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Photosynthetic characteristics of an amphibious plant, *Eleocharis vivipara*: Expression of C_4 and C_3 modes in contrasting environments

(C_4 and C_3 photosynthetic pathways/dimorphism/terrestrial and submersed aquatic environments)

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ABSTRACT **_Eleocharis vivipara_ Link, a freshwater amphibious leafless plant belonging to the Cyperaceae can grow in both terrestrial and submersed aquatic conditions. Two forms of _E. vivipara_ obtained from these contrasting environments were examined for the characteristics associated with C_4 and C_3 photosynthesis. In the terrestrial form (δ ^{13}C values = -13.5 to -15.4‰, where ‰ is parts per thousand), the culms, which are photosynthetic organs, possess a Kranz-type anatomy typical of C_4 plants, and well-developed bundle-sheath cells contain numerous large chloroplasts. In the submersed form (δ ^{13}C value = -25.9‰), the culms possess anatomical features characteristic of submersed aquatic plants, and the reduced bundle-sheath cells contain only a few small chloroplasts. ^{14}C pulse–^{12}C chase experiments showed that the terrestrial form and the submersed form fix carbon by way of the C_4 pathway, with aspartate (40%) and malate (35%) as the main primary products, and by way of the C_3 pathway, with 3-phosphoglyceric acid (53%) and sugar phosphates (14%) as the main primary products, respectively. The terrestrial form showed photosynthetic enzyme activities typical of the NAD-malic enzyme-C_4 subtype, whereas the submersed form showed decreased activities of key C_4 enzymes and an increased ribulose 1,5-bisphosphate carboxylase (EC 4.1.1.39) activity. These data suggest that this species can differentiate into the C_4 mode under terrestrial conditions and into the C_3 mode under submersed conditions.**

Photosynthetic CO_2 fixation by higher plants is thought to occur by one of three mechanisms, C_3, C_4, and CAM (crassulacean acid metabolism) (1). C_4 and CAM plants may have evolved from C_3 progenitors and this event may have occurred independently in diverse taxa (2). From an evolutionary point of view, it is of interest that certain families include C_3, C_4, and CAM species within a single genus (2), and it has been intriguing to investigate whether or not various photosynthetic modes could be found within a single species or within an individual plant. Some succulents can shift their photosynthetic mode between C_3 and CAM when subjected to water stress, NaCl stress, or photoperiod fluctuations (1). In CAM plants, the initial CO_2 fixation by phospho*enol*pyruvate (*P-e*Prv) carboxylase in the dark is followed by CO_2 fixation by the C_3 pathway that takes place in the light within a single type of green cell. In C_4 plants, the two processes are spatially compartmentalized and are carried out through the cooperation between two types of green cells, the mesophyll cells and the bundle-sheath (Kranz) cells (3, 4). Since C_4 plants possess biochemical and anatomical features conspicuously different from C_3 plants, it has generally been assumed that reversible change between the C_3 and C_4 modes would be unlikely. Ellis (5) reported that both C_3 and C_4 modes might occur within a grass species, *Alloteropsis semiallata*, although this observation needs confirmation. There is also increasing evidence for the existence of species showing various intermediate stages between the C_3 and C_4 modes (6). We report here that C_3 and C_4 photosynthesis is expressed in a dimorphic species of the Cyperaceae.

The Cyperaceae, a monocotyledonous family, includes many C_4 species (7). Most are hygrophytes growing in wet habitats and some possess the characteristics of amphibious plants (7, 8). We have investigated the photosynthetic modes of plants in this family (7) and found that *Eleocharis vivipara* Link, a freshwater amphibious plant, differentiates into a C_4 mode under terrestrial conditions and into the C_3 mode under submersed aquatic conditions.

MATERIALS AND METHODS

Plant Materials. Plants were collected from a creek in the vicinity of Tampa, FL, in August 1986. At this site, the plants grew along a gradient from terrestrial conditions to submersed conditions (at a depth of 40 cm). The terrestrial and the entirely submersed forms were transplanted into small pots (500 ml) containing sandy clay soil and grown in growth chambers (35°C during the day/30°C at night for the former and 27°C during the day/23°C at night for the latter) under sunlight for ≈3 months. The submersed form was maintained in aquaria (50 cm deep) in the growth chamber. Representative C_3 and C_4 species were grown in a greenhouse.

Anatomical Observation. Culms (stems) were transversely cut into small segments (≈1 mm thick) in 5% (vol/vol) glutaraldehyde/50 mM sodium phosphate, pH 7.3. Then these segments were transferred to fresh fixative at 4°C for 6 hr and postfixed with 2% (wt/vol) OsO_4 in the same buffer at 4°C for 12 hr. They were dehydrated through an ethanol series and embedded in Epon resin. Transverse sections were cut ≈1 μm thick with glass knives on an ultramicrotome and stained with toluidine blue O.

^{14}C Pulse–^{12}C Chase Experiments. For the terrestrial form detached culms (≈100 mg) were placed in a glass chamber (air space, 140 ml) equipped with a gas circulating system. The bases of the culms were submersed in distilled water. Temperature was maintained at 30°C by immersing the chamber in a water bath. Light intensity (incandescent) was 90 W/m^2 at the surface of the chamber. Humidified air was circulated at a flow rate of 2.5 liters/min. After 30 min of preillumination in circulating air, the gas-flow system was closed, and $^{14}CO_2$ (61.8 μCi; 1 Ci = 37 GBq) was injected into the chamber (final CO_2 concentration, 0.048%). After a

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Abbreviations: CAM, crassulacean acid metabolism; *P-e*Prv, phospho*enol*pyruvate; RbuP_2, ribulose 1,5-bisphosphate.

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30-sec (or 15-sec) ^{14}C pulse, the culms were transferred to another glass chamber in which ordinary air was circulating. At various chase times, the culms were removed and plunged into liquid nitrogen.

For the submersed form, apical segments (≈25 mg) were placed in a 5.0-ml glass tube containing 50 mM Hepes·KOH (pH 7.0). Temperature was maintained at 25°C by shaking the tube in a water bath. Light intensity was 72 W/m^2 at the surface of the tube. After 20 min of preillumination, $NaH^{14}CO_3$ was injected into the tube (final concentration, 1.0 mM; specific radioactivity, 10 $\mu Ci/\mu mol$). After a 30-sec ^{14}C pulse, the segments were removed, rinsed quickly in distilled water, and transferred into a glass beaker containing 1.0 mM $NaH^{12}CO_3$ in the same buffer. At various chase times, the segments were removed and plunged into liquid nitrogen.

The culms were ground in liquid nitrogen and extracted successively with 10% (vol/vol) acetic acid in ethanol, and then 80% and 50% (vol/vol) ethanol at 60°C. Each extraction time was 10 min. Portions of combined extracts and insoluble residues were used for determination of total ^{14}C fixed with a liquid scintillation spectrophotometer. The extracts were concentrated *in vacuo* and subjected to two-dimensional paper chromatography on Whatman no. 1 filter paper (9). After radioautography, the radioactivity in each spot was measured with a G-M counter.

Extraction and Assay of Enzymes. One gram of culms of *E. vivipara* and of leaves of other plants were ground in a mortar and a pestle with 1 g of sea sand, 0.1 g of insoluble polyvinylpyrrolidone (Polyclar AT; Gokyo Industries, Osaka, Japan), and 4 ml of grinding medium at 4°C. Grinding medium containing 50 mM Hepes·KOH (pH 7.5; pH 7.0 for *P-e*Prv carboxykinase), 0.2 mM EDTA, 2.5 mM $MgCl_2$, 2.5 mM $MnCl_2$, and 5 mM dithiothreitol was used for *P-e*Prv carboxylase (EC 4.1.1.31), NADP-malic enzyme (EC 1.1.1.40), NAD-malic enzyme (EC 1.1.1.39), and *P-e*Prv carboxykinase (EC 4.1.1.49) activity determinations. For NAD-malic enzyme assay, 50 mg of solid isoascorbate was added to the above medium before grinding. For pyruvate,P_i dikinase (EC 2.7.9.1), NADP malate dehydrogenase (EC.1.1.1.82), NAD malate dehydrogenase (EC 1.1.1.37), aspartate aminotransferase (EC 2.6.1.1), and alanine aminotransferase (EC 2.6.1.2) assays, the grinding medium contained 100 mM Tris·HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA, and 5 mM dithiothreitol. Pyruvate,P_i dikinase and NADP malate dehydrogenase were extracted under high light intensity (180 W/m^2) provided by a xenon lamp. Ribulose 1,5-bisphosphate (RbuP_2) carboxylase (EC 4.1.1.39) was extracted with 50 mM Tris·HCl (pH 8.5) containing 0.1 mM EDTA and 5 mM dithiothreitol. Homogenates were filtered through cheesecloth, treated with Triton X-100 [final concentrations, 0.5% (vol/vol)] for 5 min, and centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatants were used for enzyme assay.

Enzymes, except for RbuP_2 carboxylase, were assayed spectrophotometrically in 1-ml reaction volumes at 25°C as reported: *P-e*Prv carboxylase (10), NADP-malic enzyme (11), NAD-malic enzyme (12), *P-e*Prv carboxykinase (11), pyruvate,P_i dikinase (13), NADP- and NAD-malate dehydrogenases (14), and aspartate and alanine aminotransferases (15). RbuP_2 carboxylase was activated (16) and assayed radiometrically (17). Chlorophyll contents and a/b ratios were determined by the method of Arnon (18).

Carbon Isotope Analysis. Culm samples were collected from plants growing in the creek in the vicinity of Tampa, dried in an oven at 80°C for 2 days, and then ground. The tissue powders (10 mg) were combusted at 800°C for 8 min *in vacuo* with CuO by using the system described by Samejima (19). Isotope ratios of the purified CO_2 were measured on a mass spectrometer (Finnigan Mat model 250) and expressed in $\delta^{13}C$ units. The reference standard was Pee Dee Belemnite limestone. The reproducibility of the overall procedure was ±0.15‰ (‰, parts per thousand).

RESULTS

Fig. 1 shows the terrestrial and the submersed forms of *E. vivipara*. In all species of *Eleocharis*, the leaf blades are reduced and the culms (stems) are the main photosynthetic organ (8). The terrestrial form of *E. vivipara* exhibits a general gross morphology typical of the genus and possesses erect, firm culms. In contrast, the submersed form shows hair-like morphology consisting of slender, soft culms and reproduces new culms by proliferation from sterile spikelets at the apex of the culms.

The culms of the terrestrial form possess typical Kranz anatomy characterized by the presence of well-developed bundle-sheath cells (Kranz cells) containing numerous large chloroplasts and tightly arranged mesophyll cells (Fig. 2 *A* and *B*). As in some C_4 species of other members of the Cyperaceae, the mestome sheath lacking chloroplasts intervenes between the bundle-sheath cells and mesophyll cells (Fig. 2*B*). On the other hand, the culms of the submersed form possess one layer of spherical mesophyll cells inside the epidermis, reduced vascular bundles, and large air cavities (Fig. 2 *C* and *D*). Although small bundle sheath cells are present, they contain only a few small chloroplasts (Fig. 2*D*). These anatomical features of the submersed form cannot be regarded as Kranz type.

The pattern of ^{14}C incorporation into photosynthetic products in ^{14}C pulse–^{12}C chase experiments with the terrestrial form was typical of the C_4 pathway (Fig. 3). When the culms of the terrestrial form were exposed to air containing $^{14}CO_2$ for

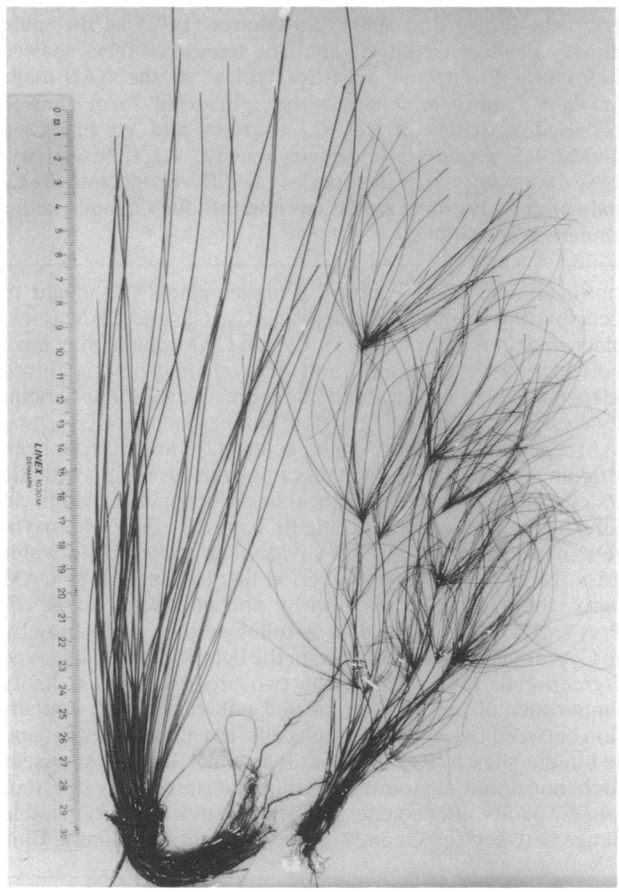

FIG. 1. Gross morphologies of the terrestrial form (to the left) and the submersed form (to the right) of *E. vivipara*. (Scale = 30 cm.)

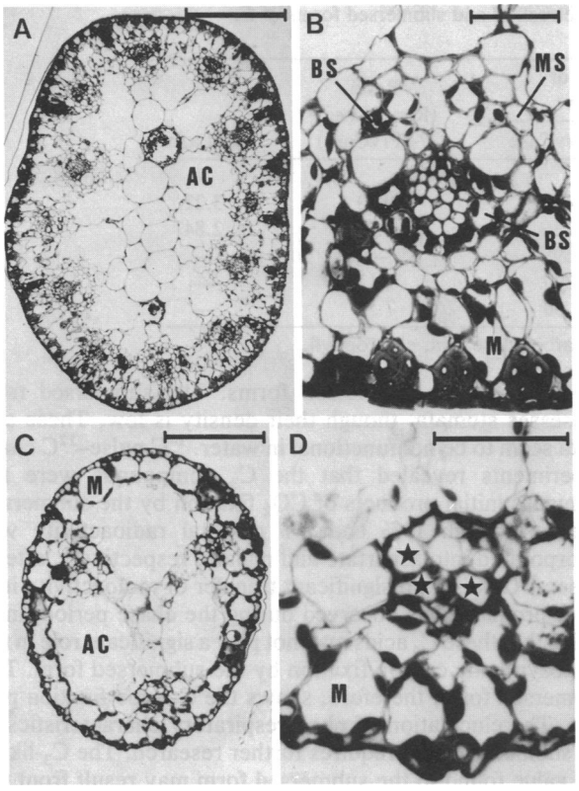

FIG. 2. Comparison of anatomical structures of culms of the terrestrial and the submersed forms of *E. vivipara*. (*A*) Cross section of a culm of the terrestrial form, showing the Kranz-type anatomy. (*B*) Vascular bundle and assimilatory tissues of the terrestrial form. Note nonchlorophyllous mestome-sheath and inner bundle-sheath cells (or Kranz cells) containing numerous chloroplasts. (*C*) Cross section of a culm of the submersed form; the anatomy is characteristic of submersed aquatic plants. (*D*) Vascular bundle and assimilatory tissues of the submersed form. Note bundle-sheath cells contain only a few small chloroplasts (see cells marked with stars). BS, bundle-sheath cell (or Kranz cell); MS, mestome-sheath; AC, air cavity; M, mesophyll cell; E, epidermis. (*A*, bar = 150 μm; *B* and *D*, bar = 25 μm; *C*, bar = 75 μm.)

30 sec, the radioactivity was mostly incorporated into aspartate (40%) and malate (35%), while only 9% of the total ^{14}C was incorporated into phosphate esters. During the chase in $^{12}CO_2$/air, the labels in aspartate and malate declined rapidly, while those in phosphate esters initially increased and then decreased. The label in sucrose showed an increase throughout the experiment. Another experiment with a 15-sec $^{14}CO_2$ pulse showed that 53% and 30% of the total ^{14}C were incorporated into aspartate and malate, respectively, whereas only 6% was incorporated into phosphate esters (data not shown).

When the apical segments of the submersed form were exposed to the labeling medium for 30 sec, the radioactivity was mostly incorporated into 3-phosphoglyceric acid (53%) and sugar phosphates (14%). On the other hand, only 13% and 8% of the total ^{14}C were incorporated into aspartate and malate, respectively (Fig. 4). During the chase, the label in 3-phosphoglyceric acid decreased rapidly while that in sugar phosphates increased up to 15–30 sec and then gradually decreased. The labels in aspartate and malate showed only slight decreases during chase. After a 90-sec chase, most of the radioactivity in 3-phosphoglyceric acid and sugar phosphates was transferred into sucrose. These labeling patterns suggest that the submersed form of *E. vivipara* fixes CO_2 mainly by the C_3 pathway. However, it is of interest that the label in glycine plus serine was maintained at a low level

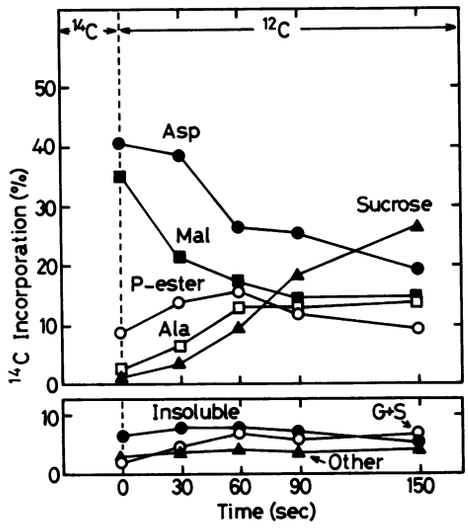

FIG. 3. Pulse–chase experiment of photosynthetic carbon fixation in the terrestrial form of *E. vivipara*. The ^{14}C pulse time was 30 sec. The rate of ^{14}C fixation during the pulse period was 56.2 μmol/mg of chlorophyll per hr. Asp, aspartate; Mal, malate; Ala, alanine; P-ester, phosphate esters; G+S, glycine plus serine.

during the experiment. Another experiment with 0.5 mM $NaH^{14}CO_3$ under the same conditions showed that there was no significant difference in the labeling patterns between 1 mM and 0.5 mM $NaHCO_3$ (data not shown).

Table 1 shows that the terrestrial form possessed lower RbuP_2 carboxylase and higher *P-e*Prv carboxylase activities than the submersed form of *E. vivipara*, and the activity ratio in the terrestrial form (0.13) was comparable to that found in a typical C_4 grass, *Eragrostis ferruginea* (0.11). The activity ratio in the submersed form (2.32) was clearly higher than in the terrestrial form and the C_4 grass, but lower than in a typical C_3 grass, *Bromus cathartianus* (7.56). Table 2 shows the activities of photosynthetic enzymes of the C_4 pathway. The terrestrial form showed higher activities of pyruvate,P_i dikinase and of enzymes involved in the NAD-malic enzyme

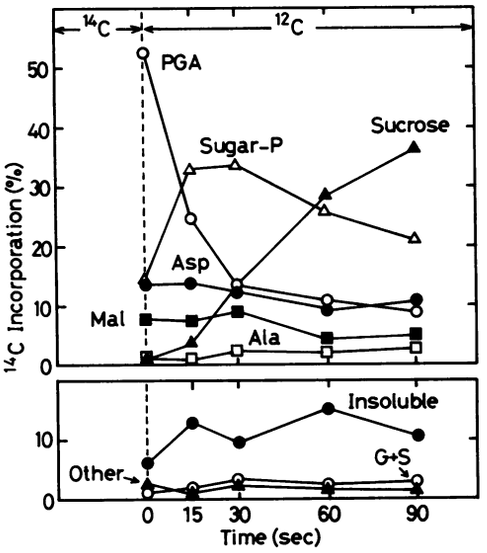

FIG. 4. Pulse–chase experiment of photosynthetic carbon fixation in the submersed form of *E. vivipara*. The ^{14}C pulse time was 30 sec. The rate of ^{14}C fixation during the pulse period was 21.2 μmol/mg of chlorophyll per hr. PGA, 3-phosphoglyceric acid; sugar-P, sugar phosphates; Mal, malate; Asp, aspartate; Ala, alanine; G+S, glycine plus serine.

Table 1. RbuP_2 carboxylase and *P-e*Prv carboxylase in terrestrial and submersed forms of *E. vivipara* and representative C_3 and C_4 species

Species	Activity, μmol/mg of chlorophyll per hr		Ratio (RbuP_2Case/ *P-e*PrvCase)	Chl a/b
	RbuP_2Case	*P-e*PrvCase		
E. vivipara				
Terrestrial form	64	499	0.13	3.21
Submersed form	153	66	2.32	2.84
Zea mays (C_4)	—	598	—	—
Eragrostis ferruginea (C_4)	152	1329	0.11	—
Bromus cathartianus (C_3)	378	50	7.56	—

RbuP_2Case, RbuP_2 carboxylase; *P-e*PrvCase, *P-e*Prv carboxylase; chl, chlorophyll.

type of decarboxylation (4), NAD-malic enzyme, NAD malate dehydrogenase, aspartate aminotransferase, and alanine aminotransferase than in the submersed form of *E. vivipara*. The submersed form had a lower chlorophyll a/b ratio than the terrestrial form (Table 1).

Analysis of δ ^{13}C values revealed that the terrestrial form of *E. vivipara* possessed C_4-like values, δ ^{13}C vs. Pee Dee Belemnite = −13.5‰ and −15.4. Its submersed form was found to have a δ ^{13}C value −25.9‰, characteristic of C_3 plants.

DISCUSSION

Amphibious plants exhibit distinctive morphological differences in leaves from the terrestrial and the submersed forms, a feature often referred to as leaf dimorphism or heterophylly (20, 21), and are generally assumed to fix CO_2 by the C_3 pathway. However, it has been reported that some plants, such as *Isoetes*, utilize a CAM-like C_4 acid metabolic system under certain submersed conditions (22). The present study shows that the terrestrial form of *E. vivipara* has the following characteristics: (*i*) ^{14}C is incorporated from CO_2 into aspartate and malate as the initial products; (*ii*) the label in C_4 acids "chases" into phosphate esters typical of C_4 plants; (*iii*) high activities of key C_4 enzymes are present; (*iv*) the ratio of RbuP_2 carboxylase/*P-e*Prv carboxylase activities is low; (*v*) the δ ^{13}C values are C_4-like; and (*vi*) the anatomy is of the Kranz type. These are characteristics of C_4 plants.

Upon submersion, *E. vivipara* developed anatomical structure characteristic of submersed aquatic plants. Its spherical mesophyll cells increased in size, while the bundle-sheath cells decreased in size, relative to those of the terrestrial form. In addition, these bundle-sheath cells contained only a few small chloroplasts, unlike those of the terrestrial form. These anatomical features suggest that the structural basis for the compartmentalization of enzymes in two types of green cells, which is essential for the operation of the C_4 pathway, is lacking in the submersed forms. The submersed form possesses stomata, though their density is low. These stomata seem to be nonfunctional in water. ^{14}C pulse–^{12}C chase experiments revealed that the C_3 compounds were the dominant initial products of CO_2 fixation by the submersed form. Although 13% and 8% of total radioactivity was incorporated into aspartate and malate, respectively, after a 30-sec ^{14}C pulse, no significant transfer of radioactivity into other products was observed during the chase period, indicating that these C_4 acids may not play a significant role in net photosynthetic carbon fixation by the submersed form. The submersed form, therefore, shows the C_3-type fixation pattern. The elucidation of photorespiratory characteristics in the submersed form requires further research. The C_3-like δ ^{13}C value found in the submersed form may result from the operation of the C_3 pathway. Other factors, such as the δ ^{13}C value of the source carbon in water and the species of inorganic carbon fixed and diffused through the aquatic phase, affect the values in the submersed aquatic plants (1, 23). The high activity of RbuP_2 carboxylase and the low activities of key C_4 enzymes in the submersed form are in general agreement with the carbon fixation pattern of C_3 type and the absence of Kranz anatomy.

Preliminary experiments showed that the culms died when the submersed form was exposed to air, but the plants would develop new photosynthetic organs with the C_4 mode. In contrast, when the terrestrial form was immersed in water the plants produced new hairlike culms with C_3 mode. *E. vivipara* is a unique plant that can reversibly change from C_4 to C_3 modes of photosynthesis depending on its environment. It seems likely that the tissue-specific expression of genes related to the photosynthetic mechanism in *E. vivipara* may be controlled by submersion or closely related factors, and this plant may offer an intriguing model system for understanding the regulation of gene expression involved in C_3 and C_4 photosynthesis.

Table 2. Key C_4 photosynthetic enzymes of the terrestrial and the submersed forms of *E. vivipara* and representative C_3 and C_4 species

Species	Activity, μmol/mg of chlorophyll per hr							
	Prv,P_iDK	NADP-ME (Mg^{2+})	NAD-ME* (Mn^{2+}, CoA)	PCK	NADP-MDHase	NAD-MDHase	Asp-ATase	Ala-ATase
E. vivipara								
Terrestrial form	102	14	123	ND	26	2983	1717	1217
Submersed form	25	—	56	ND	4	2172	439	493
Zea mays (C_4, NADP-ME)	296	634	42	ND	693	4093	366	113
Eragrostis ferruginea (C_4, NAD-ME)	213	38	164	ND	49	5862	1197	1709
Chloris gayana (C_4, PCK)	—	32	14	538	117	6752	786	755
Bromus cathartianus (C_3)	—	7	3	—	295	3794	79	370

Prv,P_iDK, pyruvate,P_i dikinase; NADP-ME, NADP-malic enzyme; NAD-ME, NAD-malic enzyme; PCK, *P-e*Prv carboxykinase; NADP-MDHase, NADP malate dehydrogenase; NAD-MDHase, NAD malate dehydrogenase; Asp-ATase, aspartate aminotransferase; Ala-ATase, alanine aminotransferase; ND, not detectable.

*For NAD-malic enzyme assay, 0.15 mM CoA was added as an activator to the reaction mixture.

It has been demonstrated that C_4 plants exhibit the best development and adaptation in tropical and subtropical savannas, where plants experience high irradiance, high temperature, and intermittent water stress (3, 24). Accordingly, C_4 plants with Kranz anatomy have not yet been found among submersed aquatic plants (25). However, *Hydrilla*, a freshwater macrophyte, shows C_4 acid metabolism without Kranz anatomy under certain environmental conditions (26). A large number of C_4 Cyperaceae species thrive in wet habitats where the C_4 mode would not be expected to be advantageous (7, 27). The adaptive significance of C_4 photosynthesis in these environments remains to be elucidated. *E. vivipara* grows on the shores of creeks and ponds. When the plants are subjected to periodic flooding and emergence, *E. vivipara* extends its ecological range from the terrestrial wet-soil environment to a submersed aquatic environment, acquiring the distinctive morphological, biochemical, and physiological adaptations associated with the C_3 and C_4 modes. The hair-like morphology of the submersed form increases the surface area to volume ratio of the plant and may be favorable for carbon uptake through epidermis (20, 21, 26). The decrease of chlorophyll a/b ratio may be related to the photosynthesis in shaded light environments under water (28). Nevertheless, the question of why the expression of C_3 metabolism in water is more advantageous for this plant than C_4 metabolism remains to be elucidated.

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