Induction of a C_4-Like Mechanism of CO_2 Fixation in *Egeria densa*, a Submersed Aquatic Species[1]

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The expression of phospho*enol*pyruvate carboxylase (PEPC) and NADP-malic enzyme (NADP-ME) in *Egeria densa* leaves was studied under low temperature and light (LTL) following incubation under high temperature and light (HTL), conditions previously shown to induce high and low CO_2 compensation points, respectively. Transfer from LTL to HTL conditions induced increases in the activities and amounts of both enzymes. One NADP-ME isoform was observed in induced and uninduced samples. Two isoforms of PEPC were expressed, with the lower M_r isoform being induced by HTL. NADP-ME showed properties similar to those of the isoform in C_3 species. The inducible PEPC isoform has a low K_m for both substrates. PEPC kinetic and regulatory properties (V_{max} and K_m for phospho*enol*pyruvate, and I_{50} for L-malate) are different in samples taken in the dark from those in the light, indicating that some modification of PEPC may be occurring during the day. Finally, abscisic acid induced the expression of PEPC and NADP-ME in a manner similar to temperature induction, except that the activities of both PEPC isoforms were increased. A different signaling system may exist in this species in response to high temperature or abscisic acid, both of which induce changes in photosynthetic metabolism.

The availability of inorganic carbon for photosynthesis differs considerably in air and in water. The supply of dissolved inorganic carbon species in water can be limiting because of the high diffusive resistance in water (Madsen and Sand-Jensen, 1991). CO_2, the substrate for Rubisco, is low in aquatic systems. When Rubisco fixes O_2, a competitive inhibitor with respect to CO_2, it initiates the process of photorespiration with the resultant loss of the fixed carbon. In this way aquatic autotrophs have developed different mechanisms to cope with limiting CO_2 and high O_2 concentrations (Bowes and Salvucci, 1989). These mechanisms include different CO_2 concentrating mechanisms and the ability to use HCO_3^- in photosynthesis (Raven, 1970; Bowes and Salvucci, 1989). Submersed aquatic macrophytes exhibit unique characteristics that are related to their environment such as low photosynthetic rates (Van and Haller, 1976), low light requirements, very high K_m (CO_2/HCO_3^-) values (Maberly, 1985), and the requirement of high CO_2 levels to saturate photosynthesis (Raven, 1970; Van and Haller, 1976; Madsen and Sand-Jensen, 1991). The most interesting characteristic is the plasticity that freshwater aquatic plants possess in relation to the biochemistry, physiology, and sometimes anatomy related to photosynthesis (Bowes and Salvucci, 1989).

Recent studies suggest that submersed aquatic macrophytes have a different photosynthetic metabolism from the ones present in terrestrial C_3, C_4, Crassulacean acid metabolism, and C_3-C_4 intermediate species. Most land plants use the C_3 pathway for carbon fixation, in which each photosynthetic cell uses Rubisco to fix CO_2 directly into C_3 compounds. In C_4 plants fully differentiated mesophyll and bundle sheath cells cooperate to fix CO_2 by the Hatch-Slack pathway (Edwards and Walker, 1983; Hatch, 1987). In these plants atmospheric CO_2 is first incorporated into C_4 acids in the mesophyll cells, which are then transported to bundle sheath cells where they are decarboxylated. The released CO_2 is in this way finally fixed by the C_3 cycle. The C_4 system is more efficient under some environmental conditions due to the increased concentration of CO_2 in bundle sheath cells that suppresses the oxygenase activity of Rubisco and, thus, photorespiration.

It has been demonstrated with a variety of submersed aquatic macrophytes that low CO_2 compensation points are induced by submergence and growth under stress conditions of low CO_2 levels, high temperatures, and long photoperiods (Salvucci and Bowes, 1981, 1983; Holaday et al., 1983; Bowes and Salvucci, 1989; Reiskind et al., 1997). There is evidence that at least three members of the Hydrocharitaceae show an appreciable Kranz-less C_4-acid metabolism in the light, including high phospho*enol*pyruvate carboxylase (PEPC) activity and fixation of radiolabeled carbon into malate and Asp (Brown et al., 1974; DeGroote and Kennedy, 1977; Browse et al., 1980; Salvucci and Bowes, 1983). These species are

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Hydrilla verticillata, *Elodea canadensis* and *Egeria densa*. For example in *E. densa* and *H. verticillata*, low CO_2 levels influence the products formed, with malate increasing at the expense of the Calvin cycle intermediates (Browse et al., 1977; Holaday and Bowes, 1980). Like the use of HCO_3^-, CO_2 fixation into C_4 acids could be part of a concentration mechanism to improve photosynthesis under carbon limiting conditions in these species. Both may be ancient mechanisms within submersed species considering that the Hydrocharitaceae is a submersed monocot family that may have its origin 100 million years ago in the Cretaceous period (Sculthorpe, 1967). This family could be more ancient than the C_4 monocots, which became more abundant in the Miocene (Sculthorpe, 1967; Ehleringer and Monson, 1993). In *H. verticillata* a C_4-like cycle has been described when this plant is grown under high temperature and light (HTL), both of which result in limiting conditions of CO_2 in water (Holaday et al., 1983). These effects produce gas-exchange and biochemical modifications that demonstrate a shift from C_3 to C_4 photosynthesis in the leaves (Bowes and Salvucci, 1989), but without having the characteristics of a typical Kranz anatomy (Reiskind et al., 1997). In this species after a 12-d induction period, the CO_2 compensation point declines, together with the O_2 inhibition of photosynthesis (Magnin et al., 1997). With this effect, PEPC, Asn, and Ala aminotransferases activities increase very rapidly (with the major increase in 3 d), with a slower but considerable increase of NADP-malic enzyme (NADP-ME) and pyruvate phosphate dikinase activities (Magnin et al., 1997). In contrast, the levels of Rubisco remain constant during this period. These results were also observed when protein levels were determined by western blotting, showing that the induction of some C_4 enzymes is consistent with a C_4 cycle concentrating CO_2 in leaves of *H. verticillata*. In *E. densa* when plants were incubated at 12°C with a 14-h photoperiod (low temperature and light, LTL), a compensation point of 43 μL CO_2 L^{-1} was determined (Salvucci and Bowes 1981). When these plants were incubated at HTL they showed a decrease in this parameter to a value of 17 μL CO_2 L^{-1}. These plants also showed maximal Rubisco activity of 76.0 and 70.6 μmol mg chlorophyll $(Chl)^{-1}$ h^{-1}, respectively; with a PEPC activity of 104.0 and 130.4 μmol mg Chl^{-1} h^{-1}, respectively. These results show a correlation in the decrease of the compensation point with the increase in temperature and PEPC activity.

In this work we studied the induction of two enzymes involved in C_4 metabolism, PEPC and NADP-ME, in *E. densa* under stress conditions of HTL. In addition we describe the effect of a plant hormone, abscisic acid (ABA), on the level and activity of these enzymes. *E. densa* is an interesting system to study the induction of C_4 photosynthesis because it has a simpler anatomy than terrestrial C_4 plants.

RESULTS

NADP-ME and PEPC Induction by HTL

E. densa shoots were transferred from conditions of LTL to HTL for up to 23 d. Different samples were taken during the period under study and PEPC and NADP-ME were measured by enzyme activity assays and western-blot analysis of protein extracts. All the assays were performed at least in duplicate in samples obtained from three distinct experiments conducted with different shoots.

Both PEPC and NADP-ME activities in shoots were markedly increased above constitutive levels after induction under HTL (Fig. 1). After 3 d of treatment, PEPC specific activity increased about 1.7 times relative to values in plants at LTL, whereas NADP-ME activity increased 1.26 times (Fig. 1). On the other hand after 23 d of treatment, the specific activity of PEPC increased about 3.7 times, and NADP-ME activity 3 times, both relative to the value determined in plants before induction (Fig. 1). Control plants did not show any change in PEPC and NADP-ME activity.

To determine whether these results were due to an increased synthesis of the protein, western-blot analysis of the samples was conducted using both an antibody raised and purified against maize NADP-ME

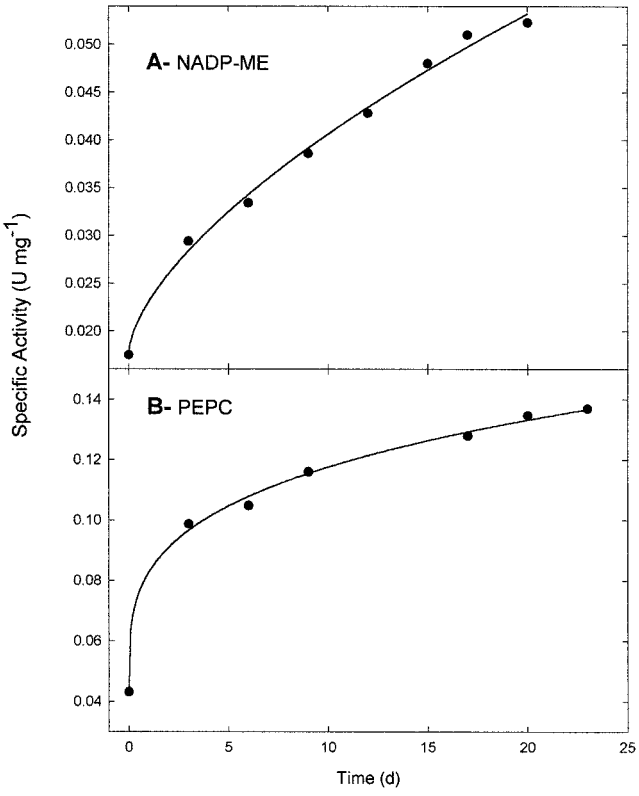

Figure 1. Effect of high temperature and high light exposure on the specific activity of NADP-ME (A) and PEPC (B) in *E. densa*. An average of the results obtained from the different repetitions is indicated. SE values were less than 5% in all cases.

and an antibody raised and purified against *Amaranthus viridis* PEPC. Figure 2 shows a typical result from one experiment. Low level expression of a 72-kD isoform of NADP-ME was evident in uninduced plants (Fig. 2A, lane 1), whereas induction of the synthesis of this protein is clearly shown after 23 d at HTL. Induction of NADP-ME was increased with days of treatment (Fig. 2A). In the case of PEPC two immunoreactive bands of 108 and 115 kD were present in plants kept under LTL (Fig. 2B, lane 1). After 15 d of induction under HTL the lower molecular mass form was clearly induced, whereas the level of the other isoform seemed not to be affected by the treatment (Fig. 2B, lane 3). The increase in PEPC activity can be related to an increase of the isoenzyme of lower molecular mass. Coomassie Blue staining was performed as a control to verify that the same amount of protein was loaded in each lane (not shown). Rubisco content appeared to remain constant during induction based on staining for the 55-kD subunit in the SDS-polyacrylamide gels, indicating that induction is specific for PEPC and NADP-ME. No morphological changes were observed in the plants during the induction time.

Isoforms of PEPC and NADP-ME in Crude Extracts of *E. densa*

Total protein was extracted from LTL and HTL *E. densa* shoots using phenol and used to perform two-dimensional PAGE coupled with western blotting (Figs. 3 and 4). Again when evaluated using antibodies against PEPC, the total protein extracted from plants at LTL exhibited two immunoreactive proteins of 108 and 115 kD (Fig. 3A). In contrast, the two-dimensional gels with proteins from HTL plants showed a major immunoreactive band corresponding to the 108-kD isoform (Fig. 3B). On the other hand, when the western blots were revealed using antibodies against NADP-ME (Fig. 4), only one isoform could be detected by this method in both uninduced and induced plants. Thus, in *E. densa* there exists at least two isoforms of PEPC, one a lower molecular mass isoform that is induced by HTL conditions. Only one isoform of NADP-ME seems to be present in this species.

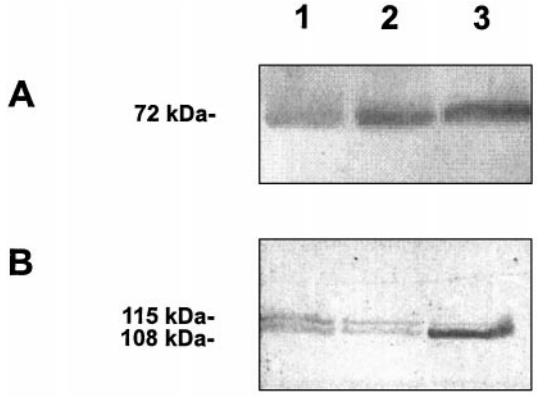

Figure 2. Effect of high temperature and high light exposure on the content of NADP-ME and PEPC in *E. densa*. Western-blot analysis of protein samples was performed with antibodies against NADP-ME (A) or PEPC (B). Thirty micrograms of total soluble protein was loaded in each lane. Lane 1, Control, d 0; lane 2, induction for 3 d; lane 3, induction for 15 d.

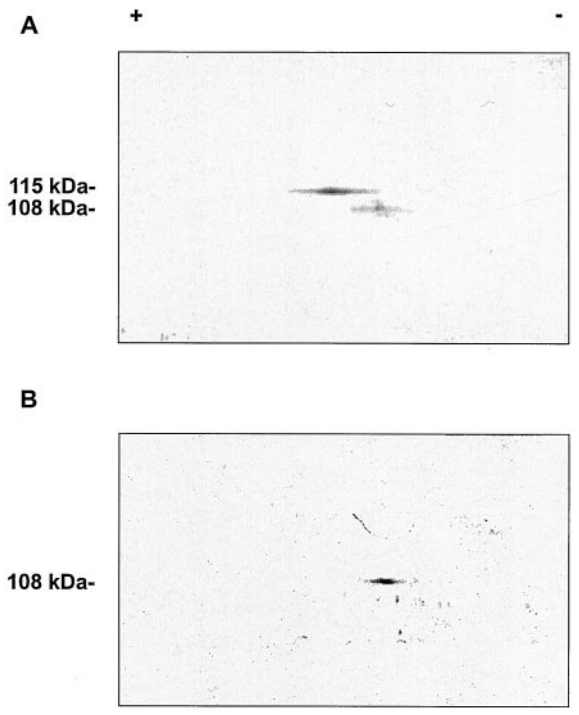

Figure 3. Two-dimensional western blots of total protein extracts (150 μg) from uninduced (A) and 23-d high-temperature induced (B) *E. densa* leaves. The membranes were treated with purified anti-*Amaranthus viridis* PEPC antibodies. The molecular masses of the immunoreactive bands are shown on the left.

Southern blotting was performed using either a 3′ terminal cDNA probe of maize NADP-ME or a 5′ terminal cDNA probe of maize PEPC. Digestion of *E. densa* genomic DNA with *Eco*RI, *Eco*RV, *Hin*dIII, and *Bam*HI produced only one band in all cases when NADP-ME cDNA was used as a probe. At least two hybridizing bands were detected after digestion with any of the restriction enzymes when PEPC cDNA was used as a probe (not shown). Again, these results suggest the presence of one gene for NADP-ME and more than one for PEPC in *E. densa*.

Subcellular Localization of NADP-ME and PEPC in *E. densa* Leaves

To study the localization of NADP-ME and PEPC in 23-d induced leaves at HTL, we separated the chloroplasts from a supernatant fraction and used them for enzyme activity assays and western blotting.

The activity (units per milligram of protein) of different enzymes in C_4 acid metabolism (NADP-ME, PEPC, and NADP-malate dehydrogenase [MDH]) was measured in both fractions. The results (Table I) in-

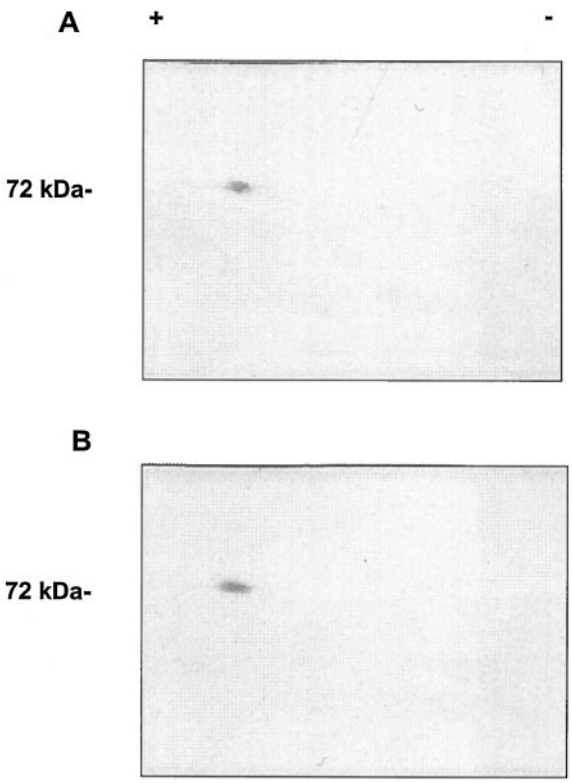

Figure 4. Two-dimensional western blots of total protein extracts (150 μg) from uninduced (A) and 23-d high temperature induced (B) *E. densa* leaves. The membranes were treated with purified anti-maize 62-kD NADP-ME antibody. The molecular masses of the immunoreactive bands are shown on the left.

dicate that the supernatant fraction had about 7.25 times higher PEPC activity than the chloroplasts. In contrast NADP-ME and NADP-MDH activities are higher in chloroplasts than in the supernatant (10.5 and 7 times higher, respectively). Western-blot analysis showed that NADP-ME is predominantly located in the chloroplasts, whereas PEPC is present in the supernatant (both isoforms) (Fig. 5). The same protein samples were also used to study the cellular localization of Rubisco, using antibodies against the spinach large subunit. The results show that Rubisco is located in the chloroplast.

Purification of NADP-ME from HTL Plants

To characterize NADP-ME in HTL *E. densa* plants (where the total amount of this enzyme is about 3

Table I. *Activity measurement of NADP-ME, PEPC, and NADP-MDH in fractions obtained from E. densa leaves*

Values are means of two preparations with SD < 0.001 in all cases.

Fraction	NADP-ME	PEPC	NADP-MDH
		units mg^{-1} total protein	
Supernatant	0.0026	0.0116	0.0046
Chloroplasts	0.0273	0.0016	0.0327

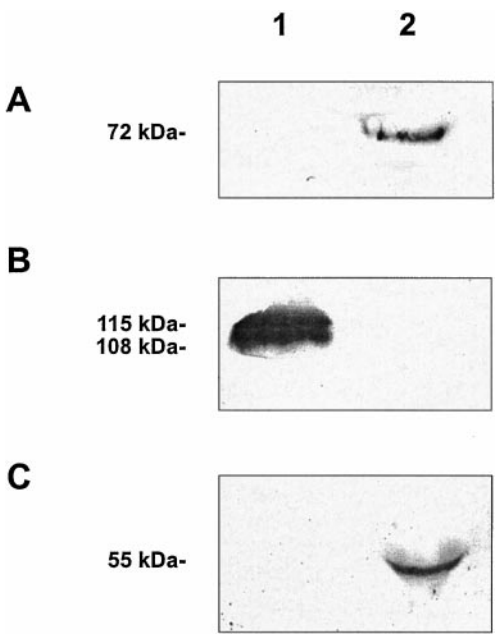

Figure 5. Western blot of protein samples (30 μg in A and B; 5 μg in C) from supernatant (lane 1) and chloroplast (lane 2) revealed with antibodies raised against maize NADP-ME (A), *Amaranthus viridis* PEPC (B), and spinach Rubisco large subunit (C). The calculated molecular masses of the immunoreactive proteins are indicated.

times higher than in plants at 12°C), a purification protocol was developed. The purified protein had a molecular mass of 72 kD, as revealed by SDS-PAGE and Coomassie Blue staining (Fig. 6).

The kinetic parameters of the purified NADP-ME were determined. At saturating concentrations of the substrates, the dependence of activity on pH revealed a maximum of activity centered at pH 7.3 (data not shown). The saturation curves obtained

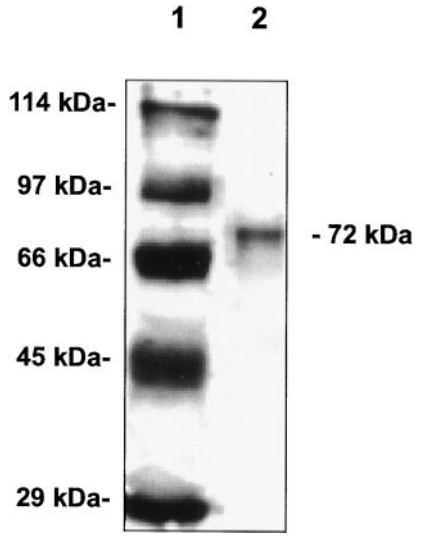

Figure 6. Coomassie Blue staining of molecular mass markers (lane 1) and the purified NADP-ME from *E. densa* (5 μg; lane 2). The molecular masses of the markers are indicated.

when the velocity of the reaction was measured as a function of free NADP in the presence of saturating concentration of L-malate (10 mM) and Mg^{2+} (10 mM) were typically hyperbolic, which was in agreement with a Hill coefficient of 1.0 obtained from Hill plots (not shown). In this way a low K_m value of 47.2 μM for NADP was calculated. In contrast the kinetics with respect to malate showed a sigmoidal response with a $S_{0.5}$ value of 4.5 mM.

As in the case of other NADP-ME from different sources (Edwards and Andreo, 1992), the enzyme required a divalent cation, Mg^{2+} or Mn^{2+}, for activity. For Mn^{2+}, a non-linear double reciprocal plot was obtained, suggesting two binding sites for this metal cofactor in the enzyme. The K_m values were 43.6 and 0.72 μM. On the other hand a typically hyperbolic response was obtained for Mg^{2+}, with a K_m value of 1.46 mM.

These results indicate that NADP-ME from *E. densa* has kinetic characteristics that are to some extent similar to those of NADP-ME from C_3 plants, but with some differences to the enzyme from terrestrial species. Moreover, the molecular mass of the protein is similar to that of the enzyme from C_3 plants.

Partial Purification of the 108-kD PEPC Isoform

The lower molecular mass isoform (108 kD) of PEPC was partially purified from HTL plants. The kinetic parameters of this PEPC isoform were determined at pH 8.0. The saturation curve obtained when the velocity of the reaction was measured as a function of free PEP was hyperbolic, and a K_m value of 48.5 μM was calculated. For the calculation of the PEPC K_m value for HCO_3^-, the integrated Michaelis-Menten equation was used. By this method a low value of 7.7 μM was obtained. Both values show a high affinity of this isoform for these substrates in comparison with the kinetic parameters of other plant PEPC enzymes. In particular *E. densa* appears to have an inducible PEPC that has a very high affinity for HCO_3^- to cope with the stress of low CO_2 concentration.

Effect of L-Malate on PEPC Activity in Crude Extracts

Studies with PEPC from C_4 plants have shown that this enzyme is regulated by a mechanism of phosphorylation/dephosphorylation of a single Ser residue located in its N terminus, and that the phosphorylation is up-regulated by light (Vidal and Chollet, 1997). In the phosphorylated state the kinetic parameters of PEPC are sometimes modified depending on the source of the enzyme. Moreover, PEPC is feedback inhibited by L-malate (Andreo et al., 1987). In the phosphorylated form PEPC is less inhibited by this metabolite (Duff et al., 1995). We studied the effect of malate, together with the kinetic parameters of PEPC, in desalted crude extracts from plants under HTL or LTL in the light or dark. Crude extracts from samples taken after the dark period or after 5 h of initiation of the light period were used to determine the kinetic parameters. Moreover, the I_{50} for L-malate at a subsaturating concentration of the substrate PEP was calculated in each case. All the experiments were performed at pH 7.3, where the largest differences exist in kinetic parameters and malate inhibition in the phosphorylated and dephosphorylated forms of PEPC.

For plants kept for 23 d under HTL (where there is a major inducible isoform of PEPC of 108 kD), the calculated values for dark samples differed from those for light samples (Table II). Both V_{max} and K_m for PEP were modified. In the first case there was an increase from dark to light of about 1.5 times in V_{max}, whereas the K_m decreased 1.7 times. Thus, the V_{max}/K_m for PEPC for samples taken in the dark was 2.6 lower than the light samples. On the other hand, the I_{50} value for L-malate at pH 7.3 and 0.2 mM PEP in illuminated samples was 2.5 times higher than the calculated value in plants in the dark. Consequently, L-malate seems to be a more potent inhibitor of PEPC from plants taken in the dark.

These results suggest that some modification of PEPC is occurring during the day. This modification may be a change in the phosphorylation state of the induced enzyme (as observed by changes in kinetic parameters and malate inhibition). However, more studies need to be done to determine if a post-translational modification is taking place.

The same experiments were done on samples taken in the dark or in the light from plants kept under LTL. We could not detect changes in the kinetic parameters and malate inhibition for the two groups of samples (not shown). Because two immunoreactive bands of PEPC with similar intensity were observed, the values obtained are an average of the parameters of both isoforms.

ABA Effect on *E. densa*

It is known that ABA is a stress hormone in plants (Hartung and Davis, 1991). Moreover, there is evidence that ABA is involved in the determination of leaf identity in aquatic plants showing dimorphism between the terrestrial and the submerged form

Table II. *Kinetic parameters for PEPC from crude extracts in E. densa plants in the light and in the dark*

V_{max}, K_m for PEP, and I_{50} values for L-malate inhibition for PEPC from crude extracts of temperature-induced *E. densa* plants in the light or in dark were assayed. Values are means of three preparations with SD < 0.01 in all cases.

Growth Condition	V_{max}	K_m (PEP)	I_{50} (Malate)
	units mg^{-1}	*mM*	
Light	0.133	0.175	1.0
Dark	0.086	0.298	0.4

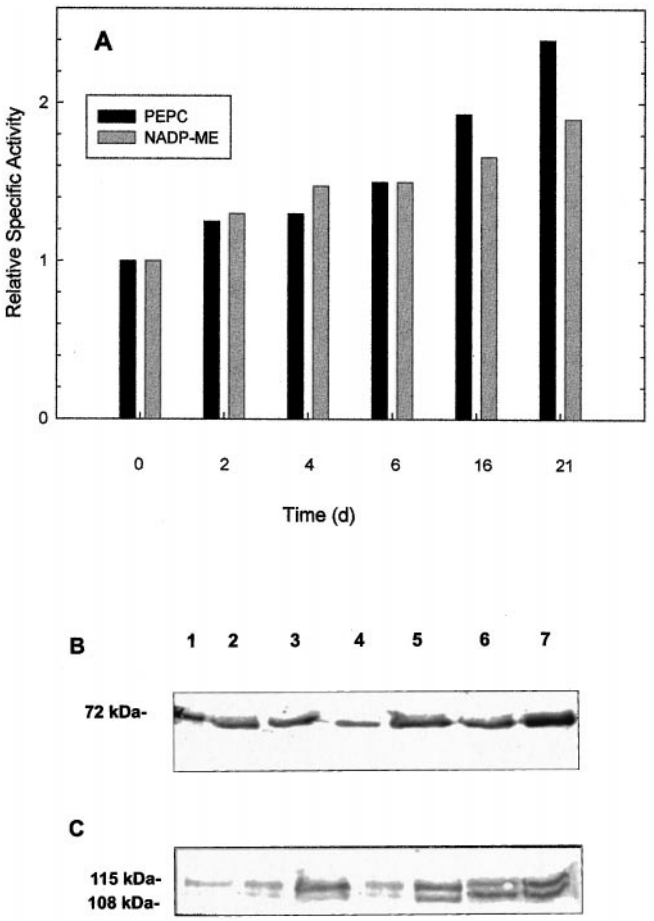

Figure 7. Effect of ABA on the specific activity and content of NADP-ME and PEPC in *E. densa* at 18°C. A, The specific activity of each sample is presented relative to the specific activity measured in control samples taken from untreated plants. An average of the results obtained from the different repetitions is indicated. SE values were less than 5% in all cases. B and C, Western-blot analysis of protein samples was performed with antibodies against NADP-ME (B) or PEPC (C). Lane 1, D 0; lane 2, 3 d without ABA; lane 3, 3 d with ABA; lane 4, 10 d without ABA; lane 5, 10 d with ABA; lane 6, 23 d without ABA; lane 7, 23 d with ABA. Thirty micrograms of total soluble protein was loaded in each lane.

(Anderson, 1978; Goliber and Feldman, 1989), as well as in the induction of Crassulacean acid metabolism in succulent plants (Dai et al., 1994; Taybi et al., 1995). ABA also induces some C_4 traits in *Eleocharis vivipara* (Ueno, 1998). This amphibious leafless sedge develops C_4-like traits as well as Kranz anatomy under terrestrial conditions, but develops C_3-like traits without Kranz anatomy under submersed conditions (Ueno et al., 1988). In the presence of ABA, photosynthetic tissues with Kranz anatomy and C_4-like biochemical traits are developed in the submerged form (Ueno, 1998).

In this study we attempted to evaluate the possible effects of ABA on the induction of C_4-like biochemical traits in *E. densa* at low temperatures (either 12°C or 18°C). Shoots were kept either at 12°C or 18°C in the absence (control) or presence of 5 μM ABA for up to 21 d. Samples were taken during this period, and PEPC and NADP-ME were measured by enzyme activity assays and western blotting. All the assays were performed at least in duplicate in different samples obtained at both temperatures.

At both temperatures, PEPC and NADP-ME activities in shoots were increased compared with the control levels after induction with ABA. After 21 d of treatment the PEPC-specific activity increased about 2.4 times relative to the control plants, whereas NADP-ME activity increased 1.9 times (Fig. 7). Induction of the appearance of both enzymes was increased with days of treatment (Fig. 7). To determine whether these results were due to increased quantity of the protein, western-blot analyses of the samples were conducted. Low expression of NADP-ME is evident in control shoots (Fig. 7B), whereas induction of the synthesis of this protein is clearly shown after induction with ABA. In the case of PEPC two immunoreactive bands of 108 and 115 kD were again present in samples from control and ABA-treated plants (Fig. 7C), In contrast with the results obtained after temperature induction, where after 15 d of induction the lower molecular mass form is clearly induced, the two isoforms seem to be induced by ABA (Fig. 7C) and the increase in the activity determined for PEPC and NADP-ME was lower than the increase measured after temperature induction. Again, during the induction time, no morphological changes were observed in the plants.

DISCUSSION

The photosynthetic features of submersed aquatic macrophytes differ from terrestrial species in several important ways. One difference is the plasticity that they show in acclimation to varying conditions. Extremes in concentrations of CO_2 cause unexpected effects on photosynthesis in these plants. In *E. densa* and *H. verticillata* grown at low CO_2 levels, the concentration of malate increases at expense of Calvin cycle intermediates (Browse et al., 1977; Holaday and Bowes, 1980). Moreover, the CO_2 compensation point decreases in this condition, with an increase in activities of C_4 enzymes (Salvucci and Bowes, 1981). We studied the expression of two C_4 enzymes, PEPC and NADP-ME, in *E. densa* leaves in conditions of high and low compensation points induced by LTL and HTL. During a 23-d induction period, both PEPC and NADP-ME activities were increased, with the major increase of PEPC activity occurring within 3 d. For NADP-ME the activity increased slowly over the induction period. Western-blot analysis shows that increased protein levels are involved in this process. The decrease of compensation point in this species, which has been determined by Salvucci and Bowes (1981), can be related to the induction of these C_4 enzymes. Moreover, we observed that PEPC, as in C_4

plants, is located in the cytosol of the photosynthetic cells, whereas NADP-ME and Rubisco are located in the chloroplasts. In this way the specific localization of these enzymes is very important for delivering inorganic carbon from the cytosol to chloroplasts via C_4 acids. The chloroplast is the site of CO_2 generation and consequently, of the concentration mechanism. Whereas only one inducible NADP-ME isoform was observed by western blotting of induced and uninduced samples, two different isoforms of PEPC seem to be present in *E. densa* leaves. The low molecular mass isoform is clearly induced after 15 d at high temperature, which may be directly related to induction of the carbon concentrating mechanism. In contrast, the 115 kD isoform was not modified after this treatment, which suggests that it may be involved in anaplerotic functions.

A 72-kD NADP-ME was purified from plants induced by HTL for 23 d. This is the only form described in leaves of C_3 terrestrial species like wheat (Casati et al., 1997), C_3 *Flaveria* sp. (Drincovich et al., 1998), and *Chenopodium album*, a C_3 species in the family of Chenopodiaceae (Casati et al., 1999). This form is also found in various organs in C_4 plants, including roots, etiolated leaves, as a minor form in green leaves of maize, and in roots, stems, and, as a minor form, leaves of C_4 *Flaveria* (Maurino et al., 1997; Drincovich et al., 1998). Analysis of the kinetic properties of the 72-kD enzyme indicates that it is similar to the isoform of the same M_r in C_3 species. The pH optimum was more acidic than the value obtained for C_4 enzymes (Edwards and Andreo, 1992), and it was not inhibited by L-malate like the C_4 enzymes (Edwards and Andreo, 1992; Casati et al., 1999).

With respect to L-malate, kinetics for NADP-ME varied from hyperbolic, to negative cooperative, to sigmoidal, depending on the source of the enzyme and the pH of the assay (Edwards and Andreo, 1992). For NADP-ME from *E. densa*, at pH 7.3, a sigmoidal response was obtained and the K_m for NADP is in agreement with K_m values previously reported for C_3 NADP-ME (Casati et al., 1997, 1999). NADP-ME from *E. densa* required either Mg^{2+} or Mn^{2+} as a cofactor. Negative cooperation was observed with respect to binding of Mn^{2+} as cofactor. On the other hand a hyperbolic response was found for Mg^{2+}, in contrast to previous results for C_4 isoforms (e.g. maize; Drincovich et al., 1991) and C_3 enzymes (e.g. wheat; Casati et al., 1997). However, in a number of studies hyperbolic saturation kinetics have been reported for other plant NADP-ME enzymes (Edwards and Andreo, 1992). In conclusion, *E. densa* has an inducible 72-kD isoform of NADP-ME with physical and kinetic properties similar to the enzyme from terrestrial C_3 plants. In this way this species would respond to a decrease in CO_2 concentration by the induction of an ancient isoform of NADP-ME similar to the one present in C_3 terrestrial species. The increase in the amount of NADP-ME after temperature induction may facilitate maintenance of high rates of decarboxylation of malate and delivery of CO_2 to Rubisco.

The inducible 108-kD PEPC isoform was also partially purified from HTL induced plants. This isoform has a low K_m for PEP, showing a hyperbolic response as a function of this substrate. Moreover, a very low K_m value for HCO_3^- of 7.7 μM was obtained. All the reported values for PEPC from different C_4 species (Bauwe, 1986) were higher than the K_m value for HCO_3^- in *E. densa.* Thus, this *E. densa* PEPC isoform has a high affinity for its substrates and is induced under conditions of low CO_2 availability.

Feedback inhibition of terrestrial isoforms of PEPC by L-malate is well established (Chollet et al., 1996). Another well-documented fact is that the enzyme is regulated by the phosphorylation/dephosphorylation of a single Ser residue (Nimmo et al., 1987; Chollet et al., 1996; Vidal and Chollet, 1997). The metabolite regulation of C_4 PEPC was shown to interact with its covalent regulation and to change the enzyme's kinetic and regulatory properties (Chollet et al., 1996). We investigated PEPC kinetic and regulatory properties in leaves of *E. densa* during light or dark. For HTL plants both V_{max} and K_m for PEP and I_{50} for malate were changed in samples taken in the dark compared with those in the light. Thus the affinity for PEP seems to be higher for the light form of the enzyme. These results suggest that some modification on PEPC could be occurring during the day. This modification could be a change in the phosphorylation state of the induced enzyme causing an increase in V_{max} and I_{50} for malate, together with a decrease in K_m for PEP as already described for PEPC from other sources (Duff et al., 1995; Vidal et al., 1997). However, in vitro and in vivo phosphorylation experiments need to be done to clarify this hypothesis.

Exogenously-supplied ABA induced the expression of both PEPC and NADP-ME. The patterns of accumulation of these photosynthetic enzymes in ABA-induced plants were similar to those after temperature induction. However, the increase in PEPC activity was related not only to an increase in the 108-kD isoform, but also in the levels of the 115-kD isoenzyme. Therefore it appears that a different signaling system may exist in this species in response to high temperature or ABA, both leading to changes in photosynthetic metabolism. This signaling mechanism may have evolved in plants that can change the mode of photosynthesis according to environmental fluctuations, like *E. vivipara*, *M. crystrallinum*, and *E. densa*.

In conclusion we have characterized the induction of a C_4-like mechanism of CO_2 fixation in *E. densa*, a submersed aquatic species. As already shown in *H. verticillata* (Magnin et al., 1997), *E. densa* has an inorganic carbon concentrating mechanism based on C_4 metabolism that takes place in a single cell. This

mechanism may represent an ancient form of C_4 photosynthesis occurring in terrestrial plants.

MATERIALS AND METHODS

Chemicals

NADP, L-malic acid, Tris [Tris(hydroxymethyl)aminomethane], MES, (2-[*N*-morpholino]ethanosulfonic acid), *N*-Tris ([hydroxymethyl] methyl-Gly), Tricine (*N*-[2-hydroxy-1,1-bis(hydroxymethyl) ethyl] Gly), MOPS (3-[*N*-morpholino] propanesulfonic acid), HEPES (*N*-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]), bovine serum albumin (BSA), M_r standards, PEP, NAD-MDH, and alkaline phosphatase-tagged goat anti-(rabbit IgG) IgG were from Sigma Chemical (St. Louis). Ampholytes were from LKB-Pharmacia (Uppsala). Nitrocellulose membrane was from Bio-Rad (Hercules, CA). All other reagents were of analytical grade.

Plant Material

Plants of *Egeria densa* were washed and maintained submersed in 5% (v/v) Hoagland solution at 12°C and for a 10-h photoperiod with a light fluence of 30 μmol m^{-2} s^{-1} for a week before the induction. After that the plants were kept under the same conditions (control, LTL) or transferred to a system at 30°C and a 14-h photoperiod with a light fluence of 350 μmol m^{-2} s^{-1} for up to 23 d (HTL). These conditions were used to mimic those present in winter and summer. For stress treatments with ABA the plants were placed in a flask containing distilled water in the absence (control) or presence of 5 μM ABA at either 12°C or 18°C with a 10-h photoperiod with a light fluence of 30 μmol m^{-2} s^{-1} for 21 d. Samples were taken after 5 h into the light period and were immediately frozen in liquid N_2 and stored at −80°C.

Protein Extraction and Gel Electrophoresis

Total protein from the different samples and subcellular fractions was extracted using a buffer containing 100 mM Tris-HCl, pH 7.3, 1 mM EDTA, 10 mM $MgCl_2$, 15 mM β-mercaptoethanol, 20% (v/v) glycerol, 1 mM phenylmethylsulphonylfluoride (PMSF), 10 μg mL^{-1} leupeptin, and 10 μg mL^{-1} chymostatin. The samples were ground completely in a cold mortar and centrifuged at 10,000*g* for 10 min at 4°C. For the assays of PEPC inhibition by malate, the supernatant of crude extracts was desalted in a cold Sephadex G-25 column pre-equilibrated with the above buffer. After that the extract was used for activity measurements or diluted in 0.25 M Tris-HCl, pH 7.5, 2% (w/v) SDS, 0.5% (v/v) β-mercaptoethanol, and 0.1% (v/v) bromphenol blue and boiled for 2 min for SDS-PAGE.

SDS-PAGE was performed with 8% (w/v) polyacrylamide gels. Proteins on the gels were either stained with Coomassie Blue or electroblotted onto a nitrocellulose membrane for immunoblotting according to Burnette (1981). Affinity purified anti-maize 62-kD NADP-ME IgG was used for detection (Maurino et al., 1997). A serum against spinach Rubisco large subunit, diluted 1:10,000, and an affinity purified anti-*Amaranthus viridis* PEPC IgG were also used. Bound antibody was visualized by linking to alkaline phosphatase-conjugated goat anti-rabbit IgG according to the manufacturer's instructions (Promega, Madison, WI). The molecular masses of the polypeptides were estimated from a plot of the log of molecular mass of marker standards versus migration distance.

For two-dimensional PAGE, the pH gradient used for isoelectrofocusing was from 4.2 to 7.5, and a gradient polyacrylamide gel (7.5%–15% [w/v]) containing SDS was used for separating proteins by size in the second dimension. After electrophoresis the proteins were electroblotted onto a nitrocellulose membrane for western-blot analysis. For this technique, total protein from *E. densa* was phenol extracted according to Van Etten et al. (1987). Protein concentration was determined by the method of Sedmak and Grossberg (1977) using BSA as a standard.

Enzyme Assay

NADP-ME activity was determined spectrophotometrically at 30°C by monitoring NADPH production at 340 nm. The standard assay medium contained 50 mM Tris-HCl, pH 7.3, 0.5 mM NADP, 10 mM L-malate, and 10 mM $MgCl_2$ in a final volume of 1 mL. One unit of enzyme activity is defined as the amount of enzyme resulting in the production of 1 μmol of NADPH min^{-1}.

PEPC activity was determined spectrophotometrically at 30°C in a coupled reaction with MDH by monitoring NADH oxidation at 340 nm. The standard assay medium contained 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 4 mM PEP, 0.15 mM NADH, and 10 units of MDH in a final volume of 1 mL. To determine malate sensitivity of PEPC, L-malate was added when the reaction was linear. The assay was performed at pH 7.3. The L-malate concentration range used in this study was chosen so as to encompass the inhibitor concentration causing 50% inhibition of the initial PEPC activity (I_{50}).

NADP-MDH activity was determined spectrophotometrically at 30°C by monitoring NADPH oxidation at 340 nm. The samples were pre-incubated in 100 mM dithiothreitol for 2 h before the assay. The standard assay medium contained 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.25 mM NADPH, and 1 mM oxaloacetate.

Enzyme Localization Procedures

About 6 g of leaves from *E. densa* were sliced into pieces at 4°C in a medium containing 1:2 (w/v) homogenizing solution of 50 mM Tris-HCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, 10 mM KH_2PO_4, 500 mM Suc, 1% (w/v) BSA, and 0.1% (w/v) polyvinylpyrrolidone-40, pH 7.6, according to the procedure described by Magnin et al. (1997). After filtering through three layers of cheesecloth, the filtrate was centrifuged at 15,900*g* for 10 min at 4°C. The supernatant was separated and kept in ice for enzyme assays or western-blot analysis, whereas the pellet was resuspended in 10 mM KH_2PO_4, 500 mM sorbitol, and 0.5% (w/v) BSA,

pH 7.2, layered onto a stepwise gradient of 50%, 45%, 40%, and 30% (v/v) Percoll, and centrifuged at 7,500*g* for 10 min. The chloroplast fraction together with the supernatant obtained were analyzed for PEPC, NADP-MDH, and NADP-ME activities and were subjected to western-blot analysis.

Purification of NADP-ME

Young shoots of *E. densa* were washed, chopped into pieces, and suspended in 300 mL of an extraction buffer A containing 100 mM Tris-HCl, pH 7.3, 10 mM $MgCl_2$, 2 mM Na_2HPO_4, 1 mM EDTA, 20% (v/v) glycerol, 10 mM 2-mercaptoethanol, 0.5% (w/v) ascorbate, and 1 mM PMSF. The shoots were homogenized using a blender. The homogenate was filtered through cheesecloth and centrifuged at 9,000*g* for 15 min. To the supernatant, crystalline ammonium sulfate was gradually added up to 30% saturation. After centrifugation at 9,000*g* for 30 min, the supernatant was brought to 70% saturation and centrifuged. The resulting precipitate was dissolved in 20 mL of purification buffer B containing 50 mM Tris-HCl, pH 7.3, 5 mM $MgCl_2$, 0.1 mM EDTA, 10% (v/v) glycerol, and 10 mM 2-mercaptoethanol and passed through a column of Sephadex G-75 previously equilibrated with buffer B. The eluate was then applied to a column of Q-Sepharose, equilibrated with buffer B connected to an FPLC system (Pharmacia). The enzyme was eluted with a linear gradient of NaCl (0–400 mM). The fractions containing NADP-ME activity were pooled and the protein was precipitated with solid ammonium sulfate at 70% saturation. The precipitate was collected by centrifugation, dissolved in buffer B, and dialyzed overnight against buffer B. The dialyzed enzyme was applied to a Matrex Blue Gel A column, equilibrated with buffer B, and attached to an FPLC system (Pharmacia). NADP-ME was eluted with a linear gradient of NaCl (0–400 mM). The fractions containing NADP-ME activity were precipitated with solid ammonium sulfate at 70% saturation. The precipitate was collected by centrifugation, dissolved in buffer B, and dialyzed overnight against the same buffer. The dialyzed enzyme was applied to a Fractogel EMD DEAE-650(S) column, equilibrated with buffer B, connected to an FPLC system (Pharmacia), and eluted with a linear gradient of NaCl (0–400 mM). Finally, the enzyme was applied to a Sephacryl S 300 HR column attached to the FPLC system and pre-equilibrated with buffer B. All steps were carried out at 4°C. Purified NADP-ME was stored at −20°C.

Purification of PEPC

Young shoots of *E. densa* were washed, chopped into pieces, and homogenized using a blender in 300 mL of a buffer C containing 100 mM Tris-HCl, pH 7.5, 5 mM L-malate, 50 mM Na_2HPO_4, 1 mM EDTA, 20% (v/v) glycerol, 15 mM 2-mercaptoethanol, 1 mM PMSF, 1 mM NaF, 10 $\mu g\ mL^{-1}$ leupeptin, and 10 $\mu g\ mL^{-1}$ chymostatin. The extract was allowed to adsorb batchwise for 30 min, shaking at 100 rpm, to a Q-Sepharose matrix previously equilibrated with purification buffer D containing 20 mM Tris-HCl, pH 8.0, 2 mM L-malate, 50 mM Na_2HPO_4, 0.1 mM EDTA, 5% (v/v) glycerol, 15 mM 2-mercaptoethanol, 1 mM PMSF, 1 mM NaF, 5 $\mu g\ mL^{-1}$ leupeptin, and 5 $\mu g\ mL^{-1}$ chymostatin. The slurry was then poured into a column connected to an FPLC (Pharmacia). The enzyme was eluted with a linear gradient of KCl (0–400 mM). The fractions containing PEPC activity were pooled and applied to a column of Phenyl-Sepharose equilibrated with the buffer D containing 400 mM KCl. The enzyme was eluted with a decreasing linear gradient of KCl (400–0 mM). The fractions containing PEPC were concentrated using a 30 PM tube (Centricon, Beverly, MA) and applied to a Sephacryl S 300 HR column, equilibrated with buffer D, and attached to the FPLC system. All steps were carried out at 4°C. PEPC was stored at −20°C.

Kinetics Studies

Initial velocity studies were performed by varying the concentration of one of the substrates around its K_m while keeping the other substrates concentrations at saturating levels. The K_m values of the substrates were calculated in terms of free concentrations by both linear and non-linear least-squares regression. Different buffer systems were used when analyzing the NADP-ME activity as a function of pH: 50 mM MES (pH 5.5–6.5), 50 mM Tricine-MOPS (pH 7.0), and 50 mM Tris-HCl (pH 7.5–8.5). The NADP-ME reaction was started by the addition of L-malate. The PEPC reaction was started by the addition of the protein. For calculation of the PEPC K_m value for HCO_3^-, the integrated Michaelis-Menten equation was used (Bauwe, 1986).

Genomic DNA Isolation and Southern-Blot Analysis

Leaves were ground in liquid nitrogen and suspended in extraction buffer: 10 mM piperazine-*N*, PIPES-KOH (*N*′-bis[2-ethanesulphonic acid]-KOH), pH 7.0, 1 M Suc, 10 mM $MgCl_2$, and 0.5% (v/v) Triton X-100. The isolated nuclei were washed twice with the above buffer containing 0.5 M Suc, and centrifuged at 3,000*g* for 10 min at 4°C. The precipitate was resuspended in 2 mL of lysis buffer: 2% (w/v) cetyltrimetylammonium bromide, 100 mM Tris-HCl, pH 8, 20 mM EDTA, 1.4 M NaCl, and 2% (w/v) 2-mercaptoethanol. After a 30-min incubation at 65°C, DNA was extracted twice with (24:1, v/v) chloroform:isoamilic and precipitated with 0.8 volume of isopropanol. The resulting pellet was washed with 70% (v/v) ethanol and dissolved in 50 mM Tris, pH 8, and 1 mM EDTA.

For Southern blotting, 15 μg of DNA was digested with *Eco*RI, *Eco*RV, *Hin*dIII, and *Bam*HI, and the resulting fragments were separated by electrophoresis in a 0.7% (w/v) agarose gel. DNA was transferred to a Hybond N+ membrane (Amersham, Buckinghamshire, UK) following the manufacturer's recommendation. Hybridization was done at 62°C, using either a 3′-terminal cDNA probe of maize NADP-ME or a 5′-terminal cDNA probe of maize PEPC.

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