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Short communication

Expression of C_3 and C_4 photosynthetic characteristics in the amphibious plant *Eleocharis vivipara*: structure and analysis of the expression of isogenes for pyruvate, orthophosphate dikinase

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Abstract

Eleocharis vivipara, a unique leafless amphibious sedge, adopts the C_4 mode of photosynthesis under terrestrial conditions and the C_3 mode under submerged aquatic conditions. To analyze the molecular basis of these responses to the contrasting environments, we isolated and characterized two full-length cDNAs for a key C_4 enzyme, pyruvate, orthophosphate dikinase (PPDK; EC 2.7.9.1). The isogenes for PPDK, designated *ppdk1* and *ppdk2*, were highly homologous to one another but not identical. The PPDK1 protein, deduced from the nucleotide sequence of the cDNA, contained an extra domain at the amino terminus which, presumably, serves as a chloroplast transit peptide, while PPDK2 lacked this extra domain. It seems likely, therefore, that the *ppdk1* and *ppdk2* genes encode a chloroplastic and a cytosolic PPDK, respectively. Genomic Southern blot analysis revealed the existence of a small family of genes for PPDK in the genome of *E. vivipara*. Northern blot analysis indicate that both chloroplastic and cytosolic genes for PPDK are expressed simultaneously in the culms, a photosynthetic organ, of *E. vivipara* and that the pattern of expression of these genes differs between the growth forms.

Although most higher plants fix CO_2 through the C_3 pathway, some plants in tropical and subtropical regions utilize the C_4 pathway [8, 14, 22]. In C_4 photosynthesis, cooperation of two types of photosynthetic cell, namely, the mesophyll cells and the bundle sheath cells, is essential [14, 21]. The enzymes involved in the C_4 pathway are strictly compartmentalized between the two types of cell [14, 21]. It has been proposed that C_4 plants evolved from C_3 ancestors [8]. This hypothesis is supported by the observation that the key C_4 enzymes, such as phosphoenolpyruvate carboxylase (PEPCase) and pyruvate, orthophosphate dikinase (PPDK), are found at lower levels in C_3 plants [1, 3, 13, 24]. Recent studies suggest that the evolution of C_4 photosynthesis occurred through the recruitment and alteration of pre-existing genes in C_3 plants [16, 24].

Although some succulent plants, such as *Mesembryanthemum crystallinum*, change their mode of photosynthesis from the C_3 to the CAM (Crassulacean acid metabolism) mode under water stress and NaCl stress [36], there are only a few reports about plants that shift from the C_3 mode to the C_4 mode. It was recently demonstrated that *Eleocharis vivipara*, an amphibious sedge, changes its photosynthetic and anatomical traits dramatically with changes in its environment [31, 32, 33]. This plant develops the C_4 traits with Kranz anatomy under terrestrial conditions and the C_3 traits with non-Kranz anatomy under submerged aquatic conditions. This species is considered, therefore, to be a useful model plant for studies of genetic and developmental aspects of C_3 and C_4 photosynthesis. It was

The nucleotide sequence data reported will appear in the EMBL, GenBank and DOBJ Nucleotide Sequence Databases under the accession numbers D86337 and D86338.

demonstrated very recently that the pattern of cellular localization of C_3 and C_4 enzymes in the terrestrial form is unique, and that this pattern and the changes in the extent of accumulation of these enzymes are the main factors responsible for the difference in the photosynthetic traits between the two forms [32].

E. vivipara lacks leaf blades and the culms perform all photosynthetic functions [31, 33]. This plant develops new culms with either the C_3 mode or the C_4 mode, depending on environmental conditions [33]. When the submerged form is exposed to air, the culms die as a result of rapid dessication. Within several days, however, the plants begin to develop new culms, which already have Kranz anatomy and the C_4 biochemical traits. By contrast, when the terrestrial form is immersed in water, the plants develop new culms with intermediate traits and, after several months, with the C_3 mode; they change gradually from the C_4 to the C_3 mode.

As part of our effort to elucidate the molecular mechanisms responsible for the development of C_3 and C_4 traits in *E. vivipara*, we focused initially on one of the key C_4 enzymes: pyruvate, orthophosphate dikinase (PPDK; EC2.7.9.1). In leaves of C_4 plants, this enzyme is located mainly in the chloroplasts of mesophyll cells where it catalyzes the conversion of pyruvate to phosphoenolpyruvate [7, 14]. The cell-specific expression of the gene for PPDK is regulated at the transcriptional level [28, 29]. PPDK is encoded by nuclear DNA and the gene is transcribed from two different initiation sites under the control of two promoters: the larger transcript produces the chloroplastic PPDK, which includes a transit peptide, and the smaller one produces the cytosolic PPDK [11, 12, 15, 18, 19, 26, 27]. The existence of PPDK has also been reported in leaves of C_3 plants and seeds of both C_3 and C_4 plants [1, 3, 13].

In this study, we isolated two full-length cDNAs that encoded PPDK from the terrestrial form of *E. vivipara* and characterized their structures. In addition, we investigated the patterns of expression of these genes in the two growth forms.

The terrestrial and submerged forms of *E. vivipara* were grown in a greenhouse and in aquaria in the greenhouse, respectively, as described elsewhere [31]. After removal of culms, the terrestrial plants were transferred to a growth chamber, which was programmed for 14 h of light at 25 °C and 10 h of darkness at 20 °C. The irradiance, provided by metal-halide lamps, was about 400 μE m^{-2} s^{-1}. After removal of culms, the submerged plants were transferred to water tanks (30 l) in the growth chamber and were grown for more than one month prior to experiments. The water in the tanks was changed at weekly intervals.

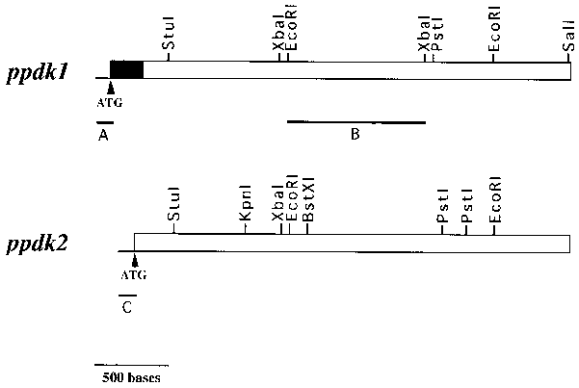

Figure 1. Restriction maps of the full-length cDNA clones, *ppdk1* and *ppdk2*. Total RNA was prepared from the culms of the terrestrial form as described by Fromm *et al.* [10], and poly(A)$^+$ RNA was isolated with Oligotex-dT30 (Roche, Tokyo, Japan). cDNAs were synthesized by reverse transcriptase from avian myeloma virus with random primers (Amersham International, Buckinghamshire, UK). A cDNA library was constructed in the λZAPII vector (Stratagene, La Jolla, CA) using adapters [37] at the termini of the cDNA instead of *Eco*RI sites. The cDNA library was screened by plaque-hybridization with a ^{32}P-labeled DNA fragment as probe. To obtain a probe for genes for PPDK, a DNA fragment was amplified by RT-PCR from RNA that had been isolated from the culms of the terrestrial form as template with degenerate primers that corresponded to highly homologous regions in the nucleotide sequences of the genes for PPDK of *Zea mays* [19] and *Flaveria trinervia* [25]. The DNA fragment was 906 bp long and corresponded to the sequence from 1966 to 2872 of *Z. mays* and from 1976 to 2882 of *F. trinervia*. The black box indicates the region that encodes the putative transit peptide. The black lines under the maps indicate the locations of probes used for nothern blot analysis (A, C) and for Southern blot analysis (B).

The cDNA clones encoding the isoforms of PPDK in *E. vivipara* were isolated from a cDNA library that was prepared from the culms of the terrestrial form, as detailed in the legend to Figure 1. Two kinds of full-length cDNA were obtained and they were designated *ppdk1* and *ppdk2* (Figure 1). The *ppdk1* and *ppdk2* cDNAs were 3053 and 2868 bp in length, and they contained single long open reading frames of 2841 and 2652 bp, respectively (Figure 1). The coding regions of the two genes were highly homologous at the nucleotide level (88%), but they were not identical. Differences in nucleotides between the two genes were found sporadically throughout the coding regions, and *ppdk1* included an extra domain of 207 bp at the amino terminus.

The open reading frames of *ppdk1*- and the *ppdk2*-encoded polypeptides, designated PPDK1 and PPDK2,

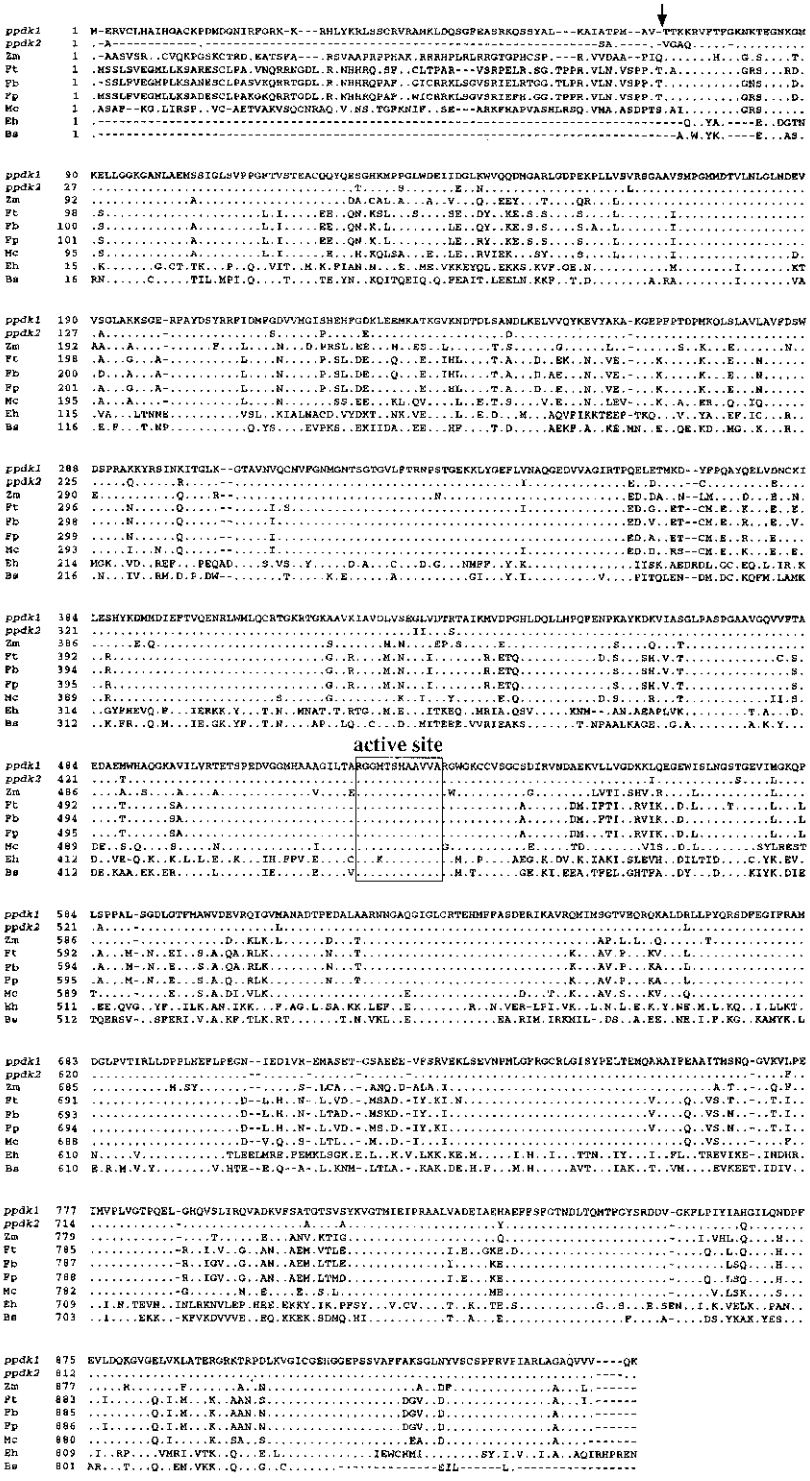

Figure 2. Alignment of the deduced amino acid sequences of PPDKs from *E. vivipara* (*ppdk1*, *ppdk2*), *Zea mays* (Zm), *Flaveria trinervia* (Ft), *F. brownii* (Fb),*F. pringlei* (Fp), *Mesembryanthemum crystallinum* (Mc), *Entamoeba histolytica* (Eh), and *Bacteroides symbiosus* (Bs). Dots indicate amino acids identical to those of *ppdk1* and bars indicate gaps introduced for maximum alignment. The arrow marks a putative site for cleavage of the precursor polypeptide, as deduced from its location in the sequence from *Z. mays*. The putative active site is boxed.

of 947 and 884 amino acids with predicted molecular masses of 103.4 kDa and 95.9 kDa, respectively. PPDK1 had an amino-terminal sequence of 69 amino acid that was missing from PPDK2. However, the amino acid sequences in the other regions of the two proteins were highly homologous (95%). The deduced amino acid sequences were aligned with those of PPDKs from other higher plants, protozoa and bacteria (Figure 2). In PPDK of *Zea mays*, a transit peptide has been identified that directs the entry of the precursor protein into chloroplasts and is then removed by proteolytic cleavage [2, 11, 12, 19, 27]. The amino-terminal additional peptide of PPDK1 was similar in size to this transit peptide and it shared a characteristic feature with the transit peptide in that its central region was rich in basic amino acids. However, we found no clear similarities among the primary sequences of these PPDKs. The deduced molecular mass of the putative mature protein, without the amino-terminal additional peptide, was calculated to be about 95 kDa, which corresponded approximately to the molecular mass that had been estimated for mature PPDK in leaves of *Z. mays* [12, 19, 30]. These characteristics suggest that the amino-terminal additional peptide of 69 amino acid residues in PPDK1 serves as a transit peptide. The absence of such an additional domain in PPDK2 suggests that PPDK2 might be a cytosolic enzyme (Figure 2).

The *ppdk1* and *ppdk2* cDNAs were closely related to the genes for PPDK of other higher plants. The homologies of *ppdk1* and *ppdk2*, respectively, to the genes for PPDK from other plants were calculated as follows: 78 and 77% for a C_4 monocot, *Z. mays* [19]; 76 and 75% for a facultative CAM dicot, *Mesembryanthemum crystallinum* [9]; 75 and 74% for a C_3 dicot, *Flaveria pringlei* [24]; 75 and 74% for a C_4-like dicot, *F. brownii* [34]; and 73 and 73% for a C_4 dicot, *F. trinervia* [25]. The homology of *ppdk1* and the *ppdk2* to the genes for PPDK of bacteria and protozoa was lower than that to genes of higher plants, as follows: 48 and 52% for *Bacteroides symbiosus* [23]; and 46 and 49% for *Entamoeba histolytica* [4]. A completely conserved amino acid sequence (GGMTSHAAVVA) was found in PPDK1 and PPDK2 (boxed in Figure 2), as it is in the deduced PPDKs of other species. This region includes histidyl and threonyl residues, which are presumed to serve as the catalytic site and the site of the phosphorylation and dephosphorylation that regulate the reversible activation of this enzyme [5, 7].

To determine the number of copies of the gene for PPDK in the genome of *E. vivipara*, we performed Southern hybridization with a probe that corresponded to a highly homologous region that included the active site and was common to the both genes (Figure 3). One or two prominant bands were detected upon digestion with three restriction enzymes. The two bands seen with *Xba*I and *Hin*dIII could represent the genomic sequences for each of the two cDNAs, *ppdk1* and *ppdk2*. This suggests that PPDK is encoded by a small family of genes, probably by two genes. In *Z. mays*, there are at least two genes for PPDK: one gene encodes a cytosolic isoform exclusively, and the other gene encodes both a chloroplastic and a cytosolic isoform [11, 15, 27]. In C_3, C_3-C_4, and C_4 species of *Flaveria* [24, 26, 34] and *M. crystallinum* [9], only one gene for PPDK is present in the genome. It has been suggested that the transcripts for the chloroplastic and the cytosolic isoforms of PPDK are produced from two different initiation sites of a single gene, under the control of two promoters [11, 26, 27]. In *E. vivipara*, the transcripts for the two isoforms are obviously derived from different genes. It appears that the differential expression of two isoforms of PPDK is regulated in a different mechanism from that in *Z. mays* [11, 27] although, at this time, we cannot exclude the possibility that the cytosolic form might also be transcribed from the *ppdk1* gene at a low level.

The organ-specificity of the expression of the two genes for PPDK was investigated by northern blot analysis, using gene-specific probes for *ppdk1* and *ppdk2* derived from 5′ regions of the genes (Figure 4). In the culms, the levels of transcripts for *ppdk1* and *ppdk2* were far more abundant in the terrestrial form than in the submerged form. This result is consistent with the previous observation that the terrestrial form accumulates higher amounts of PPDK protein than the submerged form [32, 33], and it indicates that the difference in the levels of transcripts for PPDK is one of the key factors responsible for the expression of different modes of photosynthesis between the two forms. In the roots of the terrestrial form, we only found the *ppdk2* transcript (Figure 4). Previous studies for maize [11, 15, 27] and *Flaveria* [26] indicated that the transcripts of the gene for chloroplastic PPDK were found in leaves, while those for the cytosolic PPDK were found in roots. Thus, the existence of the *ppdk2* transcript in the roots of *E. vivipara* supports the hypothesis that *ppdk2* encodes the cytosolic PPDK. The present study demonstrates that the chloroplastic *ppdk1* and the cytosolic *ppdk2* genes are expressed simultaneously in the culms, a photosynthetic organ, of *E. vivipara*.

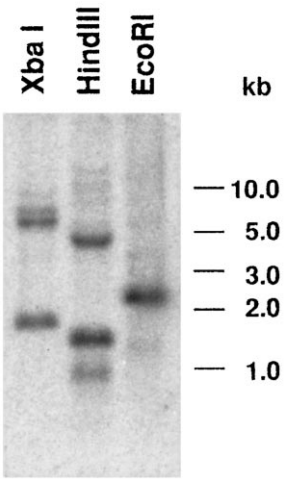

Figure 3. Genomic Southern blot analysis of the genes for PPDK in *E. vivipara*. Genomic DNA was isolated from the culms of the terrestrial form as described by Wagner *et al.* [35] with modifications. Culms were ground with a mortar and pestle and suspended in extraction buffer that contained 10% polyethylene glycol 6000, 0.35 M sorbitol, 0.1 M Tris-HCl pH 8.0, 0.5% spermidine, 0.5% spermine, and 1% 2-mercaptoethanol. The suspension was centrifuged (2800 rpm, 4 °C, 15 min), and the resulting pellet was suspended in 5 ml of the extraction buffer without polyethylene glycol 6000. N-Laurylsarcosine was added to the solution to a final concentration of 1% (w/v). After centrifugation (2800 rpm, 4 °C, 15 min), the upper aqueous phase was recovered. The solution was subjected to precipitation with cetyltrimethylammonium bromide (CTAB) and subsequent purification of genomic DNA as described by Murray and Thompson [20]. Genomic DNA (10 μg) was digested with restriction enzymes and subjected to electrophoresis on a 0.7% agarose gel. After transfer of bands of DNA to a nylon filter, the filter was hybridized with a ^{32}P-labeled DNA fragment that had been amplified by PCR from a cDNA clone for PPDK as template (see Figure 1), at 65 °C, in a hybridization buffer that contained 1% SDS, 10% sodium dextran sulfate and 1 M NaCl, and then it was washed at 65 °C in 2× SSC, 1% SDS.

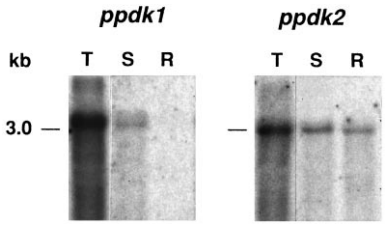

Figure 4. Northen blot analysis of transcripts of genes for PPDK in *E. vivipara*. Total RNA was isolated from culms of the terrestrial (T) and the submerged (S) forms, and from roots (R) of the terrestrial form, by the method of Murray and Thompson [20], with the exception that 1/3 volume of 8 M LiCl was added to the solution of CTAB and nucleic acids to prevent the precipitation of DNA and low-molecular-weight RNA. Total RNA (5 μg) was denatured in a mixture of 56% formamide and 12% formaldehyde, and RNAs were separated on a 1% agarose gel that contained 1.6% formaldehyde. After transfer to nylon filters, the bands of RNA were allowed to hybridize with ^{32}P-labeled cDNA probes (see Figure 1) at 60 °C in hybridization buffer that contained 1% SDS, 10% sodium dextran sulfate, and 1 M NaCl. Then filters were washed at 60 °C in 2× SSC, 1% SDS.

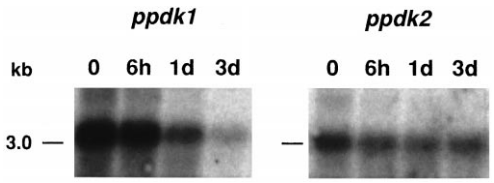

Figure 5. Northern blot analysis of transcript levels of genes for PPDK in light-to-dark transition. The terrestrial plants grown under light conditions were transferred to darkness for up to three days. Total RNA was isolated from the culms and aliquots (3 μg) were then fractionated on 1% agarose gels, blotted onto nylon filters, and hybridized with ^{32}P-labeled DNA probes, as described in Figure 4.

We also examined whether the levels of transcripts for *ppdk1* and *ppdk2* in the terrestrial plants are influenced by light-to-dark transition (Figure 5). The level of *ppdk1* transcript was reduced during this period, while the level of *ppdk2* transcript remained constant. These data suggest that the expression of *ppdk1* and *ppdk2* is controlled in different manners. In *Flaveria trinervia*, the levels of transcript for the chloroplastic gene of PPDK were also reduced in leaves and stems, when the plant was kept under dark conditions [26]. By contrast, the cytosolic gene for PPDK was expressed in the stems under dark conditions [26].

When the level of the *ppdk1* and the *ppdk2* transcript were compared within the individual growth forms, a contrasting tendency was found in the two forms (Figure 4). In the terrestrial form, *ppdk1* was expressed at somewhat higher levels than *ppdk2*. In the submerged form, by contract, *ppdk2* was expressed at higher levels than *ppdk1*. In the culms of the terrestrial form, the PPDK protein was found to accumulate to a large extent in the chloroplasts and to a lesser extent in the cytosol of the photosynthetic tissue [32]. In the culms of the submerged form, the extent of the accumulation of the chloroplastic PPDK protein was lower than that of the cytosolic PPDK [32]. Therefore, it appears that the pattern of expression of the isogenes for PPDK in *E. vivipara* reflects this cellular distribution of PPDK protein, even though the physiological role of the cytosolic PPDK remains unknown. In maize leaves, the accumulation of the PPDK protein was observed only in the chloroplasts and not in the cytosol [17].

It seems likely that osmotic stress is involved in the conversion from the C_3 mode to the C_4 mode in

E. vivipara. C_4 photosynthesis and CAM share several common photosynthetic enzymes, such as PEPCase and PPDK [22]. In several respects, the environmental responses of *E. vivipara* appear similar to those in facultative CAM plants. During the induction of CAM in *M. crystallinum*, the level of the transcripts of a CAM-specific isogene for PEPCase increases but that of a C_3 isogene remains unchanged [6]. Thus, the pattern of expression of isogenes for PPDK in *E. vivipara* seems to be similar but not identical to that of gene for PEPCase, since both isogenes for PPDK respond simultaneously to environmental changes. The present study revealed that this amphibious sedge modulates the expression of the isogenes for PPDK in response to a change in environmental conditions and, moreover, that mechanisms for regulation of such modulation are different from those in other plants. Further analysis of this phenomenon should shed more light on the molecular mechanism of the change in photosynthetic traits.

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