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RESEARCH ARTICLE

The Light-Dependent Transduction Pathway Controlling the Regulatory Phosphorylation of C4 Phospho*enol*pyruvate Carboxylase in Protoplasts from *Digitaria sanguinalis*

Nathalie Giglioli-Guivarc'h,[a] Jean-Noël Pierre,[a,1] Spencer Brown,[b] Raymond Chollet,[c] Jean Vidal,[a] and Pierre Gadal[a]

[a] Institut de Biotechnologie des Plantes, Centre National de la Recherche Scientifique, UA D1128, Université de Paris–Sud, Bâtiment 630, 91405 Orsay Cedex, France

[b] Institut des Sciences Végétales, Centre National de la Recherche Scientifique, UPR 40, avenue de la Terrasse, 91198 Gif-sur-Yvette Cedex, France

[c] University of Nebraska–Lincoln, Department of Biochemistry, G.W. Beadle Center, Lincoln, Nebraska 68588-0664

Phospho*enol*pyruvate carboxylase (PEPC) was characterized in extracts from C4 mesophyll protoplasts isolated from *Digitaria sanguinalis* leaves and shown to display the structural, functional, and regulatory properties typical of a C4 PEPC. In situ increases in the apparent phosphorylation state of the enzyme and the activity of its Ca^{2+}-independent protein-serine kinase were induced by light plus NH_4Cl or methylamine. The photosynthesis-related metabolite 3-phosphoglycerate (3-PGA) was used as a substitute for the weak base in these experiments. The early effects of light plus the weak base or 3-PGA treatment were alkalinization of protoplast cytosolic pH, shown by fluorescence cytometry, and calcium mobilization from vacuoles, as suggested by the use of the calcium channel blockers TMB-8 and verapamil. The increases in PEPC kinase activity and the apparent phosphorylation state of PEPC also were blocked in situ by the electron transport and ATP synthesis inhibitors DCMU and gramicidin, respectively, the calcium/calmodulin antagonists W7, W5, and compound 48/80, and the cytosolic protein synthesis inhibitor cycloheximide. These results suggest that the production of ATP and/or NADPH by the illuminated mesophyll chloroplast is required for the activation of the transduction pathway, which presumably includes an upstream Ca^{2+}-dependent protein kinase and a cytosolic protein synthesis event. The collective data support the view that the C4 PEPC light transduction pathway is contained entirely within the mesophyll cell and imply cross-talk between the mesophyll and bundle sheath cells in the form of the photosynthetic metabolite 3-PGA.

INTRODUCTION

The operation of C4 photosynthesis requires the cooperation of two different photosynthetic cell types that have distinct but coordinated functions. This difference involves concentrically organized leaf tissues, with the outer mesophyll cells surrounding the inner bundle sheath cells in which the C4 cycle and the classic Calvin–Benson cycle take place. In NADP–malic enzyme–type C4 plants, such as sorghum, maize, and *Digitaria sanguinalis* (hairy crabgrass), cytosolic phospho*enol*pyruvate carboxylase (PEPC; EC 4.1.1.31) catalyzes primary photosynthetic CO_2 fixation with the formation of oxaloacetate and subsequently L-malate via a coupled, chloroplastic NADPH–malate dehydrogenase. More than 98% of the PEPC is in the is in the mesophyll cell. Malate is transported to the bundle sheath cells and decarboxylated by the stromal NADP–malic enzyme, thereby generating a concentrated pool of CO_2 for refixation by ribulose 1,5-bisphosphate carboxylase/oxygenase in the Calvin–Benson cycle. Functional cooperation of these two physically separated photosynthetic cycles most likely requires complex regulatory interactions.

Regulation of C4 PEPC in the mesophyll cell cytosol involves opposing photosynthesis-related metabolite effectors, that is, glucose 6-phosphate (positive) and L-malate (negative) (Doncaster and Leegood, 1987; Echevarria et al., 1994; Duff et al., 1995), and a light-dependent, reversible phosphorylation process (Nimmo et al., 1987; Jiao and Chollet, 1988; McNaughton et al., 1991; Bakrim et al., 1992). This latter post-translational mechanism targets a single serine residue of the

[1] To whom correspondence should be addressed.

holoenzyme subunit (Jiao and Chollet, 1989; Jiao et al., 1991b) in a highly conserved N-terminal domain shared by all plant PEPCs sequenced to date (Lepiniec et al., 1993). Within the C4 leaf, phosphorylation of PEPC is completed ~1 hr after the onset of illumination and causes gradual changes in the enzyme's functional properties, namely, an increase in catalytic activity and apparent affinity for glucose 6-phosphate and a decrease in end-product inhibition by L-malate (as determined at suboptimal but near-physiological conditions of pH and PEP concentration) (Bakrim et al., 1992; Duff et al., 1995). Therefore, this modulation of PEPC is believed to enable the enzyme to cope with the high concentrations of L-malate that occur during CO_2 fixation by an illuminated C4 leaf (10 to 20 mM) (Stitt and Heldt, 1985) and might explain why this modification is critical for the functioning of C4 photosynthesis (Bakrim et al., 1993; Echevarria et al., 1994).

In initial work with sorghum and maize, the use of specific chemical inhibitors with intact leaves (Jiao et al., 1991a; McNaughton et al., 1991; Bakrim et al., 1992; Jiao and Chollet, 1992; Bakrim et al., 1993) and isolated mesophyll cell protoplasts (Pierre et al., 1992) suggested that PEPC phosphorylation occurs via a light signal transduction cascade originating in the illuminated chloroplast and involving second messengers (e.g., pH and Ca^{2+}), cytosolic protein synthesis, and a reversibly light-activated protein–serine kinase (PEPC kinase). In reconstituted assays, two types of protein kinases requiring or not requiring Ca^{2+} for activity have been shown to phosphorylate a purified preparation of recombinant C4-type sorghum PEPC specifically at the target residue, Ser-8 (Li and Chollet, 1993). The calcium-independent protein kinase, which has been highly but not completely purified from illuminated maize leaves (Wang and Chollet, 1993), is regarded as the best candidate for the physiological converter enzyme because its activity in vivo shows clear light dependency and sensitivity to inhibitors of photosynthesis and cytosolic protein synthesis (Li and Chollet, 1993).

Although many of the components of signal–response coupling described in animal cells also are present in plant cells (Morré, 1990; Neuhaus et al., 1993), few specific targets have been identified (Huber et al., 1994), and data about how these cascades are spatially and temporally organized are scarce. In this study, we investigated further the components of the light signaling process leading to the in situ phosphorylation of C4 PEPC in mesophyll cell protoplasts isolated from *D. sanguinalis* with the objectives of clarifying their specific roles in transducing the light signal as well as their sequential organization. To our knowledge, the results highlight a regulatory cross-talk between the two photosynthetic cell types of the C4 leaf, the pivotal role of mesophyll cytosol pH (pHc), and the involvement of cytosolic Ca^{2+} and a multicyclic protein kinase cascade in C4 mesophyll cells. Based on these in situ findings, we propose an integrated working model for this highly complex transduction pathway and provide insight into how this regulatory network presumably impacts the functioning and homeostasis of C4 photosynthesis.

RESULTS

Characterization of C4 PEPC in Mesophyll Cell Protoplasts from *D. sanguinalis*

A mesophyll cell protoplast from *D. sanguinalis* contains ~150 pg of total soluble protein and 2 nmol min^{-1} of extractable PEPC activity. Protein gel blotting experiments with specific C4 PEPC phosphorylation domain antibodies as probes (Pacquit et al., 1995) identified both the enzyme subunit, which had a molecular mass of ~108 kD, and the presence of the phosphorylation domain epitope (Figure 1). This latter observation was expected because all plant PEPCs sequenced to date contain this N-terminal site with the regulatory serine (Lepiniec et al., 1993). Examination of the kinetic properties of crude *D. sanguinalis* PEPC (nonphosphorylated form) extracted from noninduced protoplasts showed that enzyme activity increased sharply with increasing pH in the range 7 to 8. The 50% maximum velocity for PEP is ~1.2 mM at the suboptimal pH of 7.3, and the 50% inhibition constant for L-malate is 0.3 mM at pH 7.3 and 1.2 mM PEP. Collectively, these results established that *D. sanguinalis* photosynthetic PEPC is similar to its sorghum and maize counterparts (Jiao and Chollet, 1988; Echevarria et al., 1994; Duff et al., 1995).

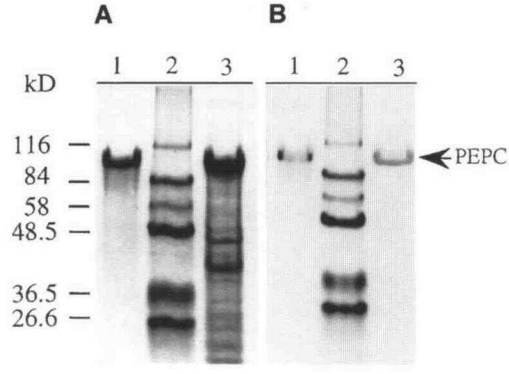

Figure 1. Immunological Characterization of C4 PEPC from *D. sanguinalis* Protoplasts.

Proteins were extracted from mesophyll cell protoplasts, separated by SDS-PAGE (12% acrylamide gel), and electroblotted onto a nitrocellulose membrane.

(A) Coomassie blue staining of the proteins. Lane 1 contains immunoaffinity-purified recombinant C4 PEPC from sorghum (10 μg); lane 2, molecular mass markers given in kilodaltons; and lane 3, desalted protein extracts (20 μg of protein) from *D. sanguinalis* protoplasts.

(B) Immunological detection using anti-phosphorylation site antibodies directed against the sorghum C4 PEPC. Contents of lanes 1 to 3 are as given in **(A)**.

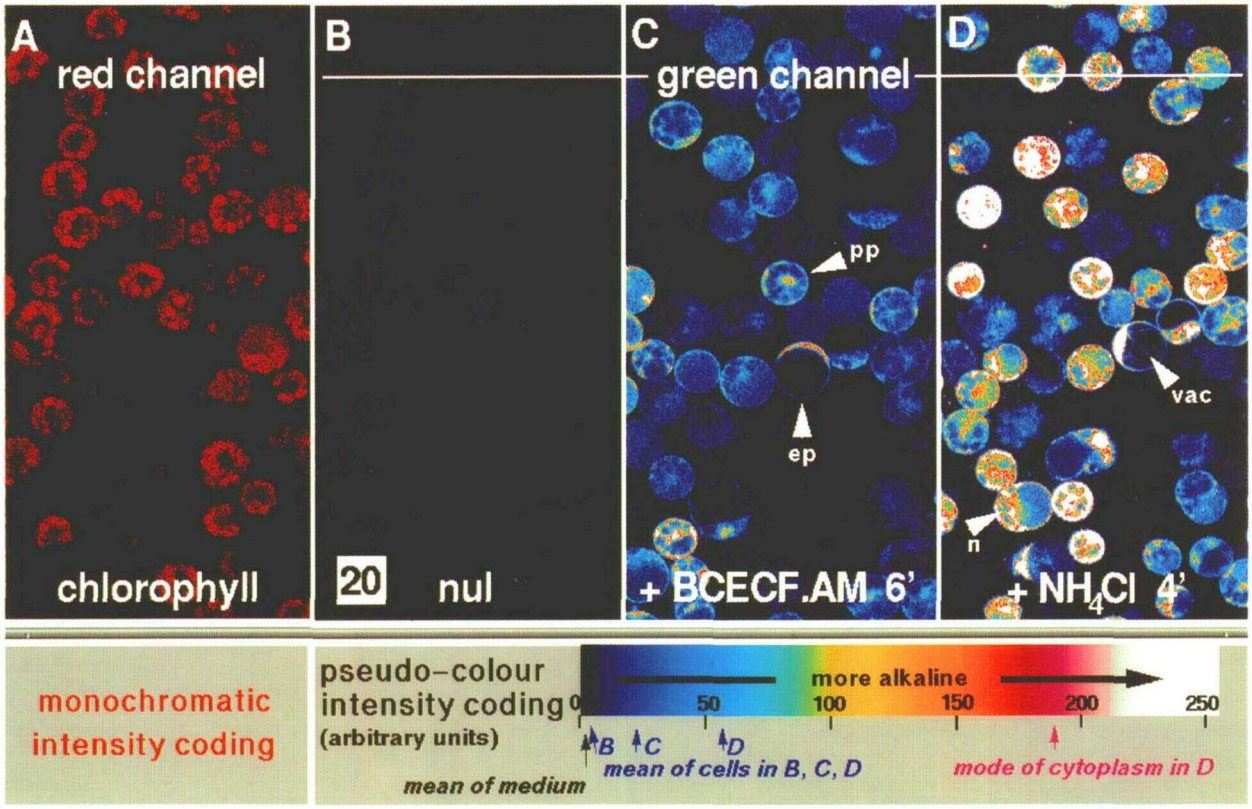

Figure 2. Fluorescence Imaging of Cytosolic pH Changes in Mesophyll Cell Protoplasts.

(A) Endogenous (red channel) fluorescence of the chlorophylls of unloaded protoplasts.

(B) Endogenous (green channel) fluorescence of unloaded protoplasts (nul). Side of the square = 20 μm.

(C) Basal fluorescence emission of BCECF-AM–loaded protoplasts (green channel). Protoplasts were preloaded with 10 nM dye in the dark at 25°C for 6 min (6′). ep, equatorial plan; pp, polar plan.

(D) Fluorescence emission of the dye (green channel) was recorded after preloaded protoplasts (from the experiment described in **[C]**) had been treated for 4 min (4′) with 20 mM NH_4Cl. n, nucleus; vac, vacuole.

Monochromatic red intensity coding is shown in **(A)**. A numeric scale of pseudo-color intensity coding is shown beneath **(B)** through **(D)**.

Phosphorylation of PEPC Is Controlled by pHc in Mesophyll Cell Protoplasts

The addition of NH_4Cl or methylamine to an illuminated suspension of mesophyll cell protoplasts from *D. sanguinalis* was required for the in situ stimulation of PEPC kinase activity and PEPC phosphorylation. These two weak bases permeate cells in their neutral form and therefore tend to increase pHc (Sanders et al., 1981; Bertl et al., 1984; Felle, 1987, 1988). Examination of protoplasts preloaded with the specific pH fluorescent probe 2′,7′-*bis*-(2-carboxyethyl)-5-(and-6)carboxyfluorescein acetoxymethyl ester (BCECF-AM) (Rink et al., 1982) demonstrated that these two compounds rapidly and efficiently alkalinized pHc (Figure 2). By using the null point method (Thomas et al., 1979; Rink et al., 1982) and flow cytometric analysis of dye fluorescence intensity (Giglioli-Guivarc'h et al., 1996), it was shown that pHc increased in mesophyll cell protoplasts from the initial value of 6.4 to ~7.3 when 20 mM NH_4Cl was added to the suspension medium. The methodology was validated elsewhere (Giglioli-Guivarc'h et al., 1996): the cytoplasmic location of the fluorescent probe (used at 10 nM) was confirmed, and intensity changes were calibrated to pH shifts. Under the conditions used, the autofluorescence was ~4% of the total green signal, and the amplitude of the BCECF signal was 200%.

Here, we determined the corresponding in situ changes in the apparent phosphorylation state of PEPC, as reflected in the malate sensitivity of the enzyme, and PEPC kinase activity. Figure 3A shows that a concentration-dependent decrease in inhibition by L-malate occurred when protoplasts were illuminated in the presence of increasing concentrations of NH_4Cl, whereas no change in this parameter was observed

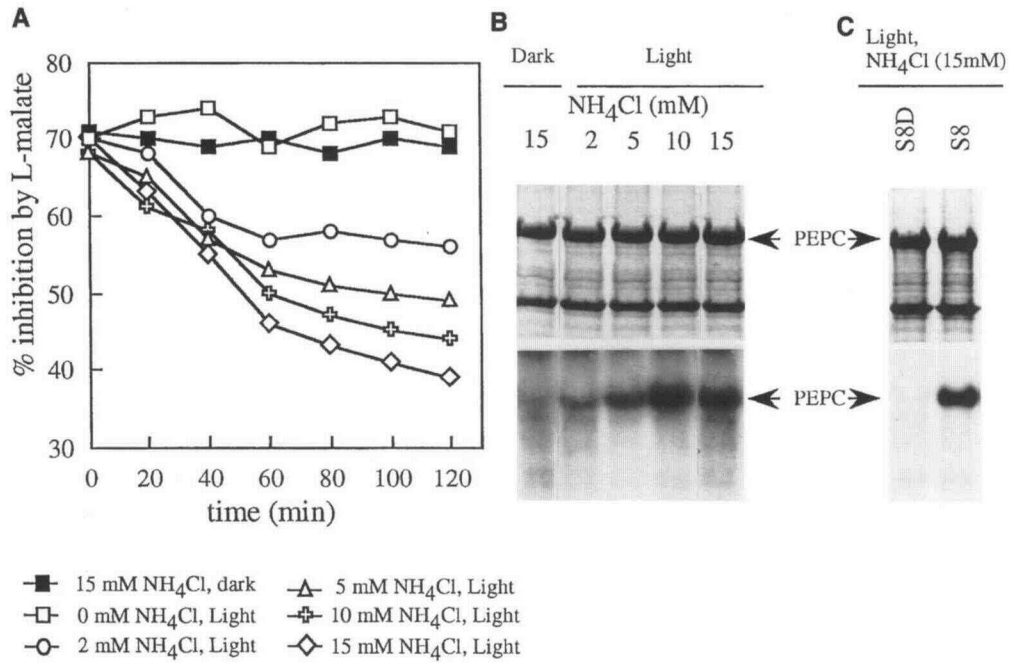

Figure 3. Induction of PEPC Kinase Activity in Situ in Mesophyll Cell Protoplasts from *D. sanguinalis*.

Darkened protoplasts were preincubated in suspension medium with different concentrations of NH_4Cl (0 to 15 mM) and subsequently maintained in darkness or illuminated at 300 μE m^{-2} sec^{-1} and 25°C for up to 120 min with gentle stirring.

(A) Aliquots of the protoplast suspensions were taken at different times and immediately mixed with the PEPC assay medium to estimate the malate sensitivity of the enzyme at pH 7.3, 1.2 mM PEP, and ±0.5 mM L-malate.

(B) After 120 min, soluble proteins were extracted, desalted on Sephadex G-25, and assayed (5 μg of protein) for PEPC kinase activity by using a ^{32}P-based radiochemical assay supplemented with 15 μg of exogenous recombinant (nonphosphorylated) C4 PEPC (S8) from sorghum. Radiolabeled proteins were analyzed by SDS-PAGE. Shown at top is a Coomassie blue–stained gel. Shown at bottom is the corresponding autoradiograph.

(C) Control phosphorylation assays contained either 15 μg of the S8D mutant sorghum PEPC or S8 wild-type enzyme and 5 μg of desalted protein extracted from mesophyll cell protoplasts treated with 120 min of light plus 15 mM NH_4Cl. Shown at top is a Coomassie blue–stained gel. Shown at bottom is the corresponding autoradiograph.

In **(B)** and **(C)**, the positions of the PEPC subunit are indicated with arrows.

in the dark at the highest concentration tested. The light- and weak base–induced change in this regulatory property of PEPC should presumably be correlated with a corresponding increase in PEPC kinase activity. To demonstrate that this actually took place in situ, proteins were extracted from the protoplasts after the various incubations were completed, desalted, and used to measure PEPC kinase activity in vitro in a reconstituted phosphorylation medium containing immunopurified, nonphosphorylated sorghum C4 PEPC (approximately four times the level of endogenous *D. sanguinalis* PEPC) as the target protein and γ-^{32}P-ATP–Mg^{2+}. A consistent increase in protoplast PEPC kinase activity, which was clearly dependent on light and inducer concentration, was detected after SDS-PAGE of the in vitro–radiolabeled proteins and autoradiography of the dried gels (Figure 3B).

The barely detectable level of PEPC phosphorylation observed in control experiments in which the recombinant S8D mutant of sorghum C4 PEPC was substituted for the wild-type protein (S8) as exogenous substrate was due to the endogenous mesophyll cell enzyme (Figure 3C). These latter results established that the inducible protein kinase present in protoplast extracts catalyzes a specific phosphorylation of Ser-8 on the target protein. Notably, this PEPC kinase activity in the induced mesophyll cell protoplasts was calcium independent, because the addition of either calcium (200 μM; data not shown) or EGTA in reconstituted assays did not modify the phosphorylation of the target PEPC (Figure 4). This is in excellent agreement with previous findings involving the reversibly light-activated C4 leaf enzyme (Echevarria et al., 1990; Bakrim et al., 1992; Li and Chollet, 1993).

Collectively, the above data indicate that the final in situ level of a calcium-independent PEPC kinase activity and the apparent phosphorylation state of its target protein depend on the pHc value of illuminated mesophyll cell protoplasts. In these experiments, an increase in pHc of ~0.6 to 1 pH unit was required to obtain a high induction of PEPC kinase activity; whether such a large variation in pHc occurs in mesophyll cells of an illuminated intact C4 leaf remains to be determined.

3-Phosphoglycerate Modulates pHc in Mesophyll Cell Protoplasts

Previous studies have demonstrated that chemical inhibition of the Calvin–Benson cycle in bundle sheath cells of an illuminated C4 leaf prevents PEPC kinase activation and PEPC phosphorylation in vivo (Samaras et al, 1988; Bakrim et al., 1992; Jiao and Chollet, 1992). A possible explanation for these findings is that an intercellular metabolic message is required for altering pHc and thereby activating the transduction pathway leading to PEPC phosphorylation in mesophyll cells. Notably, 3-phosphoglycerate (3-PGA) generated in the light by the Calvin–Benson cycle in bundle sheath chloroplasts diffuses readily to mesophyll cells, where it is transported into chloroplasts to be transformed into triose phosphate (Stitt and Heldt, 1985). Considering that, on the one hand, the rate of transport of this photosynthetic metabolite is rapid, approximating the rate of C4 photosynthesis (Leegood, 1985), and, on the other, its uptake into the stroma involves only the partially protonated (2^-) form (Heber and Heldt, 1981; Leegood, 1985), this process could conceivably account for an alkalinization of pHc in the mesophyll cell (Yin et al., 1990).

We next investigated the fluorescence intensity of BCECF-AM–preloaded mesophyll cell protoplasts and showed that it was greatly enhanced when 10 mM 3-PGA was included in the suspension medium at low light energy (30 $\mu E\ m^{-2}\ sec^{-1}$; Figure 5). This light flux intensity was required to avoid photolysis of the dye and was verified as sufficient to induce a PEPC kinase response in mesophyll cell protoplasts. In protoplasts maintained in the dark in the presence of 3-PGA or illuminated in the absence of this phosphorylated metabolite, little or no change in dye fluorescence was observed. Such fluorescence emission by the protoplasts reflected an increase in their pHc that is consistent with the aforementioned results, using light plus NH_4Cl as the inducer (Figure 2). The fact that there was no significant increase in pHc in the dark, in contrast to the weak base effect, suggested that the uptake of 3-PGA by chloroplasts is a light-dependent process.

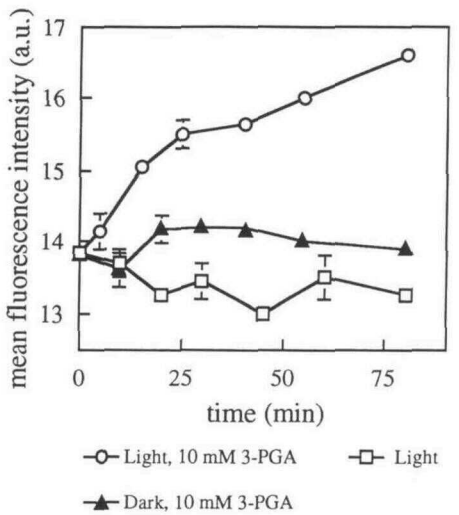

Figure 5. Effect of 3-PGA on Dye Fluorescence Intensity of Mesophyll Cell Protoplasts.

Protoplasts were preloaded in the dark with the pH-sensitive dye BCECF-AM (10 nM). Samples were then illuminated in the presence (O) or absence (□) of 10 mM 3-PGA, and aliquots were analyzed by flow cytometry to obtain the fluorescence intensity per intact protoplast. In this experiment, the light energy was set at 30 $\mu E\ m^{-2}\ sec^{-1}$, because higher levels led to partial photolysis of the probe. A dark control was performed with 10 mM 3-PGA (▲). The data represent the average ±SE of the mean of two different series of experiments. Where error bars are not indicated, the standard error is smaller than the size of the symbol. (a.u.), arbitrary units.

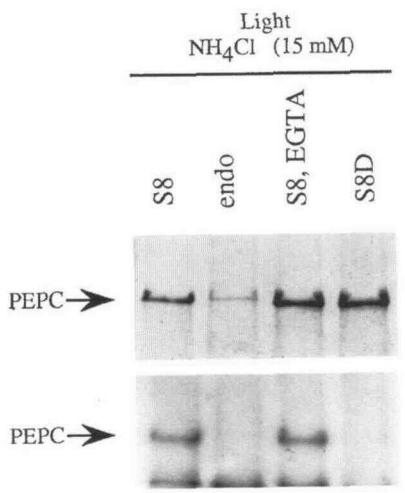

Figure 4. Influence of EGTA on PEPC Kinase Activity in Extracts from Mesophyll Cell Protoplasts.

Soluble protein extracts from light- and weak base–induced protoplasts (120 min plus 15 mM NH_4Cl) were desalted and used to estimate PEPC kinase activity, as described in Figures 3B and 3C, in the presence or absence of 1 mM EGTA. In this experiment, the amount of exogenous S8 and S8D recombinant PEPC was 5 μg, and endo corresponds to no exogenous PEPC added. Shown at top is a Coomassie blue–stained gel. Shown at bottom is the corresponding autoradiograph. The positions of the PEPC subunit are indicated with arrows.

The change in pHc in illuminated mesophyll cell protoplasts in the presence of 3-PGA was accompanied by a decrease in malate sensitivity of PEPC that was similar to that obtained in the presence of NH_4Cl (Figure 6A). In the dark (with 3-PGA), no such change was observed. In addition, in vitro phosphorylation assays showed that the activity of PEPC kinase was significantly higher in illuminated protoplasts that had been treated with 3-PGA or NH_4Cl, whereas there was little or no induction in the dark (with 3-PGA) or in the light without inducer (Figure 6B). Although these experiments do not document the uptake of 3-PGA by mesophyll cell chloroplasts in situ, clearly this metabolite induced an increase in pHc, up-

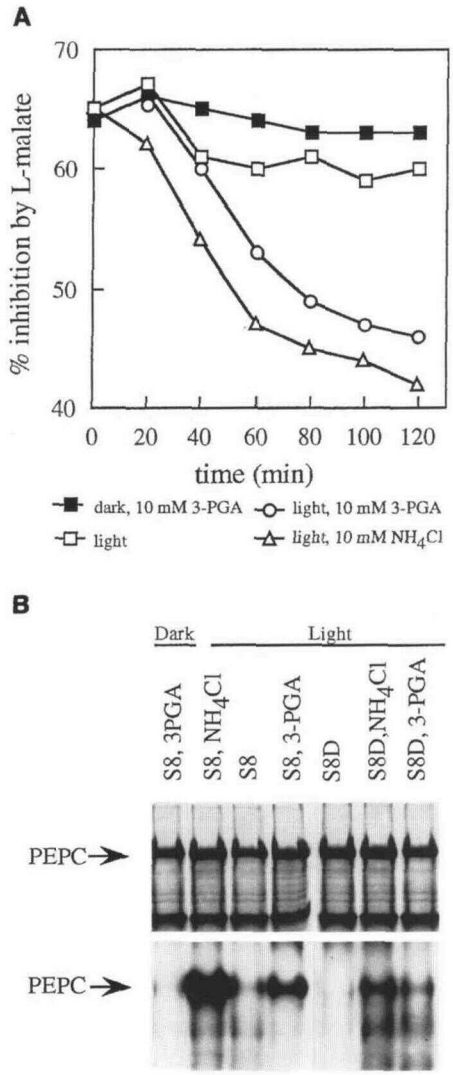

Figure 6. In Situ Effects of 3-PGA on the Induction of PEPC Kinase Activity and the Apparent Phosphorylation State of PEPC in Mesophyll Cell Protoplasts.

(A) Changes in malate sensitivity of PEPC in protoplasts treated for up to 120 min with light (□), light plus 10 mM NH_4Cl (△), light plus 10 mM 3-PGA (○), or darkness plus 10 mM 3-PGA (■). Light intensity was 300 μE m^{-2} sec^{-1}.

(B) Estimation of PEPC kinase activity in desalted protein extracts from corresponding mesophyll cell protoplasts. After 120 min, soluble proteins were extracted, desalted on Sephadex G-25, and assayed (5 μg) for PEPC kinase activity by using the radiochemical assay supplemented with 15 μg of immunopurified wild-type (S8) or mutant (S8D) C4 PEPC from sorghum. Radiolabeled proteins were analyzed by SDS-PAGE. Shown at top is a Coomassie blue–stained gel. Shown at bottom is the corresponding autoradiograph. The positions of the PEPC subunit are indicated with arrows.

regulation of PEPC kinase activity, and an increase in the apparent phosphorylation state of PEPC in illuminated mesophyll cell protoplasts.

Calcium Dependency of PEPC Phosphorylation in Mesophyll Cell Protoplasts

Calcium is often a second messenger in signal transduction cascades in animal cells, and there is growing evidence that it is involved similarly in plants (Sanders et al., 1990; Gilroy et al., 1991, 1993; Trewavas and Gilroy, 1991; Bush, 1993; Neuhaus et al., 1993). We have demonstrated previously that depletion of this cation by use of a specific ionophore (A23187) and EGTA blocks PEPC phosphorylation in situ in mesophyll cell protoplasts from sorghum (Pierre et al., 1992), and we also checked that the same was true for *D. sanguinalis* protoplasts. In contrast, EGTA alone did not prevent the in situ PEPC phosphorylation, thereby suggesting that endogenous calcium was involved in this process. To clarify further its role in the signaling pathway in *D. sanguinalis* protoplasts, the effect of various calcium channel blockers, including diltiazem, nifedipine, verapamil, and TMB-8, was determined. Among these compounds, only verapamil and TMB-8 were found to reduce significantly the weak base–induced decrease in malate sensitivity of PEPC in illuminated protoplasts at concentrations of 0.10 to 0.25 mM (Table 1).

SDS-PAGE analysis of labeled mesophyll cell proteins in reconstituted phosphorylation assays showed that the in situ PEPC kinase activity remained close to the noninduced control level (dark plus NH_4^+; e.g., Figure 3B) in illuminated

Table 1. In Situ Effect of Calcium Channel Blockers on the Relative Percentage of Decrease in Malate Sensitivity of C4 PEPC in Induced Mesophyll Cell Protoplasts of *D. sanguinalis*

Treatment	Relative Decrease in L-Malate Sensitivity (%)
Dark	0
Light	5
+ NH_4Cl (10 mM)	100[a]
+ Diltiazem	
(200 μM)	96
+ Nifedipine	
(150 μM)	92
+ Verapamil	
(100 μM)	88
(200 μM)	64
+ TMB-8	
(100 μM)	60
(200 μM)	20
(250 μM)	8

[a]Arbitrarily set at 100% for the mesophyll cell protoplasts induced for 120 min in the presence of light plus (+) NH_4Cl.

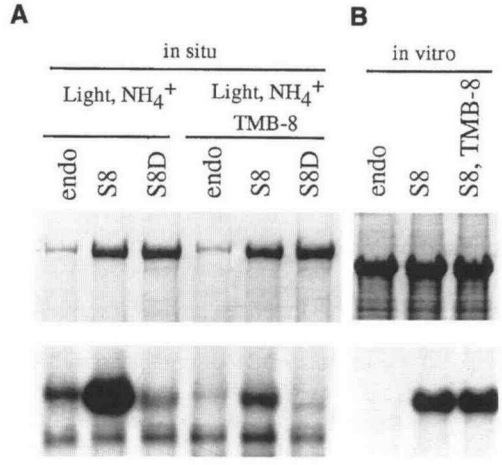

Figure 7. Effect of the Calcium Channel Blocker TMB-8 on the in Situ Induction and in Vitro Activity of PEPC Kinase in Mesophyll Cell Protoplasts.

Protoplasts were preincubated in darkness for 30 min at 25°C in suspension medium ±200 μM TMB-8. After washing thoroughly with suspension medium to remove excess TMB-8, protoplasts were illuminated (300 μE m^{-2} sec^{-1}) in the presence of 10 mM NH_4Cl. Controls were performed at the same time without pretreatment with TMB-8. After 120 min, proteins were extracted, desalted, and assayed (5 μg) in a reconstituted phosphorylation mixture in the presence of 10 μg of wild-type (S8) or mutant (S8D) C4 PEPC from sorghum or without exogenous C4 PEPC (endo).

(A) Radiolabeled proteins were analyzed by SDS-PAGE. Shown at top is a Coomassie blue–stained gel. Shown at bottom is the corresponding autoradiograph.

(B) Desalted protein extracts (5 μg) from control protoplasts incubated 120 min under light in the presence of 10 mM NH_4Cl also were used to evaluate the direct effect of TMB-8 (200 μM) on in vitro PEPC kinase activity. Radiolabeled proteins were analyzed by SDS-PAGE. Shown at top is a Coomassie blue–stained gel. Shown at bottom is the corresponding autoradiograph.

protoplasts treated with TMB-8 (Figure 7A). It was verified that the inhibitor directly alters neither the in vitro activity of PEPC kinase (Figure 7B) nor the weak base–induced increase in pHc of illuminated mesophyll cell protoplasts, as analyzed by flow cytometry (data not shown). Furthermore, given that (1) diltiazem and nifedipine are considered to be specific for calcium channels present in the plasmalemma (Godfraind, 1987), (2) TMB-8 is a potent blocker (Malagodi and Chiou, 1974) of the inositol 1,4,5-trisphosphate ($InsP_3$)–gated calcium channel that resides in the tonoplast membrane (Schumaker and Sze, 1987; Ranjeva et al., 1988), and (3) verapamil decreases the activity of a voltage-dependent calcium channel in vacuole membranes from red beet roots and *Candida albicans* (Alexandre et al., 1990; Calvert and Sanders, 1995), it is inferred that the PEPC phosphorylation cascade in induced *D. sanguinalis* mesophyll cell protoplasts involves activation of calcium channels in the tonoplast membrane and calcium release into the cytosol. Thus, it also is suggested that a calcium-dependent step acts upstream of the Ca^{2+}-independent PEPC kinase (Figure 4) in the PEPC phosphorylation pathway.

The Transduction Pathway Is Multicyclic

An additional problem to be examined in situ with the *D. sanguinalis* protoplasts was how to link calcium mobilization with the reversibly light-activated C4 PEPC kinase that was known not to require this cation for activity in in vitro assays from work with maize and sorghum leaves (Echevarria et al., 1990; Bakrim et al., 1992; Li and Chollet, 1993; Wang and Chollet, 1993). When added to an illuminated mesophyll cell protoplast suspension containing inducer (3-PGA or NH_4Cl), the calcium/calmodulin naphthalenesulfonamide antagonists W5 and W7 (Hidaka et al., 1981) and compound 48/80 (an *N*-methyl-*p*-methoxyphenethylamine polymer) were found to decrease strongly the change in malate sensitivity (phosphorylation) of PEPC in a concentration-dependent manner (Table 2). As expected, this was accompanied by an inhibition of the increase in PEPC kinase activity (Figure 8), which was also dose dependent. It should be noted that there was neither a direct inhibition of PEPC kinase activity by W7 in vitro, in agreement with the fact that this protein kinase is calcium/calmodulin independent, nor a decrease in protoplast pHc, as determined by flow cytometric analysis (data not shown). Moreover, these drugs had no apparent toxic effect on the viability of protoplasts, because an extensive light activation of the chloroplastic

Table 2. In Situ Effect of Calcium/Calmodulin–Dependent Protein Kinase Antagonists on the Relative Percentage of Decrease in Malate Sensitivity of C4 PEPC in Induced Mesophyll Cell Protoplasts

Treatment	Relative Decrease in L-Malate Sensitivity (%)
Dark	0
Light	5
+ 3-PGA	
(10 mM)	100[a]
+ W7	
(150 μM)	85
(200 μM)	30
(250 μM)	5
+ W5	
(200 μM)	75
(250 μM)	60
+ Compound 48/80	
(5 μg/mL)	20

[a]Arbitrarily set at 100% for the mesophyll cell protoplasts induced for 120 min in the presence of light plus (+) 3-PGA.

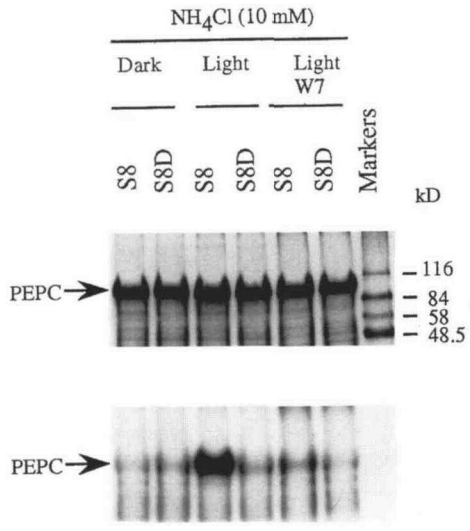

Figure 8. Effect of W7 on the in Situ Induction of PEPC Kinase Activity in Mesophyll Cell Protoplasts.

Protoplasts were preincubated in darkness for 20 min at 25°C in suspension medium ±250 μM W7. After washing thoroughly with suspension medium to remove excess W7, protoplasts were illuminated (*300 μE m^{-2} sec^{-1}) in the presence of 10 mM NH_4Cl*. A control without W7 was performed in the dark. After 120 min of incubation, proteins were extracted, desalted, and assayed (5 μg) in a reconstituted phosphorylation medium in the presence of 15 μg of wild-type (S8) or mutant (S8D) C4 PEPC. Radiolabeled proteins were analyzed by SDS-PAGE. Shown at top is a Coomassie blue–stained gel, with molecular mass markers given in kilodaltons. Shown at bottom is the corresponding autoradiograph. The positions of the PEPC subunit are indicated with arrows.

NADP–malate dehydrogenase, which reflects electron transfer capacity of the chloroplast, was still observed after the treatment. These in situ results lend support to the view that the PEPC signaling pathway occurs via a multicyclic protein kinase cascade.

The decrease in malate sensitivity of PEPC after NH_4Cl treatment of illuminated *D. sanguinalis* protoplasts, which reflects the in situ increase in the phosphorylation state of the enzyme, was prevented by the cytosolic protein synthesis inhibitor cycloheximide (Table 3). Flow cytometric analysis assured us that the weak base–dependent increase in pHc was not affected by this antibiotic (data not shown). These results with isolated C4 mesophyll cell protoplasts confirmed previous observations made using intact C4 leaves, suggesting that the light-dependent increase in PEPC kinase activity involves cytosolic protein synthesis (Jiao et al., 1991a; Bakrim et al., 1992, 1993). In contrast, the RNA polymerase II inhibitors α-amanitin and actinomycin D had no inhibitory effect on the light- and weak base–dependent phosphorylation of PEPC in situ (Table 3). Thus, it appears that the increase in PEPC kinase activity in these induced mesophyll cell protoplasts results from the activated translation of preexisting mRNA.

Is Light a Signal for the PEPC Phosphorylation Pathway? Influence of Photosynthesis Inhibitors on PEPC Phosphorylation in Mesophyll Cell Protoplasts

Both light and an increase in pHc, as induced by NH_4Cl or 3-PGA, are required for the regulatory phosphorylation of PEPC in isolated mesophyll cell protoplasts (Figures 3 and 6). In intact leaves of sorghum and maize, the photosynthesis inhibitors gramicidin (ATP synthesis), DCMU (photosystem II electron transport), and DL-glyceraldehyde (Calvin–Benson cycle) markedly inhibit the light-dependent increase in PEPC kinase activity and the concomitant increase in the apparent phosphorylation state of PEPC (McNaughton et al., 1991; Bakrim et al., 1992; Jiao and Chollet, 1992). When used with an illuminated suspension of *D. sanguinalis* protoplasts in the presence of NH_4Cl, gramicidin and DCMU acted in a similar way, blocking both the induction of PEPC kinase activity (Figure 9) and the decrease in malate sensitivity of its target protein (Table 4). In contrast, DL-glyceraldehyde was without effect (Table 4), consistent with the fact that C4 mesophyll cell protoplasts do not contain the Calvin–Benson cycle. In addition, these various photosynthesis inhibitors affected neither the increase in protoplast pHc in the presence of NH_4Cl nor PEPC kinase and PEPC activity directly in the in vitro assays (data not shown). Overall, these data support the view that, in addition to the cytosolic alkalinization of the mesophyll cell protoplast, the production of ATP and/or NADPH by the illuminated mesophyll cell chloroplast is required for this regulatory process to be achieved.

Table 3. In Situ Effect of Cytosolic Protein Synthesis and Transcriptional Inhibitors on the Relative Percentage of Decrease in Malate Sensitivity of C4 PEPC in Induced Mesophyll Cell Protoplasts

Treatment	Relative Decrease in L-Malate Sensitivity (%)
Dark	0
Light	5
+ NH_4Cl (10 mM)	100[a]
+CHX[b]	
(0.2 μM)	60
(5 μM)	18
+α-Amanitin	
(5 μg/mL)	100
(50 μg/mL)	120
+Actinomycin D	
(20 μg/mL)	122

[a] See Table 1 for details.
[b] CHX, cycloheximide.

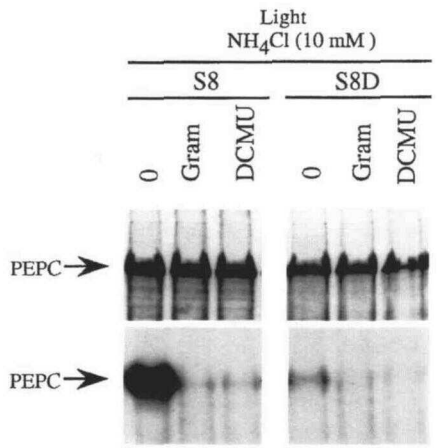

Figure 9. Effect of Photosynthesis Inhibitors on the in Situ Induction of PEPC Kinase Activity in Mesophyll Cell Protoplasts.

Protoplasts were preincubated in darkness for 20 min at 25°C in suspension medium ±50 μM gramicidin (Gram) or DCMU. After washing thoroughly with suspension medium to remove excess inhibitor, protoplasts were illuminated (300 μE m^{-2} sec^{-1}) in the presence of 10 mM NH_4Cl. A control without inhibitor was performed at the same time (0). After 120 min of incubation, proteins were extracted, desalted, and assayed (5 μg) in a reconstituted phosphorylation medium containing 15 μg of recombinant wild-type (S8) or mutant (S8D) C4 PEPC from sorghum. Radiolabeled proteins were analyzed by SDS-PAGE. Shown at top is a Coomassie blue–stained gel. Shown at bottom is the corresponding autoradiograph. The positions of the PEPC subunit are indicated with arrows.

DISCUSSION

Our results suggest that the light-dependent signaling pathway leading to the regulatory phosphorylation of C4 PEPC in leaves of *D. sanguinalis* involves intercellular cross-talk between the two photosynthetic cell types. In addition, pH, calcium, and at least two different protein kinases, including PEPC kinase and an unidentified upstream kinase, are implicated as components in the mesophyll cell cytosol.

In mesophyll cells of an illuminated C4 leaf, pHc could be the cardinal event controlling, in a concerted manner, both the functioning of the transduction chain as a second messenger and an environmental factor influencing some committed enzymatic step(s) directly. Along these lines, our data demonstrate unequivocally that the activity state of PEPC kinase (or a putative activation protein factor of this enzyme) and, thus, the phosphorylation state of PEPC depend on the amplitude of the pHc jump in mesophyll cells, reaching a maximum at a pH value close to 7.3. On the other hand, a change in pHc would dramatically affect both PEPC kinase and PEPC activities because these two enzymes strongly respond in vitro to H^+ concentrations in the range of pH 7 to 8 (Wang and Chollet, 1993; Echevarria et al., 1994). In addition, metabolite control of PEPC activity by opposing allosteric effectors, which both fine-tunes the enzyme and affects its phosphorylation by PEPC kinase, is particularly sensitive to a small shift in pH at ~7.3 (Wang and Chollet, 1993; Echevarria et al., 1994). Alkalinization of the pHc in C4 mesophyll cells in the light might result from the uptake of bundle sheath cell–generated 3-PGA, in its partially protonated form, into the chloroplast stroma (Yin et al., 1990). This mechanism does not preclude a contribution in the establishment of the pHc value by H^+-ATPase (V-type) or H^+-pyrophosphatase, which have been shown to pump protons into the vacuole in the light (Sanders et al., 1992).

Felle (1989) emphasized the fact that pHc, besides being highly regulated, is essential as an intracellular messenger in plants. For example, a modest increase in pHc (from 7.1 to 7.3) has been shown to be induced by abscisic acid and to be part of a transduction pathway in barley aleurone protoplasts (Van der Veen et al., 1992). Alkalinization of guard cells also precedes stomatal movements in epidermal strips from the orchid *Paphiopedilum tonsum* (Irving et al., 1992). Unfortunately, we have no experimental evidence to date concerning a possible light-dependent increase in C4 mesophyll cell pHc in the plant. However, there are indications that this, indeed, might occur in a variety of illuminated C4 and C3 plants (Yin et al., 1990; Raghavendra et al., 1993; Rajagopalan et al., 1993). Because the production of the photosynthesis-related metabolite message 3-PGA in bundle sheath cells is dependent on light intensity, corresponding variations in the pHc of mesophyll cells would influence the level of PEPC kinase activity and thus the phosphorylation state of PEPC, thereby providing, via pH, the coupling between the two photosynthetic cell types in a C4 leaf. This long-term covalent regulation of the enzyme would impact its short-term regulation by metabolite effectors, as discussed in a previous report (Echevarria et al., 1994), ultimately to adjust the carbon flux through this initial CO_2 fixation step to the demand of the Calvin–Benson cycle in the bundle sheath cells.

Our results also point to a role for cytosolic [Ca^{2+}] as a relay in the light signal transduction pathway of C4 PEPC. Various

Table 4. In Situ Effect of Photosynthesis Inhibitors on the Relative Percentage of Decrease in Malate Sensitivity of C4 PEPC in Induced Mesophyll Cell Protoplasts

Treatment	Relative Decrease in L-Malate Sensitivity (%)
Dark	0
Light	5
+NH_4Cl (10 mM)	100[a]
+DCMU (50 μM)	16
+Gramicidin (50 μM)	12
+DL-Glyceraldehyde (20 mM)	96

[a] See Table 1 for details.

calcium channels have been described in plant cells (Graziana et al., 1988; Johannes et al., 1992; Bush, 1993). Inhibition of PEPC phosphorylation by TMB-8 in *D. sanguinalis* mesophyll cell protoplasts suggests that the tonoplast $InsP_3$-gated channel could account for the observed regulation by calcium (Schumaker and Sze, 1987; Ranjeva et al., 1988; Sanders et al., 1992). Furthermore, in red beet microsomes of predominantly vacuolar origin, this type of calcium channel has been found to be sensitive to pH in the range of 7 to 7.5 (Brosnan and Sanders, 1993). This observation is consistent with the view that a change in pHc is needed to trigger calcium efflux from the mesophyll cell vacuole and the ensuing response of PEPC in *D. sanguinalis* protoplasts. A highly selective, voltage-gated channel also has been shown to reside in the tonoplast of red beet vacuoles, the activation of which is dependent on pH and leads to long-term regulation (Sanders et al., 1992; Bush, 1993). In vacuolar membranes of *C. albicans*, a voltage-sensitive pathway for calcium release that is inhibited markedly by verapamil also has been reported (Calvert and Sanders, 1995). Currently, experiments are in progress to identify the calcium channels in the vacuolar membrane from *D. sanguinalis* mesophyll cell protoplasts and to clarify their proposed role in controlling cytosolic $[Ca^{2+}]$ with respect to the regulatory phosphorylation of C4 PEPC by the Ca^{2+}-independent PEPC kinase.

Light provides the energy required for photosynthetic carbon assimilation in bundle sheath cells, which, in turn, produces the intercellular metabolite message 3-PGA and a pHc shift in the adjoining mesophyll cells. However, light also is required, in addition to a weak base or exogenous 3-PGA, to activate PEPC kinase and the in situ phosphorylation of PEPC in mesophyll cell protoplasts. Thus, light presumably provides ATP and/or NADPH via photosynthesis for some step(s) in the transduction pathway, particularly if a protein synthesis event related to the photoregulation of PEPC kinase is involved. On the other hand, it could also act as a signal per se, for example, via the thioredoxin pathway (Buchanan et al., 1994). Another possibility for light acting as a signal in this process comes from the fact that an $InsP_3$-gated (TMB-8 sensitive) channel has been suggested to be a chain component. A light-dependent increase in $InsP_3$ has been shown to occur in plants (Morse et al., 1989). Our results are similar to those reported on the abscisic acid–induced alkalinization of barley aleurone protoplasts in that a change in pHc is necessary but not sufficient to induce the physiological response, that is, activation of abscisic acid–regulated genes (Van der Veen et al., 1992). In this latter case, however, the change in pHc is due to the activation of a plasma membrane H^+-ATPase.

Finally, the reversibly light-activated C4 PEPC kinase is a calcium-independent serine–threonine protein kinase (Figure 4; Echevarria et al., 1990; Li and Chollet, 1993) whose activity is unaffected by the naphthalenesulfonamide W7 in vitro; yet, this agent inhibits PEPC phosphorylation in situ in induced mesophyll cell protoplasts. A consistent hypothesis is that the signaling pathway is perhaps multicyclic, involving more than one protein kinase. This putative, upstream kinase would require calcium and calmodulin for activity. The new class of protein kinases (calcium-dependent protein kinase), which contains both a protein kinase catalytic domain and a calcium binding regulatory domain similar to calmodulin within the same polypeptide, is now well documented in plants (Roberts and Harmon, 1992). A calcium/calmodulin–dependent pathway recently has been reported to transduce a light signal, via phytochrome, and contribute to chloroplast development in a tomato phytochrome-deficient mutant (Neuhaus et al., 1993). As is well documented (Jiao et al., 1991a; Bakrim et al., 1992) and confirmed in this work, the light-dependent stimulation of C4 PEPC kinase activity is due to neosynthesis of the protein kinase itself or of a putative activation factor. Thus, an attractive hypothesis is that this upstream, calcium-activated protein kinase would stimulate synthesis of either by selectively activating mRNA translation via phosphorylation of some

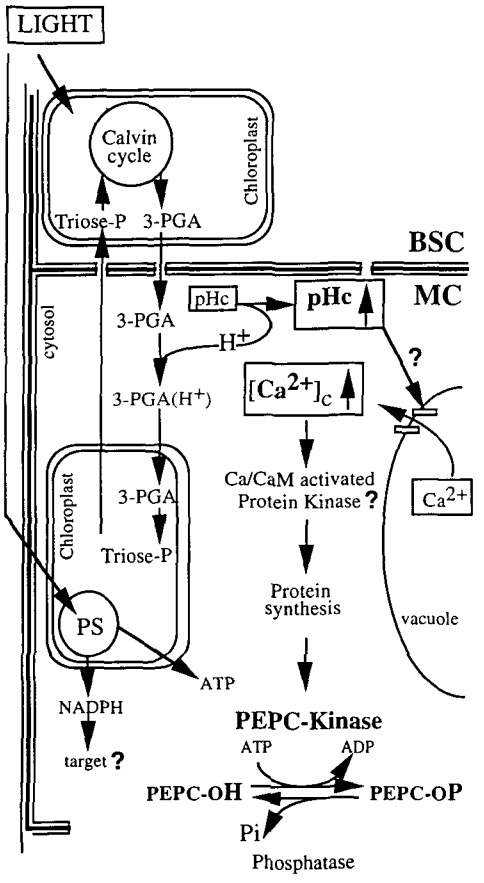

Figure 10. Schematic Working Model of the Sequential Organization of the Various Components of the C4 PEPC Phosphorylation Cascade in an Illuminated Leaf of *D. sanguinalis*.

BSC, bundle sheath cell; $[Ca^{2+}]_c$, cytosolic calcium; CaM, calmodulin; MC, mesophyll cell; P, phosphate; Phosphatase, type 2A protein phosphatase (McNaughton et al., 1991); PS, photosystems. Major points that remain to be clarified by additional experimentation are indicated by question marks.

protein factor. Activation of protein synthesis has been demonstrated to require both an increase in intracellular pH and a calcium release in unfertilized sea urchin eggs (Winkler, 1982).

Based on the collective in situ and in vitro findings presented in this report and the related literature, an integrated view of the C4 PEPC light transduction chain is presented in Figure 10 as a working model and framework for future experiments.

METHODS

Plant Material

Hairy crabgrass (*Digitaria sanguinalis*) was grown from seed (Valley Seed Co., Fresno, CA) in a controlled climate chamber (16 hr light at 27°C and 8 hr dark at 17°C). The intensity of illumination at plant level was 800 $\mu E\ m^{-2}\ sec^{-1}$, and relative humidity was 70%. Plants were watered daily with standard nutrient solution. Mesophyll cell protoplasts were isolated from fully expanded leaves (the third leaf on the main axis) harvested from 1-month-old plants at the end of the dark period.

Protoplast Isolation and Purification

Viable mesophyll cell protoplasts, devoid of significant contamination by bundle sheath and liberated organelles, were prepared according to the protocol of Pierre et al. (1992) with slight modifications. Approximately 2 g of leaf fragments (0.5- to 1-mm^2 sections) was floated on 20 mL of buffered osmoticum (0.7 M sorbitol, 10 mM Mes-KOH, pH 5.5) containing 1.5% (w/v) cellulase Onozuka-RS (Yakult Honsha Co., Tokyo), 0.3% (w/v) macerase (Calbiochem, La Jolla, CA), and 0.2% (w/v) BSA for 1.5 hr in the dark at 27°C. After incubation, the digestion medium was removed; leaf fragments were washed thoroughly with suspension medium containing 0.7 M sorbitol, 10 mM Hepes-KOH, pH 7.6. The wash solutions were pooled, filtered through a 100-μm nylon mesh, and centrifuged at 110*g* for 5 min. The pellet was resuspended in 5 mL of medium containing 0.7 M sucrose, 10 mM Hepes-KOH, pH 7.6, and 7.5% (w/v) Ficoll 400 (Pharmacia, Uppsala, Sweden). The suspension was loaded into 12-mL centrifuge tubes and overlaid with 1 mL of suspension medium. This sucrose–Ficoll/sorbitol discontinuous gradient was centrifuged at 600*g* for 5 min, and the mesophyll cell protoplasts were collected at the interface. Protoplasts were washed twice with suspension medium, pelleted by centrifugation at 110*g*, resuspended in this medium to a concentration of 2.5×10^6 protoplasts mL^{-1}, and kept in darkness at room temperature. Protoplast integrity was evaluated by light microscopy by estimating the exclusion of 0.2% (w/v) phenosafranin. More than 95% intact protoplasts were obtained routinely. Protoplasts released by this protocol were essentially all of mesophyll cell origin; any bundle sheath strands or single cells were eliminated by the 100-μm filter and the sucrose–Ficoll/sorbitol gradient. However, some leaf epidermal cells (negative for both chlorophyll and phospho*enol*pyruvate carboxylase [PEPC] activity) did remain.

Induction of PEPC Phosphorylation in Mesophyll Cell Protoplasts

Protoplast suspensions (1 mL) were maintained with gentle stirring at 25°C in 2-mL thermoregulated glass chambers. Protoplasts were either darkened or illuminated with a light source ($300\ \mu E\ m^{-2}\ sec^{-1}$), and 10 mM NH_4Cl, methylamine, or 3-phosphoglycerate (3-PGA) was added simultaneously to the suspension medium. Induction of PEPC phosphorylation was performed over a 2-hr period. Aliquots (15 μL; $\sim 3.5 \times 10^4$ protoplasts) of the protoplast suspension were removed at 20-min intervals to determine the L-malate sensitivity of PEPC activity and to estimate the activity of PEPC kinase in desalted extracts, using a ^{32}P-based assay (see below).

Assays of PEPC Activity and L-Malate Sensitivity

Optimal PEPC activity was measured spectrophotometrically (ultraviolet/visible spectrophotometer model 3000; Hitachi, Tokyo) at 340 nm and 30°C in a 1-mL assay medium containing 100 mM Hepes-KOH, pH 8, 5 mM phospho*enol*pyruvate (PEP; Boehringer Mannheim), 5 mM $MgCl_2$, 5 mM $NaHCO_3$, 0.2 mM NADH, and 5 units of NAD–malate dehydrogenase (Boehringer Mannheim). One unit of PEPC activity corresponds to the transformation of 1 μmol of substrate min^{-1} at 30°C.

Malate sensitivity (or malate test) was determined spectrophotometrically at 340 nm and 30°C in a 1-mL assay mixture containing 100 mM Hepes-KOH, pH 7.3, 1.2 mM PEP (suboptimal conditions), 5 mM $MgCl_2$, 5 mM $NaHCO_3$, 0.2 mM NADH, and 5 units of NAD–malate dehydrogenase, ±0.5 mM L-malate (Sigma). Results are expressed as a percentage of inhibition corresponding to 1 minus the ratio of PEPC activity with or without L-malate. A 15-μL aliquot of the protoplast suspension ($\sim 3.5 \times 10^4$ protoplasts) was mixed immediately with the assay medium and stirred vigorously to rupture the protoplasts. Typical malate inhibition values for PEPC activity from protoplasts maintained in the dark or after illumination (see below) were routinely ~70 and 50%, respectively.

In Vitro Phosphorylation Assays

PEPC kinase activity, extracted from mesophyll cell protoplasts according to Pierre et al. (1992), was estimated by a radiometric assay with immunopurified, nonphosphorylated recombinant C4 PEPC from sorghum as exogenous substrate (Crétin et al., 1991). In controls, a nonphosphorylatable mutant PEPC, in which the target serine at position 8 was replaced with an aspartate residue (S8D), was used to verify the specificity of PEPC phosphorylation (Wang et al., 1992; Li and Chollet, 1993). The phosphorylation reaction was performed for 45 min at 30°C; assays (50 μL) were carried out in 50 mM Tris-HCl, pH 8, 10% (w/v) sucrose, 5% (v/v) glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (Sigma), 1 μg/mL chymostatin (Sigma), 1 μg/mL leupeptin (Sigma), 0.1 μg/mL okadaic acid (Sigma), 5 mM $MgCl_2$, components of an ADP-scavenging system (4 mM phosphocreatine/10 units of creatine phosphokinase), 0.25 mM P^1P^5-di(adenosine-5′)-pentaphosphate (an adenylate kinase inhibitor), 5 to 15 μg of recombinant PEPC (S8 or S8D), 5 μg of G-25 desalted protein extract from mesophyll cell protoplast, and 1 μCi of γ-^{32}P-ATP (1 to 3 Ci/mmol, 6.7 to 20 μM). The reaction was stopped by the addition of 10 μL of dissociation buffer (250 mM Tris-HCl, pH 8, 10% [w/v] SDS, 20% [v/v] glycerol, 10% [v/v] 2-mercaptoethanol, 0.01% [w/v] bromophenol blue). The mixture was heated for 3 min at 100°C and subjected to SDS-PAGE followed by autoradiography at −80°C.

Administration of Chemical Treatments

Protoplasts were preincubated in darkness at 25°C in the presence of various pharmacological reagents (Sigma): the chemical channel blockers verapamil, TMB-8, diltiazem, or nifedipine (30 min); the cyto-

solic protein synthesis inhibitor cycloheximide (10 min); the calmodulin antagonists W7, W5, or compound 48/80 (20 min); the transcription inhibitors α-amanitin or actinomycin D (10 to 30 min); and the photosynthesis inhibitors gramidicin or DCMU (20 min). Drug concentrations are indicated in the corresponding figures 7 to 9 and tables 2 to 4. Protoplasts were washed to remove excess drug, resuspended in suspension medium, and treated with light and weak base or 3-PGA, as indicated above. Minimal (dark) and maximal (light) decreases in malate sensitivity of PEPC, as determined after a 2-hr incubation of control protoplasts without the drug, are taken as 0 and 100%, respectively. Samples were used to prepare desalted protein extracts for the determination of PEPC kinase activity (see above).

Flow Cytometric Analyses

2′,7′-*bis*-(2-Carboxyethyl)-5-(and-6)carboxyfluorescein acetoxymethyl ester (BCECF-AM), a specific pH fluorescent probe (Rink et al., 1992), was purchased from Molecular Probes (Eugene, OR). Protoplasts (2.5×10^6 protoplasts mL^{-1}) were preloaded with 10 nM BCECF-AM in suspension medium for 40 min at 25°C in the dark. The suspension was rinsed once with the same medium without dye and stored on ice for up to 5 hr in the dark. Aliquots of these preloaded protoplasts were incubated at 25°C in the dark for 10 to 30 min with the various compounds indicated in the legend to the corresponding figures 2 and 5 and subsequently analyzed with an EPICS V flow cytometer (Coulter, Hialeah, FL) at room temperature, using a 100-μm nozzle, water as sheath fluid, and an argon laser (model 2025-05; Spectra-Physics, Mountain View, CA), set at 488 nm, 300 mW. Particle rate was typically 300 protoplasts sec^{-1}. The following signals were collected: forward-angle and wide-angle light scatter and fluorescence emission through two 515-nm long-path filters (interference and absorbance filters) subsequently split with a 560-nm dichroic filter to obtain the red component of endogenous chlorophyll that was passed through a 685 ± 5–nm bandpass filter and a 560-nm shortpass filter, with 525 ± 10–nm bandpass filters to select the green emission of the fluorescein analog BCECF. Time also was retained as a parameter. Analyses usually lasted 254 sec. Kinetics were visualized with both logarithmic and linear intensity scales, the former offering an adequate dynamic range and the latter providing simple linear statistics. Unless otherwise noted, the BCECF histograms correspond to the chlorophyll-containing mesophyll cells only, because the protoplasts lacking chlorophyll were eliminated by gating out red-negative events. Furthermore, forward-angle and wide-angle light scatter were used to eliminate broken cells and debris. This methodology has been validated with weak permanent acids and bases (nigericin in high K^+ buffers at various pH values), and fluorescence intensity changes have thereby been calibrated to pH (Giglioli-Guivarc'h et al., 1996). The technique responds to reversible pH change with no detectable lag time.

Confocal Microscopy

A Sarastro 2000 confocal microscope (Molecular Dynamics, Evry, France) was used with a Nikon (Tokyo) ×40 (0.7 numerical aperature, 2-mm working distance) water immersion lens (without coverslip) to follow the BCECF fluorescence in various cellular compartments. Images were simultaneously taken in two channels, fluorescein and chlorophyll, using the following configuration: excitation at 488 nm, green emission at 530 ± 10 nm, and red emission at 610 nm with a long pass filter. Effectors, such as NH_4Cl, could be added directly to the drop of protoplasts during observation; stability of the mesophyll cell protoplasts was improved by using Petri dishes pretreated with 1.5 μg/mL polyornithine.

Immunoblotting

Immunopurified recombinant (dephosphorylated) C4 PEPC from sorghum (Crétin et al., 1991) and desalted extracts from *D. sanguinalis* mesophyll cell protoplasts were subjected to SDS-PAGE (12% polyacrylamide gel) at 100 V for 1 hr. Proteins were electroblotted onto a nitrocellulose membrane at 9 V cm^{-1} overnight at 4°C. Protein bands were immunochemically labeled by incubating the membrane for 3 hr in 10 mL of Tris-buffered saline with 10 μg of affinity-purified rabbit IgG directed against the N-terminal phosphorylation domain of the sorghum C4-type PEPC (Pacquit et al., 1995), and detection was performed with a peroxidase assay (affinity-purified goat anti-rabbit IgG horseradish peroxidase conjugate from Bio-Rad).

SDS-PAGE Analysis and Autoradiography

Denaturing electrophoresis in 12% acrylamide gels was performed as described by Laemmli (1970). Gels were stained with Coomassie Brilliant Blue R 250, dried, and autoradiographed at −80°C by using Hyper film-MP (Amersham) and an intensifying screen.

Protein Determination

Soluble proteins were measured in protoplast extracts by using the Bio-Rad assay kit according to Bradford (1976), with BSA as the standard.

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