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Cold-tolerant C_4 photosynthesis in *Miscanthus x giganteus*

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Introduction

Some of our most important agronomic species (*e.g.*, maize, sorghum, sugar cane, and switchgrass) are extremely efficient producers when grown at high temperatures and otherwise optimal conditions, but both early season growth and the extent of their growing range are limited by poorer performance at low temperatures. These crops are highly productive because they use the C_4 photosynthetic pathway, which has the highest efficiency of photosynthesis known; however, this efficiency is lost below 20ºC (reviewed, Long 1999). This is of particular importance to the maize crop, which is limited in northern areas of cultivation by low temperatures during seedling development (Greaves 1996, Miedema *et al.* 1987). Specifically, the development of key photosynthetic enzymes and photosynthetic rates are both reduced in maize grown at 14ºC (e.g., Nie *et al.* 1995).

The rhizomatous perennial grass *Miscanthus x giganteus* (Greef et Deu., Greef & Deuter 1993) is from the same taxonomic group as sugar cane, sorghum and maize and uses the same C_4 photosynthetic pathway (NADP-ME form). However, in contrast to these other species it performs efficiently at temperatures as low as 5ºC (Beale *et al.* 1996). Thus, this species shows a far greater resilience to low temperatures, and is unaffected by chilling temperatures which severely limit photosynthesis in its relative, maize.

In C_4 species other than *Miscanthus*, chilling sensitivity may depend on the sensitivity of key C_4 photosynthetic enzymes. In particular, the C_4 photosynthetic isoform of pyruvate orthophosphate dikinase (PPDK) has been shown to be low temperature labile, with a sharp transition in activation energy requirement around 10ºC (Du *et al.* 1999, Shirahashi *et al.* 1978). Furthermore, photosynthetic efficiency and biomass accumulation in maize is highly-correlated with PPDK activity, but not Rubisco (Sugiyama & Hirayama 1983, Ward 1987), suggesting that PPDK activity may be the rate-limiting step of C_4 photosynthesis in maize. In support of this hypothesis, PPDK enzymes extracted from C4 plants usually have activities just sufficient to support *in vivo* rates of photosynthesis (reviewed Long 1983) and often exhibit cold-sensitivity (Shirahashi *et al.* 1978, Sugiyama 1973). We hypothesize that a potential mechanism for cold tolerance in *Miscanthus* may be improved efficiency of PPDK at low temperature.

Our objective was to investigate the physiological basis for effective low-temperature C_4 photosynthesis in *Miscanthus* and to begin to identify the genetic components responsible. Photosynthesis was measured in maize and *Miscanthus* plants grown under optimum and low temperatures and the levels of key photosynthetic enzymes, PPDK, PEPc and Rubisco, determined using Western blot analysis.

Materials and Methods

Miscanthus x giganteus clones were propagated from rhizomes into 1.2L pots and *Zea mays* (genotype Fr1064, a commercial inbred line generously provided by Illinois Foundation Seeds) plants were germinated from seed in 0.3L pots in soil:sand:clay (1:1:1 v/v). Plants were grown in controlled environment chambers (Conviron E15, Controlled Environments Limited, Winnipeg, Manitoba, Canada) under 400 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD), 70% relative humidity, and 25/20 (warm) or 14/11 (cold) °C day/night temperature. Plants were kept well-watered and fertilized once/week with Peter's Professional 20:20:20 (N:P:K) plus micronutrients, commercial fertilizer (The Scotts Co., Marysville, OH, USA) at the recommended rate. All measurements were made on the youngest fully-expanded (after ligule formation) leaf on a shoot and confined to the second leaf formed.

The temperature response of photosynthesis was measured at 1000 µmol m^{-2} s^{-1} PPFD following acclimation at 500 µmol m^{-2} s^{-1} PPFD at 5, 10, 15, 20, 25, 30, 35, and 38 °C, using an open gas exchange system (LI-6400, LI-COR, Inc., Lincoln, NE, USA). The chamber was modified for extreme temperature control as described in (Bernacchi *et al.* 2001). Replicate sizes were 8-15 leaves at each temperature with each leaf being measured at 3-8 different temperatures.

Western blot analysis was performed on plants grown under similar conditions in a previous experiment (AL-Shoaibi 2001). Leaf tissue was collected from the youngest fully-expanded leaf and frozen in liquid nitrogen prior to protein extraction according to (Nie *et al.* 1993). Total leaf proteins were separated by SDS-PAGE and gels were blotted onto nitrocellulose. Samples were loaded on an equal leaf area basis. Blots were incubated for with the appropriate primary, polyclonal antibodies* after blocking with 6% (w/v) skimmed milk in phosphate buffered saline containing 0.0005% (v/v) Tween-20 (Sigma, UK) (PBS-T) and then washed with PBS-T. The blot was then incubated with sheep anti-rabbit secondary antibody (1:5000 dilution)

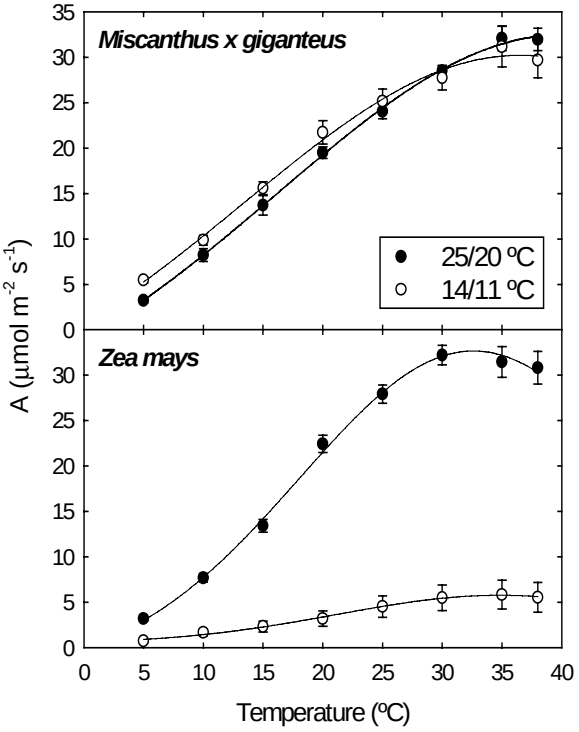

Fig. 1. Temperature response of photosynthesis for *Miscanthus* and maize grown under two temperature regimes. Error bars are ± 1 SE of the mean (n=8-15).

* Primary antibodies were provided by the following:
Rubisco: C. Raines; PEPc: R C. Leegood, Univ. of Sheffield, UK; PPDK: J N. Burnell, James Cook Univ., Australia

conjugated to horseradish peroxidase (Serotec, UK), washed repeatedly in PBS-T, and the proteins detected using Enhanced Chemiluminescence according to the manufacturer's directions (Amersham Life Science, Little Charlfont, UK).

Results

Photosynthesis of warm-grown leaves rose from 5˚C to an optimum near 35˚C for *Miscanthus* and near 30˚C for maize (Fig. 1). Cold-grown leaves of *Miscanthus* exhibited the same temperature optimum and a temperature-response curve similar to warm-grown *Miscanthus* leaves, although rates were higher at measurement temperatures of about 5-20˚C and lower above 35˚C. In contrast, cold-grown maize leaves exhibited an approximately 80% reduction in photosynthesis at all measurement temperatures.

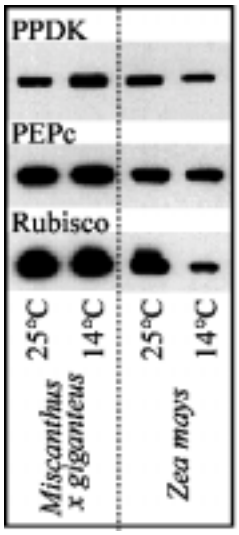

Fig. 2. Western blots of *Miscanthus* and maize leaves

Western blot analysis showed that amounts of PPDK protein in extracts from cold-grown *Miscanthus* leaves were greater than in extracts from warm-gown leaves (Fig. 2). Amounts of PEPc and Rubisco did not differ between leaves grown at different temperatures. This contrasts remarkably with maize, which has reductions in all three photosynthetic enzymes.

Discussion

The photosynthetic data presented here support previous results demonstrating that *Miscanthus x giganteus* is cold-tolerant. Photosynthetic rates of *Miscanthus* leaves grown at 14˚C were equal to or surpassed rates of leaves grown at 25˚C, suggesting that this species may be better adapted to low temperatures. The latter is interesting considering its presumed origins in warm temperate parts of South-East Asia. These data are in remarkable contrast to maize which shows marked decreases in photosynthesis when grown at 14˚C. Western blot analysis of Rubisco, PPDK and PEPC, key photosynthetic enzymes of the C_4 pathway, suggest that all three may play some role in enhancing cold-tolerance in *Miscanthus*. These data support the results of Du *et al.* (1999) which suggest that multiple enzymes play a role in determining cold-tolerance of sugarcane species. The levels of both Rubisco and PEPc are maintained at low temperature in *Miscanthus*, in contrast to maize, in which amounts of these enzymes are reduced in comparison to warm-grown leaves. The role of PPDK is particularly intriguing as amounts of this enzyme are reduced in cold-grown maize but are increased in *Miscanthus*. These data support the hypothesis that PPDK may be primarily responsible for cold-tolerant photosynthesis in *Miscanthus*, although increased stability of the other photosynthetic enzymes may also play a role.

We have yet to determine whether the increase in PPDK at cold temperatures is due to increased transcription or increased protein stability. The former hypothesis is currently being investigated using RT-PCR analysis of *Miscanthus* PPDK genes. Differences in protein structure and function between PPDK from maize and *Miscanthus* are also being examined by sequence analysis and expression of functional enzymes in *E. coli*.

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