maximum activities of PPDK expressed on a leaf area basis were measured at 25°C as in Figure 4. Values are the means (± 1 SE) of five plants.

Because light activation of PPDK is tightly associated with dephosphorylation, regulated by the bifunctional PPDK regulatory protein, the phosphorylation level of PPDK from warm-grown (25°C) and chilling-acclimated (14°C) *M. × giganteus* and maize was also examined using PPDK Thr-P-specific antibodies during the dark to light transition, in parallel with enzyme activity assays. The antibodies used here were previously shown to be specific to phosphorylated PPDK with a very low background for nonphosphorylated PPDK, such as the recombinant form expressed in *E. coli* (Chastain et al., 1996; Chastain et al., 2000). The phosphorylation level of PPDK declined progressively following illumination in both species (Fig. 8, A and B). As observed in the enzyme assays, acclimation at 14°C reduced the rate of PPDK activation in both species, as indicated by a significantly greater $t_{1/2}$ for dephosphorylation of PPDK (Table II). The $t_{1/2}$ for dephosphorylation of PPDK from chilling-acclimated *M. × giganteus* leaves increased to 2.3-fold compared to warm-grown leaves, while it increased 3.1-fold in maize (Table II). By the end of 1-h illumination, the phosphorylation level of PPDK was 15% higher in chilling-adapted *M. × giganteus* leaves than that in warm-grown leaves, while it was 25% higher for maize (Fig. 8, A and B).

DISCUSSION

Exposure to chilling temperatures is a key limitation to the production of maize both in the early and late growing season, particularly near the high-latitude limits of current cultivation (Miedema, 1982; Allen and

Ort, 2001). Chilling events can occur after planting or before completion of grain fill. These result in a loss of photosynthetic capacity, which is exacerbated by photoinhibition and photooxidation (Long et al., 1983, 1994; Baker et al., 1989). The chilling tolerance of *M. × giganteus* is shown by comparison here to an inbred maize line selected for the corn belt. This inbred line also parallels responses observed previously for hybrids selected for use at the cold temperature limit of maize cultivation in Europe (Miedema, 1982; Long et al., 1983; Nie et al., 1992). While *M. × giganteus* retains a high photosynthetic rate in leaves transferred to 14°C ($18.3 \mu\text{mol m}^{-2} \text{s}^{-1}$), the rate in maize declined to almost one-quarter of that value ($5.3 \mu\text{mol m}^{-2} \text{s}^{-1}$), even though these leaves had very similar rates of CO₂ uptake to those of *M. × giganteus* prior to being transferred to 14°C.

Both Rubisco and PPDK have been suggested as the potential control points limiting C₄ photosynthesis at chilling temperatures (Furbank et al., 1997; Kubien et al., 2003). Upon transfer of the chilling-tolerant NADP-malic enzyme C₄ grass *M. × giganteus* grown at 25°C to 14°C, mature leaves show a small transient loss of photosynthetic capacity. Their recovery and maintenance of a high photosynthetic rate under these cool conditions correlated with a marked increase in PPDK protein and mRNA. By contrast, Rubisco content remained constant during this treatment. Similarly, Naidu et al. (2003) showed that leaves of *M. × giganteus* developed at 14°C accumulated higher amounts of PPDK protein than leaves grown at 25°C but had similar levels of Rubisco as those grown at 25°C, in contrast to maize, where both PPDK and Rubisco declined. The dramatic increase of PPDK protein concentration in *M. × giganteus* leaves may be the mechanism by which PPDK activity in vivo is maintained in cool temperatures. Alteration of the stromal environment by concentrating this protein may enhance the level of active tetramer association (Hatch, 1979). This is supported by observations of increased cold stability of PPDK as protein concentra-

Table II. Activation kinetics of PPDK from warm-grown and chilling-adapted *M. × giganteus* (MG) and maize (ZM) grown continuously at 25°C (25) or grown at 25°C and then transferred to 14°C (14) for 14 d

| Species | $V_{\text{max,ppdk}}^{\text{a}}$ | Time to 50% $V_{\text{max,ppdk}}$ ($t_{1/2}^{\text{b}}$) | Time to 50% Dephosphorylation ($t_{1/2}^{\text{c}}$) |
|---------|--------------------------------------|---|--|
| | $\mu\text{mol s}^{-1} \text{m}^{-2}$ | s | s |
| MG25 | 31.3 ± 1.7 | 270 ± 66 | 222 |
| MG14 | 37.3 ± 1.2 | 696 ± 60 | 522 |
| ZM25 | 34.3 ± 1.4 | 252 ± 48 | 210 |
| ZM14 | 17.2 ± 1.1 | $1,182 \pm 204$ | 648 |

^aThe maximum in vitro PPDK activity in crude leaf extracts made after at least 2-h illumination. ^bValues were calculated by fitting data from enzyme assays of PPDK activation in Figure 7. ^cValues were calculated by fitting data from western blot of phospho-PPDK in Figure 8B.

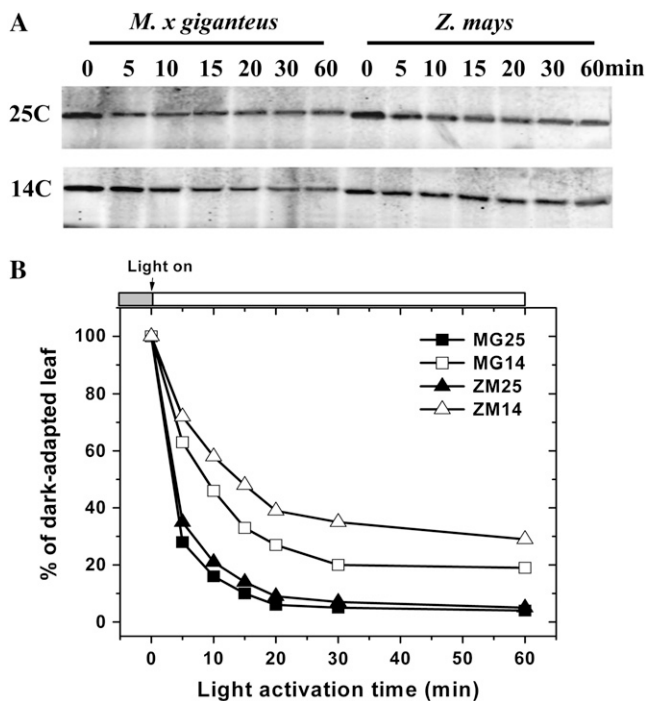


Figure 8. Changes in phosphorylation level of PPDK upon illumination from leaves of *M. × giganteus* (MG) and maize (ZM) grown continuously at 25°C (25) or grown at 25°C and then at 14°C (14) for 14 d. The leaf samples were collected as described in Figure 7. The phospho-PPDK was probed with an affinity-purified rabbit polyclonal antibodies raised against a synthetic phosphopeptide conjugate corresponding to the Thr-phosphorylation domain of maize C₄ PPDK. A representative immunoblot is shown (A). The band density shown in Figure 8A was quantified by densitometry (Odyssey V1.2 application software). The changes in phosphorylation level of PPDK at illumination times were expressed as percentage of that from dark-adapted leaves at time 0 (B).

tions are increased, as shown both here and in previous studies (Shirahashi et al., 1978; Salahas et al., 1990). Is there any evidence that increased expression of PPDK can increase photosynthetic rate in C₄ plants at chilling temperatures? Ohta et al. (2006) recently over-expressed PPDK from the moderately cold-tolerant C₄ species *Flaveria brownii* in maize and found that the transgenic plants could maintain a photosynthetic rate 23% greater than the untransformed line down to a leaf temperature of 8°C. This is consistent with the inference from the present study, where the ability to maintain photosynthetic rate during chilling correlates with increases in the amount of PPDK. In addition, a stress-induced (drought-induced and cold-induced) increase in PPDK expression has been shown in previous studies of other C₄ species (Michalowski et al., 1989; Moons et al., 1998; Nogueira et al., 2003). For example, a RNA profile of cold-responsive genes using filter arrays showed that PPDK transcripts significantly increased on transfer of a sugar cane hybrid (*Saccharum* sp. 'SP80-3280') from 26°C to 4°C (Nogueira et al., 2003). In summary, these previous reports coupled with our findings suggest an important role for PPDK in acclimation to chilling in C₄ species.

Not only is PPDK expression increased, but the apparent kinetics and activation of the enzyme are altered. In crude extracts, PPDK from *M. × giganteus* shows lower activation energy at low temperature and so shows less loss of activity as temperature is decreased. However, this does not appear to result from differences in the primary polypeptide, because when PPDK was cloned and expressed in *E. coli*, the recombinant-expressed protein from *M. × giganteus* showed no difference in activation energy to that from maize. This is consistent with the observation that there are few amino acid changes that distinguish C₄ PPDK sequences among *M. × giganteus*, maize, and cold-intolerant sugar cane (Naidu et al., 2003). Previous analyses of sugar cane lines with different cold sensitivity (Du et al., 1999a) and *Echinochloa crus-galli* ecotypes from contrasting climates (Simon, 1996) also concluded that cold tolerance in those species is not due to the intrinsic properties of PPDK. The difference in crude extracts is therefore the result of either posttranslational modification or the presence of activators or inhibitors that affect the activity of the enzyme in leaf extracts. Divalent cations (Mn²⁺, Mg²⁺, and Ca²⁺), PEP, polyols (glycerol and sorbitol), and free amino acids (Pro) or their derivatives have been shown to protect PPDK against cold inactivation, possibly by preventing the dissociation of tetrameric active enzyme (Krall et al., 1989; Salahas et al., 2002).

Low temperature decreased the activation of PPDK in both *M. × giganteus* and maize, which is consistent with previous reports on maize and *E. crus-galli* (Edwards et al., 1980; Simon and Hatch, 1994). Early analyses showed that activation of PPDK from the C₄ grass *E. crus-galli* subjected to cold treatment was faster in ecotypes from cool climates compared to those native to warmer climates (Simon and Hatch, 1994). Activation is also faster in *M. × giganteus* than maize. This may be critical in protecting the leaf against photoinhibition. When carbon metabolism is inhibited, utilization of absorbed light energy is decreased, which increases the potential for photoinhibition and photooxidative damage (Long et al., 1983; Powles and Bjorkman, 1983). Maize is particularly vulnerable to such damage on cool mornings with clear skies (Baker et al., 1989; Long et al., 1994). Slow induction of PPDK at low temperature could exacerbate this damage.

The findings presented here, coupled with those from the PPDK overexpressor transformants of maize (Ohta et al., 2006), provide strong evidence that this enzyme is critical to maintaining C₄ photosynthesis at low temperature. Rubisco has previously been suggested as the most likely candidate for limiting C₄ photosynthesis at low temperatures (Sage, 2002; Kubien et al., 2003). However, Rubisco levels remained constant in *M. × giganteus* leaves at low temperature, whereas PPDK increased, which is consistent with the suggestion by Sage (2002) that C₄ leaves cannot physically accommodate more Rubisco. But restriction at Rubisco cannot explain the observed decline and

recovery in leaf photosynthetic rates for *M. × giganteus* upon transfer to low temperature nor the increased photosynthetic rate measured at low temperature in transgenic maize that overexpresses PPDK.

One major difference in the *C₄* photosynthetic pathway between *M. × giganteus* and chilling-sensitive maize appears to be higher steady-state PPDK mRNA accumulation, which may result from either increased transcriptional activity of the *C₄* PPDK gene or greater stability of its mRNA. The basis of this increase is unknown but may reflect a pattern of acclimation to other stresses where one or more transcription factors are up-regulated (Thomashow et al., 2001). Discovering the regulatory network underlying increased *C₄* PPDK expression will be critical to gaining a more complete picture of the mechanistic basis for successful acclimation of *C₄* photosynthesis to low temperature, as realized in *M. × giganteus*.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Miscanthus × giganteus and maize (*Zea mays*) FR1064 (a commercial inbred line, Illinois Foundation Seeds) was grown in soil-less potting media (Sunshine Mix LC1²; Sun Gro Horticulture) following the procedure of Naidu and Long (2004). Plants were grown at 14°C/12°C (cold) or 25°C/20°C (warm) day/night temperature and 14-h-day/10-h-night cycle under 500 μmol photons m⁻² s⁻¹ in controlled-environment chambers equipped with a mixture of high-pressure mercury and sodium lamps (E-15H; Conviron). To examine the ability of leaves to maintain photosynthetic capacity on chilling, plants originally grown in a controlled-environment chamber with 25°C/20°C (warm) day/night temperature were transferred to an identical chamber but with 14°C/12°C (cold) day/night temperature, and all other environmental conditions were unchanged. Leaves that had just completed expansion, as judged by ligule emergence, were collected after 3 h of illumination for assays based on crude extracts or time specified for light activation assays. The leaf discs were cut from fully expanded leaves and immediately frozen into and then stored in liquid nitrogen. Light-saturated leaf CO₂ assimilation (*A*_{CO₂}) and ΦPSII were measured on fully expanded leaves that had been illuminated for 3 to 4 h.

RNA Extraction and Quantitative Real-Time RT-PCR

Total RNA from leaf samples was isolated using RNeasy kit (Qiagen) according to the manufacturer's instructions. First-strand cDNAs were synthesized from DNaseI-treated total RNA using RT (Superscript II RNase H⁻; Invitrogen). For real-time quantitative PCR, reaction mixes of 25 μL were prepared including 12.5 μL of the SYBR Green PCR MasterMix (Applied Biosystems), 0.4 pmol of a primer pair, and 1 μL of 10-fold diluted first-strand cDNA. PCR amplifications were carried out on a Cepheid Smart Cycler system. The reactions were started with a step of 50°C for 2 min and 95°C for 10 min to activate the polymerase, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Dissociation kinetics analysis of the amplification products were performed to ensure specific amplification. The PCR for ubiquitin gene was used as the internal control. The primer pairs for PPDK genes are: for *M. × giganteus* PPDK (accession no. AY262272), forward primer 5'-GCGCCGATTGCGACGAAAAAGAG-3' and reverse primer 5'-CCCAGCAGTTCCTTCATGCTCTTGT-3'; and for maize PPDK (accession no. J03901), forward primer 5'-CGCCGATACAGACGACCAAAAAGAGG-3' and reverse primer 5'-CCCAGCAGTTCCTTCATGCTCTTGT-3'. The primers of each pair contained the sequences of the ends of two contiguous exons to avoid amplification of genomic DNA. The primer pairs for the ubiquitin gene are: for *M. × giganteus* ubiquitin, forward primer 5'-CCTCTGACACCATCGACAATGTGAA-3' and reverse primer 5'-GCTGCTTGCCGGCAAGATG-3'; and for maize ubiquitin, forward primer 5'-GCTCTGACACCATCGACAACGTGAA-3' and reverse primer 5'-GCTGCTTGCCGGCAAGATC-3', respectively.

Expression and Purification of Recombinant His-Tagged PPDK

The cDNA of *MgPPDK13* (Naidu et al., 2003) was cloned into *Bam*HI and *Eco*RI sites of pRSETa (Invitrogen) with a pair of primers: forward primer TTGGATCCTCCGACGCCGCGCGGGACGG and reverse primer ATGAATTCACAGACAAGCACCTGAGCTGCAGC with the restriction sites *Bam*HI and *Eco*RI underlined, respectively. The plasmid construction was confirmed by sequencing. The plasmid construction for maize PPDK cDNA (*zmPPDK*) was a gift from Dr. Chastain (Minnesota State University). The plasmids were transformed into BL21 (DE3) competent strains of *Escherichia coli*. The overexpression and purification of recombinant His-tagged PPDK were performed as described previously (Chastain et al., 1996).

Chlorophyll Contents, Chlorophyll Fluorescence, and Gas Exchange

Chlorophyll contents were measured and calculated according to Porra et al. (1989). The light-saturated CO₂ uptake and transpiration were measured simultaneously with modulated chlorophyll fluorescence on fully expanded attached leaves using a LI-COR 6400 portable photosynthesis system as described previously (Naidu and Long, 2004; Wang and Portis, 2007). All measurements were conducted in ambient air under 1,000 μmol m⁻² s⁻¹ of light illumination. Five replicate plants were measured for each measurement temperature and treatment combination. Actinic light was supplied by light-emitting diodes (90% red light, 630 nm; 10% blue light, 470 nm) to record the steady-state chlorophyll fluorescence level (*F_s*). A pulse-modulated measuring light (630 nm; 1 μmol photons m⁻² s⁻¹) was used to determine the minimum chlorophyll fluorescence at the open PSII center. An 800-ms saturating pulse of about 5,000 μmol m⁻² s⁻¹ was applied to measure the maximum chlorophyll fluorescence at the closed PSII center in the dark (*F_m*) or during actinic light illumination (*F_m'*). The ΦPSII of illuminated leaves was calculated as (*F_m'* - *F_s*)/*F_m'* (Genty et al., 1989).

PPDK Extraction and Activity Assays

Extraction of PPDK followed the procedure of Crafts-Brandner and Salvucci (2002) with the following modifications. The extraction buffer contained 50 mM HEPES-NaOH, pH 8.0, 10 mM MgCl₂, 5 mM dithiothreitol, 1 mM EDTA, 1% (w/v) casein, 1% (w/v) polyvinylpyrrolidone, 0.05% (v/v) Triton X-100, 20 mM NaF (for phospho-PPDK immunoassays only), 2 μM orthovanadate (for phospho-PPDK immunoassays only), and one protease inhibitor cocktail tablet (EDTA-free) per 10 mL of extraction buffer (Roche Applied Science). Two leaf discs (1.1-cm diameter) were ground rapidly in 500 μL of extraction buffer using a Tenbroeck tissue homogenizer at room temperature. The extract was then centrifuged for 30 s at 15,000g.

The activity of PPDK was measured by coupling the production of PEP to NADH oxidation via PEPc and malate dehydrogenase modified from Ashton et al. (1990). Except where noted below, all enzymes and chemicals were obtained from Sigma-Aldrich. NADH oxidation was monitored for 1 min in a dual-beam UV/VIS spectrophotometer at a wavelength of 340 nm in temperature-controlled cuvettes (Cary I and Temperature Control Accessory; Varian). The measurement temperatures were 6°C, 8°C, 10°C, 12°C, 15°C, 18°C, 20°C, 25°C, and 28°C. A 20-μL aliquot of crude extract supernatant was added to the cuvette (pre-equilibrated at assay temperature in the spectrophotometer) containing 1 mL of assay buffer and was incubated for 5 min at each measuring temperature. The assay buffer consisted of 100 mM HEPES-NaOH, pH 8.0, 15 mM MgCl₂, 0.15 mM EDTA, 5 mM NaHCO₃, 0.3 mM NADH, 5 mM NH₄Cl, 2.5 mM K₂HPO₄, 5 mM dithiothreitol, 1 mM Glc-6-P, 1.5 mM ATP, and 10.5 units malate dehydrogenase. The reaction was initiated by the addition of pyruvate to 1.25 mM final concentration and 3 units of purified maize PEPCase (Bio-Research Products).

Immunoblot Analysis

Total soluble proteins on a leaf area basis were separated by 10% Tris-Gly SDS-PAGE and blotted onto the polyvinylidene difluoride membrane. PPDK and Rubisco proteins were detected with rabbit polyclonal antibodies raised against recombinant maize PPDK (Budde and Chollet, 1986) and native spinach (*Spinacia oleracea*) Rubisco. The phospho-PPDK was probed with the affinity-purified rabbit polyclonal antibodies raised against a synthetic phosphopeptide conjugate corresponding to the Thr-phosphorylation domain of

maize C₄ PPKD (Chastain et al., 2000). The blots were incubated with fluorescent-conjugated rabbit secondary antibodies (IRDye; LI-COR). The polyvinylidene difluoride membranes were scanned by using an Odyssey Infrared Imaging system (LI-COR), and relative protein expression levels were quantified with the Odyssey V1.2 application software as described previously (Wang and Portis, 2006).

Calculations

PPDK activity was calculated from the change in absorbance and the extinction coefficient of NADH ($6,221 \mu\text{L} \mu\text{mol}^{-1} \text{cm}^{-1}$), accounting for the stoichiometry of the reactions linking each enzyme from NADH to CO₂: i.e. 1 mol of PEP is consumed for each mol of NADH and 1 mol of bicarbonate for each of PEP. The maximum enzyme activity was reported as V_{max} for PEP formation. To calculate the E_a , $\log V_{\text{max}}$ versus the inverse of the measuring temperature (K) times 1,000 was plotted (Arrhenius plot), and the slope of the line determined by linear regression (Origin 7.0). The slopes were determined separately at high (15°C–28°C or 18°C–28°C) and low (6°C–15°C or 6°C–15°C) temperature, because a break point, reflecting a shift in E_a for PPKD, was apparent at 15°C. Fitting two lines on either side of the break at 15°C accounted for significantly more residual variation than use of a single line (F , statistic). E_a was calculated as the product of the slope, 2.3 (to convert from \log_{10} to \ln), and the ideal gas constant ($R = 8.3143 \text{ J/mol K}$) to yield E_a in units of kJ/mol.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Quantification of PPKD in crude leaf extract of *M. × giganteus* grown continuously at 25°C (25) and or grown at 25°C and then transferred to 14°C (14) for 14 d, before sampling.

Supplemental Figure S2. Changes in PPKD protein contents in leaves of *M. × giganteus* (MG) and maize (ZM) grown continuously at 25°C (25) and then at 14°C (14) for 14 d upon illumination.

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