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Induction of Phosphoenolpyruvate Carboxylase from *Hydrilla verticillata* Grown Under CO_2 Stress

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The goal of this research was to better understand the mechanism of environmental control of PEPC expression from *Hydrilla verticillata* grown under CO_2 stress. When PEPC concentration was plotted against CO_2 compensation point (Γ), a linear increase in PEPC concentration was seen with a decrease in CO_2 compensation point. Γ was measured on each day of the time course induction. *Hydrilla* plants exhibited an initially large decrease in Γ between day 0 and day 1; thereafter, the Γ declined in a fairly linear trend for the remaining 5 day time course. PEPC activity varied greatly over the time course. PEPC activity increased daily from day 0 through day 5 of the time course. Activity of PEPC increased almost 4-fold over the time course induction. The two upper bands with the approximately 100 kD mass are the PEPC subunits and PEPC was barely detectable in day 0 but increased through day 4 with day 5 being approximately equal to day 4. We provide evidence of increased PEPC protein concentration, and increased PEPC enzyme activity in as little as 24 h after beginning of induction.

Key words : *Hydrilla verticillata*, environmental induction, phosphoenolpyruvate carboxylase

INTRODUCTION

Most plants capture carbon through the photosynthetic carbon reduction cycle (PCR) or C_3 pathway. CO_2 is fixed by ribulose 1, 5-bisphosphate carboxylase/oxygenase (Rubisco) and enters the PCR cycle. However, Rubisco also catalyzes an oxygenase reaction in which O_2 enters the photorespiratory carbon oxidation (PCO) cycle. Therefore, CO_2 and O_2 compete as substrates for Rubisco. This PCO cycle results in a subsequent loss of CO_2 and reduces photosynthetic efficiency by as much as 40% under atmospheric conditions (Ku *et al*., 1999). C_4 plants have effectively overcome O_2 inhibition of photosynthesis by elevating CO_2 levels around Rubisco with an additional biochemical pathway and Kranz anatomy. Under conditions of high light intensities and high temperature that result in lower internal CO_2 concentrations, C_4 plants have higher rates of photosynthesis than C_3 plants (Furbank and Taylor, 1995). C_4 plants growing in an environment in which internal CO_2 concentration are limited possess a selective advantage over C_3 plants in the same environment.

The submersed freshwater environments that are densely populated suffer from low CO_2 and high O_2 concentrations relative to air (Spencer *et al*., 1994). Submersed species have adapted to this environment by evolving a variety of CO_2 concentrating mechanisms (CCM) (Boston, 1986; Bowes and Salvucci, 1989; Keeley, 1990). *Hydrilla verticillata*, a submersed aquatic angiosperm, exhibits facultative C_4-like photosynthetic metabolism under limiting CO_2 where its photosyn-

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thetic phenotype switches from C_3 to C_4–like photosynthetic metabolism (Holaday and Bowes, 1980; Salvucci and Bowes, 1981; Spencer *et al*., 1994). This C_4–like state is characterized by a lower CO_2 compensation point (Γ), decreased O_2 inhibition of photosynthesis, and lower photorespiratory rates than the C_3 phenotype (Salvucci and Bowes, 1981). The dissolved inorganic carbon (DIC) concentrations in C_4–like *Hydrilla* leaves are up to 5 times higher than that of the bathing medium, while C_3 leaves had DIC concentrations similar to the bathing medium (Reiskind *et al*., 1997). This evidence suggests the presence of an inducible CCM. HCO_3^- utilization cannot account for this CCM because C_4–like leaves retain their CO_2 concentrating abilities in a low pH medium (Reiskind *et al*., 1997). The C_4–like photosynthetic phenotype exhibits increased activity and concentrations of key C_4 enzymes (Salvucci and Bowes, 1981; Magnin *et al*., 1997; Rao *et al*., 2002). However, *Hydrilla* lacks Kranz anatomy and is not a CAM plant. This leads to the question of how a plant would avoid futile cycling of carboxylase/decarboxylase activities. Magnin *et al*. (1997) and Reiskind *et al*. (1989) found PEPC activity to be located in the cytoplasm, while NADP–ME activity was located in the chloroplasts. This is consistent with an organelle compartmentation model in which CO_2 is fixed into an organic acid (C_4 acid) in the cytosol. This organic acid is transported into the chloroplast where it is decarboxylated to elevate CO_2 around Rubisco as first proposed by Salvucci and Bowes (1981).

Previous research has shown that PEPC reached maximal activity around day five of induction from C_3 to C_4–like photosynthetic phenotype (Magnin *et al*., 1997). Our goal is to better understand the mechanism of environmental control of PEPC expression in *Hydrilla*. We provide the evidence of increased PEPC protein concentration, and increased PEPC enzyme activity in as little as 24 h after beginning of induction.

MATERIALS AND METHODS

1. Plant material and photosynthetic phenotype induction

Hydrilla verticillata (L.f.) Royle was obtained from Newnans Lake, Alachua County, Florida and washed visibly free of epiphytes. The plants were maintained in continuous culture in tanks in a greenhouse using flowing water pumped in from Kentucky Lake. To induce C_4 biochemistry, the plants were treated as in Spencer *et al*. (1994). Three terminal shoots (5–10 cm length) were immersed in a 3.5×20 cm test tube containing 5% (v/v) Hoagland solution. These tubes were placed in a growth chamber with a 14 h photoperiod under a quantum flux of 600 µmol $m^{-2}s^{-1}$, 30°C and 10 h dark period at 20°C for up to 5 days. The Hoagland solution was changed every other day. Tubes were taken from the growth chamber as necessary for experiments every day for 5 days.

Measurement of the Γ was accomplished using an infrared gas analyzer in a closed system of Li–COR (Li–6400) (Van *et al*., 1976). Measurements were made at a quantum flux of 600 µmol $m^{-2}s^{-1}$, pH 5.5 in 20 mM MES and 5% (v/v) Hoagland solution at 25°C water temperature.

2. Soluble protein extraction and PEPC activity assay

Plants were selected during the induction phase at 0, 1, 2, 3, 4, and 5 d. The plants were harvested in the light, rinsed, and approximately 2 g fresh mass (exact mass recorded) was homogenized in a Ten Broeck glass tissue grinder on ice in 10 mL of extraction buffer. The extraction buffer contained 200 mM HEPES (pH 7.0), 5 mM DTT, 10 mM $MgCl_2$, 2% (w/v) PVP–40. The samples were centrifuged at 10,000 g for 5 min at 4°C and the supernatants were collected. An aliquot of the supernatant was taken for protein concentration determination using a dye–binding protein assay (Bradford, 1976). For PEPC activity assays, an aliquot was taken from the extraction and 1% (w/v) bovine serum albumin was added to reduce effect of proteases on PEPC.

PEPC activity was assayed by a coupled reaction with NADH malate dehydrogenase. The reaction was measured in a spectrophotometer at A_{340} and ambient room temperature (Schuller *et al*., 1990). The final concentration in the assay mixture was: 90 mM Tris–HCL pH 8.0, 9 mM $MgCl_2$, 1.8 mM phosphoenolpyruvate, 450 µM $NaHCO_3$, 126 µM NADH, 5 units of malate dehydrogenase. A 100 µL aliquot of the protein extract was added to the reaction mixture for final volume of 1 mL. The reaction was initiated by the

addition of the enzyme extract.

3. ELISA

Plants were selected before induction and at various Γ. The plants were harvested in the light, rinsed, and homogenized in a Ten Broeck glass tissue grinder on ice in 10 mL of extraction buffer containing 50 mM Tris (pH 8.0), 5 mM DTT, 10 mM $MgCl_2$, 5 mM isoascorbate, 1.5% (w/v) PVP−40, 1 mM PMSF, and 0.1 mM leupeptin. The samples were centrifuged at 10,000 g for 5 minutes at 4°C and the supernatants were collected. An aliquot of the supernatant was taken for protein concentration determination using a dye−binding protein assay (Bradford, 1976).

A direct ELISA was developed to quantitate PEPC in crude extracts of *Hydrilla verticillata* leaf tissue (Crowther, 1995). Optimal PEPC protein standard and antibody−conjugate concentrations were determined by the criss−cross method of the serial dilution analysis. The PEPC standard used was obtained from corn (Biozyme, Inc., San Diego, CA). The antibody−conjugate used was anti−PEPC of corn conjugated to horseradish peroxidase (Biodesign International, Inc., Saco, ME).

For the ELISA, serial dilutions of corn PEPC standard were loaded in the wells of a microtiter plate with ranged from 2.5 to 100 μg mL^{-1}. The crude extract of *Hydrilla verticillata* was diluted 1 : 10 in TBST. The plate was coated at room temperature overnight and then washed 3 times with TBST. The wells were then blocked with blocking buffer containing 10 mM Tris−HCl, 150 mM NaCl, 0.05% (v/v) Tween 20, 1 mM EDTA, 0.25% (w/v) bovine serum albumin at pH 7.6 and incubated for 30 min at room temperature. The plates were rinsed 3 times in TBST. Anti−PEPC −horseradish peroxidase was added to the wells and incubated for 2 h at room temperature. The plate was rinsed 3 times in TBST and the substrate TMB was added. The reaction mixture was incubated for 30 min at room temperature and then stopped with 3 M H_2SO_4. The solution of each well was transferred to a microcuvette and measured in a spectrophotometer at 340 nm.

4. Western Blotting

For western blotting, an aliquot of the protein extract was mixed with an equal part sample treatment buffer containing 125 mM Tris−HCl pH 7.0, 4% w/v SDS, 25% glycerol, 10% v/v β−mercaptoethanol, and 0.02% bromophenol blue and boiled for 1 min. The samples and molecular weight marker were subjected to SDS−PAGE on precast 4−20% acrylamide, Tris−glycine gels. The proteins were transferred to a nitrocellulose membrane using a Trans−blot apparatus (Bio−Rad Lab., Hercules, CA). This transfer was carried out in Towbin Transfer at 300 mA and 100 V for 2.5 h (Towbin *et al.*, 1979). The membrane was rinsed twice in TBS and blocked with 1% blocking solution (Chemiluminescence Blotting Substrate Kit, Roche Diagnostics Corporation, Indianapolis, IN) at 4°C overnight. The blot was incubated with 4 μg mL^{-1} rabbit anti−PEPC polyclonal antibody (Biodesign International, Saco, ME) in 0.5X blocking solution at room temperature for 1h. The blot was then washed twice with TBST for 10 min each, and twice with 0.5% blocking solution for 10 min each. The blot was then incubated with 2 μg mL^{-1} goat anti−rabbit IgG−horseradish peroxidase conjugate in 0.5X blocking solution at room temperature for 30 min and washed four times in TBST for 15 min each. The blot was incubated in the detection solution for 60 sec and exposed to X−ray film. The film was then developed and analyzed.

For a more objective, quantitative measurement of the PEPC band on the western blots was carried out in the software program Quantity One (BioRad Lab., Hercules, CA) used to measure the peak density of each band. Two western blots from two independent time course inductions were measured and the results for each day were averaged.

RESULTS

1. Direct ELISA and CO_2 compensation points (Γ)

A direct ELISA was developed in order to quantitate the amount of PEPC in crude extracts of *Hydrilla verticillata* at various CO_2 compensation points. When PEPC concentration is plotted against CO_2 compensation point, a linear increase in PEPC concentration is seen with a decrease in CO_2 compensation point (Fig. 1). The concentration of PEPC increased more than two fold when CO_2 compensation point decreased from 118 μL L^{-1} to 10 μL L^{-1}, and reached the

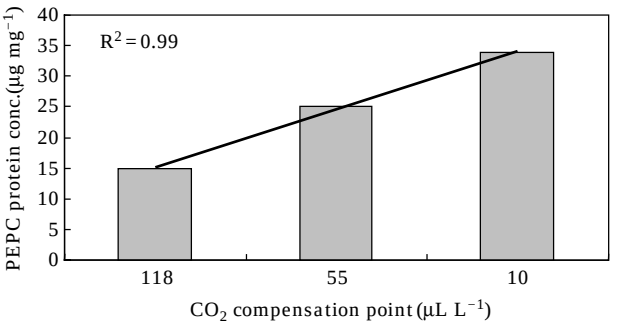

Fig. 1. Direct ELISA was used to determine the relative concentration of PEPC (µg PEPC mg^{-1} soluble protein) in crude extracts of *H. verticillata* at various CO_2 compensation points (Γ, µL L^{-1}).

highest concentration at a Γ of 10 µL L^{-1}. The direct ELISA were exhibited a significant negative relationship with Γ.

2. Γ, PEPC activity, and western blot analysis

Two independent time course inductions (two replications) were performed and the CO_2 compensation points (Γ), PEPC activity, and optical densities of Western blot bands were averaged for graphical representation. Averaging of the data was used to ascertain trends in the graphs.

the CO_2 compensation points (Γ) was measured on each day of the time course induction. *Hydrilla verticillata* exhibited an average Γ of 259 µL CO_2 L^{-1} on day 0, and an average largely decrease by 100 µL CO_2 L^{-1} in Γ between day 0 and day 1; thereafter, the µL declined in a fairly linear trend to from an average 100 µL CO_2 L^{-1} on day 1 to an average 42 µL CO_2 L^{-1} on day 5 (Fig. 2A). The average Γ for day 0 was skewed by a very high measurement of 259 µL L^{-1} with unknown reason

The level of expression of PEPC over the time course induction was assayed by activity levels of PEPC and western blot analysis. Activities of PEPC ranged from 0.20 ΔA_{340} min^{-1} mg^{-1} protein at day 0 to 0.75 ΔA_{340} min^{-1} mg^{-1} protein at day 5, and increased almost 4−fold over the time course induction (Fig. 2B).

The relative amount of PEPC protein was determined from two western blots and showed ranged from 0.023 to 0.18 dA min^{-1} g^{-1}. PEPC protein increased the almost 6 fold from day 0 to day 2, but was little increased for the remaining days. It was the highest value at day 4 (Fig. 2C).

Western blot analysis was performed on protein

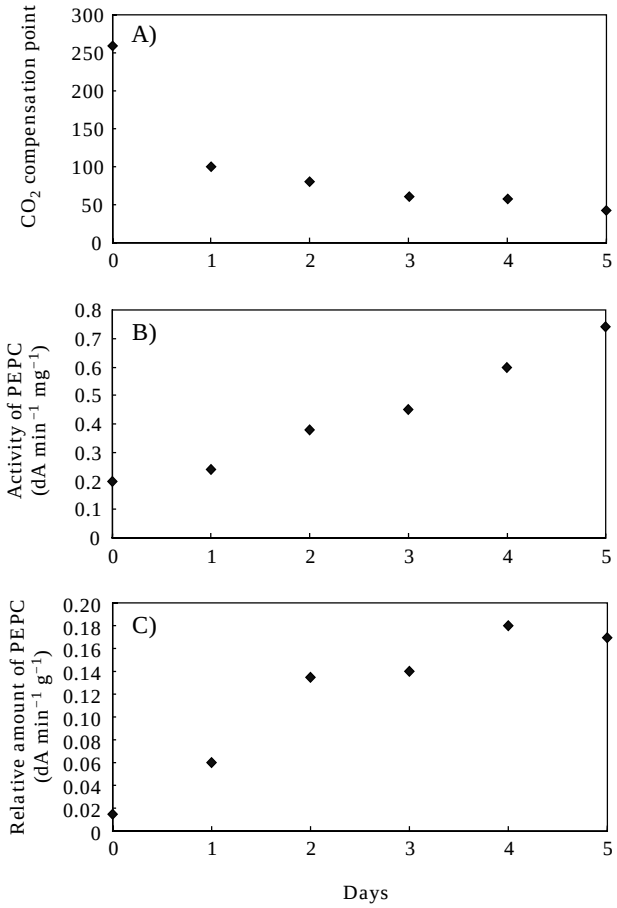

Fig. 2. (A) CO_2 compensation point (Γ) in µL L^{-1} of *H. verticillata* over a 5 day induction period, (B) PEPC activity of *H. verticillata* over a 5 day induction period, (C) averaged optical density of PEPC band from western blot analyses of *H. verticillata* over a 5 day induction period.

extracts from the time course induction to correlate activity values with the relative amounts of PEPC. The two upper bands with the approximately 100 kD mass were the PEPC subunits and the level of PEPC was barely detectable in day 0 but increased through day 4 with day 5 being approximately equal to day 4 (Fig. 3). Initially, PEPC protein increased rapidly but it reached a plateau by day 5. However, activity of PEPC increased slowly from day 0 to day 1; thereafter, activity increased at a more rapid rate without reaching a plateau. The increase in PEPC amount paralleled the increase in activity of PEPC.

The identity of the approximately 70 kD polypeptide (Fig. 3) that was recognized by the PEPC

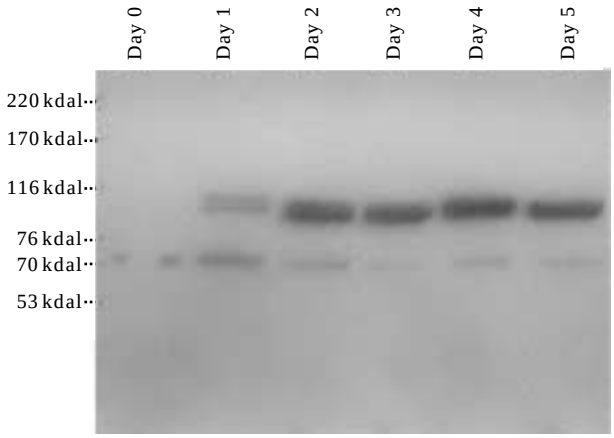

Fig. 3. Western analysis of crude extracts of *H. verticillata* over a 5 day time course induction using PEPC antibodies.

antibody remains in question. But there are several possible assumptions for the presence of these lower mass polypeptides. Because of its size, the lower, approximately 70 kD band on western blot was presumed to be degradation products of PEPC. A similar result has been reported by Harpster and Taylor (1986); where the PEPC antibodies bound to 77 and 84 kD polypeptide bands. The presence of the 70 kD polypeptide did not mask the measured increase of the 100 kD PEPC subunit during the induction of C_4−like photosynthesis.

DISCUSSION

Hydrilla verticillata has previously been shown to display a large range of CO_2 compensation points (Γ) values both in the laboratory and in the field (Spencer *et al*., 1996). A time course induction showed a linear decline in CO_2 compensation points (Γ) over 12days period (Magnin *et al*., 1997). However, they started their time course with lower Γ plants, typical of the summer months (Salvucci and Bowes, 1981). Our results do not show a linear decline over the 5 day period, but plants with initial higher CO_2 compensation points (Γ) were used along with a shorter induction period. It is possible and probable that a longer induction period would yield a linear decline in CO_2 compensation points (Γ) because PPDK and NADP−ME take up to 12 days to reach maximum activity levels (Magnin *et al*., 1997).

In our study of PEPC protein during induction, our western blot data showed a large increase of the level of PEPC after only one day of induction. Previously, Rao *et al*. (2002) had shown an increase the level of PEPC after four days after induction. Here, we show that an increase of PEPC protein can be measured at a much earlier time period, which indicates that *Hydrilla* plants sense changes in ambient CO_2 concentration very quickly with PEPC transcription beginning soon afterwards. These results may be helpful as research is directed toward understanding the initial events that occur during the transition from C_3 to C_4−like photosynthesis in *Hydrilla*.

We assume that the increased amount of PEPC found during the induction is due to increased amounts of the photosynthetic isoform. This assumption is reasonable given that Rao *et al*. (2002) found an increase in the photosynthetic isoform, but no increase in the anapleurotic isoform after 4 days of induction.

Increases in PEPC protein was confirmed by both direct ELISA assays and western blotting. Western blot analysis was barely able to detect PEPC before induction began, but showed a large increase in PEPC levels by day 1 of induction. These data suggest that the PEPC gene is switched on rapidly with accumulation of mRNA and subsequent translation to protein (Sugiharto *et al*., 1989). PEPC protein continued to increase up to day 4 of the induction period indicating that the transcript remained active for at least this amount of time. However, large increases in PEPC activity were not seen until after day 2 of induction. The lag in PEPC activity may reflect an additional step of PEPC activation. Bowes *et al*. (2002) has demonstrated that PEPC isolated from C_4−like *Hydrilla* plants is light activated. It is possible that PEPC enzyme activation is partially decoupled from PEPC protein production.

Given the strong evidence of inducible C_4−like photosynthesis in *Hydrilla* (Magnin *et al*., 1997; Reiskind *et al*., 1997) together with PEPC inhibitor data that demonstrate dependence of low Γ and low O_2 inhibition of photosynthesis upon PEPC activity (Spencer *et al*., 1996; Magnin *et al*., 1997), the rapid production of PEPC protein reported here represents an important advance in our understanding of inducible C_4−like photosynthesis in *Hydrilla*.

Hydrilla exhibits inducible C_4 biochemistry

within a single cell, which makes it of great interest regarding the possibility of genetically engineering C_3 agricultural crops to exhibit C_4–like photosynthesis (Bowes *et al*., 2002). Incorporating single–cell C_4 biochemistry into crop plants would be a much simpler strategy than transferring the complement of genes required for full C_4 biochemistry together with Kranz anatomy. This strategy appears feasible given the recent report that Kranz anatomy is not required for C_4 photosynthesis (Voznesenskaya *et al*., 2001). Realization of this single–cell strategy requires identification of the genes and the mechanisms of their induction that result in C_4–like physiology in *Hydrilla*.

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＜국문적요＞

CO_2 스트레스에서 생장한 검정말로부터 Phosphoenolpyruvate carboxylase 유도

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본 연구는 CO_2 스트레스 하에서 생장한 검정말로부터 PEPC 단백질 발현에 대한 환경조절 기작을 잘 이해하도록 하는데 그 목적이 있다. PEPC 농도와 CO_2 보상점과의 관계에서, PEPC농도는 CO_2 보상점(Γ)이 감소할 때 직선상으로 증가하였다. Γ를 유도하는 과정 동안 매일 측정하였다. 검정말은 초기 0일과 1일 사이에 Γ를 급감시키며, 이후 5일까지는 완만한 감소를 나타내었다. PEPC 활성은 0일에서 5일까지 유도 과정 동안 매일 증가하였으며, 5일 동안 4배 이상 증가하였다. PEPC는 100 KD으로 상부에 두 개의 band를 나타내었다. 본 연구의 결과 유도 시작 후 24시간 내에 PEPC 단백질과 효소활성이 증가하였다.