Species Having C_4 Single-Cell-Type Photosynthesis in the Chenopodiaceae Family Evolved a Photosynthetic Phospho*enol*pyruvate Carboxylase Like That of Kranz-Type C_4 Species[1]

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Spatial and temporal regulation of phospho*enol*pyruvate carboxylase (PEPC) is critical to the function of C_4 photosynthesis. The photosynthetic isoform of PEPC in the cytosol of mesophyll cells in Kranz-type C_4 photosynthesis has distinctive kinetic and regulatory properties. Some species in the Chenopodiaceae family perform C_4 photosynthesis without Kranz anatomy by spatial separation of initial fixation of atmospheric CO_2 via PEPC from C_4 acid decarboxylation and CO_2 donation to Rubisco within individual chlorenchyma cells. We studied molecular and functional features of PEPC in two single-cell functioning C_4 species (*Bienertia sinuspersici*, *Suaeda aralocaspica*) as compared to Kranz type (*Haloxylon persicum*, *Salsola richteri*, *Suaeda eltonica*) and C_3 (*Suaeda linifolia*) chenopods. It was found that PEPC from both types of C_4 chenopods displays higher specific activity than that of the C_3 species and shows kinetic and regulatory characteristics similar to those of C_4 species in other families in that they are subject to light/dark regulation by phosphorylation and display differential malate sensitivity. Also, the deduced amino acid sequence from leaf cDNA indicates that the single-cell functioning C_4 species possesses a Kranz-type C_4 isoform with a Ser in the amino terminal. A phylogeny of PEPC shows that isoforms in the two single-cell functioning C_4 species are in a clade with the C_3 and Kranz C_4 *Suaeda* spp. with high sequence homology. Overall, this study indicates that *B. sinuspersici* and *S. aralocaspica* have a C_4-type PEPC similar to that in Kranz C_4 plants, which likely is required for effective function of C_4 photosynthesis.

In C_4 plants having Kranz anatomy, fully differentiated mesophyll cells (MCs) and bundle sheath cells (BSCs) cooperate to fix CO_2 by the C_4 pathway (Edwards and Walker, 1983). In these plants, atmospheric CO_2 is first fixed into C_4 acids in the MC by phospho*enol*pyruvate (PEP) carboxylase (PEPC). These C_4 acids are then transported to BSCs where they are decarboxylated and the CO_2 is concentrated and donated to the C_3 cycle.

Since the discovery of C_4 photosynthesis, the spatial compartmentation of terrestrial C_4 plants was consistently linked to the occurrence of Kranz-type anatomy. Recently, three succulent species in the Chenopodiaceae family, *Bienertia cycloptera* Bunge ex Boiss., *Bienertia sinuspersici* Akhani, and *Suaeda aralocaspica* (Bunge) Freitag and Schütze (formerly classified as Borszczowia), were found to have a unique mechanism of C_4 photosynthesis, which occurs within individual photosynthetic cells by intracellular partitioning of enzymes and organelles (including dimorphic chloroplasts) into two compartments (Voznesenskaya et al., 2003; Edwards et al., 2004; Akhani et al., 2005). *S. aralocaspica* has a single layer of elongated, cylindrical chlorenchyma cells in which functions of C_4 photosynthesis are spatially separated between opposite ends of the cells. The model for photosynthesis from studies on cell structure and compartmentation of photosynthetic enzymes is that atmospheric CO_2 enters the chlorenchyma cell at the distal end and carbon is assimilated into C_4 acids by the action of PEPC located in the cytosol. This is accomplished through use of the PEP generated in chloroplasts by pyruvate orthophosphate dikinase (PPDK) in this part of the cell. C_4 acids diffuse from the distal to the proximal part of the cell through a thin, peripheral cytoplasmic space in the middle of the cell. There, C_4 acids are decarboxylated by the NAD-malic enzyme (NAD-ME) in mitochondria, which are localized in the proximal end, and the CO_2 captured by Rubisco in the chloroplasts (Voznesenskaya et al., 2003). *B. cycloptera*

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and *B. sinuspersici* have an unusual development of two cytoplasmic compartments in chlorenchyma cells, which consist of a large central cytoplasmic compartment packed with chloroplasts and mitochondria and a peripheral layer of cytoplasm with chloroplasts (Voznesenskaya et al., 2002, 2005; Akhani et al., 2005). The central compartment, which is surrounded by vacuoles, is connected to the peripheral cytoplasm by cytoplasmic channels. From our studies, the model for C_4 photosynthesis in *Bienertia* spp. is that atmospheric CO_2 enters the cell around the periphery and is incorporated into C_4 acids by PEPC. C_4 acids then diffuse to the central compartment through cytoplasmic channels and are decarboxylated by NAD-ME in the mitochondria specifically located there. Rubisco in the chloroplasts in the central compartment fixes the released CO_2. A three-carbon product from this decarboxylation diffuses to the peripheral chloroplasts, where PPDK generates PEP from pyruvate for the PEPC reaction (Voznesenskaya et al., 2002).

The specialized organelle and enzyme compartmentation in these single-cell C_4 functioning chenopods, and the particular features of the connecting cytoplasmic compartments, mimic the organization of BSCs and MCs, where there is intercellular spatial separation (Edwards et al., 2004). However, in the single-cell functioning C_4 species, the cytosolic enzyme PEPC is not exclusively confined to the site of entry of atmospheric CO_2. In the chlorenchyma cells of *S. aralocaspica*, PEPC is found throughout the cytoplasm (Voznesenskaya et al., 2001). In Bienertia, immunolabeling for PEPC shows the most intensive labeling in the peripheral cytoplasm, with some labeling in the central cytoplasmic compartment (Voznesenskaya et al., 2002). Selective function of the enzyme is necessary to prevent a futile cycle in the Rubisco-containing cellular compartment. In Bienertia and *S. aralocaspica*, PEPC catalysis in the central and proximal compartments, respectively, may be limited by (1) less PEPC protein because there is lower cytosolic space due to the high density of mitochondria and chloroplasts in these compartments; (2) selective control of PEPC activity by metabolites or covalent modification (Edwards et al., 2004); or (3) unavailability of PEP because PPDK is selectively localized in the peripheral and distal chloroplasts, respectively.

PEPC catalyzes the β-carboxylation of PEP by HCO_3^- in the presence of a divalent cation to yield oxaloacetate and inorganic phosphate (O'Leary, 1982). It plays a cardinal role in the initial fixation of atmospheric CO_2 in leaf tissue of plants performing C_4 photosynthesis and Crassulacean acid metabolism (CAM). PEPC also participates in diverse anaplerotic functions in basic plant metabolism, such as gluconeogenesis and nonautotrophic CO_2 fixation in C_3 leaves and nonphotosynthetic tissues (Andreo et al., 1987; Chollet et al., 1996). In Kranz-type C_4 and CAM plants, PEPC is highly regulated in vivo with L-malate and Glc-6-P acting as negative and positive allosteric effectors, respectively. In addition, the enzyme is regulated by phosphorylation/dephosphorylation of a single Ser residue, located in its N terminus, resulting in an increase in catalytic activity by phosphorylation and, more notably, a decrease in L-malate sensitivity. PEPC is phosphorylated by a specific Ca^{2+}-independent protein kinase closely related to plant calcium-dependent protein kinases and dephosphorylated by protein phosphatase 2A. The phosphorylation state of PEPC is highly controlled by the PEPC kinase, but not through changes in phosphatase activity (Nimmo et al., 1987; Carter et al., 1991; Chollet et al., 1996; Vidal and Chollet, 1997). In C_4 plants, PEPC phosphorylation is triggered by illumination (Nimmo et al., 1987; Jiao and Chollet, 1991; Duff et al., 1995; Vidal and Chollet, 1997), whereas in CAM plants, enzyme phosphorylation occurs during the dark period according to circadian rhythm (Hartwell et al., 1996). Additionally, regulatory phosphorylation of a C_4 form of PEPC in mature maize (*Zea mays*) plants is controlled not only by a light signal, but also by some other metabolic signals such as nitrogen status (Ueno et al., 2000; Echeverria and Vidal, 2003; Nimmo, 2003). PEPC kinase transcript levels from CAM plants are regulated in response to a circadian oscillator (Hartwell et al., 1999; Taybi et al., 2000), and those in C_3 and C_4 plants have been shown to be induced by light (Tsuchida et al., 2001; Fontaine et al., 2002).

Isoforms of enzymes of the C_4 pathway, including PEPC, are present in C_3 plants. The evolution of C_4 plants was facilitated by a set of genes that already existed in ancestral C_3 species. New expression patterns and regulatory elements of the genes were acquired to make them more efficient and spatially regulated, and genes modified in the region were transcribed to give forms with kinetic properties different from those in C_3 (Lepiniec et al., 1994; Svensson et al., 2003).

The aim of this work was to determine whether PEPC from *B. sinuspersici* and *S. aralocaspica* are regulated in such a way as to control day/night activity and to analyze whether these single-cell functioning C_4 species possess a C_4 PEPC isoform, as occurs in Kranz-type C_4 plants. For comparison, we also studied PEPC from *Suaeda linifolia* (C_3)—phylogenetically very close to *S. aralocaspica*—and *Haloxylon persicum*, *Suaeda eltonica*, and *Salsola richteri* (C_4 species), also succulent species belonging to the Chenopodiaceae family. We biochemically characterized PEPC in crude extract from leaves and isolated and sequenced cDNAs of some members of the PEPC family. The sequence characteristics of each were studied and analyzed in an evolutionary context.

RESULTS

Western Blots for Enzymes Related to Carbon Fixation

To study PEPC from the single-cell functioning C_4 plants *B. sinuspersici* and *S. aralocaspica*, and to compare

the enzyme's molecular and biochemical features with those of other C_3 and C_4 species belonging to the Chenopodiaceae family, we analyzed leaf extracts from *S. linifolia*, *H. persicum*, *S. richteri*, and *S. eltonica*.

S. linifolia has been described as a C_3 species according to its carbon isotope composition and leaf anatomy (Akhani et al., 1997; Kapralov et al., 2006); however, there are no biochemical data regarding the carbon fixation enzymes. Western-blot analysis carried out with total protein from this species (Fig. 1, lane 7) indicates that C_3-type photosynthetic metabolism is operating in this plant, as shown by high levels of Rubisco (Fig. 1D, lane 7), very low levels of PEPC—consistent with it having a housekeeping function (Fig. 1C, lane 7)—and no reaction with antibodies against the malate decarboxylating enzymes NAD-ME and NADP-ME (Fig. 1, A and B, respectively).

With respect to the other species, the occurrence of C_4-type metabolism is indicated by the presence of high levels of a 110-kD immunoreactive PEPC (Fig. 1C, lanes 2–6) as compared to the C_3 species *S. linifolia* (Fig. 1C, lane 7), by the presence of lower levels of the large subunit of Rubisco (*rbcL*; Fig. 1D, lanes 2–6) than in the C_3 species (Fig. 1D, lane 7), and by high levels of the decarboxylating enzymes NAD-ME or NADP-ME (Fig. 1, A and B, lanes 2–6).

Both of the non-Kranz, single-cell functioning C_4 species, *B. sinuspersici* and *S. aralocaspica*, are NAD-ME type, displaying an immunoreactive band of 65 kD with antibodies against NAD-ME (Fig. 1A, lanes 2 and 3) and no reaction with antibodies against NADP-ME (Fig. 1B, lanes 2 and 3). This subclassification has been previously reported for *S. aralocaspica* (Voznesenskaya et al., 2001), but not for *B. sinuspersici*, which was more recently classified as a single-cell functioning C_4 plant (Akhani et al., 2005).

S. eltonica is classified as an NAD-ME-type C_4 species as evidenced by the presence of the α-NAD-ME subunit of 65 kD (Fig. 1A, lane 5) and lack of NADP-ME. In agreement with previous work (Casati et al., 1999; Pyankov et al., 2000), *H. persicum* and *S. richteri* are classified as NADP-ME-type C_4 species, as shown by the presence of an immunoreactive band with antibodies against maize NADP-ME (Fig. 1B, lanes 4 and 6). In addition, both species show strong immunoreactive bands in western blots with anti-α-NAD-ME from *Amaranthus hypochondriacus* (Fig. 1A, lanes 4 and 6). For *S. richteri* and *H. persicum*, the presence of both NADP and NAD decarboxylating enzymes is not surprising because in several species within the Chenopodiaceae family coexistence of both enzymes has been previously reported (Pyankov et al., 2000).

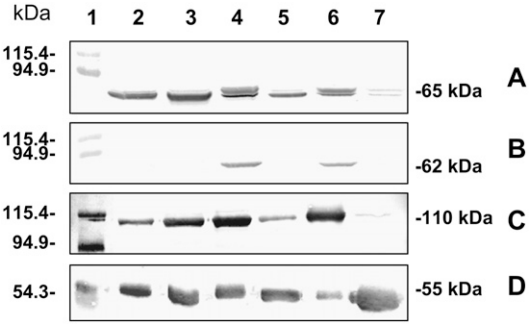

Figure 1. Western blots of photosynthetic enzymes in the single-cell functioning C_4 species *B. sinuspersici* and *S. aralocaspica* compared to Kranz C_4 and C_3 chenopod species. Western blots were revealed with antibodies against α-NAD-ME (A), NADP-ME (B), PEPC (C), and Rubisco (D). Twenty micrograms (A–C) or 5 μg (D) of total protein from *B. sinuspersici* (2), *S. aralocaspica* (3), *H. persicum* (4), *S. eltonica* (5), *S. richteri* (6), and *S. linifolia* (7) were loaded. Molecular masses of the M_r markers loaded in lane 1 are shown on the left. Molecular masses of the immunoreactive bands are shown on the right.

Biochemical Characterization of PEPC

PEPC Kinetic Parameters

Prior to measurements of PEPC activity, native gels were run followed by activity staining to check the oligomerization state of the enzyme. It was found that inclusion of 20% (v/v) glycerol in the extraction buffer and in the gels was necessary to maintain the enzyme in the tetrameric form and to obtain maximal activity. Glycerol and other compatible solutes are known to stabilize PEPC and they may affect the kinetic properties by protecting against deoligomerization and/or as osmoprotectants (Krall and Edwards, 1993; Ogawa et al., 1997). With glycerol, PEPC proteins from the different species displayed similar mobility, corresponding to that of a molecular mass of 440 kD. Because a similar PEPC subunit size (110 kD) was present in all samples (see western-blot analysis; Fig. 1C), the aggregation state in native gels indicates that the enzyme in single-cell functioning C_4 and other species is a tetramer. When glycerol was omitted, some additional bands, corresponding to dimer forms, were observed (data not shown). To prevent dissociation of the enzyme during kinetic analysis, 20% (v/v) glycerol was included in extraction and activity media assay.

To biochemically characterize PEPC from different Chenopodiaceae species, we determined the kinetic parameters of the enzyme [apparent maximal specific activity of PEPC at saturating substrates (apparent V_{max}), $K_{m\ (PEP)}$, and $I_{50\ (malate)}$] using desalted soluble protein from leaf extracts collected in the light period and the dark period (Table I). In all cases, when assayed at the optimal pH (8.0), the curve linking initial velocity to substrate (PEP) concentration was a rectangular hyperbola.

As anticipated for a C_3-type PEPC, the enzyme from *S. linifolia* had much lower apparent V_{max} values and 2- to 4-fold lower $K_{m\ (PEP)}$ than those of PEPC from the Kranz C_4 species *H. persicum*, *S. eltonica*, and *S. richteri*. Interestingly, the highest values for apparent maximal specific activity of PEPC were from the non-Kranz, single-cell functioning C_4 species. With respect to $K_{m\ (PEP)}$ for PEPC, in the case of *S. aralocaspica*, the values were 2-fold higher than C_3 *S. linifolia* and comparable

Table I. *Kinetic parameters of PEPC of the single-cell functioning C_4 species B. sinuspersici and S. aralocaspica and related chenopod species*

Leaves were collected after 5 h into the light and 5 h into the dark period. Activity was assayed at pH 8.0. Values represent the mean of at least three independent determinations (SES are shown).

Species	Photosynthetic Type	Parameter	Light	Dark
B. sinuspersici	Single-cell C_4	Apparent V_{max} (units/mg protein)	1.96 ± 0.01	1.78 ± 0.04
		$K_{m\ (PEP)}$ (mM)	0.06 ± 0.01	0.06 ± 0.01
S. aralocaspica	Single-cell C_4	Apparent V_{max} (units/mg protein)	1.67 ± 0.03	1.06 ± 0.04
		$K_{m\ (PEP)}$ (mM)	0.10 ± 0.01	0.12 ± 0.02
H. persicum	Kranz C_4	Apparent V_{max} (units/mg protein)	1.27 ± 0.04	0.87 ± 0.04
		$K_{m\ (PEP)}$ (mM)	0.12 ± 0.03	0.17 ± 0.03
S. eltonica	Kranz C_4	Apparent V_{max} (units/mg protein)	0.83 ± 0.03	0.67 ± 0.08
		$K_{m\ (PEP)}$ (mM)	0.09 ± 0.02	0.10 ± 0.03
S. richteri	Kranz C_4	Apparent V_{max} (units/mg protein)	0.93 ± 0.03	1.00 ± 0.04
		$K_{m\ (PEP)}$ (mM)	0.21 ± 0.04	0.22 ± 0.04
S. linifolia	C_3	Apparent V_{max} (units/mg protein)	0.09 ± 0.01	0.08 ± 0.01
		$K_{m\ (PEP)}$ (mM)	0.05 ± 0.01	0.05 ± 0.01

to that of the C_4 plants *H. persicum* and *S. eltonica*. In contrast, $K_{m\ (PEP)}$ values of PEPC from *B. sinuspersici* were intermediate between those of the enzyme from the C_3 *S. linifolia* and from the C_4 species.

When comparing the kinetic parameters of samples collected during the day and night, it is clear that PEPC from *H. persicum* is differentially regulated during the photoperiod, showing higher apparent maximal activity (1.5 times) and higher affinity for the substrate PEP during the day than at night. In crude extracts of *S. eltonica* (C_4), although apparent V_{max} estimated during the day was higher than that at night, PEP affinity remained almost unchanged. In the case of PEPC from *S. aralocaspica*, the enzyme parameters were affected in the same manner as those of PEPC from the C_4 species *S. eltonica*, showing a 1.6 times increase in apparent V_{max} and a 0.9 times decrease in $K_{m\ (PEP)}$ during the day versus night. In contrast to the other species, PEPC kinetic parameters from the Kranz C_4 *S. richteri*, C_3 *S. linifolia*, and single-cell functioning C_4 *B. sinuspersici* remained almost unchanged.

Varying the L-malate concentration in the reaction mixture at suboptimal PEP concentration and pH 7.2 resulted in a progressive decrease of enzyme activity in all cases. The concentration of malate causing 50% inhibition of initial activity of PEPC [$I_{50\ (malate)}$] was measured in desalted crude extracts from samples collected during the day and night (Table II). Measurements were performed at a PEP concentration (0.1 mM) that was similar to estimated $K_{m\ (PEP)}$ values for most species (Table I). PEPC from *S. linifolia* did not show differences in malate sensitivity during the day and night periods, as revealed by essentially similar $I_{50\ (malate)}$ values, which is expected for a C_3-type PEPC (Gupta et al., 1994; Svensson et al., 2003).

In *B. sinuspersici* and *S. aralocaspica*, malate sensitivity of PEPC was similar to that from Kranz-type C_4 species. When comparing samples collected during the light and dark periods, in all PEPCs from Kranz-type C_4 plants analyzed during the day, malate sensitivity was lower compared to the night, as shown by an increase in the $I_{50\ (malate)}$ value ranging from 1.7 (*S. richteri*) to 4 times (*H. persicum*). In the same manner, the $I_{50\ (malate)}$ value of PEPC from the single-cell functioning C_4 species *S. aralocaspica* collected during the day is twice that measured during the night, indicating that the enzyme is more sensitive to malate at night. However, in the case of *B. sinuspersici*, only a slight increase (1.1 times) in the $I_{50\ (malate)}$ was obtained for samples collected during the day. Nevertheless, when

Table II. *Inhibition of PEPC by malate [$I_{50\ (malate)}$] in soluble protein extracts from leaves of the single-cell functioning C_4 species B. sinuspersici and S. aralocaspica and related chenopod species*

Samples were taken after 5 h into the light and dark periods. PEPC from crude extracts was assayed at pH 7.2, 0.1 mM PEP. Values represent the mean of at least three independent determinations (SES are shown).

Species	Photosynthetic Type	$I_{50\ (malate)}$		$I_{50\ (malate)}$ Light/Dark
		Light	Dark	
		mM		
B. sinuspersici	Single-cell C_4	2.22 ± 0.03	2.00 ± 0.25	1.11
		0.60 ± 0.02[a]	0.23 ± 0.02[a]	2.61[a]
S. aralocaspica	Single-cell C_4	0.69 ± 0.07	0.33 ± 0.06	2.09
H. persicum	Kranz C_4	1.91 ± 0.04	0.47 ± 0.05	4.06
S. *eltonica*	Kranz C_4	0.89 ± 0.05	0.26 ± 0.01	3.42
S. richteri	Kranz C_4	2.17 ± 0.11	1.25 ± 0.14	1.74
S. linifolia	C_3	3.93 ± 0.06	3.90 ± 0.11	1.01

[a]With *B. sinuspersici*, PEPC was also assayed at pH 7.2 with 0.055 mM PEP.

malate sensitivity was evaluated at a lower level of PEP (0.55 mM), which is the $K_{m\,(PEP)}$ value estimated for the enzyme in this species (Table I), there was a 2.6 times increase in the $I_{50\,(malate)}$ values for samples collected in the light compared to those collected in the dark. In addition, the $I_{50\,(malate)}$ values for PEPC for *B. sinuspersici* and *S. aralocaspica* were similar when determined around the respective $K_{m\,(PEP)}$ values for the two species.

PEPC Isoelectric Point

Data obtained from kinetic and malate inhibition studies of PEPC (on day versus night isolations) from *B. sinuspersici* and *S. aralocaspica* indicate there may be posttranslational modification of the enzyme. To evaluate this possibility, native isoelectric focusing (IEF) was conducted followed by activity staining, using samples taken during the night versus the day. In both species, PEPC from samples collected during the night showed a more alkaline pI than from samples harvested during the day. Representative results are shown in Figure 2, with two single-cell C_4 species in lanes 1 (*B. sinuspersici*) and 2 (*S. aralocaspica*), two Kranz-type species in lanes 3 (*H. persicum*) and 4 (*S. eltonica*), and the C_3 species (*S. linifolia*) in lane 5. The average pI values (from three replications) for samples collected from the day versus night, respectively, were 5.41 ($\pm$0.05) versus 5.55 ($\pm$0.05) for *B. sinuspersici*, and 5.47 ($\pm$0.03) versus 5.62 ($\pm$0.05) for *S. aralocaspica*. Similar results were obtained for the Kranz-type C_4 chenopod species *S. richteri* (data not shown), *H. persicum*, and *S. eltonica* with average day versus night values for the three species of 5.52 ($\pm$0.04) and 5.63 ($\pm$0.03), respectively. In contrast, no light versus dark changes in the pI values (5.14 $\pm$ 0.02 versus 5.11 $\pm$ 0.05) were observed for PEPC from C_3 *S. linifolia*. The light versus dark changes in pI in the single-cell C_4 species provides additional evidence that their PEPC undergoes regulatory modifications during the day/night periods.

PEPC Phosphorylation Studies

Based on changes in the kinetic properties and pI of PEPC during the day/night periods, *B. sinuspersici* and *S. aralocaspica* leaves were investigated to determine whether the enzyme undergoes a diel pattern of phosphorylation/dephosphorylation. For this purpose, we used samples collected during the day and night periods and performed western-blot analysis with antibodies raised against phosphorylated PEPC (Fig. 3B), which cross-reacts with PEPC from a number of C_4 species (T. Furumoto and K. Izui, personal communication). As controls, crude extracts from the previously characterized species, *Flaveria trinervia* (C_4) and *Flaveria pringlei* (C_3), were included (Fig. 3, lanes 3 and 4). In *F. trinervia* leaves, the *ppcA* gene encoding the C_4 isoform is the PEPC gene having the highest level of expression (Westhoff et al., 1997). Thus, the immunoreactive band observed in crude extracts of *F. trinervia*, which undergoes phosphorylation in the light, is considered the C_4-type isoform. In *F. pringlei*, all the genes encoding PEPCs are expressed in low levels in leaves, and the antiphosphorylated PEPC antibody did not detect light/dark changes in the phosphorylation state, although *F. pringlei* PEPC-A has a phosphorylatable Ser near the N terminus (Westhoff et al., 1997; Bläsing et al., 2000). Figure 3, A and C, shows western blots with anti-PEPC from *Amaranthus viridis* and the corresponding Coomassie Blue SDS-PAGE carried out with the same samples with the same amount of protein loaded in each lane. The results indicate that the same levels of PEPC protein are present in samples collected during the day and night for each plant.

Using the antibody with specificity to the phosphorylated form of PEPC, in the Kranz-type C_4 species *F. trinervia* (used as a control; Fig. 3A, lane 3L), PEPC is phosphorylated during the day and dephosphorylated during the night. Likewise, PEPC in the single-cell functioning C_4 species *B. sinuspersici* (lane 5L) and *S. aralocaspica* (lane 6L), and in all C_4 Kranz species studied from Chenopodiaceae (lanes 2L, 7L, and 8L), is phosphorylated during the day and dephosphorylated during the night.

In addition, studies were done to establish whether the changes in the PEPC phosphorylation state in the single-cell functioning C_4 species respond to light stimulus or to endogenous circadian rhythm. For these experiments, samples were collected at different times in the day/night cycle and also after 17 and 41 h of complete darkness (which includes time corresponding to that of the day period for the usual sampling). Western blots with anti-*A. viridis* PEPC showed that similar levels of PEPC protein were loaded in each lane (Fig. 4, A and C). On the other hand, western blots with antiphosphorylated PEPC, using *B. sinuspersici* (Fig. 4B) and *S. aralocaspica* (Fig. 4D) crude extracts, indicate that PEPC is phosphorylated under light conditions (lanes 2–5), showing a major proportion of phosphorylated PEPC at 1 and 5 PM (Fig. 4D, lanes 4 and 5, respectively) in the case of *S. aralocaspica* and at 1 PM in the case of *B. sinuspersici* (Fig. 4B, lane 4). Immunoreaction with antiphosphorylated PEPC was not observed in samples collected from plants during the night period (lanes 6 and 7) or during the extended dark periods when it would usually be phosphorylated

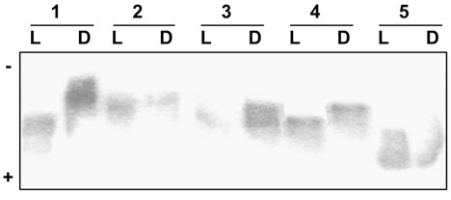

Figure 2. Native IEF protein gel revealed by activity staining for PEPC in the single-cell functioning C_4 species *B. sinuspersici* and *S. aralocaspica* and related chenopod species. Leaf samples were taken after 5 h into the light (L) and 5 h into the dark (D) period. Approximately 4 milliunits of PEPC from leaf extracts of *B. sinuspersici* (1), *S. aralocaspica* (2), *H. persicum* (3), *S. eltonica* (4), and *S. linifolia* (5) were loaded.

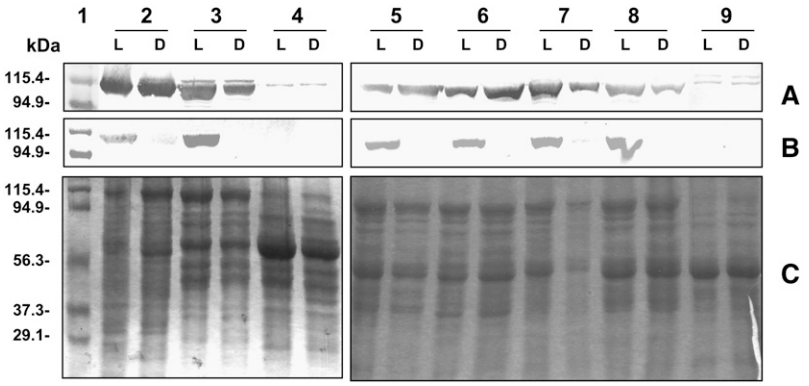

Figure 3. Western blots for PEPC protein and for the phosphorylated form of PEPC from leaves harvested in the light versus dark. Samples were taken after 5 h into the light (L) and 5 h into the dark (D) period. Fifteen micrograms of total protein from *S. richteri* (2), *F. trinervia* (3), *F. pringlei* (4), *B. sinuspersici* (5), *S. aralocaspica* (6), *H. persicum* (7), *S. eltonica* (8), and *S. linifolia* (9) were loaded for western-blot analysis with antitotal PEPC (A) or with antiphosphorylated PEPC (B). Molecular masses of the M_r markers loaded in lane 1 are shown on the left. Twenty micrograms of total protein were loaded in each lane (2–9) for Coomassie Blue staining (C).

under illumination (lanes 8 and 9). These results indicate that, as in the case of C_4-type PEPC from Kranz species, phosphorylation of the enzyme from *B. sinuspersici* and *S. aralocaspica* is light dependent.

Molecular Characterization of PEPC

To gain insight into PEPC molecular characterization and evolution within the Chenopodiaceae in relation to the single-cell functioning C_4 species, we isolated by reverse transcription (RT)-PCR the *ppc* transcripts expressed in major proportions in leaves of *B. sinuspersici* and *S. aralocaspica*, in the Kranz C_4 plant *S. eltonica*, and in the C_3 species *S. linifolia*. In the case of *B. sinuspersici*, the complete cDNA (2,909 bp; GenBank accession no. DQ538352) encoding PEPC was isolated. The nucleotide sequence obtained has 85% homology to the C_4 PEPC from the dicot *A. hypochondriacus* (GenBank accession no. L4917). In the case of *S. aralocaspica*, *S. eltonica*, and *S. linifolia*, partial cDNAs were isolated (GenBank accession nos. DQ538353, DQ538354, DQ538355), where the nucleotides encoding for the first 116, 136, and 296 amino acid residues of the sequence were missing. Figure 5 shows the sequence alignment from the deduced amino acids from the PEPC sequences isolated in this work compared with that from maize, which was used for analysis of the three-dimensional structure of the enzyme (Kai et al., 2003).

A phylogenetic tree constructed from amino acid sequences of PEPC, from both monocot and dicot species, shows that sequence information on the forms of PEPC isolated from Chenopodiaceae and Amaranthaceae species form a separate clade (Fig. 6). In our tree, the other C_4 PEPC of grasses such as maize, *Sorghum vulgare*, sugar cane (*Saccharum officinarum*), and *Sorghum spontaneum* are basal. Surprisingly, both single-cell functioning C_4 species, *B. sinuspersici* and *S. aralocaspica*, are grouped together in a subclade with C_3 *S. linifolia*, C_4 *S. eltonica*, and C_4 *A. hypochondriacus*.

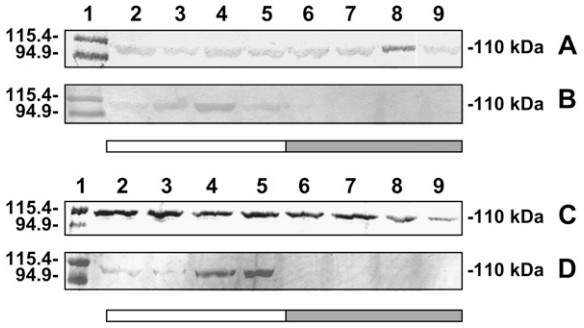

Figure 4. Western-blot analyses showing PEPC phosphorylation time course in the single-cell functioning C_4 species *B. sinuspersici* and *S. aralocaspica*. *B. sinuspersici* (A and B) and *S. aralocaspica* (C and D) samples were taken at 7 AM (2), 10:30 AM (3), 1 PM (4), 5 PM (5), 8:30 PM (6), and 12:30 PM (7), or after 17 (8) or 41 (9) h of complete darkness. In the case of *B. sinuspersici*, lights were turned on at 6 AM and turned off at 8 PM, whereas for *S. aralocaspica* the light period was 6 AM to 6 PM. Fifteen micrograms of total protein were loaded in lanes 2 to 9 for western-blot analysis with anti-PEPC (A and C) or with antiphosphorylated PEPC (B and D). Molecular masses of the M_r markers loaded in lane 1 are shown on the left. Molecular masses of the immunoreactive bands are shown on the right.

DISCUSSION

In this study, we have characterized PEPC from leaves of different Chenopodiaceae species, including a photosynthetic PEPC from single-cell functioning C_4 species. Although more than one isoform may be present in leaves of the species under study, as previously seen in Arabidopsis (*Arabidopsis thaliana*), *Flaveria* spp., *Alternanthera* spp., and maize, among others (Westhoff et al., 1997; Dong et al., 1999; Sánchez and Cejudo, 2003; Gowik et al., 2006), activity on native IEF gels shows only one activity band for each species (Fig. 2). Even if there are multiple isoforms in leaves, the major activity band observed indicates that either there is one isoform that is expressed at higher levels or there is an isoform that displays higher specific activity.

Previously, it was shown with PEPC from Kranz-type C_4 plants that the photosynthetic isoform is predominant in quantity and has a higher specific activity than the C_3 isoform (O'Leary, 1982). In the case of *S. linifolia*, a C_3 plant, the form of PEPC present in leaves should have a housekeeping, anaplerotic function. As is typical for C_3-type PEPC, the enzyme from *S. linifolia* exhibited lower $K_{m\ (PEP)}$ and apparent V_{max}

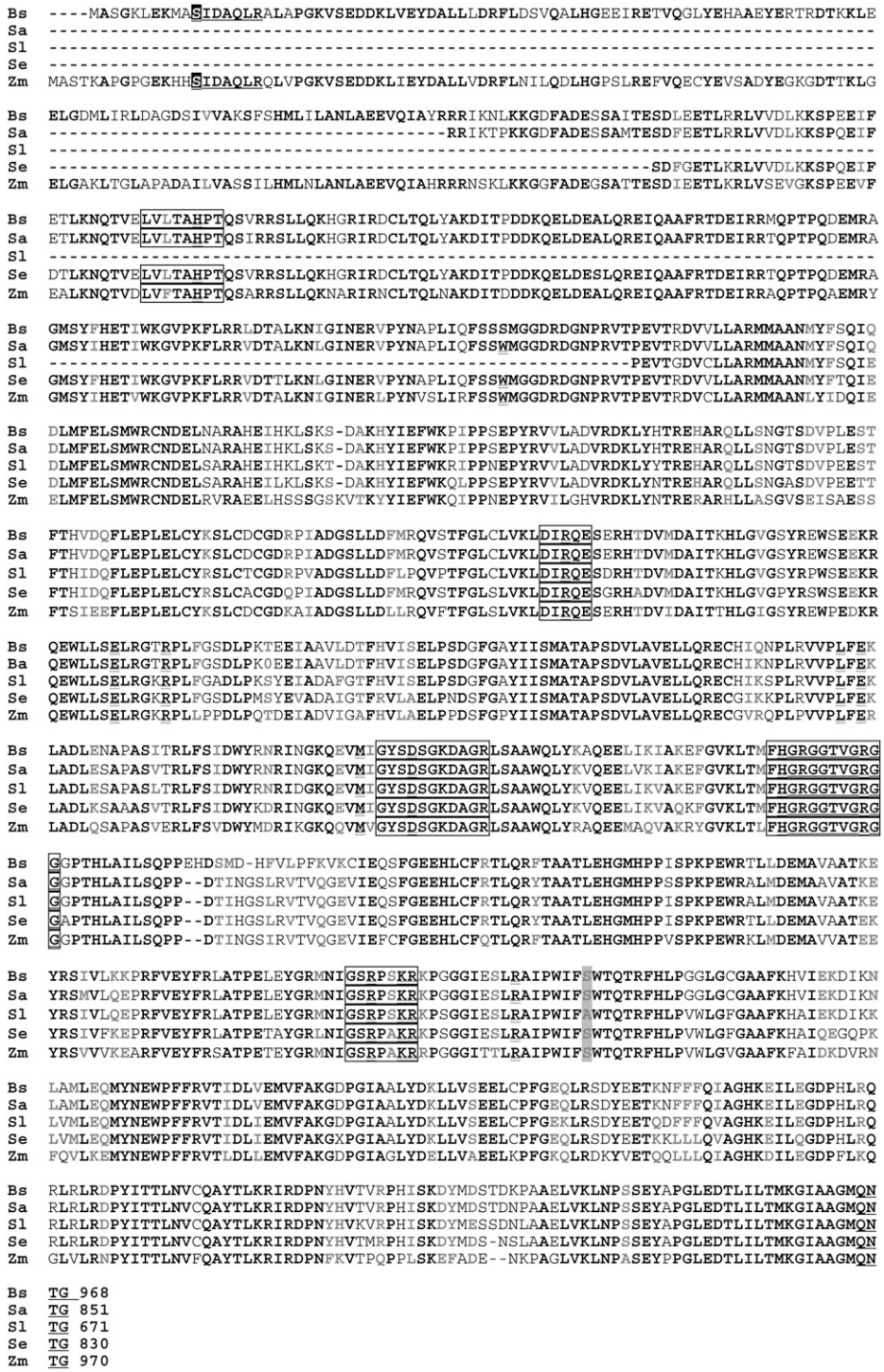

Figure 5. The deduced amino acid sequence of PEPC from *B. sinuspersici* (Bs), *S. aralocaspica* (Sa), *S. linifolia* (Sl), and *S. eltonica* (Se) aligned with the C_4 PEPC from maize (Zm) using ClustalW analysis. Identical amino acid residues are highlighted in black, whereas highly conserved amino acids are highlighted in light gray. The strictly conserved, nonphosphorylatable Ala residue in PEPC near position 774, which when changed to Ser comprises a necessary, but not sufficient, determinant of C_4-specific kinetics of C_4 PEPCs (Svensson et al., 2003), and the corresponding Ser are shaded in a light background. The Ser residue located in the amino-terminal region subjected to day/night regulatory phosphorylation is shown in white and shaded in a dark background. Motifs are underlined, whereas conserved residues are double underlined. Boxes mark domains important for enzyme catalysis.

(Table I) and did not show differential day-night sensitivity to the malate inhibitor (Table II). Even though we were not able to isolate the 5′ end of this *ppc* and evaluate whether a Ser or Ala residue is present in the plant enzyme conserved motif (S/A) IDAQLR, no regulatory changes in the protein were observed by using antibodies against phosphorylated PEPC (Fig. 3) or IEF analysis (Fig. 2).

With respect to *H. persicum*, *S. eltonica*, and *S. richteri*, the higher apparent V_{max} and $K_{m\ (PEP)}$ values for the enzyme compared to *S. linifolia* indicate that a C_4-type isoform is responsible for these differences (Table I). Using purified proteins, considerable species-to-species variability has been reported in these parameters, but C_3 PEPCs always exhibited a lower $K_{m\ (PEP)}$ than their C_4 counterparts (Svensson et al., 2003). Changes

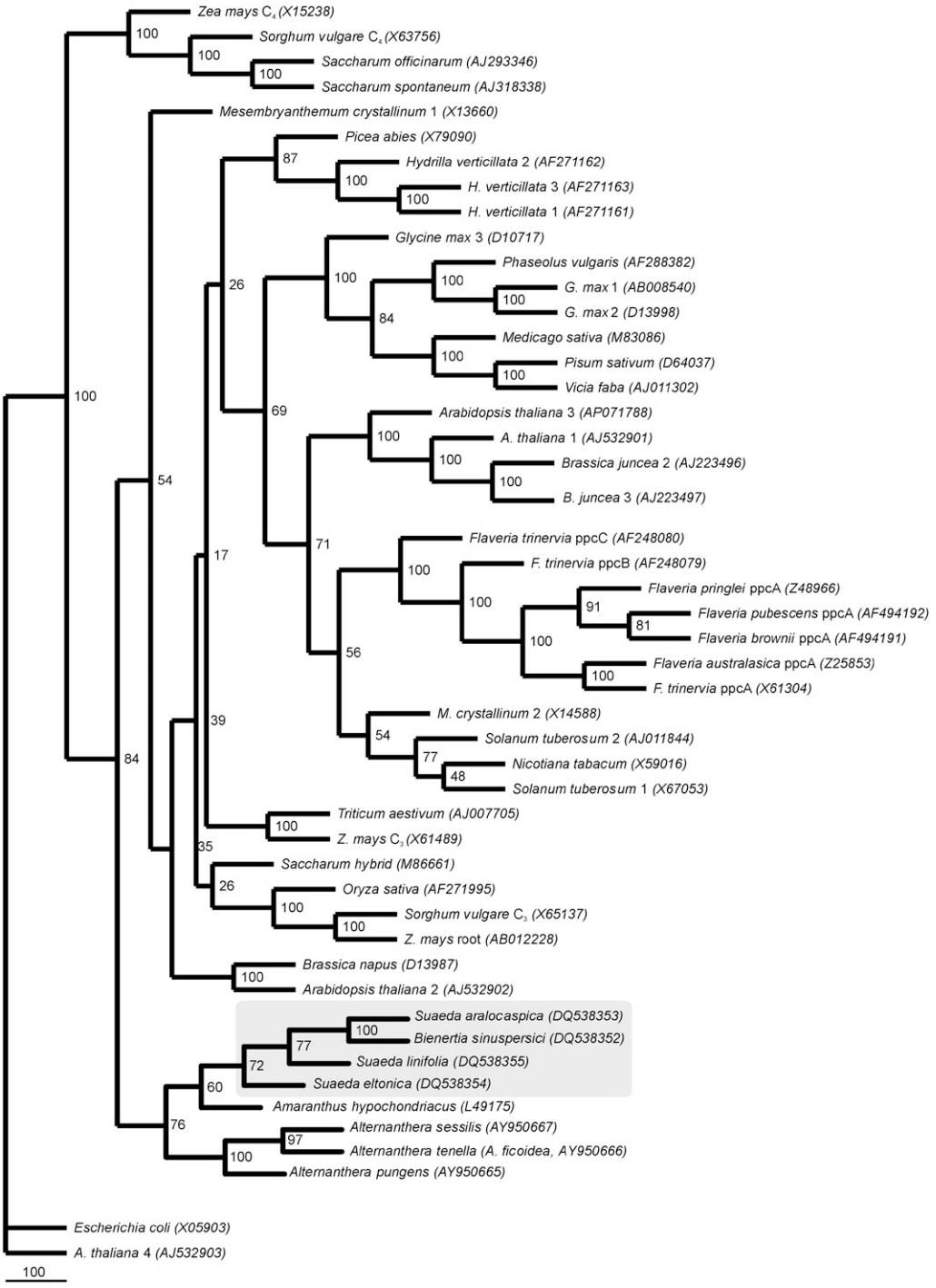

Figure 6. Phylogenetic analysis of various plant PEPC isoforms (based on the last 70 amino acid residues of the 3′ side of the coding region) using a neighbor-joining consensus tree including the single-cell functioning C_4 species *B. sinuspersici* and *S. aralocaspica*. The tree was oriented with PEPC amino acid sequence from *Escherichia coli* and Arabidopsis bacterial-type PEPC as functional outgroups. The gray box indicates the distribution of members of the Chenopodiaceae family whose sequences have been obtained in this work. The GenBank accession numbers of the sequences used to construct the phylogenetic tree are shown in the figure. Bootstrap analysis was carried out with 1,000 replicates. Bootstrap values of the branches are indicated in a 100 basis.

in apparent V_{max} and $K_{m\ (PEP)}$ during the day versus night periods, as well as a decrease in malate sensitivity of up to 4 times during the day (Table II), are a consequence of regulatory phosphorylation occurring during the day. Native IEF (Fig. 2) and western blot with antibodies against phosphorylated PEPC (Fig. 3) support this conclusion.

The single-cell functioning C_4 species *B. sinuspersici* and *S. aralocaspica* have the highest apparent V_{max} values (Table I) among the species tested, whereas their $K_{m\ (PEP)}$ values and changes in malate sensitivity during the day and night periods (Table II) are similar to those of the PEPCs from the Kranz-type C_4 species (in the case of *B. sinuspersici*, PEP affinity is only slightly lower than that of C_3 *S. linifolia*). Together with native IEF (revealed by activity; Fig. 2), the use of antibodies that react with the phosphorylated amino terminal of the protein shows that PEPC from both species is subject to regulatory phosphorylation (Fig. 3), which occurs during the day and peaks around noon (Fig. 4). Future studies are needed with purified or recombinant PEPC from single-cell C_4 species and related photosynthetic chenopods to provide detailed analyses of their properties.

In general, the PEPC sequences from the chenopods have the typical conserved features of plant PEPCs for residues involved in catalysis, mobile loops, and regulation. This is the case for residues from maize, R456, R759, and R773, which have been shown to be catalytically essential; residues W288, L564, and M598 in the hydrophobic pocket around PEP; E566 and D603, which bind Me^{2+}; and the basic residues K762 and R763, involved in the binding of bicarbonate, which are all also conserved in the sequences analyzed. As in maize PEPCs, residues R498 and E493, which are responsible for the formation of the dimer-of-dimer homotetrameric structure, are also conserved in the chenopod sequences presented here. The functionally essential R647 residue in the ^{640}GRGGTVGRGG^{649} motif and the H177 residue involved in protein movements directly participating in the carboxylation reaction and in Asp inhibition, as well as the H639 residue of central importance found in maize and other plant PEPCs, are also present in the chenopod PEPC sequences we studied. In addition, the conserved C-terminal QNTG motif, indispensable for maximal catalytic activity of PEPC (Dong et al., 1999), is also found (Fig. 5).

Sequence comparison of plant PEPCs revealed that C_4 enzymes investigated to date, of both monocot and dicot origin, harbor a Ser residue at a position corresponding to 774 of *F. trinervia* PEPC, 775 of *Alternanthera pungens*, or 780 in maize C_4-PEPC (Blässing et al., 2000; Gowik et al., 2006), whereas this position is occupied by an Ala in all nonphotosynthetic and CAM PEPCs. As expected for C_4-type PEPC, the deduced amino acid sequence obtained from *S. eltonica* displays a Ser residue in the equivalent position, whereas the equivalent position in C_3 *S. linifolia* PEPC has an Ala. In agreement with kinetic assays performed in this work, a Ser residue is found in the deduced sequences from *B. sinuspersici* and *S. aralocaspica* (Fig. 5). It has been proposed that the substitution of Ser for Ala at position 774 in Flaveria PEPCs or in the equivalent position in other C_4 PEPCs was essential to create a fully functional C_4 isoform, and that this occurred as one of the last steps in the evolutionary transformation of a C_3 PEPC to the C_4 isoform. The comparison of ppcA PEPCs from various *Flaveria* spp. indicates that, in addition to the C_3 species, both the C_3-C_4 intermediate and the C_4-like species still have an Ala at position 774 (Svensson et al., 2003).

In the case of *B. sinuspersici*, the deduced amino acid sequence from the cDNA shows a Ser residue in the motif of ^{11}SIDAQLR^{17} of the amino-terminal region of the enzyme, which is only found in plant proteins. It is involved in the regulation of activity by light or darkness in C_4 and CAM PEPCs (Lepiniec et al., 1994) through a phosphorylation/dephosphorylation process. Even though the presence of Ser in the SIDAQLR motif has been described in some PEPCs from C_3 species, such as Arabidopsis (Atppc1-3; Sánchez and Cejudo, 2003), the occurrence of an Ala residue instead of a Ser residue in a position equivalent to 774 from Flaveria PEPCs seems to be a constant. Thus, molecular analysis of PEPC from the single-cell functioning C_4 species indicates that these isoforms are really highly evolved C_4-type proteins, supporting the biochemical data obtained. The day/night modification of the enzyme by phosphorylation/dephosphorylation selectively maintains a more active state in the light during photosynthesis, and the C_4-type kinetic properties, with respect to apparent V_{max} (even higher than related Kranz-type C_4 species) and $K_{m\ (PEP)}$, may provide optimal function in the C_4 pathway.

The clustering of PEPC sequences confirms the hypothesis that the C_4 isoform of grasses could have evolved earlier than that of dicots (Fig. 6; Lepiniec et al., 1994; Gehring et al., 1998). Among the dicot families having C_4 species, the Chenopodiaceae seems to be the earliest C_4 lineage in accordance with the phylogenetic relationships in angiosperms (Angiosperm Phylogeny Group, 2003). The eight species of Amaranthaceae and Chenopodiaceae in the phylogeny in Figure 6 include single-cell and Kranz-type C_4 and C_3 species. They form a monophyletic clade, which is consistent with previous phylogenetic studies on these families (Kadereit et al., 2003; Müller and Borsch, 2005). The C_4 and non-C_4 PEPCs of grasses have been shown to be well differentiated, whereas it appears that, in dicotyledonous plants, C_4 PEPCs are more related to non-C_4 PEPCs. The phylogenetic tree constructed here is consistent with this hypothesis, as one can see well-defined branches of the C_4 and C_3 PEPC isoforms of the monocots maize and sorghum in the Poaceae family, whereas all PEPCs from the dicots Flaveria in the Asteraceae and Amaranthaceae s. l. family are grouped together (Fig. 6), consistent with phylogenetic studies in these families (Angiosperm Phylogeny Group, 2003).

The cluster of Amaranthaceae/Chenopodiaceae is branched into two subclades, one including Alternanthera and another including Amaranthus, Suaeda, and Bienertia. All species of Chenopodiaceae are sister to

A. hypochondriacus. Suaeda and Bienertia belong to the Suaedoideae subfamily, each representing monotypic tribes Suaedeae and Bienertieae (Schütze et al., 2003; Kapralov et al., 2006). However, Amaranthus belongs to the Amaranthoideae subfamily in Amaranthaceae s. str. and Alternanthera belongs to the Gomphrenoideae subfamily in the same family (Müller and Borsch, 2005). The two single-cell functioning C_4 species, *B. sinuspersici* and *S. aralocaspica*, provide a monophyletic clade for PEPC phylogeny with 100% bootstrap support. This is interesting because phylogenetic results obtained from various chloroplast and nuclear markers, including Rubisco large subunit (*rbcL*), maturase K (*matK*), the atpB/rbcL intergenic spacer (*atpB-rbcL*), psbB gene, psbB-psbT intergenic spacer, psbT gene, psbT-psbN intergenic spacer, psbN gene, psbN-psbH intergenic spacer (*psbB-psbH*), the trnL intron, the trnL exon 2, the trnL/trnF intergenic spacer (*trnL-trnF*), and the tRNA-Lys intron with matK inserted in the middle of the trnK intron (*matK-trnK*), indicate that two types of single-cell functioning C_4 photosynthesis evolved independently (Schütze et al., 2003; Müller and Borsch, 2005; Kapralov et al., 2006). However, they have very similar C_4 isoforms of PEPC, suggesting their *ppc-C*$_4$ gene was either derived from a common ancestor or arose independently by convergence. Both species are NAD-ME-type C_4 and both have remarkably similar PEPCs. Thus, their C_4 biochemistry appears very conserved, whereas they evolved independent, novel means of spatial separation of functions in C_4 photosynthesis. Future phylogenetic studies of the PEPC isoforms in various species in the Suaedoideae subfamily are needed to gain insight into evolution of the *ppc-C*$_4$ gene in relation to the different structural forms of C_4 photosynthesis.

C_4 plants have evolved independently several times from ancestral C_3 plants due to selective environmental conditions. This required adaptation of enzymes for effective functioning of the C_4 cycle, which involved changes in the enzyme's kinetics and regulation. C_4 photosynthesis evolved much earlier in monocots than in dicots (Cerling, 1999). In the case of PEPC, C_4-type isoforms are derived from non-C_4 isoforms. The C_4 and non-C_4 PEPCs of grasses have been shown to be well differentiated, whereas it appears that, in dicotyledonous plants, C_4 PEPCs are more related to non-C_4 PEPCs. Gehring et al. (1998) noted that PEPC isoforms are valuable molecular markers, which can be used to visualize trends in evolution and provide a useful tool to help answer taxonomic questions. Thus, further sequencing of other PEPC isoforms from *B. sinuspersici* and *S. aralocaspica*, as well as from other species from the family, may allow deciphering of the origin and evolutionary development of C_4 photosynthesis. Overall, biochemical and molecular data obtained in this work suggest that PEPCs from the single-cell functioning C_4 species, *B. sinuspersici* and *S. aralocaspica*, share the same characteristics as those from Kranz-type C_4 plants. Analyses carried out with Flaveria PEPCs reveal that only small changes are required to convert a C_3 PEPC into C_4 (Blässing et al., 2000). In addition, C_4 photosynthesis is of polyphyletic origin. Thus, perhaps it is not surprising that, in the single-cell species *B. sinuspersici* and *S. aralocaspica*, evolution of C_4 photosynthesis was accomplished by the expression of C_4-type PEPC isoforms with development of a completely different structural means of spatial compartmentation of the C_4 process. The polyphyletic origin of C_4 photosynthesis implies that, in genetic terms, it was comparatively simple for C_4 plants to evolve from C_3 ancestors (Westhoff and Gowik, 2004). However, genetic control of development of the spatial separation of functions of C_4 photosynthesis in both the Kranz and single-cell C_4 systems in terrestrial plants remains to be elucidated.

MATERIALS AND METHODS

Plant Material

The species used in the study were *Bienertia sinuspersici* Akhani, *Suaeda aralocaspica* (Bunge) Freitag and Schütze (=*Borszczowia aralocaspica* Bunge; Schütze et al., 2003), *Haloxylon persicum* Bunge, *Suaeda eltonica* Iljin, *Salsola richteri* (Moq.) Karel. ex Litv., and *Suaeda linifolia* Pall. With the exception of *S. aralocaspica*, plants were grown from seeds and kept in the greenhouse under natural sunlight (summer) plus supplemental light from sodium vapor lamps (supplying a photosynthetic photon flux density of 400 μmol m^{-2} s^{-1}), with a 14-h light/10-h dark photoperiod and a 25°C day/15°C night regime. The *S. aralocaspica* plants were kept in a growth chamber under the same conditions, except the photoperiod was 12-h light/12-h dark. Samples were taken after 5 h into the light period (day samples) or after 5 h into the dark period (night samples). After collection, the leaves were immediately frozen in liquid N_2 and stored at −80°C prior to analysis.

Protein Extraction

Total protein from the different samples 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 NaF, 50 mM KH_2PO_4, 1 mM phenylmethylsulfonylfluoride, 10 μg mL^{-1} leupeptin, 10 μg mL^{-1} chymostatin, and 10 μL/mL of protease inhibitor cocktail (Sigma) extraction buffer. The samples were ground completely in a cold mortar and centrifuged at 10,000*g* for 10 min at 4°C. The supernatant of crude extracts was desalted in a cold Sephadex G-25 column preequilibrated with the above buffer according to Penefsky (1977). This 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. For IEF gels, the extract was diluted in 0.25 M Tris-HCl, pH 6.8, 0.05% (v/v) bromphenol blue, and 50% (v/v) glycerol. Protein concentration was determined in desalted crude extracts by the method of Bradford (1976) using Bio-Rad protein assay reagent and bovine serum albumin as standard.

Enzyme Assay

PEPC activity was determined at 30°C in a coupled reaction with malate dehydrogenase by monitoring NADH oxidation at 340 nm. The standard assay medium contained 50 mM Tris-HCl, pH 8.0, 20% (v/v) glycerol, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 0.15 mM NADH, 10 units of malate dehydrogenase, and PEP in a final volume of 1 mL. The reaction was started by addition of PEPC. The assay with enzyme extract was also performed in the absence of PEP to correct for any oxidation of NADH independent of PEPC. One unit of enzyme activity is defined as the amount of enzyme resulting in the consumption of 1 μmol of NADH min^{-1}. Initial velocity studies were performed by varying the concentration of the substrate PEP around its K_m while keeping the other substrate concentrations at saturating levels. The K_m values of the substrates were calculated by nonlinear least-squares regression using Sigma Plot.

To determine L-malate sensitivity of PEPC, the assay was performed at pH 7.2, 20% (v/v) glycerol, and 0.10 mM or 0.55 mM PEP. L-malate was added within a range where the reaction was linear, after enzyme addition, and to

encompass the inhibitor concentration causing 50% inhibition of the initial PEPC activity (I_{50}). I_{50} was estimated according to Brooks (1992).

Gel Electrophoresis

SDS-PAGE was performed in 8% or 10% (w/v) polyacrylamide gels according to Laemmli (1970). Proteins were visualized with Coomassie Blue or electroblotted onto a nitrocellulose membrane for immunoblotting according to Burnette (1981). Bound antibodies were located by linking to alkaline phosphatase-conjugated goat anti-rabbit IgG according to the manufacturer's instructions (Sigma). The antibodies used for detection were the following: 1:200 anti-*Amaranthus viridis* PEPC (Colombo et al., 1998); 1:1,000 antiphosphorylated PEPC (the antibody was prepared in the same way as described by Ueno et al.,2000, except for the use of a synthetic phosphonopeptide as an antigen; T. Furumoto and K. Izui, unpublished data); 1:200 anti-maize (*Zea mays*) 62-kD NADP-ME (Saigo et al., 2004); serum against the α-subunit of NAD-ME (diluted 1:1,000) from *Amaranthus hypochondriacus* (Long et al., 1994); 1:10,000 against spinach (*Spinacia oleracea*) *rbcL* (provided by Dr. A. Viale). The molecular masses of the polypeptides were estimated from a plot of the log of molecular mass of marker standards versus migration distance.

Native IEF was carried out on precast polyacrylamide slab gels (pH range 5–8). Gels were prerun for 30 min at 200 V with 20 mM NaOH in the cathode and 10 mM H_3PO_4 in the anode, and then run for 1.5 h at a constant voltage of 200 V followed by 1.5 h at 400 V and 6°C. After electrophoresis, PEPC was detected by incubating the gel in a solution containing 50 mM Tris-HCl, pH 8.0, 20% (v/v) glycerol, 10 mM $NaHCO_3$, 10 mM $MgCl_2$, and 2 mM PEP for 1 h at 25°C. The gel was washed with distilled water and incubated in the dark at room temperature with Fast Violet B salt (1 mg mL^{-1}) until red bands developed where oxaloacetic acid was produced. Excess dye was removed by rinsing with distilled water (Vidal et al., 1976).

RNA Isolation

Total RNA from leaves of *B. sinuspersici*, *S. aralocaspica*, *S. eltonica*, and *S. linifolia* was isolated from 1 g of tissue using the lithium chloride method, according to the manufacturer's instructions (Ambion). Following extraction of total RNA, a DNase treatment (DNA-free; Ambion) was performed to eliminate contamination with genomic DNA. The integrity of the RNA was verified by agarose electrophoresis. The quantity and purity of RNA were determined spectrophotometrically according to the method described by Sambrook et al. (1989).

RACE and RT-PCR

One microgram of RNA was converted into first-strand cDNA using alfalfa mosaic virus reverse transcriptase following the manufacturer's instructions (Promega). A nested PCR was performed using the forward 296F primer (5′-GGATGGGTGGTGACCGTGATGGCA-3′) and reverse M3RT primer (5′-GGTAGATGAAACCTGGTTTGTGTCC-3′) and the cDNA template generated was employed as a template. For the 3′ terminal, a nested PCR was performed using the forward 35RT primer (5′-ACCCATCTTGCCATTTTGTCTCA-ACC-3′) and the reverse 33RT primer (5′-TAACCGGTGTTCTGCATTCC-3′) and the cDNA template generated before was employed as template. The 5′ end of PEPC was amplified with the 5′/3′-RACE system (Roche) according to the manufacturer's instructions using gene-specific primers RACE53 (5′-GCC-ATCATTCTGGCTAGCAAACT-3′) for the first-strand synthesis and 296R (5′-GCCATCACGGTCACCACCCATCCA-3′) for the PCR amplification of dA-tailed cDNA. The amplified products were purified using the QIA quick extraction kit (Qiagen) and both strands fully sequenced using the ABI Prism 3730 genetic analyzer and the ABI Prism big dye terminator cycle sequencing ready reaction kit (PE Biosystems) at Washington State University sequencing facilities.

DNA and Protein Sequence Comparison and Phylogenetic Analysis

DNA sequence data were analyzed using the DNA Star sequence analysis programs. Database searches were conducted using the National Center for Biotechnology Information network version of BLAST 2.2.13 (Altschul et al., 1997). Multiple alignment of complete amino acid sequences of PEPC from different sources accessible from public databases was performed with the ClustalW multiple alignment program (Thompson et al., 1994), removing gap-only columns. This alignment was used to construct the phylogenetic tree with the neighbor-joining method (protein distance after Kimura) using the Phylip software package (Felsenstein, 1989). Bootstrap analysis was computed with 1,000 replicates and excluding positions with gaps. Trees were orientated with PEPC amino acid sequences from *Escherichia coli* and Arabidopsis (*Arabidopsis thaliana*) bacterial-type PEPC as functional outgroups.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers DQ538352 to DQ538355.

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