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Phospho*enol*pyruvate Carboxylase Kinase in Tobacco Leaves Is Activated by Light in a Similar but Not Identical Way as in Maize[1]

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We have previously reported the partial purification of a Ca^{2+}-independent phospho*enol*pyruvate carboxylase (PEPC) protein-serine/threonine kinase (PEPC-PK) from illuminated leaves of N-sufficient tobacco (*Nicotiana tabacum* L.) plants (Y.-H. Wang, R. Chollet [1993] FEBS Lett 328: 215–218). We now report that this C_3 PEPC-kinase is reversibly light activated in vivo in a time-dependent manner. As the kinase becomes light activated, the activity and L-malate sensitivity of its target protein increases and decreases, respectively. The light activation of tobacco PEPC-PK is prevented by pretreatment of detached leaves with various photosynthesis and cytosolic protein-synthesis inhibitors. Similarly, specific inhibitors of glutamine synthetase block the light activation of tobacco leaf PEPC-kinase under both photorespiratory and nonphotorespiratory conditions. This striking effect is partially and specifically reversed by exogenous glutamine, whereas it has no apparent effect on the light activation of the maize (*Zea mays* L.) leaf kinase. Using an in situ "activity-gel" phosphorylation assay, we have identified two major Ca^{2+}-independent PEPC-kinase catalytic polypeptides in illuminated tobacco leaves that have the same molecular masses (approximately 30 and 37 kD) as found in illuminated maize leaves. Collectively, these results indicate that the phosphorylation of PEPC in N-sufficient leaves of tobacco (C_3) and maize (C_4) is regulated through similar but not identical light-signal transduction pathways.

PEPC is a ubiquitous cytosolic enzyme in plants, catalyzing the irreversible carboxylation of PEP to oxaloacetate and Pi. The enzyme plays a key photosynthetic role in primary CO_2 fixation by C_4 and CAM leaves (Ting, 1985; Andreo et al., 1987; Hatch, 1987). In addition, PEPC has a variety of anaplerotic, nonphotosynthetic functions in C_3 plants, including the replenishment of oxaloacetate for the citric acid cycle and amino acid biosynthesis, and the provision of C_4 dicarboxylic acids in support of N_2 fixation by legume root nodules (Latzko and Kelly, 1983; Vance et al., 1994).

It is well established that PEPC in C_4 and CAM leaves is regulated by reversible protein phosphorylation and that this process in turn is controlled by a highly regulated protein-Ser/Thr kinase (Jiao and Chollet, 1991; Nimmo, 1993; Huber et al., 1994; Lepiniec et al., 1994). Two seemingly different scenarios exist for modulating the activity of PEPC-PK in C_4 and CAM leaves. In the former, light activates and darkness inactivates the kinase in vivo (Echevarria et al., 1990; Jiao and Chollet, 1991; Nimmo, 1993). Moreover, treatment of C_4 leaves with inhibitors of photosynthesis or cytosolic protein synthesis prevents this light activation of PEPC-PK (Jiao et al., 1991a; Bakrim et al., 1992, 1993; Jiao and Chollet, 1992; Li and Chollet, 1993), implicating the involvement of photosynthesis and protein turnover in the light-signal transduction pathway. Cytosolic levels of Ca^{2+}, pH, and certain photosynthetic metabolites also have been suggested as signaling elements in the light activation of C_4 PEPC-PK in situ (Pierre et al., 1992; Duff et al., 1996; Giglioli-Guivarc'h et al., 1996). In CAM leaves, however, PEPC-kinase activity is up-/down-regulated through an endogenous circadian rhythm rather than by a light signal (Carter et al., 1991; Nimmo, 1993; Li and Chollet, 1994). As a result, the CAM leaf kinase exhibits a higher activity during the night than during the day. In the leaves of the common ice plant, a facultative CAM species, PEPC-PK is induced concomitantly with its target protein when the plant switches its photosynthetic metabolism from C_3 to CAM during salt stress (Li and Chollet, 1994). The mechanisms underlying the seemingly distinct regulation of C_4 and CAM leaf PEPC-PKs by environmental signals are presently unclear.

There is now increasing in vivo evidence that indicates that C_3 PEPCs are similar to their C_4 and CAM counterparts with respect to regulatory phosphorylation. Van Quy et al. (1991) and, more recently, Duff and Chollet (1995) found that illumination and nitrate feeding of excised, N-deficient wheat leaves increased the activity and I_{50}(L-malate) of PEPC, which was accompanied by an increased in vivo incorporation of ^{32}P from radioactive Pi into the enzyme. The regulation of PEPC by reversible phosphorylation in *Vicia* guard cells (Zhang et al., 1994) and soybean

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Abbreviations: BDA, blue dextran-agarose; CHX, cycloheximide; I_{50}, 50% inhibition constant; MSX, Met sulfoximine; PEPC, PEP carboxylase; PEPC-PK, PEPC protein-Ser/Thr kinase; PPT, phosphinothricin.

root nodules (Zhang et al., 1995) also has been reported. However, information concerning how the phosphorylation of the C_3 isoforms is regulated in response to environmental signals is limited. For example, although previous studies (Van Quy et al., 1991; Duff and Chollet, 1995) showed that PEPC in N-deficient wheat leaves is subject to regulatory phosphorylation, it is unclear whether the enzyme in leaves of N-sufficient C_3 plants is also influenced by this same mode of regulation (Rajagopalan et al., 1993; Gupta et al., 1994; Duff and Chollet, 1995). Similarly, although there has been speculation that the phosphorylation state of PEPC in C_3 leaves is controlled largely by PEPC-PK (Champigny, 1995), as it is in C_4 and CAM plants, its experimental basis is weak. Except in one recent investigation in which PEPC-kinase activity was surveyed in N-deficient wheat leaves (Duff and Chollet, 1995), neither protein-kinase nor protein-phosphatase activity has been studied directly in C_3 plants; instead, changes in the activity and/or L-malate sensitivity of PEPC have been used as an indicator for apparent changes in PEPC-PK activity (Van Quy et al., 1991; Manh et al., 1993). Obviously, direct and more detailed studies of C_3 PEPC-kinase are needed to document that this protein-Ser/Thr kinase, indeed, controls the phosphorylation state of PEPC in C_3 plants and to begin to understand the signal-transduction pathway leading to the reversible phosphorylation of this target protein in N-sufficient C_3 leaves.

In a previous study, Wang and Chollet (1993a) partially purified a Ca^{2+}-independent PEPC-PK from illuminated leaves of tobacco (*Nicotiana tabacum* L.) grown under standard greenhouse conditions. We now report that the activity of this kinase in leaves of these N-sufficient tobacco plants is reversibly regulated in vivo by light/dark signals. Light activates the kinase and, consequently, causes an increase in the activity and decrease in the L-malate sensitivity of PEPC. Similar to C_4 leaf PEPC-PK, light activation of the tobacco kinase is sensitive to both photosynthetic and cytosolic protein-synthesis inhibitors. However, the tobacco leaf kinase differs from the maize (*Zea mays* L.) enzyme in that light activation of the former is blocked by two different Gln synthetase inhibitors, whereas the latter is not affected.

MATERIALS AND METHODS

Chemicals

[γ-^{32}P]ATP (specific radioactivity, 3 Ci/mmol) was purchased from Amersham, microcystin-LR came from Calbiochem, and prestained and calibrated SDS-PAGE protein standards were obtained from Bio-Rad. Recrystallized DCMU was a gift from DuPont. All other biochemical reagents were purchased from Sigma.

Plant Material

Tobacco (*Nicotiana tabacum* L.) and maize (*Zea mays* L.) plants were grown in a local greenhouse with supplemental lighting under standard cultural practices. Plants were treated daily with water and two to three times each week with nutrient solution (Peters Professional; NPK, 20/20/20). Four- to 6-week-old plants were used for the experiments. Before use, the plants were transferred from the greenhouse to a darkened chamber, in which they were maintained for 12 h at 25°C. Mature, fully expanded leaves were then excised at the leaf base and recut under water. For most experiments each detached leaf blade was immersed individually in a 250-mL beaker containing 150 mL of a one-third-strength modified Hoagland nutrient solution with or without various inhibitors (for details, also see Jiao and Chollet, 1992). The leaves were then either illuminated at a PPFD of 800 to 1000 μmol m^{-2} s^{-1} or placed in darkness at 25°C for the indicated period in air. At the end of the various treatments the leaves were either processed immediately for PEPC or PEPC-PK activity assays or quickly frozen in liquid N_2 and stored at −80°C until they were used for PEPC-PK studies.

For experiments in which treatment with MSX in air was compared to that in low O_2, individual detached tobacco leaves were placed inside a 1.25-L, clear Plexiglas infrared gas analyzer chamber with the cut petiole immersed in degassed, one-third-strength nutrient solution, ±2 mM MSX. The sealed chamber was flushed continuously with a humidified stream of compressed air (21% O_2) or 2% O_2 (containing 350 μL L^{-1} CO_2) for 3 h in the light (chamber PPFD of 500–800 μmol m^{-2} s^{-1}) or dark at 25°C before the pretreated tissue was quickly frozen in liquid N_2 for subsequent PEPC-kinase determinations.

Enzyme Preparation

All procedures were performed at 0 to 4°C. To extract tobacco PEPC, two leaf discs (2 cm^2 $disc^{-1}$) were taken from each of three illuminated or darkened excised leaf blades (avoiding large veins) and immediately ground in a chilled mortar and pestle with 0.8 mL of buffer A (100 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM fresh PMSF, 10 μg/mL chymostatin, 10 μg/mL leupeptin, 1 μM microcystin-LR, 5 mM NaF, 10 mM L-malate [pH adjusted], 5% [v/v] glycerol, 14 mM 2-mercaptoethanol) plus 2% (w/v) insoluble PVP. About 0.5 mL of the homogenate was clarified by centrifugation at 16,000*g* for 5 min. The supernatant fluid was then rapidly desalted on a Sephadex G-25 column (1 × 10 cm) equilibrated with buffer B (50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM fresh PMSF, 10 μg/mL chymostatin, 10 μg/mL leupeptin, 1 μM microcystin-LR, 5 mM NaF, 5% [v/v] glycerol, 1 mM DTT). The desalted extract was used immediately to determine the activity and sensitivity of PEPC to L-malate under suboptimal assay conditions as described below.

For the preparation of PEPC-PK, the pretreated tobacco or maize leaves (5 g fresh weight) were ground in buffer A lacking microcystin-LR, NaF, and L-malate (1:5, w/v). After the sample was centrifuged at 20,000*g* for 15 min, protein in the supernatant fraction was precipitated by adding ultrapure ammonium sulfate to 60% saturation (4°C) and stirring for 15 min. The precipitate was collected by centrifugation for 20 min at 20,000*g*, resuspended in 2 mL of buffer C (50 mM Tris-HCl, pH 7.5, 5% [v/v] glycerol, 1 mM DTT), and desalted on a Sephadex G-25 column (1.5 × 20 cm) equilibrated with this same buffer. These ammonium

sulfate-concentrated and desalted samples were used directly for the in vitro estimation of maize leaf PEPC-kinase activity. Because of the much lower activity of PEPC-PK in tobacco leaf samples, a further enrichment of the kinase was performed by chromatography on a column (2.5 × 4 cm) of BDA, which was equilibrated with buffer C (Wang and Chollet, 1993a, 1993b). After the column was washed thoroughly with this buffer to remove unbound protein, the PEPC-kinase-containing fractions were step eluted with 0.5 M NaCl in buffer C following the methods described by Wang and Chollet (1993a). Except where noted otherwise, the tobacco PEPC-PK preparations were concentrated and desalted into buffer C with a Centricon-10 microconcentrator prior to assay.

Dephospho maize PEPC was purified by fast protein liquid chromatography from dark-adapted (12 h) leaves according to a standard protocol (Jiao et al., 1991b).

Enzyme Assays

Both the activity and sensitivity of tobacco PEPC to L-malate inhibition were estimated at suboptimal levels of PEP and pH (Wang and Chollet, 1993a; Duff et al., 1995). PEPC activity was measured spectrophotometrically by following the rate of PEP-dependent NADH oxidation at 340 nm and 30°C by coupling to exogenous malate dehydrogenase. The 1-mL reaction mixture contained 50 mM Hepes-NaOH, pH 7.3, 5 mM $MgCl_2$, 1 mM $NaHCO_3$, 0.2 mM NADH, 4 IU porcine-heart mitochondrial malate dehydrogenase, and 50 μL of desalted leaf extract, with or without pH-adjusted PEP (0.2 mM) and L-malate (0.05 mM). The PEPC-containing sample was injected into the assay cuvette to initiate the reaction. Tobacco leaf PEPC activity was not completely stable under these assay conditions in that the decrease in A_{340} was linear only within the first 1.5 min after initiation of the reaction. Therefore, only this linear phase was used for the calculation of PEPC activity.

The in vitro activity of PEPC-PK was measured by ^{32}P incorporation from [γ-^{32}P]ATP into purified dephospho maize leaf PEPC. The maize leaf kinase activity in the ammonium sulfate-concentrated and desalted extracts was assayed in a 40-μL reaction mixture containing 50 mM Hepes-NaOH, pH 8.0, 5 mM $MgCl_2$, 10 μg of purified maize leaf PEPC, 0.4 mM EGTA, 0.25 mM P^1P^5-di(adenosine-5′)-pentaphosphate, a phosphocreatine (0.25 mM)-creatine phosphokinase (0.5 IU) ADP-scavenging system, 10 nM microcystin-LR (a type 1/2A protein-phosphatase inhibitor), 3 μL [γ-^{32}P]ATP (about 6 μCi), and approximately 25 μg of kinase sample. The same 40-μL reaction mixture was used to determine the activity of approximately 4-μg samples of the tobacco leaf PEPC-PK partially purified by BDA chromatography, except that the adenylate-kinase inhibitor P^1P^5-di(adenosine-5′)-pentaphosphate and the ADP-scavenging system were omitted. After a 30-min incubation at 30°C, the phosphorylation reaction was terminated by adding concentrated SDS sample buffer, followed by immediate boiling for 2 min. The approximately 110-kD PEPC subunit was separated from other polypeptides by 10% SDS-PAGE (Laemmli, 1970). The gel was stained with Coomassie brilliant blue R-250, thoroughly destained, and dried. The stained PEPC subunit band was excised from the gel and ^{32}P was determined by Cerenkov counting. Alternatively, the destained and dried gel was analyzed by either phosphorimaging (Li and Chollet, 1994) or autoradiography with Kodak X-Omat AR film and an intensifying screen at −70°C (4–10 h for maize kinase; 2–4 d for tobacco PEPC-PK).

The procedures for the in situ "in-gel" assay of PEPC-PK activity following SDS-PAGE and subsequent renaturation were essentially the same as described previously by Wang and Chollet (1993b), except that the 12% SDS-PAGE minigels were polymerized in either the presence or absence of 0.5 mg/mL maize dephospho-PEPC. The tobacco and maize kinase preparations were both partially purified by chromatography on BDA for this analysis (see above protocol for tobacco PEPC-PK) and assayed in situ in the presence of 0.4 mM EGTA.

Protein Assay

Soluble protein concentration was measured by the method of Bradford (1976) using the Bio-Rad dye reagent and BSA as standard.

RESULTS

Light Activates Both PEPC-Kinase and PEPC Activities in N-Sufficient Tobacco Leaves

Our preliminary studies indicated that tobacco leaf PEPC-PK activity is too low to measure accurately in desalted crude extracts or ammonium sulfate-concentrated and desalted samples (data not shown). However, based on these estimates, the level of PEPC-kinase activity is at least 10 times lower in illuminated (PPFD of 800-1000 μmol photons m^{-2} s^{-1}) tobacco leaves than in light-adapted maize leaves. To overcome this experimental limitation, chromatography on BDA was used to enrich for the tobacco leaf kinase. Previous experiments by Wang and Chollet (1993a) demonstrated that the tobacco enzyme, like C_4 (Bakrim et al., 1992; Wang and Chollet, 1993b) and CAM (Carter et al., 1991; Li and Chollet, 1994) PEPC-kinases, binds tightly to this "affinity" matrix and is eluted with buffered 0.5 M NaCl. Figure 1 depicts typical chromatographic elution profiles of tobacco PEPC-PK activity from a small column of BDA following extraction from illuminated or darkened leaves, ammonium sulfate concentration, and desalting. Clearly, darkened tobacco leaves have a very low activity of PEPC-kinase that is increased markedly after 3 h of illumination of the tissue. This increased protein-kinase activity in the light is Ca^{2+} independent because 0.4 mM EGTA is included in the routine PEPC-PK assay medium. In contrast, no significant difference in the amount of total soluble leaf protein or in the A_{280} of the peak PEPC-kinase fractions (Fig. 1) was detected between the illuminated and darkened samples.

Light activation of PEPC-PK is a rather sluggish process in tobacco leaves. PEPC-kinase activity became detectable after about 30 min of illumination at 800 to 1000 μmol photons m^{-2} s^{-1} and gradually attained its maximum level in about 2 h (Fig. 2, top). Concomitant with this increase in

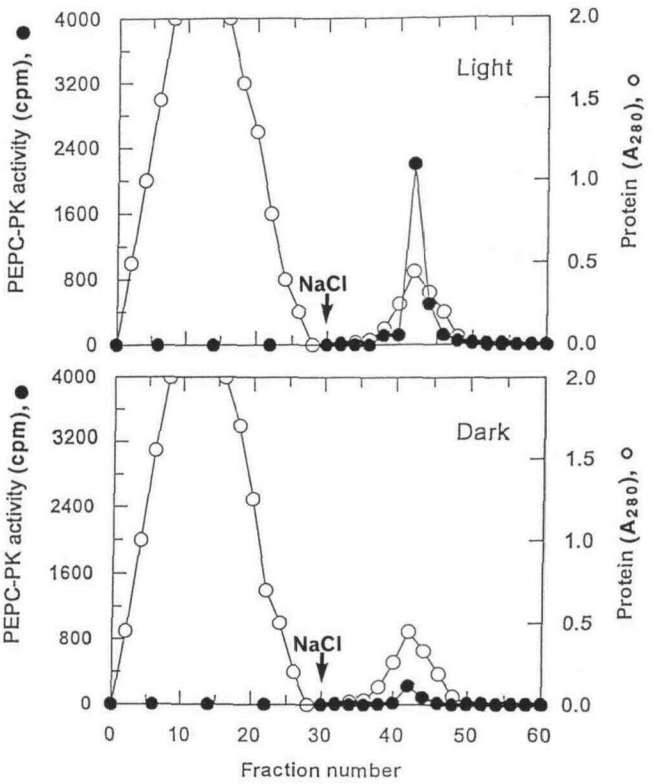

Figure 1. Light activation of tobacco leaf PEPC-kinase in vivo. Detached leaves from N-sufficient plants were either illuminated or darkened for 3 h. Following homogenization, ammonium sulfate precipitation, and desalting, the concentrated light (top) and dark (bottom) extracts were loaded onto separate 20-mL BDA columns. Step elution of PEPC-PK activity with 0.5 M NaCl in buffer C was initiated at fraction 30. Protein was estimated by A_{280} (○). Each fraction contained 0.6 mL, and 3-μL samples were assayed directly for PEPC-kinase activity (●).

PEPC-PK activity were changes in both PEPC activity and its sensitivity to inhibition by L-malate when the target protein was assayed at suboptimal levels of pH and PEP (Fig. 2, bottom). After 3 h of illumination the activity of PEPC increased by about 47% and its inhibition by 50 μM L-malate decreased from 72% to about 50%. Similarly, the I_{50}(L-malate) value for darkened leaf tobacco PEPC was about 34 μM, increasing to 73 μM after 3 h of illumination.

Light Activation of Tobacco Leaf PEPC-Kinase Is Reversible and Sensitive to Various Inhibitors of Photosynthesis and Cytosolic Protein Synthesis

When detached illuminated tobacco leaves were returned to darkness for 3 h, PEPC-PK activity decreased markedly (70%), indicating that the light activation of the kinase was largely reversible (Fig. 3). Similarly, light activation of tobacco PEPC-kinase was sensitive to several photosynthesis inhibitors. Treatment of the detached leaves with either 0.2 mM DCMU or methyl viologen for 3 h in the light almost completely abolished kinase activity (Fig. 3). DL-Glyceraldehyde, a Calvin cycle inhibitor, and CHX, a cytosolic protein-synthesis inhibitor, also largely prevented the activation of PEPC-PK in illuminated tobacco leaves (Fig. 3). However, it must be emphasized that the detached tobacco leaves became severely wilted soon after application of 5 μM CHX. This overt symptom of severe water loss was previously observed neither with leaves excised from maize (Jiao et al., 1991a), sorghum (Bakrim et al., 1993), wheat (Bakrim et al., 1993; Duff and Chollet, 1995), and spinach (Weiner et al., 1992) plants nor with any of the other inhibitor treatments used in the present study.

Tobacco Leaves Contain Two Major, Light-Activated PEPC-Kinase Polypeptides

Previous studies by Wang and Chollet (1993a, 1993b) indicated that the partially purified tobacco and maize PEPC-kinases are similar with respect to various chromatographic properties, substrate specificity, and Ca^{2+} independence. Thus, it was important to identify the tobacco PEPC-PK catalytic polypeptide(s) as was done previously for the maize and facultative CAM kinases (Li and Chollet, 1993, 1994).

For direct comparative purposes we analyzed the tobacco and maize leaf PEPC-kinases by an in situ activity-gel method following SDS-PAGE and subsequent renaturation (Wang and Chollet, 1993b). To minimize possible interference from the PEPC-independent autophosphory-

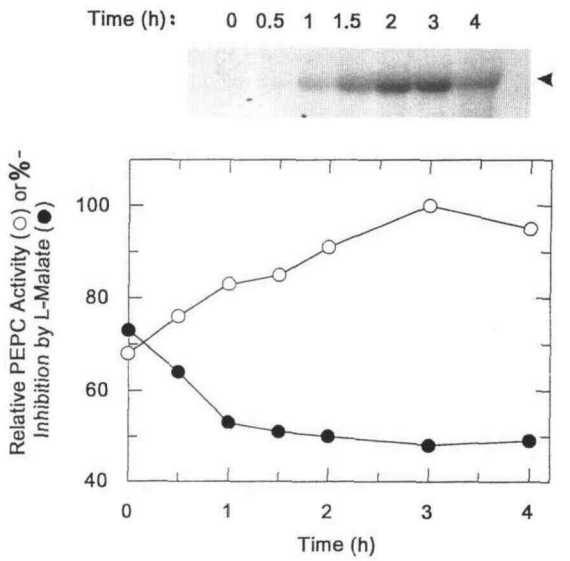

Figure 2. Time course of the in vivo changes in tobacco PEPC-kinase activity and the activity and L-malate sensitivity of PEPC. Detached leaves were illuminated for the indicated time. The autoradiograph indicates the time course of PEPC-PK activation, as shown by the ability to phosphorylate exogenous maize PEPC in vitro. PEPC-PK was prepared by partial purification on BDA as described in Figure 1. The arrowhead indicates the approximately 110-kD PEPC polypeptide. As shown in the bottom panel, rapidly desalted leaf extracts were used to determine the activity (○) and percentage inhibition by L-malate (●) of PEPC under suboptimal assay conditions (pH 7.3, 0.2 mM PEP, ±50 μM L-malate). The activity value of the 3-h sample was arbitrarily set at 100. Data represent the average of three separate experiments and were reproducible to within ±20% SE.

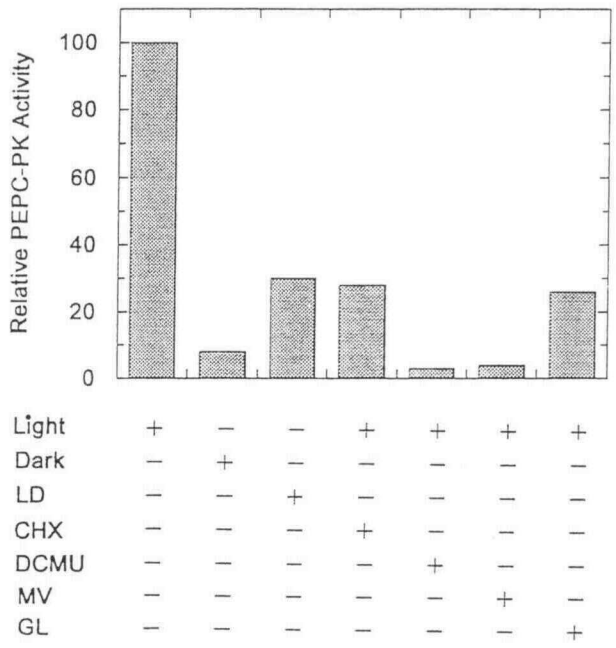

Figure 3. Inhibition of PEPC-kinase light activation by treating detached tobacco leaves with inhibitors of photosynthesis and cytosolic protein synthesis. Leaves were darkened or illuminated for 3 h in the absence (control, −) and presence (+) of 5 μM CHX, 0.2 mM DCMU, 0.2 mM methylviologen (MV), or 0.1 M DL-glyceraldehyde (GL). LD represents a light-adapted leaf transferred to darkness for 3 h. After each treatment PEPC-PK samples were prepared by the methods described in Figure 1. The in vitro ^{32}P incorporation into the approximately 110-kD maize PEPC subunit was determined quantitatively by phosphorimaging following SDS-PAGE.

lation of other leaf protein kinases and/or the co-migration of kinases with potential substrate proteins during minigel SDS-PAGE, both PEPC-PK preparations were separated from most other soluble proteins by chromatography on BDA. As shown in Figure 4, maize and tobacco leaves contain two major, light-enhanced and Ca^{2+}-independent PEPC-kinase catalytic polypeptides with molecular masses of about 37 and 30 kD (Fig. 4, a and b, respectively). Whereas the activity of the approximately 30-kD kinase polypeptide was found repeatedly to be absolutely dependent on light, the extent of light activation of the approximately 37-kD polypeptide varied among experiments, with a typical 4- to 8-fold higher activity in illuminated leaves (determined by phosphorimager analysis). The approximately 30- and 37-kD kinase polypeptides displayed no in-gel autophosphorylation activity in that neither was radiolabeled when purified dephospho maize PEPC was omitted from the separating gel (Fig. 4c). In the tobacco PEPC-PK preparations a prominent approximately 53-kD kinase polypeptide was repeatedly detected; however, its activity was largely independent of the presence of PEPC in the separating gel (Fig. 4, b and c). In addition, there were at least two minor polypeptides, with molecular masses of about 45 and 47 kD, in both the maize and tobacco preparations that displayed PEPC-dependent and light-activated kinase activity. However, this activity was considerably lower than that of the approximately 37- and 30-kD polypeptides and was largely lost following further purification of PEPC-kinase by hydrophobic chromatography on phenyl-Sepharose (data not shown; for methods, see Li and Chollet [1994]).

Gln Is Involved in the Light Activation of Tobacco-Leaf PEPC-Kinase

There has been considerable speculation that certain amino acids, most notably Gln, may act as second messengers in modulating C_3 and C_4 leaf PEPC phosphorylation by PEPC-PK in N-deficient seedlings (Manh et al., 1993; Foyer et al., 1994; Champigny, 1995). Given the fact that PEPC gene expression also is greatly influenced by N status and the level of Gln in these leaves (Sugiharto et al., 1992; Manh et al., 1993), it is not certain whether the changes in PEPC activity observed in the former studies resulted from changes in its phosphorylation state and/or PEPC-PK activity. Therefore, a direct assessment of the possible role of Gln in the light activation of PEPC-PK activity in leaves of N-sufficient tobacco and maize plants was performed.

Two different Gln synthetase inhibitors, MSX (Martin et al., 1983) and PPT (Wendler et al., 1990), were potent antagonists of the light activation of tobacco leaf PEPC-PK in vivo. Treatment with 2 mM MSX (Fig. 5A) or 1 mM PPT (data not shown) for 3 h in the light under photorespiratory conditions (21% O_2) completely abolished the light activation of the kinase. Notably, the inhibition by MSX was the same when the illuminated leaves were treated in normal air or 2% O_2 (data not shown). In contrast, the inhibitory effect of MSX was partially reversed by simultaneous feeding of 20 mM Gln (Fig. 5A). This striking effect of exogenous Gln in the presence of MSX appeared to be quite

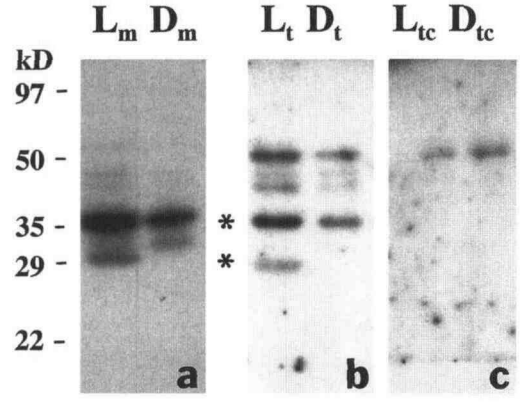

Figure 4. Comparative autoradiographic analysis of tobacco and maize leaf PEPC-kinase catalytic polypeptides by in situ renaturation and assay of PEPC-PK activity following SDS-PAGE. PEPC-PK was partially purified by BDA chromatography from illuminated (L_m) or darkened (D_m) maize (a) and illuminated (L_t) or darkened (D_t) tobacco (b) leaves. About 2 μg of the maize or 10 μg of the tobacco kinase sample were applied to each lane. Illuminated (L_{tc}) and darkened (D_{tc}) tobacco-kinase samples were also analyzed following SDS-PAGE in a PEPC-minus separating gel to assess the autophosphorylation activity of the renatured kinases (c). The asterisks indicate the light-enhanced approximately 30- and 37-kD PEPC-PK polypeptides.

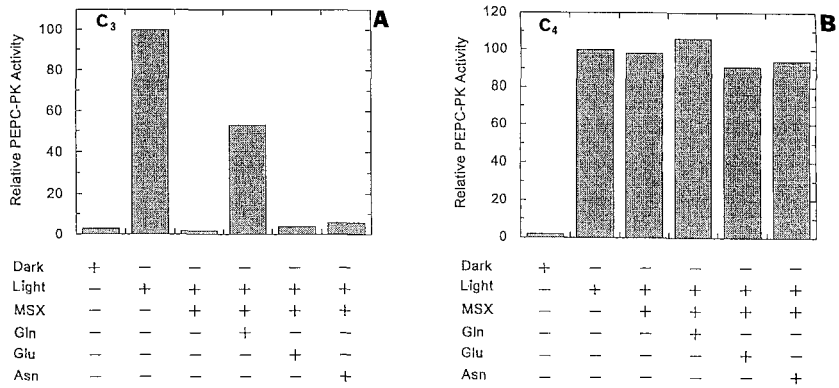

Figure 5. In vivo effects of MSX and selected amino acids on the light activation of PEPC-kinase. Detached tobacco (A, C_3) and maize (B, C_4) leaves were darkened or illuminated in an atmosphere of normal air for 3 h in the absence (control, −) and presence (+) of 2 mM MSX alone or MSX plus 20 mM Gln, Glu, or Asn. The tobacco leaf PEPC-PK samples were partially purified by BDA chromatography as described in Figure 1, whereas the maize leaf samples were prepared by 0 to 60% ammonium sulfate precipitation and desalting. The in vitro ^{32}P incorporation into the approximately 110-kD maize PEPC subunit was determined quantitatively by phosphorimaging following SDS-PAGE.

specific in that equimolar concentrations of Glu or Asn had little or no influence on the recovery of PEPC-PK activity from MSX inhibition. Importantly, MSX alone or in combination with exogenous Gln, Glu, or Asn had no apparent effect on the light activation of PEPC-kinase activity in detached maize leaves (Fig. 5B).

Given that the reversible light activation of tobacco leaf PEPC-PK is also blocked by various photosynthesis inhibitors (Fig. 3), an additional experiment was performed to determine whether exogenous Gln also antagonized the inhibitory effect of such compounds. In marked contrast to the in vivo experiment with MSX (Fig. 5A), little or no recovery of the kinase activity from inhibition by DCMU was observed when 20 mM Gln was included in the treatment solution (Fig. 6).

DISCUSSION

Several reports have documented that C_3 PEPC is regulated by reversible protein phosphorylation, in a light- and/or N-modulated manner, in leaves of N-deficient wheat seedlings (Van Quy et al., 1991; Manh et al., 1993; Duff and Chollet, 1995). The present study with tobacco has established that the regulatory phosphorylation of this ubiquitous plant enzyme also occurs in leaves of an N-sufficient C_3 plant. Light and darkness up-/down-regulate C_3 PEPC-kinase activity, thereby modulating the activity and L-malate sensitivity of the target protein (measured at suboptimal but near-physiological assay conditions). Light is obviously sufficient to induce an increase in the apparent phosphorylation state of PEPC in leaves of a high-N-grown C_3 plant, as in leaves of C_4 species. However, it is noteworthy that the magnitude of these light-induced changes in C_3 PEPC in leaves of N-sufficient tobacco and wheat plants are significantly less than those of N-deficient wheat leaves. For example, whereas the I_{50}(L-malate) value of PEPC was about doubled upon illumination of the former C_3 plants, this parameter increased 4- to 5-fold in illuminated leaves from N-deficient wheat seedlings (this study; Duff and Chollet, 1995). These comparative results suggest that leaf N status influences the regulatory phosphorylation of C_3 PEPC in vivo, but its role is not absolutely essential.

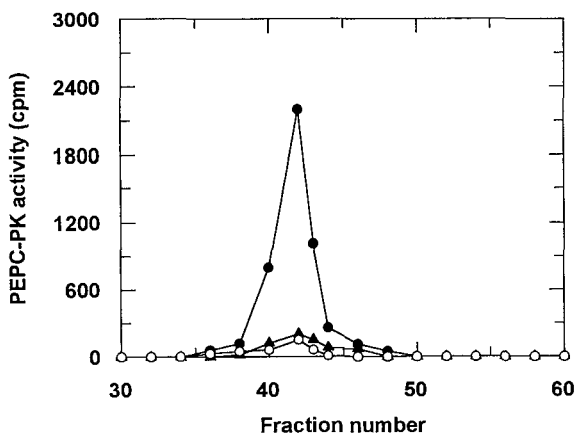

Figure 6. Effect of DCMU with or without exogenous Gln on the light activation of tobacco leaf PEPC-kinase in vivo. Detached leaves were illuminated for 3 h in the absence (●) and presence of 0.2 mM DCMU alone (○) or DCMU plus 20 mM Gln (▲). PEPC-PK samples were prepared by BDA chromatography, and 3-μL aliquots of the indicated fractions were assayed directly for kinase activity (see Fig. 1 for details).

Several previous studies failed to detect any significant light-induced changes in the activity and/or L-malate sensitivity of PEPC in leaves of N-sufficient C_3 plants (Chastain and Chollet, 1989; Rajagopalan et al., 1993; Gupta et al., 1994). The reason(s) for this apparent discrepancy has not been investigated in detail and could, in fact, vary among studies. We emphasize that the following steps, among others, must be taken to ensure an accurate estimation of the apparent phosphorylation state of plant PEPC when measuring PEPC activity and/or L-malate sensitivity. First, suboptimal assay conditions must be determined for measuring these parameters. The levels of PEP and pH are two critical factors; at optimal conditions (saturating [PEP] at pH 8.0) both C_3 and C_4 leaf PEPCs show little or no light/dark changes in activity and L-malate sensitivity (Echevarria et al., 1994; Duff and Chollet, 1995; Duff et al., 1995; data not shown). Along these lines, it must be noted that C_3 leaf PEPC has a much lower K_m(PEP) than the C_4 enzyme (Wang and Chollet, 1993a; Duff and Chollet, 1995; Duff et al., 1995), and, thus, a correspondingly lower PEP concentration must be selected for the assay of C_3 PEPC

activity. In general, we routinely use a PEP concentration equivalent to $2 \times K_m$ to $3 \times K_m$ (at pH 8.0) and an assay pH of approximately 7.3 as suboptimal conditions for assaying both C_4 (Jiao and Chollet, 1988) and C_3 (Wang and Chollet, 1993a; Duff and Chollet, 1995) PEPCs. Second, proteinase inhibitors, including most especially chymostatin, are required during enzyme preparation. Proteinase(s) frequently cause N-terminal truncation of plant PEPC, and the resulting modified enzyme possesses an unaltered maximum velocity but a much decreased sensitivity to L-malate (Duff et al., 1995, and refs. therein). Finally, a protein phosphatase type 2A inhibitor such as microcystin-LR or okadaic acid should be included during enzyme extraction to minimize in vitro dephosphorylation of phospho-PEPC.

As has been observed in C_4 and CAM leaves (Echevarria et al., 1990; Carter et al., 1991; Jiao et al., 1991a; Bakrim et al., 1992; Jiao and Chollet, 1992; Li and Chollet, 1994), the regulatory phosphorylation of PEPC in C_3 leaves from tobacco appears to be largely influenced by the activity state of PEPC-PK. However, a simultaneous assessment of the activity of the native PEPC-phosphatase heteromeric protein is necessary before a final conclusion can be drawn. There are, indeed, several striking similarities between the regulation of tobacco and C_4 leaf PEPC-kinases. First, both kinases are reversibly light activated in vivo, and this light activation is effectively blocked by various photosynthesis inhibitors (Figs. 2 and 3; Echevarria et al., 1990; Bakrim et al., 1992; Jiao and Chollet, 1992). Clearly, the light signal is initially perceived by the illuminated chloroplast. Second, photosynthetic C metabolism appears to be somehow involved in the regulation of PEPC-PK activity because light activation of both kinases is inhibited by DL-glyceraldehyde, a Calvin cycle inhibitor (Fig. 3; Bakrim et al., 1992; Jiao and Chollet, 1992). At this point, it is difficult to conclude that cytosolic protein turnover also is involved in the light activation of tobacco leaf PEPC-kinase. Despite the fact that application of CHX caused a significant depression in PEPC-PK activity in the light (Fig. 3), this effect may have resulted from the severe water stress observed and, thus, an inhibition of photosynthesis. Third, the light activation of both tobacco and C_4 leaf PEPC-kinases is a sluggish process, taking 90 to 120 min for maximal activation at high PPFD (Fig. 2; Echevarria et al., 1990; Bakrim et al., 1992). It is presently unclear which step(s) along the light-signal transduction pathway is rate limiting under these conditions. Fourth, partially purified preparations of tobacco and maize leaf PEPC-PK show similar chromatographic and substrate-protein properties and Ca^{2+} independence (Fig. 1; Wang and Chollet, 1993a, 1993b). Finally, both tobacco and maize leaves contain two approximately 30- and 37-kD PEPC-kinase catalytic polypeptides whose activities are highly regulated by light and darkness in vivo (Fig. 4; Li and Chollet, 1993). It is notable in this context that darkened leaves from a facultative CAM species also contain two PEPC-PK polypeptides with similar molecular masses (about 39 and 32 kD) and other properties (Li and Chollet, 1994). It is thus likely that the general features of PEPC-kinase have been highly conserved during the course of evolution that led to the separation of CAM and C_4 species from a presumably common C_3 ancestor.

In spite of these similarities in the light activation of C_3 and C_4 leaf PEPC-PK, the details of their respective light-signal transduction pathways are likely to differ. For example, light activation of the tobacco kinase is inhibited strikingly by specific inhibitors of Gln synthetase (MSX, PPT), whereas such compounds have no detectable effect on the light activation of maize PEPC-PK (Fig. 5; data not shown). These comparative results indicate that a disruption of leaf N metabolism does not have the same impact on the regulatory phosphorylation of PEPC in illuminated C_3 and C_4 leaves. Although the mechanism(s) underlying this difference is unclear at present, it does not appear to be related to perturbation of the photorespiratory N cycle and, thus, photosynthetic C assimilation in these illuminated C_3 leaves (Martin et al., 1983; Wendler et al., 1990). In this regard it is notable that MSX inhibited the activation of PEPC-PK in illuminated tobacco leaves to the same extent under both photorespiratory (21% O_2 [Fig. 5A]) and nonphotorespiratory (2% O_2 [data not shown]) conditions. On the other hand, the photosynthetic and nonphotosynthetic isoforms of plant PEPC have vastly different physiological functions in green leaves, fixing atmospheric CO_2 during photosynthesis by C_4 plants (Hatch, 1987) and coordinating cytosolic N and C partitioning in conjunction with Suc phosphate synthase and nitrate reductase in C_3 leaves (Huber et al., 1994; Champigny, 1995). Thus, the existence of differentiated light-signal transduction pathways in illuminated C_3 and C_4 leaves could be beneficial for the post-translational regulation of PEPC to fulfill these distinct metabolic functions.

Gln specifically antagonizes the inhibitory effect of MSX on the light activation of PEPC-PK in tobacco leaves (Fig. 5A), suggesting that this product of catalysis by Gln synthetase may be a critical component in the C_3 light-signal transduction chain. Gln has been implicated previously as a positive and negative modulator in the control of gene expression of leaf PEPC (Sugiharto et al., 1992) and nitrate reductase (Deng et al., 1991; Vincentz et al., 1993), respectively. However, it is unclear exactly how Gln regulates expression of these nuclear genes and whether it also regulates gene expression of tobacco leaf PEPC-kinase. Clearly, additional studies are warranted to define the exact role of Gln in regulating C_3 leaf PEPC-kinase activity. For example, this amide amino acid did not replace light in effecting the activation of tobacco PEPC-PK in vivo (data not shown), and it failed to reverse the inhibitory effect of DCMU on the light activation of this highly regulated protein-Ser/Thr kinase (Fig. 6).

In conclusion, the data from this study demonstrate unequivocally that C_3 PEPC is regulated by reversible protein phosphorylation in leaves of N-sufficient tobacco plants. Light increases the phosphorylation state of PEPC by activating a Ca^{2+}-independent PEPC-kinase. It appears that the C_3 and C_4 PEPC-PKs are regulated by similar but not identical light-signal transduction pathways. Further research is needed to elucidate the specific signal-transducing elements and to define the exact physiological signifi-

cance of PEPC phosphorylation in illuminated leaves of N-sufficient C_3 plants.

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