Photosynthetic and Other Phosphoenolpyruvate Carboxylase Isoforms in the Single-Cell, Facultative C₄ System of *Hydrilla verticillata*¹

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The submerged monocot *Hydrilla verticillata* (L.f.) Royle is a facultative C₄ plant. It typically exhibits C₃ photosynthetic characteristics, but exposure to low [CO₂] induces a C₄ system in which the C₄ and Calvin cycles co-exist in the same cell and the initial fixation in the light is catalyzed by phosphoenolpyruvate carboxylase (PEPC). Three full-length cDNAs encoding PEPC were isolated from *H. verticillata*, two from leaves and one from root. The sequences were 95% to 99% identical and shared a 75% to 85% similarity with other plant PEPCs. Transcript studies revealed that one isoform, *Hvpepc4*, was exclusively expressed in leaves during C₄ induction. This and enzyme kinetic data were consistent with it being the C₄ photosynthesis isoform. However, the C₄ signature serine of terrestrial plant C₄ isoforms was absent in this and the other *H. verticillata* sequences. Instead, alanine, typical of C₃ sequences, was present. Western analyses of C₃ and C₄ leaf extracts after anion-exchange chromatography showed similar dominant PEPC-specific bands at 110 kD. In phylogenetic analyses, the sequences grouped with C₃, non-graminaceous C₄, and Crassulacean acid metabolism PEPCs but not with the graminaceous C₄ and formed a clade with a gymnosperm, which is consistent with *H. verticillata* PEPC predating that of other C₄ angiosperms.

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enzymes involved in C₄ photosynthesis. A goal of such research is to introduce C₄ cycle components into C₃ crop species with the hope that the transformants, similar to C₄ and CAM plants, would have improved performance under adverse conditions (Matsuoka and Sanada, 1991; Ku et al., 1999; Mann, 1999). In this context, *H. verticillata* provides a higher plant example, albeit an aquatic one, of how the C₄ and Calvin cycle components might co-exist in the same cell and still function in series to concentrate CO₂.

As part of a molecular approach to understand how the C₄ system in *H. verticillata* is induced and regulated, we have focused attention on the PEPC isoforms that we have found in this plant. We present evidence that one is induced and operates in C₄ leaf isoforms that we have found in this plant. We present regulated, we have focused attention on the PEPC isoforms with those of members of other species possessing C₃, C₄, and CAM isoform types are also shown.

As part of a molecular approach to understand how the C₄ system in *H. verticillata* is induced and regulated, we have focused attention on the PEPC isoforms that we have found in this plant. We present evidence that one is induced and operates in C₄ leaf photosynthesis. Multiple isoforms are commonly reported for PEPC gene families (Ernst and Westhoff, 1997). However, this is the first report of three full-length PEPC cDNAs isolated from a plant that is normally C₃, but has evolved an inducible C₄ system to combat the adverse environmental conditions of low [CO₂] and high [O₂], temperature, and irradiance (Bowes and Salvucci, 1989). The phylogenetic relationships of *H. verticillata* PEPC isoforms with those of members of other species possessing C₃, C₄, and CAM isoform types are also shown.

**RESULTS**

**Isolation, Cloning, and Sequencing of Three Full-Length cDNAs Encoding *H. verticillata* PEPC**

*Hvpepc* 3 and 4 were culled from 40 C₄ leaf-derived RACE clones that screened positively for either the 3F or 4F oligoprobe. Subsequent isolations using C₃ leaf material yielded only clones of *Hvpepc* 3. A similar number of positive RACE clones tested positively only to the probe 3F, and from these clones, *Hvpepc* 5 was isolated and sequenced. The salient features of these cDNAs and their encoded PEPCs are summarized in Table I. The 5’ region in all of the isoforms had two ATG triplets that are candidates for translation initiation; the two different coding sequence lengths that would occur with each of the ATG triplets are also shown. These data indicate that the encoded proteins were very similar in terms of Mₚ and pl.

A comparison of the nucleotides (nt) from the 5’- and 3’-untranslated regions (UTR) of the three *H. verticillata* PEPC cDNA sequences indicates that *Hvpepc* 3 and *Hvpepc* 5 were very similar but not identical and that they differed from *Hvpepc* 4. The 5’-UTR of *Hvpepc* 5 showed 1 bp deletion and one substitution compared with *Hvpepc* 3, whereas there were 2 bp substitutions in the 3’-UTR and 4 bp substitutions in the coding region. The *Hvpepc* 5 sequence downstream of the stop codon (TAA) was 116 bp shorter than that of *Hvpepc* 3. All three sequences contained a single polyadenylation signal motif.

A comparative multiple alignment of the deduced amino acid sequences of the three *H. verticillata* PEPCs with those of two other monocots and one eudicot representing C₃, C₄, and CAM isoform sequences is shown in Figure 1. The monocot maize contains both C₃ and C₄ PEPCs, whereas the monocot *Vanilla planifolia* has a CAM isoform. The C₄ PEPC from the C₄ eudicot *F. trinervia* was also included in the comparison because this sequence bears a phylogenetic resemblance to those of *H. verticillata*. The conserved regions for both eukaryotic and prokaryotic PEPCs are indicated, as well as the specific catalytic and regulatory binding locales and two putative C₄ signature sites. Homology among the *H. verticillata* sequences was high (95%–99%), and they showed the closest resemblance to the C₃ PEPC from maize (85%). Identity with the CAM PEPC was 83%, with the *F. trinervia* C₄ PEPC 81%, and with the maize C₄ PEPC 78%. In a comparison with *Hvpepc* 3, *Hvpepc* 5 had three substitutions resulting from the 4 bp changes, whereas *Hvpepc* 4 had 44 substitutions and

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<th>Table I. Characteristics of the cDNAs and the predicted amino acid sequences of the three PEPC isoforms from <em>H. verticillata</em></th>
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<tr>
<td><strong>cDNA (bp)</strong></td>
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two deletions. The three substitutions found in Hvpepc5 were Ser-196 for Cys, Val-777 for Ile, and Arg-891 for Glu. The substitutions in Hvpepc4 occurred mostly in the variable regions; the Met-150 appears to be a unique change, replacing Leu, which is found in all other PEPCs listed in the database. The signature C4 Ser, Ser-774 of F. trinervia (Bla¨sing et al., 2000), was also present in the C4 PEPC of maize, but it was notably absent from all of the H. verticillata sequences. Instead, Ala was found at the correspond-

Figure 1. Multiple alignment of the deduced amino acid sequences of three H. verticillata PEPCs and those of maize (Zea mays; C3 and C4), Vanilla planifolia (CAM), and Flaveria trinervia (C4). Only residues that differ among the sequences are shown. Gaps (-) and identical (.) bp are indicated. Boxed residues indicate the most conserved regions among prokaryotes and eukaryotes. Putative regulatory and catalytic sites are also shown. ᵗ, the Ser residue that is common to all plant PEPCs and that is the target for phosphorylation; ᵕ, the unique Hvpepc4 Met residue; ᵜ, the unique Hvpepc5 Val; and ᵣ, the position of the C4 signature Ser.
ing position. A putative C₄-determinant Lys-347, as described for the \textit{F. trinervia} C₄ PEPC, occurred in \textit{Hvpepc4} at the same position, whereas the putative C₃-marker Arg occurred in the other \textit{H. verticillata} isoforms (Hermans and Westhoff, 1992; Bläsing et al., 2000). It should be noted that Lys-347 is not an absolute C₄ marker, because it also occurs in CAM and some C₃ sequences and not in the graminaceous C₄ PEPC isoforms. None of the other reported C₄-determinant residues described by Hermans and Westhoff (1992) were found in the \textit{H. verticillata} deduced sequences.

### Differential Expression of \textit{H. verticillata} Isoforms

To compare the specific expression of \textit{Hvpepc3} and \textit{Hvpepc4}, northern analyses were performed using C₃ and C₄ leaves of \textit{H. verticillata} (Fig. 2). The samples were analyzed several times throughout the C₄ induction period, starting at zero time when all the leaves had C₃ photosynthetic characteristics. When isoform-specific RNA probes were used, \textit{Hvpepc4} was expressed exclusively in C₄-induced leaves, after 96 and 264 h into the induction period. This isoform notably was not expressed in any other samples. In contrast, \textit{Hvpepc3} was expressed in C₃ and C₄ leaf samples, except at the 264-h C₃ sampling time. The results of consensus probing were similar to those using the \textit{Hvpepc3} probe. The results represent a 1-µg total RNA loading scheme, which is the maximum recommended (Roche Diagnostics/Roche Applied Science, Indianapolis). The loading of greater quantities of total RNA (2 and 5 µg) did not change the detection threshold. The probe to the \textit{Hvpepc3} isoform was specifically synthesized from its 3'-UTR, however the similarity between these regions of \textit{Hvpepc3} and \textit{Hvpepc5} suggests that the probe could not discriminate between these two isoforms. Therefore, an \textit{Hvpepc5} signal in the C₃ leaves cannot be excluded.

The activity of PEPC was followed in the same samples used for the northern analyses. Figure 3 shows the specific activity over time of PEPC in desalted extracts from the C₃- and C₄-induced leaves and shows the times when RNA was sampled for the northern analyses. The PEPC activity in the C₃ leaves remained essentially constant and low. In contrast, that of the C₄-induced leaves increased in a linear fashion, reaching values more than 10-fold greater than in the C₃ leaves.

### Partial Purification of PEPC, Kinetic Characterization, and Western Analyses

Data for the purification of PEPC from extracts of C₃ and C₄ leaves (harvested in the light at 288 h into the induction period) and roots, using ammonium sulfate fractionation and Q-Sepharose FF anion-exchange chromatography, are summarized in Table II. The PEPC activities were assayed at the optimal pH of 8.0 with saturating substrate concentrations. The root extract did not bind to the column but eluted as a single peak in the buffer wash. However, the leaf extracts did bind and were eluted with a linear salt gradient. The elution profiles of each of these extracts were characterized by a single peak, but with elution at slightly different salt concentrations. The specific PEPC activities in both the crude and chromatographed C₃ leaf extracts were substantially higher than the corresponding C₃ values, and leaf values were much higher than those of the roots. The crude activities were similar to those described previously (Fig. 3). The purification factors were greater for the leaf extracts than for the root.

Kinetic data for the C₃ and C₄ leaf peak PEPC fractions are presented in Figure 4. The activities were assayed at a cytosolic-like pH of 7.3, where PEPC kinetic effects are more pronounced. A plot of activity versus [PEP] produced a hyperbolic curve for the C₃ leaf enzyme that followed Michaelis-Menten kinetics \((r^2 = 0.957)\), whereas that of the C₄ was sigmoid and fitted the Hill equation \((r^2 = 0.998)\). The Hill coefficients for the two extracts differed considerably, 1.8 and 3.8 for the C₃ and C₄ leaves, respectively. The specific activities, calculated from the Michaelis-Menten and Hill equations, were several-fold different, with the C₄ value the higher (2.51 versus 0.37 \(\mu\)mol mg\(^{-1}\) protein min\(^{-1}\)). In contrast, the \(K_{0.5}\) PEP values did not differ substantially,
whether estimated from the graph or calculated by the Hill equation, and in addition, they were relatively high (Fig. 4).

Western analyses showed two prominent immunoreactive bands in both leaf extracts, with the second being much more pronounced in the C4 leaves (Fig. 5). In addition, a third, faster running band was evident only in the C4 leaf extract. The distribution of these bands was in the Mr range of 105,000 to 111,000.

Phylogenetic Analyses

Figure 6 shows the results of a phylogenetic analysis of deduced amino acid sequences using the Phylogenetic Inference Package (version 3.57c, Department of Genetics, University of Washington, Seattle) using the parsimony algorithm. In addition to the three H. verticillata PEPC sequences, 28 other full-length sequences from GenBank representing 17 different taxa were included. Particular emphasis was placed on selecting species with a set of two or more isoforms, so that diversity of isoform function was represented. Using the PHYLIP or the PAUP package (Phylogenetic Analysis Using Parsimony, version 4.0, Sinaur Associates, Sunderland, MA), both the protein distance and protein parsimony methods gave consensus trees that were very similar. For these analyses, the four prokaryotic sequences were taken as the outgroup, showing similarity with the seed plant sequences in the range of 26% to 39%. In all, 943 total characters were considered, and 608 of them were parsimony informative. The consistency and retention indices were 0.71 and 0.63, respectively, indicating low homoplasy or background noise because of convergence or reversion events. The root PEPC isoforms of the graminaceous plants; maize, sorghum (Sorghum vulgare), and rice (Oryza sativa); and the C4 sequences of maize and sorghum apparently diverged independently.

From this analysis, it appeared that PEPC isoforms can be grouped into three distinct groups that likely

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<th>Source</th>
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<td></td>
<td>µmol mg⁻¹ protein min⁻¹</td>
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<tr>
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<td>262</td>
<td>4.8</td>
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<tr>
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<td>N.D.</td>
<td>0.080</td>
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<td>2.7</td>
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Phosphoenolpyruvate Carboxylase Isoforms in *Hydrilla verticillata*

**Figure 4.** The effect of PEP concentration on the specific activity of PEPC in the C3 and C4 leaf peak fractions from anion-exchange chromatography (see Table II). The PEPC was extracted from the *H. verticillata* leaves harvested during the light period. The C4 sample was taken 288 h into the induction period. The assay was run at pH 7.3 in the absence of dithiothreitol. The apparent $K_{0.5}$ PEP values were determined from the curves. Data are means ± se, $n$ = 3.

**Figure 5.** Western analyses of PEPC from leaves of *H. verticillata*. Leaves of *H. verticillata* were harvested midway through their light cycle, C3 at 0 h and C4 at 288 h into the induction period. Six micrograms of protein from the C3 and C4 leaf peak fractions from anion-exchange chromatography (see Table II) was resolved by 5% (w/v) SDS-PAGE and transblotted to a nitrocellulose membrane. The membrane was probed with antibodies raised against maize PEPC. The PEPC signals from C3 and C4 leaves are shown. The kilodalton value of the prominent PEPC band is indicated at the right.

share a common ancestor: I, C4 graminaceous; II, graminaceous roots; and III, PEPC isoforms with varying functions from a variety of taxa. Although group III was monophyletic, relationships within it were largely unresolved because the component branches lacked statistical support. Nonetheless, there was good support for several clades, namely Brassica spp., Flaveria spp., *Hydrilla* spp., and a Sorghum spp./Zea spp. group (C3 PEPC). Within the genus *Flaveria*, the C3 species *Flaveria pringlei* ppcA showed a clear divergence from the C4 *F. trinervia* ppcA, with 100% support. However, the sequences from both the C3 and C4 *Flaveria* spp. fell into the same group as those from *H. verticillata*. Within the genus *Flaveria*, the C4 species *Flaveria pringlei* ppcA showed a clear divergence from the C3 *F. trinervia* ppcA, with 100% support. However, the sequences from both the C3 and C4 *Flaveria* spp. fell into the same group as those from *H. verticillata*. In the case of *H. verticillata*, Hoipepc3 and Hoipepc5, and Hoipepc4 diverged from a unique common C3 ancestor. It is intriguing that the gymnosperm Norway spruce (*Picea abies*) along with the root nodule pea (*Pisum sativum*) and the *H. verticillata* sequences appear to form a clade that is present regardless of tree construction methods.

**DISCUSSION**

Photosynthesis in *H. verticillata* is unique in that, against a C3 background, a C4 cycle is induced but without the development of specialized anatomy that occurs in terrestrial C4 species. This “minimalist” system represents something of a paradox in our concept of C4 photosynthesis. Since the classical C3 × C4 *Atriplex* spp. hybridization experiments of Björkman et al. (1970), it has been accepted that for a C4 system to concentrate CO2 and to avoid its futile cycling, the biochemical components need to be segregated in specific cell types. *H. verticillata* was the only exception, but others have been reported recently (Bowes et al., 2002), including *Borszczowia aralocaspica*, a terrestrial NAD-ME C4 species in which the C4 and Calvin cycles appear to co-exist in different regions of the same cell (Voznesenskaya et al., 2002).

The inducible *H. verticillata* system provides an excellent opportunity to study the minimum essential biochemical elements to operate a C4 photosynthetic system, such as might be needed to transform a C3 crop plant. Its facultative nature also enables us to examine the genes involved in both the C3 and C4 states, differences in their expression, and variations in the regulatory and catalytic domains of their products.

We have previously described the major physiological and biochemical features of the system (Bowes and Salvucci, 1989; Magnin et al., 1997). Thus, the purpose of this study was to begin to elucidate the molecular mechanisms involved, particularly those associated with the induction and role(s) of PEPC.
the first element in the $C_4$ pathway. The genes encoding PEPC isoforms in terrestrial plants have been well described, and distinctions can be made among the $C_3$ (non-photosynthetic forms), $C_4$, and CAM isoforms (Lepiniec et al., 1993; Ernst and Westhoff, 1997; Svensson et al., 1997; Cushman and Bohnert, 1999).

Figure 6. Phylogenetic analysis of deduced amino acid PEPC sequences. The PHYLIP package was used to construct a consensus tree with 100 bootstrap replications using the parsimony method. The four prokaryotic species served as the outgroup. The stars at the fork of the tree represent >85% support. The three groupings are: I, $C_4$ graminaceous; II, graminaceous roots; and III, sequences of other higher plants. Deduced amino acid sequences other than $H. verticillata$ were obtained from GenBank/SwissProt.
Of the three PEPC isoforms from *H. verticillata* *Hvpepc4* was expressed solely in *C*₄ leaves. Several lines of evidence point to this isoform as the photosynthetic PEPC operating in the light. It was only isolated from *C*₄ leaf RNA and was only expressed in *C*₄ leaves, and its expression coincided with the rise in PEPC activity as the *C*₄ system was induced. In addition, its sequence least resembled those of *Hvpepc3* or *Hvpepc5* that were isolated from *C*₃ leaves and roots, respectively. It also contained the *F. trinervia* *C*₄ Lys-347, though as noted earlier, this residue is not a very specific determinant of a *C*₄ PEPC isoform. The *C*₄-signature Ser residue was absent from all *H. verticillata* sequences, and instead, Ala, which is typical of *C*₃ sequences, occurred at this position. Ser appears to be ubiquitous at this position among the *C*₄ isoforms of terrestrial *C*₄ plants, and it plays a role in determining the kinetic characteristics (Blasing et al., 2000). How the *H. verticillata* PEPC functions kinetically as a *C*₄ photosynthetic isoform with the *C*₃ Ala at this position, instead of Ser, is an interesting issue that deserves further study.

We recently reported that PEPC in desalted extracts from *C*₃ and *C*₄ *H. verticillata* leaves differed kinetically in that the *C*₄ leaf enzyme is light activated and is over 10-fold more sensitive to malate inhibition (Bowes et al., 2002). In the present study, the specific activity of *C*₄ leaf PEPC was substantially higher than that from *C*₃ leaves. Among terrestrial plants, PEPC in *C*₄ leaves is light-activated, and its activity is similarly severalfold higher than that from *C*₃ leaves when assayed at a cytosolic-like pH (Gupta et al., 1994).

The *Kₘ* PEP values for PEPC differ among terrestrial plant *C*₃ and *C*₄ enzymes (O’Leary, 1982). This, however, was not the case for PEPC from *C*₃ and *C*₄ *H. verticillata* leaves, which had similar *Kₘ* PEP values that were high and *C*₄-like, confirming much earlier measurements with crude extracts (Nakamura et al., 1983). Blasing et al. (2000) showed that in site-directed mutagenesis and chimeric constructs of ppcA PEPC from *C*₃ *F. pringlei* and *C*₄ *F. trinervia*, the replacement of Ala-774 with Ser increases the *Kₘ* PEP of the recombinant proteins. They concluded that Ser-774 is a key determinant of *C*₄-like kinetics, including a high *Kₘ* PEP. Thus, the similarity of *H. verticillata* *Kₘ* PEP values might be expected, because the sequences are identical at this site. In contrast, the presence of Ala and high *Kₘ* values does not support a ubiquitous need for Ser at this position to obtain a *C*₄-like *Kₘ* PEP.

Hill coefficients for recombinant ppcA PEPCs from *C*₃ and *C*₄ *F. pringlei* spp. indicate the *C*₄ enzyme has greater positive cooperativity (Blasing et al., 2000). The *H. verticillata* data parallel this, in that the *C*₄ leaf PEPC was strongly homotropic with PEP acting as a positive modulator. A similar situation exists with maize (Tovar-Mendez et al., 1998). The in vivo role for allosteric regulation of the *C*₄ photosynthetic isoform is undetermined. However, PEPC operating in a *C*₄ CCM may need enhanced capacity to respond rapidly as metabolites fluctuate with transient changes in the environment.

The expression pattern and kinetic data point to *Hvpepc4* as the *C*₄ photosynthetic PEPC. What then is the role for *Hvpepc3* in the leaves? *H. verticillata* leaves can fix CO₂ in the dark, at 12% of the light rate in the case of *C*₄ leaves, and they accumulate malate (Reiskind et al., 1997; J.B. Reiskind, S.K. Rao, and G. Bowes, unpublished data). The ability of a PEPC that is not light-regulated to scavenge inorganic carbon at night when concentrations rise could be another factor in the plant’s carbon economy in habitats where dissolved CO₂ becomes a major daytime limitation.

The sequence similarity between *Hvpepc3* and 5 might suggest that the same gene encodes them both. However, this is unlikely because all of the *3'-UTR* sequences analyzed to date from independent clones of three organ sources, i.e. leaf, root, and subterranean and axillary tufts, revealed (a) three distinct *3'-UTR* categories; (b) that the *Hvpepc5*-like sequences were the same length and were 99% homologous; and (c) that a specific polyadenylation signal site at a common position (nt 3,198 to 3,203 in *Hvpepc3* and *Hvpepc5*) is present.

The three full-length cDNA *H. verticillata* sequences were all very similar (95%-99%). A comparable situation is seen in *Kalanchoe blossfeldiana* where two pairs of isoforms encode highly similar *C*₃- and CAM-specific PEPC isoforms, with the slight deviations being attributed to gene duplication or the hybrid status of the plant in which the parental genomes are expressed (Gehrig et al., 1995). Gene duplication could be the case in *H. verticillata*, because the plants in Florida are dioecious female diploids (2n = 16) and are materlineal (Langeland et al., 1992). Variable length and base pair differences of the UTRs, particularly at the 3’ end where message stability is an issue, may determine functional properties of encoded proteins (Ingelbrecht et al., 1989). These could be elements governing functional differences among the *H. verticillata* isoforms. In addition, there were two initiation codons downstream of the leader sequence, which are seen in other PEPC sequences (Relle and Wild, 1996). If translation is initiated from the second Met, then the motif upstream of the Ser residue is absent and the interaction of this residue with PEPC-protein kinase and the subsequent phosphorylation would not occur.

All but two plant PEPCs in GenBank contain a Cys residue at position 196, but Ser occurred in *Hvpepc5*. At 891, Arg is the residue most commonly found, and it was conserved in *Hvpepc5*, but in both *Hvpepc3* and *Hvpepc4*, Glu was substituted. The Met-150 in *Hvpepc4* was also unusual, because the conserved residue is Leu. It is not clear whether these divergences influence the kinetic and regulatory characteristics of the isoforms. As noted earlier, the absence...
of the C4 signature Ser is a very unusual feature of the *H. verticillata* photosynthetic PEPC sequence.

The deduced amino acid sequences of the three full-length PEPC isoforms indicated that they had similar pIs and Ms. This may be why Q-Sepharose chromatography of C4 leaf extracts did not yield two peaks, even though northern analyses showed the presence of two isoforms. Of the immunoreactive bands resolved on SDS-PAGE, only the second corresponded with the deduced Mr of the three identified isoforms. The others may be cross-reacting proteins or other isoforms. Similar banding patterns for PEPC have been observed in *Egeria* spp. and *Sorghum* spp. with the conclusion that they represented different PEPC isoforms (Casati et al., 2000; Nhiri et al., 2000).

The phylogenetic analyses indicated that the *H. verticillata* sequences, including *Hyovepc4*, were divergent from the C4 graminaceous PEPCs. The C4 *F. trinervia* PEPC similarly grouped with C3 and CAM PEPCs from monocots and eudicots. Thus, the functional diversity of PEPC isoforms was not fully reflected in the branching pattern. It is possible that the C4 form of PEPC diverged before the monocot/eudicot split 200 million years ago (mya) but after the gymnosperm and angiosperm divergence 330 mya (Wolfe et al., 1989; Relle and Wild, 1996). The PEPC from Norway spruce, which is suggested to be part of the N-fixation system in spruce roots (Relle and Wild, 1996) and, thus, is likely related to the pea root nodule PEPC, was potentially a sister to the *H. verticillata* isoforms and was closer to them than to other monocot C3 or CAM PEPCs. If so, the *H. verticillata* PEPCs may represent ancestral sets of genes that emerged before angiosperm divergence and may provide clues to C4 evolution in monocots. It should be noted that monocot PEPC genes have diverged early into the C4 type and were not necessarily accompanied by C4 photosynthesis (Kawamura et al., 1992).

Members of the Hydrocharitaceae, to which *H. verticillata* belongs, were adapted to an aquatic environment 120 mya (Sculthorpe, 1967). Aquatic habitats may experience very low daytime CO2 to O2 ratios, particularly in heavily vegetated areas (Bowes and Salvucci, 1989), so submersed species likely experienced lower [CO2] before terrestrial plants encountered such conditions. Some submersed species show evidence of C4 photosynthesis (Bowes et al., 2002), and it is possible an early selection pressure led to its presence in submersed species, like *H. verticillata*, before its advent in terrestrial plants.

MATERIALS AND METHODS

**Plant Material**

*Hydrilla verticillata* (L.f.) Royle sprigs 6 cm long were incubated with a photon irradiance of 300 μmol m−2 s−1 under a 14-h 30°C photoperiod/22°C scotoperiod to limit daytime [CO2] and induce C4 photosynthesis, or a 10-h 15°C photoperiod/10°C scotoperiod regime to maintain the C3 state (Magnin et al., 1997). Induction was followed over time by determining the increase in PEPC activity, and leaves in the C3 and C4 state were harvested at intervals. Rooting of *H. verticillata* sprigs was achieved by planting them in sand under a 12-h 25°C photoperiod/25°C scotoperiod. Roots were harvested 3 or 4 weeks after planting.

**PEPC Assay, Western-Blot Analyses, and Protein Purification**

Enzyme activities for maximal activity and western blots were performed as previously described (Magnin et al., 1997). For the latter, polyclonal antibodies raised against maize PEPC were used. Km and Vmax values and maximal velocities were assessed at pH 7.3 in the absence of diithiothreitol with [PEP] ranging from 0 to 2 mM. Protein was determined by the Bradford method with γ-globulin as the standard (Bradford, 1976). PEPC was purified by (NH4)2SO4 (25%–55% [w/v]) followed by desalting on PD-10 columns (Amersham Biosciences AB, Uppsala) equilibrated with 20 mM PIPES, pH 7.0, 10 mM MgCl2, 10% (v/v) glycerol, and 10 mM β-mercaptoethanol. The resulting eluate was applied to a 1-mL Q-Sepharose FF column (Amersham Biosciences AB) equilibrated with running buffer (RB: 20 mM PIPES, pH 7.0, and 10 mM β-mercaptoethanol). After a RB wash, the bound protein was eluted with a 30-μL linear KCl gradient (0–400 mM) in RB and collected in 0.5-μL fractions for PEPC assay.

**Cloning and cDNA Sequencing**

Total RNA was extracted from C4 leaves, roots, subterranean and axillary turions (Qiagen, RNeasy Kit, Qiagen USA, Valencia, CA), and RACE-ready cDNA was prepared from it using the SMART RACE cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA). PEPC-specific primers RF (5’-CGCAAGCAATATGGTGAAGTGA-3’; corresponding to nt 79–103) and 11R (5’-TTGTACATTGTACCCTGGGTCCCTT-3’; nt 933–957) were designed from the partial cDNA sequence *Hyovepc2*, obtained previously (Rao et al., 1998). TA cloning of the RACE products was performed with the TOPO-PCR Cloning Kit (Invitrogen, Carlsbad, CA). PEPC-specific inserts were initially confirmed by screening with the DIG-oligo tailed primer 11R (Roche Diagnostics/Roche Applied Science). From partial sequencing of the 5’ end of several of these clones, two primers, 3F (5’-CCGCCCTGCTGT TCTGATGCGCTC-3’; nt 47–67) and 4F (5’-TGGCGAGTGTCCCGATGG3’; nt 47–65), were designed and DIG-tailed to aid in further screening. Clones from the screening were partially sequenced to identify the extreme 5’- and 3’-DNA ends, so that specific primers could be designed to amplify full-length cDNAs encoding PEPC isoforms. A primer walking strategy was used for sequencing. The full-length sequence data reported here are in the GenBank at the National Center for Biotechnology Information under the accession numbers AF271761 (*Hyovepc3*), AF271762 (*Hyovepc4*), and AF271763 (*Hyovepc5*).

**Northern Analyses**

For northern analyses, a total of 1 μg of RNA per lane, extracted from leaves (RNeasy Plant Kit, Qiagen USA), was separated on a 1.2% (w/v) agarose formaldehyde gel (Maniatis et al., 1982). A downward capillary blotting method was employed to transfer the RNA to a positively charged nylon membrane using 10× SSC as the transfer buffer (Roche Diagnostics/Roche Applied Science). The bound RNA was UV cross-linked for 3 min and hybridized overnight with the appropriate DIG-labeled RNA probe in standard hybridization buffer with 50% (v/v) formamide. The stringency washes and detection were carried out following the DIG-System User’s Guide (Roche Diagnostics/Roche Applied Science). For stripping the probes from the hybridized membranes, two washes at 80°C for 1 h each were performed with 50% (v/v) formamide and 5% (w/v) SDS in 50 mM Tris-HCl at pH 7.5.

**Syntheses of Antisense RNA Probes**

Three different antisense RNA probes were synthesized following the protocol of the DIG RNA labeling kit (Roche Diagnostics/Roche Applied Sciences). PCR amplified regions from either full- or partial-length cDNA clones were inserted into the vector pCR-XL-TOPO (Invitrogen) in a manner...
such that the transcription template included the T7 promoter/priming site at the 3' end. The specific probes for Hpepc3 (nt 2948–3366) and Hpepc4 (nt 2948–3197) were derived from their respective full-length cDNA clones with the primer pairs PRB-3P (5'–TCTCCTGACTACCCGACACTGCGTAAAC-3') and T7-PCR primer (5'-TAATACGACTCACTATAGGG-3'). The region (nt 47–1799) of the consensus probe was PCR amplified from a 1.8-kb partial cDNA clone of Hpepc3 with the aid of primer pairs 3F (5'-CCGGCTCTGCTCGATGGCCTGC-3') and T7-PCR primer.

Sequence Analyses and Phylogeny Inference

Standard sequence compiling and analyses, including pair-wise comparison of nt and deduced amino acids, was performed using the Wisconsin package (v10.1, Genetics Computer Group, Madison, WI). For phylogenetic analysis, PHYLIP v3.57 (Felsenstein, 1989) and PAUP programs were used. The deduced amino acid sequences from the three full-length H. verticillata PEPc isoforms and 28 previously published PEPc sequences from GenBank were used to build the tree. The species and accession numbers of the 28 PEPc sequences are: Anacystis nidulans (M11198), Anabaena variabilis (M80541), Norway spruce (Picea abies; X79090), pea (Pisum sativum; D60437), rice (Oryza sativa; AF271995), tobacco (Nicotiana tabacum; X9016), common ice plant (Mesembryanthemum crystallinum; spc; X1,4588; spc1; X1,4587), Vanilla planifolia (spcV1; X87148; spcV2; X87149), maize (Zea mays; C0; X61489; root, AB012228; C2; X63613), sorghum (Sorghum vulgare; CP21; X53755; CP24; X96925), Flaviera trinervia (spcA; X64143; spcB; AF240870; spcC; AF240880), Flaviera pringlei (spcA; Z1,48966), sugarcane (Saccharum officinarum; C0; A1,293346), Saccharum hybrid var H32–8560 (C0; M866611), tomato (Lycopersicon esculentum; spc; A1,243416; spc2; A1,243417), brown mustard (Brassica juncea; spc2; A1,223496; spc3; A1,223497), Streptomyces coelicolor (CA959220), and Escherichia coli (AE000469).

The predicted protein sequences were aligned using the CLUSTAL program (Thompson et al., 1994), and the sequences were edited to include only the unambiguously aligned sections. Two different methods in the PHYLIP package, NEIGHBOR (neighbor-joining based on the output file from PROTDIST distance matrix analysis program) and PROTPARS (maximum parsimony), were used with a bootstrap analysis of 100 replications to determine the unambiguously aligned sections. Two different methods in the PHYLIP package, NEIGHBOR (neighbor-joining based on the output file from PROTDIST distance matrix analysis program) and PROTPARS (maximum parsimony), were used with a bootstrap analysis of 100 replications to determine and compare the confidence level of branches within the phylogenetic tree.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requester.

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LITERATURE CITED


