A Minimal Serine/Threonine Protein Kinase Circadianly Regulates Phospho*enol*pyruvate Carboxylase Activity in Crassulacean Acid Metabolism-Induced Leaves of the Common Ice Plant[1]

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Plant phospho*enol*pyruvate carboxylase (PEPc) activity and allosteric properties are regulated by PEPc kinase (PPcK) through reversible phosphorylation of a specific serine (Ser) residue near the N terminus. We report the molecular cloning of PPcK from the facultative Crassulacean acid metabolism (CAM) common ice plant (*Mesembryanthemum crystallinum*), using a protein-kinase-targeted differential display reverse transcriptase-polymerase chain reaction approach. *M. crystallinum* PPcK encodes a minimal, Ca^{2+}-independent Ser/threonine protein kinase that is most closely related to calcium-dependent protein kinases, yet lacks both the calmodulin-like and auto-inhibitory domains typical of plant calcium-dependent protein kinase. In the common ice plant PPcK belongs to a small gene family containing two members. McPPcK transcript accumulation is controlled by a circadian oscillator in a light-dependent manner. McPPcK encodes a 31.8-kD polypeptide (279 amino acids), making it among the smallest protein kinases characterized to date. Initial biochemical analysis of the purified, recombinant McPPcK gene product documented that this protein kinase specifically phosphorylates PEPc from CAM and C_4 species at a single, N-terminal Ser (threonine) residue but fails to phosphorylate mutated forms of C_4 PEPc in which this specific site has been changed to tyrosine or aspartate. McPPcK activity was specific for PEPc, Ca^{2+}-insensitive, and displayed an alkaline pH optimum. Furthermore, recombinant McPPcK was shown to reverse the sensitivity of PEPc activity to L-malate inhibition in CAM-leaf extracts prepared during the day, but not at night, documenting that PPcK contributes to the circadian regulation of photosynthetic carbon flux in CAM plants.

The cytosolic enzyme phospho*enol*pyruvate carboxylase (EC 4.1.1.31; PEPc) catalyzes the initial fixation of atmospheric CO_2 into C_4-dicarboxylic acids during C_4 photosynthesis and Crassulacean acid metabolism (CAM) (Chollet et al., 1996; Vidal and Chollet, 1997; Nimmo, 2000). Additional plant PEPc isoforms perform anaplerotic roles in C_3 leaves, non-photosynthetic tissues, as well as specialized functions in stomatal guard cells, legume root nodules, developing seeds, and ripening fruits (Chollet et al., 1996; Vidal and Chollet, 1997; and references therein). Photosynthetic and non-photosynthetic isoforms of plant PEPc undergo reversible phosphorylation of a single, strictly conserved Ser residue near the N terminus, resulting in a striking up-/down-regulation of the enzyme's allosteric properties (Chollet et al., 1996; Vidal and Chollet, 1997; Nimmo, 2000). Phosphorylation renders the enzyme considerably less sensitive to inhibition by negative effectors (e.g. L-malate) but both more active and more sensitive to activation by positive effectors (e.g. Glc-6-P, triose-P; Duff et al., 1995).

PEPc phosphorylation is controlled largely by changes in the activity of PEPc kinase (PPcK), a dedicated, Ca^{2+}-insensitive, monomeric protein kinase in the 30- to 39-kD size range (Li and Chollet, 1993, 1994; Chollet et al., 1996; Zhang and Chollet, 1997a, 1997b). In C_4 leaves PPcK activity is activated in the light by photosynthesis and indirectly by a putative, upstream Ca^{2+}-dependent protein kinase (CDPK; Giglioli-Guivarc'h et al., 1996; Vidal and Chollet, 1997), but this up-regulation apparently does not require a functional Calvin cycle (Smith et al., 1998). In addition, C_4 PPcK activity correlates with de novo synthesis of PPcK translatable mRNA and protein (Jiao et al., 1991a; Hartwell et al., 1996, 1999b). In CAM plants however, PPcK activity is regulated by a circadian oscillator that controls the temporal separation of C_3

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and C_4 carboxylation reactions by Rubisco and PEPc, respectively, thereby avoiding futile cycling of CO_2 (Nimmo, 1998, 2000). In salinity-stressed common ice plant (*Mesembryanthemum crystallinum*), a facultative CAM species, PPcK activity is induced concomitantly with its target protein with an approximately 8-fold increase in night versus day activity (Li and Chollet, 1994). In *Kalanchoë fedtschenkoi*, a constitutive CAM plant, PPcK activity is much more abundant at night than during the day, which correlates with PPcK translatable mRNA abundance and protein synthesis (Carter et al., 1991, 1996; Hartwell et al., 1996, 1999a). In contrast, the down-regulation/dephosphorylation of PEPc is catalyzed by a constitutive mammalian-type protein phosphatase 2A that does not appear to be regulated by any of the conditions that alter PPcK activity (Carter et al., 1990, 1991).

PPcK expression and activity in CAM plants is thought to be regulated, in part, by the cytosolic malate concentration and/or the processes that control malate transport into and out of the vacuole (Borland et al., 1999; Nimmo, 2000). Recently, elevated levels of total-leaf L-malate were shown to reduce increases in both PPcK mRNA and activity in *Kalanchoë daigremontiana* (Borland et al., 1999), suggesting that the circadian expression pattern of CAM PPcK is mediated through malate abundance. However, the exact mechanism(s) by which PPcK activity is regulated is not fully understood. L-Malate inhibits PPcK activity indirectly through its interaction with PEPc (Wang and Chollet, 1993a; Li and Chollet, 1994). In addition, malate may affect the expression of PPcK via a mechanism of feedback repression involving circadian control of malate uptake and release at the tonoplast (Nimmo, 2000).

The extreme low abundance of PPcK protein in leaves and root nodules has thwarted direct biochemical attempts to isolate the gene by obtaining partial amino acid sequence data (Carter et al., 1991; Wang and Chollet, 1993a; Li and Chollet, 1994; Li et al., 1996; Zhang and Chollet, 1997a; Hartwell et al., 1999a). However, a very recent report described the successful isolation of a CAM PPcK gene by screening in vitro transcription/translation products for PPcK activity from successively more enriched pools of a cDNA library from *K. fedtschenkoi* (Hartwell et al., 1999a). Here we report the first molecular cloning of PPcK from the facultative CAM common ice plant using a protein-kinase-targeted differential display reverse transcriptase PCR (DDRT-PCR) approach by virtue of PPcKs night- and salinity stress-specific activity profile in salt-stressed, CAM-induced leaves (Li and Chollet, 1994). We also provide the first initial functional characterization of recombinant PPcK activity using the affinity-purified kinase and present unequivocal evidence that this gene product regulates the allosteric properties of CAM PEPc via reversible phosphorylation, which, in turn, contributes to the circadian pattern of CO_2 fixation in these plants.

RESULTS AND DISCUSSION

Molecular Cloning of McPPcK by Targeted Differential Display

In salinity-stressed, CAM-induced common ice plant leaves increased PEPc protein is accompanied by a concomitant induction of Ca^{2+}-insensitive PPcK activity that is only present at night (Li and Chollet, 1994). We exploited this CAM- and night-specific expression pattern, combined with a DDRT-PCR strategy using a degenerate, sense protein-Ser/Thr kinase primer that targets the well-conserved RDLK-PEN subdomain VIb (Donohue et al., 1995; Hanks and Hunter, 1995; Sessa et al., 1996) and various combinations of one-base-anchored primers to clone a 440-bp cDNA encoding PPcK (see "Materials and Methods"). Semiquantitative RT-PCR was used to confirm the salt and night up-regulated expression pattern of this *McPpck1* display product (Fig. 1A), similar to that of authentic PPcK activity in leaves of CAM-induced common ice plant (Li and Chollet, 1994). In contrast, the expression of a control amplification product generated from *Fnr1*, a gene-encoding ferredoxin-NADP reductase (Michalowski

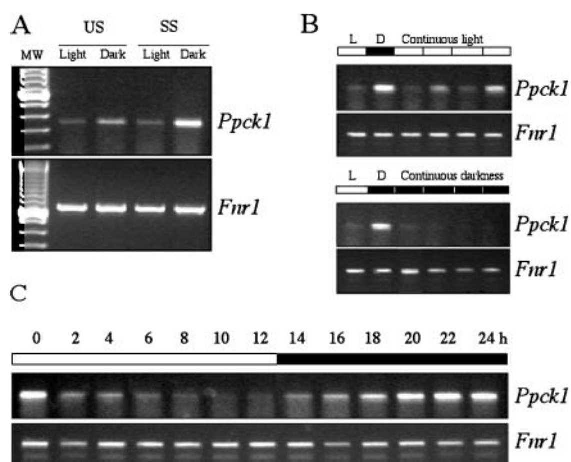

Figure 1. Control of *McPpck1* expression by a circadian rhythm in leaves of salinity-stressed, CAM-induced common ice plant. A, Ethidium bromide-stained gel of RT-PCR products from total RNA, isolated from leaves of unstressed (US) or NaCl-stressed (SS) plants during the middle of the 12-h light or dark period, using gene-specific primers for *McPpck1* (337 bp) and *Fnr1* (600 bp). M_r marker (MW, 100-bp ladder, Life Technologies/Gibco-BRL, Rockville, MD) indicates size of RT-PCR products. B, Circadian control of *McPpck1* expression in salinity-stressed plants exposed to two consecutive 24-h periods of continuous light (white bar/top) or continuous darkness (black bar/bottom) following a normal entrainment pattern of 12-h light (L) and 12-h darkness (D). C, Time course of RT-PCR analyses of *McPpck1* and *Fnr1* expression using leaf tissue samples from salt-stressed plants collected every 2 h during the course of a normal 12-h day (white bar)/12-h night (black bar) cycle. The 0- and 24-h samples represent duplicate samples.

et al., 1989), remained constant. *Fnr1* expression also served as a convenient internal control to document equivalent amounts of input RNA analogous to ethidium bromide staining of RNA gels used for northern-blot analysis (Taybi and Cushman, 1999).

Circadian Control of *McPpck* Expression

Analysis of *McPpck1* mRNA abundance under conditions of constant light in leaves of salinity-stressed plants revealed that *McPpck1* transcripts showed a circadian oscillation in abundance that reached a maximum during the subjective night period (Fig. 1B). These observations are consistent with the persistent circadian rhythm in the reversible phosphorylation of CAM PEPc and its interconversion between two different malate-sensitivity forms in leaves of *K. fedtschenkoi* (Nimmo et al., 1987). In plants held in constant darkness *McPpck*1 transcript abundance declined and remained below the level of detection (Fig. 1B). This decline is consistent with the disappearance of changes in apparent K_i for L-malate of PEPc and in net CO_2 release observed in *K. fedtschenkoi* leaves maintained in continuous darkness and normal air, but not under CO_2-free air (Nimmo et al., 1987). This disappearance of *McPpck1* transcript abundance may be an indirect consequence of the depletion of energy reserves in the darkened leaves, which may curtail the active transport of L-malate from the cytosol to the vacuole, thus causing a rise in the cytosolic malate concentration (Wilkins, 1983). Under CO_2- and O_2-free air conditions, elevations in total-leaf malate levels have recently been shown to reduce both PPcK mRNA and activity accumulation in *K. daigremontiana* (Borland et al., 1999). Thus, the light dependency of the maintenance of a persistent circadian rhythm for CAM PPcK expression appears to be mediated through malate abundance. Metabolites may also modulate light-induced *Ppck* transcript accumulation in C_4 leaves (Hartwell et al., 1999b). Changes in L-malate concentrations in the cytosol and vacuole are, in turn, controlled by permeability or transport at the tonoplast during CAM (Grams et al., 1997). Therefore, circadian control of CAM is likely to be mediated by tonoplast membrane components that regulate the uptake and/or release of L-malate, which in turn may act as a feedback repressor of *Ppck* expression (Nimmo, 2000).

Detailed examination of the diurnal expression pattern of *McPpck1* at 2-h intervals over a 24-h time course revealed that *McPpck1* expression was most abundant near the end of the normal 12-h dark period and least abundant near the end of the light cycle (Fig. 1C). This expression pattern is consistent with the persistent circadian rhythm in the interconversion of PEPc forms that exhibit differing malate sensitivities and phosphorylation states observed in common ice plant and other CAM plants (Winter, 1982; Nimmo et al., 1987). The rapid, diurnal accumulation and disappearance of *McPpck1* transcripts reported here is similar to the induction kinetics of *Ppck* mRNA, translatable *Ppck* mRNA, and PPcK activity observed in the constitutive CAM plant *K. fedtschenkoi* grown under a 8-h day/16-h night photoperiod (Hartwell et al., 1996, 1999a). However, the exact mechanism(s) controlling transcript abundance has not been established unequivocally. The rapid changes in mRNA abundance suggest that PPcK transcripts might be targeted for destabilization. Consistent with this suggestion, the 3′-untranslated region of *McPpck1* contains multiple, canonical downstream and downstream-like elements (GGA, ATAGATT, and GTA sub-domain elements; data not shown) that are likely to contribute to mRNA instability in plants (Gutiérrez et al., 1999).

There are many reports of circadian clock-regulated transcripts with peak expression during the day (Piechulla et al., 1998) or early within the subjective night (Heintzen et al., 1997; Staiger et al., 1999). In contrast, Ppck transcripts exhibit peak expression after approximately 8 h into the night (Fig. 1C; Hartwell et al., 1996, 1999a). Analysis of the *McPpck* promoter will be needed to characterize the 5′-flanking region(s) controlling circadian rhythmicity and the cognate transcription factors mediating the maximal nocturnal accumulation of *McPpck* transcripts. Entrainment studies using *K. fedtschenkoi* (*Bryophyllum)* have shown that phytochrome serves as the major photoreceptor in CAM leaves for the regulation of the circadian rhythm in CO_2 exchange (Harris and Wilkins, 1978a, 1978b). Therefore, it will be of interest to determine if *McPpck* expression is controlled by cis-elements and *CCA1*-related, Myb-like trans-acting factor elements similar to those described for the phytochrome-responsive *Lhc* genes (Wang et al., 1997; Wang and Tobin, 1998). Such information will be crucial in determining how temporal information from a circadian clock is phased for different output rhythms or if multiple clocks operate concurrently within the plant (Millar, 1999).

McPpck cDNA Sequence Analysis

Sequence analysis of the original 440-bp differential display product revealed that it did not encode a full-length cDNA. Thus, both 5′- and 3′-RACE procedures were used to recover the full-length transcript (see "Materials and Methods"). The full-length *McPpck1* cDNA contained a single open reading frame of 840 bp flanked by 5′- and 3′-untranslated sequences of 77 and 669 bp, respectively (accession no. AF158091). The predicted polypeptide of 279 amino acids has a calculated molecular mass of 31.8 kD. This predicted mass is in close agreement with that of a partially purified, approximately 32-kD isoform of PPcK isolated from CAM-induced leaves of common ice plant (Li and Chollet, 1994). Analysis of

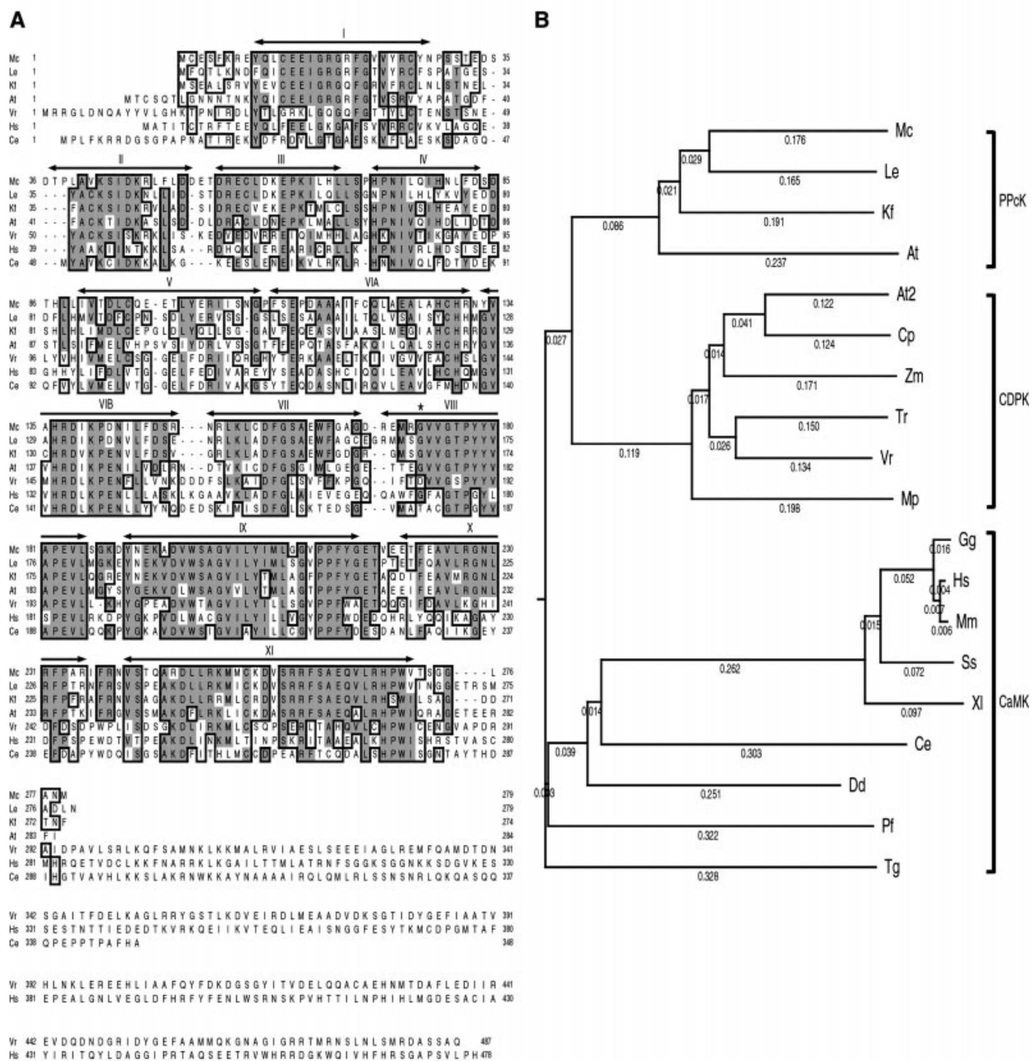

Figure 2. Amino acid alignment and CLUSTALX analysis of CaM/Ca^{2+}-dependent protein kinases related to the McPPcK protein. A, The deduced amino acid sequence of PPcK from CAM-induced common ice plant (Mc, AF158091) was aligned with PPcKs from tomato (Le, AF203481), *K. fedtschenkoi* (Kf, AF162661), and Arabidopsis (At, AF162660), a CDPK from mung bean (Vr, U08140), a CaM-dependent protein kinase II-α from humans (Hs, AF145710), and a CaM-dependent protein kinase from *Caenorhabditis elegans* (Ce, AB021864). Identical amino acid residues are highlighted in dark gray, whereas conserved amino acids are highlighted in light gray. The 12 conserved catalytic subdomains (I–XI) of the eukaryotic protein kinase superfamily (Hanks and Hunter, 1995) are shown in Roman numerals. The asterisk (*) in subdomain VIII indicates the strictly conserved, non-phosphorylatable Gly residue in PPcK near position 170 normally targeted in phosphorylation-activated kinases. B, Pairwise and multiple analysis of deduced amino acid sequences was performed using CLUSTALX (Thompson et al., 1997) to generate a dendrogram to show relationships among closely related protein-Ser/Thr kinases. The PPcK sequence from common ice plant, Mc (AF158091), was aligned with those from tomato, Le (AF203481), *K. fedtschenkoi*, Kf (AF162661), and Arabidopsis, At (AF162660). In addition, CDPK sequences from Arabidopsis, At2 (U31833), squash, Cp (U90262), maize, Zm (AJ007366), *Tortula ruralis,* Tr (U82087), mung bean, Vr (U08140), and *Marchantia polymorpha*, Mp (AB017515); and CaMK sequences from *Gallus gallus*, Gg (AF109069), human, Hs (AF145710), *Mus musculus*, Mm (S04365), *Sus scrofa*, Ss (AAC48715), *Xenopus laevis*, Xl (U18196), *C. elegans*, Ce (AB021864), *D. discoideum*, Dd (P25323), *Plasmodium falciparum*, Pf (CAA68090), and *Toxoplasma gondii,* Tg (AF43629) were aligned. The distance between nodes is indicated in phylogentic units with a value of 0.1 corresponding to a difference of 10% between two sequences.

the deduced amino sequence indicated that the protein is most closely related to PPcKs very recently described from Arabidopsis (accession no. AF162660), tomato (accession no. AF203481), and *K. fedtschenkoi* (accession nos. AF162661 and AF162662; Hartwell et al., 1999a). Alignment of the deduced common ice plant PPcK shows that it shares 57%, 60%, and 67% amino acid sequence identity with the Arabidopsis

(C_3), *K. fedtschenkoi* (CAM), and tomato (C_3) sequences, respectively, over the entire length of the kinase catalytic domain (Fig. 2A).

All four PPcKs contain kinase domains most similar to plant CDPKs and animal calmodulin (CaM)-dependent protein kinases (CaMK group; Hanks and Hunter, 1995; Hardie, 1999) but lack the N-terminal variable, autoinhibitory (pseudosubstrate), and CaM-like domains typically present in plant CDPKs (Fig. 2) as noted previously by Hartwell et al. (1999a). Thus, PPcKs comprise the lowest M_r-class of protein-Ser/Thr kinases characterized to date, containing between 274 and 284 amino acids, and their activity is unlikely to be regulated by CaM or Ca^{2+} as they lack these regulatory domains. Nonetheless, CLUSTALX analysis (Thompson et al., 1997) indicates that these PPcKs form a new group of calcium-independent protein kinases that are more closely related to CDPK than any other protein kinases characterized so far. The PPcK group shares 38% to 43% amino acid identity and 58% to 60% similarity with the plant CDPK group (Fig. 2B; see also Nimmo, 2000). Overall, McPPcK shares slightly lower amino acid identity (36%–38%) and similarity (56%–59%) with the animal CaMK group of protein kinases. PPcKs are unlikely to undergo activation by phosphorylation as they contain a strictly conserved, non-phosphorylatable Gly residue near position 170 (indicated by an asterisk in Fig. 2A) in the "activation loop" normally involved in the up-regulation of many kinases (Johnson et al., 1996; Hartwell et al., 1999a). The PPcK members also do not appear to form functional complexes with regulatory subunits (Li and Chollet, 1993, 1994; Zhang and Chollet, 1997a; Hartwell et al., 1999a), suggesting that the primary means of controlling their activity is based on their expression patterns at the levels of transcription and/or translation.

McPpck1 Belongs to a Small Gene Family

To determine the complexity of the *McPpck1* gene family in common ice plant, genomic Southern-blot analysis was performed using the original 440-bp DDRT-PCR product as a probe. The resulting hybridization pattern obtained under high-stringency conditions indicated that *McPpck1* is encoded by a small gene family of two members in that the products of each restriction enzyme digestion reaction hybridized to two bands (Fig. 3). The more intensely labeled band likely represents the CAM-induced isogene described here, whereas the more weakly hybridizing band may represent a PPcK that targets the product of the C_3 or "housekeeping" PEPc isogene present in the common ice plant genome (Cushman et al., 1989). The existence of two PPcK isogenes is also consistent with the approximately 32- and approximately 39-kD isoforms of PPcK previously described in CAM-induced leaves of common ice plant (Li and Chollet,

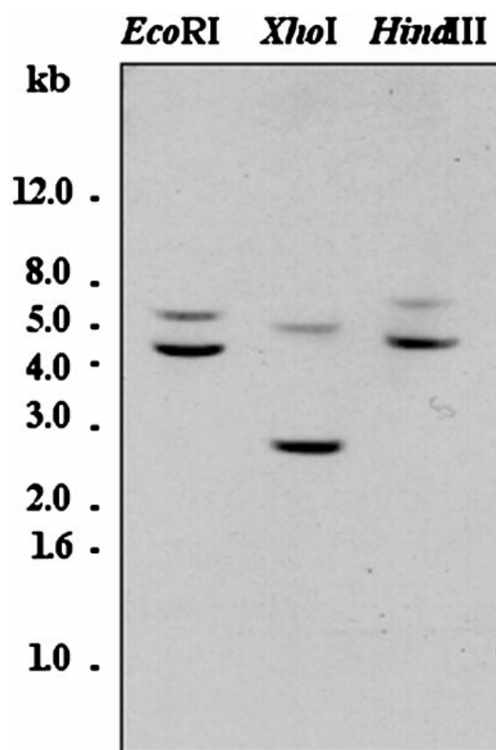

Figure 3. Genomic Southern-blot analysis of *McPpck1*. Total genomic DNA isolated from salt-stressed ice plant leaf tissue was digested with *Eco*RI, *Xho*I, or *Hind*III. The DNA samples (5 μg/lane) were then separated on a 0.9% (w/v) agarose gel, blotted onto a nylon membrane, and hybridized with a 440-bp PCR-generated probe for *McPpck1*. The sizes of the DNA markers (1-kb ladder, Life Technologies/Gibco-BRL) are indicated in kb.

1994). The existence of a second PEPcK isogene in common ice plant has recently been confirmed by PCR amplification (M. Nomura and J. Cushman, unpublished data).

Initial Biochemical Characterization of Recombinant McPPcK1

Although a very recent report has described the isolation and characterization of PPcK cDNA clones from *K. fedtschenkoi* and Arabidopsis, only a limited functional characterization of the resultant protein kinase was conducted using in vitro transcription-translation products rather than an active, purified recombinant protein (Hartwell et al., 1999a). Furthermore, the phosphorylation site(s) in CAM PEPc targeted by this cloned kinase was determined indirectly by one-dimensional phosphopeptide mapping (Carter et al., 1991; Hartwell et al., 1999a). To directly assess the activity and biochemical properties of PPcK and unequivocally define the phosphorylation site(s) in PEPc targeted by this cloned kinase, the *McPpck1* cDNA was overexpressed in *Escherichia coli* and affinity-purified. The molecular mass of the soluble, recombinant protein corresponded to the predicted size of the 6×-His-fusion protein (32.6 kD) as

determined by SDS-PAGE and Coomassie staining (data not shown). Recombinant McPPcK was then used to perform in vitro phosphorylation assays in the presence of purified forms of wild-type and mutant PEPc from common ice plant (CAM), *Sorghum vulgare* (C_4), and dark-leaf maize (C_4; Li and Chollet, 1994; Li et al., 1997). The results documented that the recombinant McPPcK was able to phosphorylate these substrates, including the sorghum S8T mutant enzyme (Fig. 4A). However, other phosphorylation-site mutant forms of recombinant sorghum C_4 PEPc (S8D and S8Y) were unable to serve as substrates for the common ice plant PPcK. Furthermore, Suc synthase purified from soybean root nodules, a tetrameric CDPK-target enzyme that harbors an homologous phosphorylation domain (basic-X-X-Ser-hydrophobic) near the N terminus like PEPc (Zhang and Chollet, 1997b; Zhang et al., 1999), was also unable to serve as a substrate, identical to the results with authentic plant PPcK (Zhang and Chollet, 1997b). These findings strongly suggest that the invariant Ser residue near the N terminus of plant PEPc (e.g. Ser-8, -11, and -15 in sorghum [C_4], common ice plant [CAM], and maize [C_4] PEPc, respectively [Chollet et al., 1996; Vidal and Chollet, 1997]) is the only phosphorylation target for the cloned common ice plant PPcK. Control phosphorylation reactions were performed with a commercial protein kinase preparation from porcine heart (Beavo et al., 1974) to verify substrate integrity. It should be noted that this kinase preparation differs from that used in previous experiments (Sigma bovine protein kinase A [P-2645], St. Louis) which, like PPcK, does not phosphorylate the S8D and S8Y mutants of sorghum C_4 PEPc (Duff et al., 1995; Li et al., 1997). The differences observed between these two commercial protein kinase A preparations may be accounted for by variations in their degree of purity or by species-specific differences. Recombinant PPcK failed to significantly phosphorylate the common kinase substrates casein and histone III-S (Fig. 4B) in agreement with previous observations with authentic PPcK from leaves and root nodules (Wang and Chollet, 1993a; Li and Chollet, 1994; Zhang and Chollet, 1997a). Using purified, dark-leaf (dephospho) *Z. mays* PEPc as substrate, the recombinant kinase also displayed a pH-activity profile with maximal activity at pH 8.0 (Fig. 4C), identical to that of the partially purified common ice plant leaf PPcK (Li and Chollet, 1994). Finally, the activity of the recombinant McPPcK was found to be Ca^{2+} insensitive in that neither 0.3 mM $CaCl_2$ nor 0.3 mM EGTA had any effect on its phosphorylation of purified dark-leaf maize PEPc (Fig. 4D). Detailed determination of the specific activity and other properties of the purified, recombinant McPPcK is currently under way.

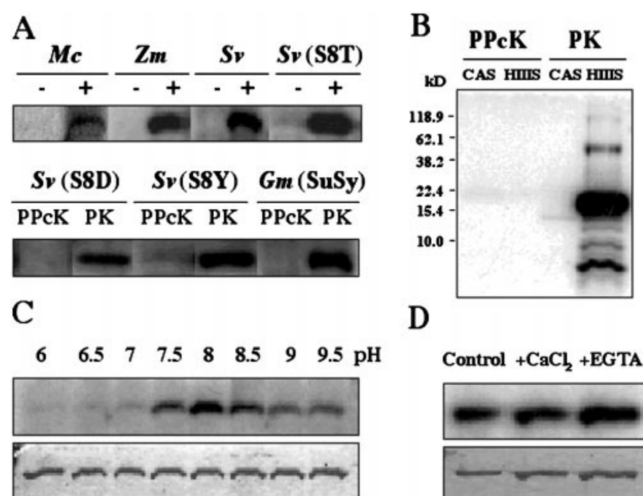

Figure 4. *McPpck1* encodes a functional PPcK. A, In vitro substrate specificity for wild-type and mutant PEP carboxylases by the purified, recombinant McPPcK1. Top, Phosphorylation of recombinant or leaf PEPc from CAM common ice plant (*Mc*), C_4 maize (*Zm* [dark-leaf enzyme]), and C_4 *Sorghum vulgare* wild-type (Ser-8 [*Sv*]), and S8T mutant (*Sv* [S8T]) in the absence (−) or presence (+) of recombinant McPPcK1. Bottom, Negative control substrates treated with McPPcK1 (PPcK) included two different non-phosphorylatable recombinant forms of mutated *S. vulgare* C_4 PEPc, *Sv* (S8D) and *Sv* (S8Y), and Suc synthase purified from soybean root nodules, *Gm* (SuSy). To verify the integrity of these control substrates, each was also treated with porcine protein kinase (PK) under reaction conditions identical to those used for McPPcK1. B, Comparison of the in vitro activity of purified, recombinant common ice plant PPcK1 (PPcK) and porcine protein kinase (PK) with histone type-IIIS (HIIIS) and casein (CAS). C, pH dependence of in vitro activity of purified, recombinant McPPcK1. Top, Autoradiogram; bottom, corresponding Coomassie-stained gel. D, Insensitivity of McPPcK to free [Ca^{2+}] using purified, dark-leaf maize PEPc as substrate in the presence of the standard phosphorylation buffer (control), or with the addition of 0.3 mM $CaCl_2$ or 0.3 mM EGTA. Top, Autoradiogram; bottom, corresponding Coomassie-stained gel.

McPPcK Reverses L-Malate Inhibition of Day-Form (Dephospho) CAM PEPc

To document the physiological relevance of McPPcK, the effect of phosphorylation by this recombinant Ser/Thr kinase on the allosteric inhibition of CAM PEPc activity by L-malate was measured in soluble extracts of salt-stressed common ice plant leaves collected during the day or night. Extracts prepared at the middle of the normal 12-h day period contain dephosphorylated PEPc (Weigend, 1994) and thus exhibit a high degree of inhibition (approximately 80%) of endogenous PEPc activity by L-malate, whereas extracts prepared at night show a much lower degree of inhibition (approximately 43%) due to the greater phosphorylation state of the target enzyme in these extracts (Fig. 5). Notably, phosphorylation of day-prepared extracts with purified, recombinant common ice plant PPcK resulted in a significant reduction in the L-malate inhibition of endogenous PEPc activity (Fig. 5). In contrast, extracts prepared during the middle of the ensuing night period, which are enriched in phosphorylated PEPc (Weigend, 1994), showed no significant change in sensitivity to L-malate. These collective results pro-

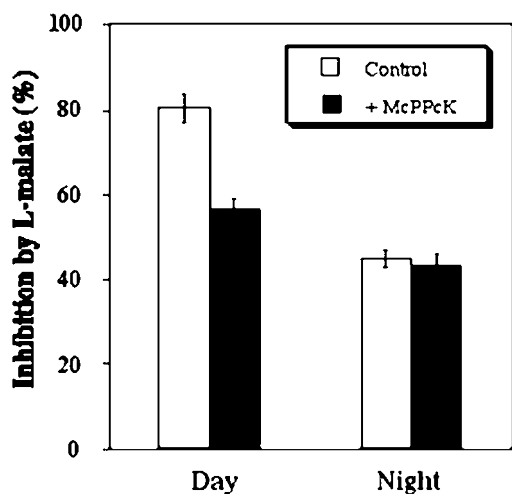

Figure 5. In vitro phosphorylation by McPPcK1 reverses L-malate inhibition of day-form (dephospho) McPEPc. Soluble extracts were prepared from salt-stressed common ice plant leaf material collected at the middle of the normal 12-h day and night periods in phosphorylation buffer. Control phosphorylation reactions (white bars) contained 5 mM $MgCl_2$ and 50 μM ATP, whereas +McPPcK reactions (black bars) contained 5 mM $MgCl_2$/50 μM ATP plus purified, recombinant common ice plant PPcK1 (200 ng). The sensitivity of endogenous PEPc activity to 1 mM L-malate was subsequently determined at pH 7.3 and 1.2 mM PEP as described (Giglioli-Guivarc'h et al., 1996), and plotted relative to control PEPc assays performed in the absence of L-malate.

vide unequivocal evidence that the common ice plant cDNA clone described herein encodes a minimal Ser/Thr protein kinase that specifically phosphorylates PEPc and thereby alters its allosteric properties, which, in turn, contributes to the regulation of nocturnal CO_2 fixation, malate accumulation, and metabolic flux through the CAM pathway under the control of a circadian rhythm. Furthermore, the biochemical properties of the recombinant kinase are in complete agreement with those described previously for authentic leaf (CAM, C_4, and C_3) and root nodule PPcK (Carter et al., 1991; Li and Chollet, 1993, 1994; Wang and Chollet, 1993a, 1993b; Li et al., 1996, 1997; Zhang and Chollet, 1997a, 1997b).

CONCLUDING REMARKS

We describe the molecular cloning and initial biochemical analysis of recombinant PPcK, the enzyme responsible for the seryl-phosphorylation of PEPc, one of the most intensively studied examples of regulatory protein phosphorylation in plants (Chollet et al., 1996; Vidal and Chollet, 1997; Nimmo, 1998, 2000). PEPc phosphorylation is a fundamental regulatory event in plant metabolism in that it influences both photosynthetic CO_2 fixation by C_4 and CAM plants and diverse non-photosynthetic processes. The targeted DDRT-PCR approach described here to clone *McPpck* has been used successfully to isolate other protein kinases having similarly distinct expression profiles (Donohue et al., 1995; Sessa et al., 1996) and thus appears to be generally applicable to cloning any differentially expressed protein kinase. Currently, no evidence exists to suggest that the monomeric PPcK protein (Li and Chollet, 1993, 1994; Zhang and Chollet, 1997a) or its activity (Chollet et al., 1996; Vidal and Chollet, 1997; Hartwell et al., 1999a) is regulated directly by any mechanism other than by its expression level. This characteristic makes PPcK unique among members of the protein kinase superfamily, which are typically regulated by second messengers (e.g. Ca^{2+}, cAMP), reversible phosphorylation or some other means of covalent modification, and/or non-covalent mechanisms such as interaction with ligands, regulatory subunits, or large multimeric complexes (Johnson et al., 1996; Hardie, 1999). Thus, McPPcK1 and its homologs (Fig. 2; Hartwell et al., 1999a) define a new group of minimal calcium-independent protein kinases that are most closely related to plant CDPKs yet are distinguished from this group by their lack of both autoregulatory (pseudosubstrate) and CaM-like domains and their regulation by developmental and environmental stimuli and/or a circadian clock (Hartwell et al., 1999a). Modeling studies based on the predicted amino acid sequence of PPcK and the three-dimensional structure of PEPc from *E. coli* (Kai et al., 1999; Matsumura et al., 1999) should lead to a better understanding of the secondary-tertiary interactions involved in substrate recognition by PPcK (Li et al., 1997) and the relationships between reversible phosphorylation of plant PEPc and its allosteric control by opposing metabolite effectors.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Plants of the common ice plant (*Mesembryanthemum crystallinum*) were grown with a 12-h photoperiod (450–500 μmol photons m^{-2} s^{-1}) at 26°C as previously described (Taybi and Cushman, 1999). All experiments were conducted with 5-week-old plants. Potting medium-grown plants were salinity stressed by irrigation once daily with 0.5 M NaCl in 0.5× Hoagland solution 2. Alternative photoperiod conditions are indicated in the figure legends. Leaf samples were collected at the times indicated in the legends, immediately frozen in liquid nitrogen, ground to a fine powder, and stored at −80°C until use.

mRNA Differential Display RT-PCR

Total RNA, isolated as previously described (Taybi and Cushman, 1999) from unstressed or salinity-stressed leaves 6 h into either the 12-h light or dark period, was treated with DNAase I using the MessageClean kit from GenHunter Corporation. Targeted RNA DDRT-PCR was conducted using the RNAimage Kit 1 according to the manufacturer's instructions (GenHunter Corporation, Nashville, TN) (Liang et al., 1993) with various combinations of

1-base-anchored primers (H-T_{11}M), where M is C, A, or G (e.g. 5′-AAGCTTTTTTTTTTTC-3′), except that a degenerate primer (5′-GNGAYYTNARCCNGARAA-3′), specific to subdomain VIb (RDLKPEN) of the protein-Ser/Thr kinase catalytic domain (Hanks and Hunter, 1995), was used in place of arbitrary primers. [α-^{33}P]dATP-labeled reaction products were resolved on 6% (w/v) denaturing polyacrylamide sequencing gels and visualized by autoradiography using x-ray film (Biomax MS, Eastman-Kodak, Rochester, NY). Following autoradiography at room temperature, selected bands were marked, recovered from the dried gel, and re-amplified according to the manufacturer's instructions using the same primer pairs. Re-amplified products were cloned into a TA cloning vector (pCR2.1-TOPO, Invitrogen, Carlsbad, CA) and sequenced on a 373A automated DNA sequencing system (Perkin-Elmer Applied Biosystems, Foster City, CA) using the Prism Ready Reaction Dyedeoxy Terminator Cycle Sequencing kit (Perkin-Elmer Applied Biosystems).

RNA Isolation, RACE, and Semiquantititative RT-PCR Assays

Total RNA was isolated as previously described (Taybi and Cushman, 1999). The 5′ end of *McPpck1* cDNA was amplified with the 5′-RACE system (Life Technologies/Gibco-BRL) according to the manufacturer's instructions using gene-specific primers, GSP1 (5′-ACTGGTTACCCAAGGATG-3′) and GSP2 (5′-GTGGTCGTGAAGGTGTGTA-3′). The 3′ end of *McPpck1* cDNA was amplified with the 3′-RACE system (Life Technologies/Gibco-BRL) according to the manufacturer's instructions using two different gene-specific primers, GSPa (5′-TCGGAAGCAGGAGACAG-3′) and GSPb (5′-AACCAGTGGAGGATTGGC-3′). The identity of each of these products was confirmed by DNA sequence analysis.

Semiquantitative RT-PCR assays were conducted as previously described (Taybi and Cushman, 1999) using 50 and 100 ng of DNase I-treated RNA for *McPpck1* and *Fnr1* products, respectively. A 337-bp amplicon was obtained using *McPpck1*-specific primers: 5′-TCGGAAGCAGGAGACAG-3′ (forward, melting temperature [Tm] = 56°C) and 5′-ACTGGTTACCCAAGGATG-3′ (reverse, Tm = 54°C). A 600-bp amplicon was obtained using *Fnr1*-specific primers: 5′-ATTGCCAGCAGGCCCTTG-3′ (forward, Tm = 54°C) and 5′-GAACCAGTCAATACCATCT-3′ (reverse, Tm = 54°C). After amplification, the reaction products were resolved by electrophoresis on a 1.2% (w/v) agarose gel and stained with ethidium bromide. Images were captured using a Gel-Doc 1000 DNA Gel Analysis and Documentation System (Bio-Rad Laboratories, Hercules, CA). All semiquantitative RT-PCR experiments were repeated twice and representative data are shown.

Genomic Southern-Blot Analysis

Genomic Southern gel-blot analysis was performed as described (Sambrook et al., 1989) using nylon membranes (Hybond N+, Amersham Pharmacia Biotech, Piscataway, NJ) and hybridized with the original 440-bp fragment of *McPpck1* obtained by DDRT-PCR. Probe labeling, hybridization, and detection reactions were conducted using the AlkPhos direct system (Amersham Pharmacia Biotech) and the chemiluminescent substrate reagent (CDP-Star, Amersham Pharmacia Biotech) according to the manufacturer's instructions.

DNA and Protein Sequence Analyses

DNA sequence data were analyzed using the MacVector/AssemblyLIGN sequence analysis programs (Oxford Molecular Group, Oxford). Database searches were conducted using the National Center for Biotechnology Information network version of BLAST 2.0 (Altschul et al., 1997). Multiple sequence alignments were conducted with the CLUSTALX multiple alignment program (Thompson et al., 1997).

Overexpression and Affinity Purification of Recombinant McPPcK

The entire open reading frame of the *McPpck1* cDNA was amplified using *Pfu* polymerase (Promega, Madison, WI) and a forward (5′-ATATCTAGAAGAAGGAGATATACATATGTGTTGAGAGCTTCAAGAG-3′) and reverse (5′-ATACTCGAGCATGTTGGCCAATCCTC-3′) primer pair and subsequently cloned into the *Xba*I and *Xho*I sites of the *E. coli* expression vector pET30b(+) (Novagen, Madison, WI). Sequence analysis of both strands of this product confirmed its identity to the original DDRT-PCR product and to the 5′- and 3′-RACE products. The McPPcK-6×-His·Tag fusion protein (tagged at the C terminus) was overexpressed in *E. coli* BL21 (DE3) cells grown in Luria-Bertani medium plus 50 μg/mL kanamycin at 28°C for 6 to 12 h. After 1 to 2 h of induction by 1 mM isopropylthio-β-galactoside at 28°C, cells were harvested by centrifugation and lysed at 4°C by sonication in binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, pH 8.0) containing one Complete protease inhibitor cocktail tablet (Roche Molecular Biochemicals, Indianapolis) per 10 mL of buffer. The extracts were clarified by centrifugation at 15,000*g* for 15 min at 4°C. Recombinant McPPcK was purified at 4°C by His·Bind affinity chromatography on iminodiacetic acid-agarose according to the manufacturer's instructions (Novagen), and desalted by gel-filtration chromatography using Sephadex G-50 (Pharmacia) equilibrated in phosphorylation buffer (0.1 M Tris [tris(hydroxymethyl) aminomethane]-HEPES [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid] buffer, pH 8.0, 1 mM dithiothreitol, and 5 mM $MgCl_2$). The concentration of the purified protein was determined by the Bradford method (Bradford, 1976), adjusted to 0.2 mg/mL, and stored at −20°C in 33% (v/v) glycerol until use.

In Vitro Phosphorylation and PEPc Malate-Inhibition Assays

Phosphorylation assays were conducted using affinity-purified, recombinant McPPcK. The CAM isoform of PEPc

from common ice plant was produced in a coupled in vitro transcription/translation system (TnT, Promega) and immunoprecipitated using a common ice plant anti-PEPc polyclonal antibody (kind gift of Bryan Arundel, University of Arizona, Tucson) prior to conducting phosphorylation assays. The antigen-antibody complexes were captured on protein A-agarose beads (Pharmacia), washed four times with phosphorylation buffer, and used directly in the kinase assays. Wild-type and mutant forms of recombinant sorghum C_4 PEPc were expressed in *E. coli* and purified as described (Li et al., 1997). Dephospho maize PEPc was extracted from dark-adapted (10–12 h) leaves and purified by FPLC as described (Jiao et al., 1991b). Suc synthase was purified from soybean root nodules as described (Zhang et al., 1999). Control phosphorylation reactions, designed to verify the integrity of the various protein substrates, were performed using 2.5 units/reaction of a protein kinase preparation (no. P-8289, Sigma) from porcine heart (Beavo et al., 1974). Histone type III-S (H-5505) and casein (C-7164) were purchased from Sigma. Phosphorylation assays were conducted in a 30-μL reaction volume containing phosphorylation buffer (pH 8.0) supplemented with 1 mM NaF, 0.25 μM okadaic acid, 25 μM ATP, 10 μCi [γ-^{32}P]ATP, and 2 μg of protein substrate. Reactions were initiated by adding 0.2 μg of McPPcK, incubated at 30°C for 10 min, and then stopped by the addition of 10 μL of hot 4× SDS gel-loading buffer (1× buffer is 50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% [v/v] SDS, 0.1% [v/v] bromphenol blue, and 10% [v/v] glycerol). The denatured samples were boiled for 3 min and subjected to electrophoresis in 12% (v/v) SDS-polyacrylamide gels (Laemmli, 1970). The gels were stained with Coomassie Brilliant Blue R-250 to confirm equal loading of protein in each lane and then dried and subjected to autoradiography. The pH dependence of McPPcK activity was determined using a range of pH values from 6.0 to 9.5 and dark-leaf maize PEPc as substrate as previously described (Li and Chollet, 1994). To determine Ca^{2+} sensitivity of McPPcK activity, 0.3 mM $CaCl_2$ or EGTA was added to phosphorylation reactions conducted at pH 8.0 with maize PEPc.

For PEPc activity assays, soluble extracts were prepared from common ice plant leaves, harvested in the middle of the 12-h day and night periods, in phosphorylation buffer containing one Complete protease inhibitor cocktail tablet (Roche Molecular Biochemicals) per 10 mL of buffer, 50 μM ATP, 1 mM phosphocreatine, and 1 unit of creatine phosphokinase. Aliquots were preincubated in the presence or absence of 0.2 μg of affinity-purified McPPcK at 30°C for 15 min. Sensitivity of endogenous PEPc activity to inhibition by 1 mM L-malate was subsequently determined at pH 7.3 and 1.2 mM PEP as described (Giglioli-Guivarc'h et al., 1996). All in vitro phosphorylation assays were performed twice with similar results.

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