Plant Physiology, February 1999, Vol. 119, pp. 511–520, www.plantphysiol.org © 1999 American Society of Plant Physiologists

Evidence for a Slow-Turnover Form of the Ca^{2+}-Independent Phospho*enol*pyruvate Carboxylase Kinase in the Aleurone-Endosperm Tissue of Germinating Barley Seeds[1]

Lidia Osuna, Jean-Nöel Pierre, María-Cruz González, Rosario Alvarez, Francisco J. Cejudo, Cristina Echevarría*, and Jean Vidal

Departamento de Biología Vegetal, Facultad de Biología, Universidad de Sevilla, Avenida Reina Mercedes no. 6, 41012 Sevilla, Spain (L.O., R.A., C.E.); Instituto de Bioquímica Vegetal y Fotosíntesis, Centro de Investigaciones Científicas "Isla de la Cartuja," Avda Américo Vespucio s/n, 41092 Sevilla, Spain (M.-C.G., F.J.C.); and Institut de Biotechnologie des Plantes, Unité Associée Centre National de la Recherche Scientifique, D 1128, Bâtiment 630, Université de Paris-Sud, Centre d'Orsay, cedex, France (J.-N.P., J.V.)

Phospho*enol*pyruvate carboxylase (PEPC) activity was detected in aleurone-endosperm extracts of barley (*Hordeum vulgare*) seeds during germination, and specific anti-sorghum (*Sorghum bicolor*) C_4 PEPC polyclonal antibodies immunodecorated constitutive 103-kD and inducible 108-kD PEPC polypeptides in western analysis. The 103- and 108-kD polypeptides were radiolabeled in situ after imbibition for up to 1.5 d in ^{32}P-labeled inorganic phosphate. In vitro phosphorylation by a Ca^{2+}-independent PEPC protein kinase (PK) in crude extracts enhanced the enzyme's velocity and decreased its sensitivity to L-malate at suboptimal pH and [PEP]. Isolated aleurone cell protoplasts contained both phosphorylated PEPC and a Ca^{2+}-independent PEPC-PK that was partially purified by affinity chromatography on blue dextran-agarose. This PK activity was present in dry seeds, and PEPC phosphorylation in situ during imbibition was not affected by the cytosolic protein-synthesis inhibitor cycloheximide, by weak acids, or by various pharmacological reagents that had proven to be effective blockers of the light signal transduction chain and PEPC phosphorylation in C_4 mesophyll protoplasts. These collective data support the hypothesis that this Ca^{2+}-independent PEPC-PK was formed during maturation of barley seeds and that its presumed underlying signaling elements were no longer operative during germination.

Higher-plant PEPC (EC 4.1.1.31) is subject to in vivo phosphorylation of a regulatory Ser located in the N-terminal domain of the protein. In vitro phosphorylation by a Ca^{2+}-independent, low-molecular-mass (30–39 kD) PEPC-PK modulates PEPC regulation interactively by opposing metabolite effectors (e.g. allosteric activation by Glc-6-P and feedback inhibition by L-malate; Andreo et al., 1987), decreasing significantly the extent of malate inhibition of the leaf enzyme (Carter et al., 1991; Chollet et al., 1996; Vidal et al., 1996; Vidal and Chollet, 1997). These metabolites control the rate of phosphorylation of PEPC via an indirect target-protein effect (Wang and Chollet, 1993; Echevarría et al., 1994; Vidal and Chollet, 1997).

Several lines of evidence support the view that this protein-Ser/Thr kinase is the physiologically relevant PEPC-PK (Li and Chollet, 1993; Chollet et al., 1996; Vidal et al., 1996; Vidal and Chollet, 1997). The presence and inducible nature of leaf PEPC-PK have been established further in various C_3, C_4, and CAM plant species (Chollet et al., 1996). In all cases, CHX proved to be a potent inhibitor of this up-regulation process so that apparent changes in the turnover rate of PEPC-PK itself or another, as yet unknown, protein factor were invoked to account for this observation (Carter et al., 1991; Jiao et al., 1991; Chollet et al., 1996). Consistent with this proposal are recent findings about PEPC-PK from leaves of C_3, C_4, and CAM plants that determined activity levels of the enzyme to depend on changes in the level of the corresponding translatable mRNA (Hartwell et al., 1996).

Using a cellular approach we previously showed in sorghum (*Sorghum bicolor*) and hairy crabgrass (*Digitaria sanguinalis*) that PEPC-PK is up-regulated in C_4 mesophyll cell protoplasts following illumination in the presence of a weak base (NH_4Cl or methylamine; Pierre et al., 1992; Giglioli-Guivarc'h et al., 1996), with a time course (1–2 h) similar to that of the intact, illuminated sorghum (Bakrim et al., 1992) or maize leaf (Echevarría et al., 1990). This light- and weak-base-dependent process via a complex transduction chain is likely to involve sequentially an increase in pHc, inositol trisphosphate-gated Ca^{2+} channels

[1] This research was supported by grants from the Dirección General de Investigación Científica y Técnica (no. PB 92-0675), the Dirección General de Ensenan za Superior e Investigacion Científica (no. B1097-1205-C02-02), and the Acción Integrada Hispano-Francesa (no. HF96-0200), and by the Grupo de Investigacion de Fisiología Vegetal from La Junta de Andalucía. This work was also partially supported by the Rhone-Poulenc Agro S.A., Torre de la Reina, Sevilla, Spain.

* Corresponding author; e-mail echeva@cica.es; fax 34–95–461–5780.

Abbreviations: APS, antiphosphorylation site; BDA, blue dextran-agarose; CHX, cycloheximide; PEPC, PEP carboxylase; PK, protein kinase; pHc, cytosolic pH; TMB-8, 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride; W5, *N*-(6-aminohexyl)-1-naphthalenesulfonamide hydrochloride; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride.

of the tonoplast, an increase in cytosolic Ca^{2+}, a Ca^{2+}-dependent PK, and PEPC-PK.

Considerably less is known about the up-regulation of PEPC-PK and PEPC phosphorylation in nongreen tissues. A sorghum root PEPC-PK purified on BDA was shown to phosphorylate in vitro both recombinant C_4 PEPC and the root C_3-like isoform, thereby decreasing the enzyme's malate sensitivity (Pacquit et al., 1993). PEPC from soybean root nodules was phosphorylated in vitro and in vivo by an endogenous PK (Schuller and Werner, 1993; Zhang et al., 1995; Zhang and Chollet, 1997). A Ca^{2+}-independent nodule PEPC-PK containing two active polypeptides (32–37 kD) catalyzed the incorporation of phosphate on a Ser residue of the target enzyme and was modulated by photosynthate transported from the shoots (Zhang and Chollet, 1997). Regulatory seryl phosphorylation of a heterotetrameric ($\alpha_2\beta_2$) banana fruit PEPC by a copurifying, Ca^{2+}-independent PEPC-PK was shown to occur in vitro (Law and Plaxton, 1997). Although phosphorylation was also detected in vivo and found to concern primarily the α-subunit, PEPC exists mainly in the dephosphorylated form in preclimacteric, climacteric, and postclimacteric fruit.

In a previous study we showed that PEPC undergoes regulatory phosphorylation in aleurone-endosperm tissue during germination of wheat seeds (Osuna et al., 1996). Here we report on PEPC and the requisite PEPC-PK in germinating barley (*Hordeum vulgare*) seeds. PEPC was highly phosphorylated by a Ca^{2+}-independent Ser/Thr PEPC-PK similar to that found in other plant systems studied previously (Chollet et al., 1996); however, the PK was already present in the dry seed and its activity did not require protein synthesis during imbibition.

MATERIALS AND METHODS

Materials

Barley (*Hordeum vulgare* cv Beka) seeds were sterilized in 2% (v/v) NaOCl for 20 to 30 min and washed sequentially with sterile water, 10 mM HCl, and sterile water. Whole or de-embryonated seeds were placed on filter papers soaked with sterile water in a glass Petri dish and allowed to imbibe for the necessary time at room temperature and in the presence or absence of different inhibitors, as indicated in the figure legends. APS-IgG and polyclonal antibodies were raised against a synthetic peptide encompassing the N-terminal regulatory domain of C_4 PEPC from sorghum (*Sorghum bicolor*) and the native enzyme, respectively (Vidal et al., 1981; Pacquit et al., 1995). [γ-^{32}P]ATP (10 Ci/mmol) and [^{32}P]Pi (200 Ci/mol) were purchased from Amersham, Cellulase-RS was from Yakult Honsha (Tokyo, Japan), Macerase pectinase was from Calbiochem, goat anti-rabbit IgG horseradish peroxidase conjugate was from Bio-Rad, and protein A-Sepharose was from Pharmacia. All other reagents were from Sigma.

Seed Extracts

Eight de-embryonated seeds were chopped and ground thoroughly in a chilled mortar with washed sand and 1 mL of buffer A (0.1 M Tris-HCl, pH 8.0, 5% [v/v] glycerol, 1 mM EDTA, 10 mM $MgCl_2$, 10 μg mL^{-1} chymostatin, 10 μg mL^{-1} leupeptin, 1 mM PMSF, 1 μM microcystin-LR, 50 mM KF, and 14 mM 2-mercaptoethanol). The homogenate was centrifuged at 15,000*g* for 4 min at 4°C, and the supernatant fluid (20 μL) was used as a crude extract.

For in vitro phosphorylation of seed PEPC by endogenous PEPC-PK, proteins were extracted from 15 de-embryonated seeds in 2 mL of buffer A. After centrifugation at 45,000*g* for 10 min, proteins were precipitated from the supernatant fraction by the addition of $(NH_4)_2SO_4$ to 60% saturation, sedimented by centrifugation at 45,000*g* for 5 min, and resuspended in 400 μL of buffer A. The protein preparation was clarified by centrifugation at 45,000*g* for 5 min before use.

Isolation of Protoplasts

De-embryonated seeds were cut in half longitudinally, sterilized, and soaked for 3 d in distilled water at 30°C in the dark. Endosperm, testa, and pericarp were removed, and the aleurone layers were incubated for 20 to 22 h at 28°C in the dark in 5 mL of digestion medium containing 20 mM Mes, pH 5.5, 0.5 M mannitol, 4% (w/v) Cellulase, 2% (w/v) Macerase pectinase, and 10 μg mL^{-1} chloramphenicol, with gentle shaking. The digestion medium was sterilized by filtration through a 0.22-μm filter (Millipore). After digestion the seed halves were washed thoroughly with wash medium containing 0.5 M mannitol and 20 mM Hepes, pH 7.0. Protoplasts were pooled and filtered through a 75-μm nylon net and pelleted by centrifugation at 600*g* for 5 min. The pellet was resuspended in 5 mL of wash medium, centrifuged, and washed again. Finally, the pellet was resuspended in 0.25 mL of buffer A, and soluble proteins were extracted by passing the protoplast suspension several times through the needle of a microliter syringe (Hamilton, Reno, NV).

Partial Purification of PEPC-PK from De-Embryonated Seeds and Aleurone Protoplasts

All procedures were carried out at 4°C. Crude extracts from de-embryonated seeds were prepared (100 seeds per 12 mL) in buffer B (50 mM Tris-HCl, pH 8.0, 5% [v/v] glycerol, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF). The homogenate was centrifuged at 150,000*g* for 15 min in an ultracentrifuge. The supernatant fraction (44 mg of protein) was loaded at a flow rate of 0.2 mL min^{-1} on a small column containing 2 mL of BDA equilibrated with buffer C (50 mM Tris-HCl, pH 8.0, 5% [v/v] glycerol, and 1 mM DTT). After the column was rinsed with 40 mL of buffer C, bound proteins were eluted with 0.5 M NaCl in buffer C. Eluted proteins were precipitated by $(NH_4)_2SO_4$ to 60% saturation for 30 min and then centrifuged at 15,000*g* for 15 min. The protein pellet was resuspended in 200 μL of buffer D (50 mM Tris-HCl, pH 7.8, 20% [v/v] glycerol, and 1 mM DTT) and then desalted by dialysis against two changes (100 mL each) of this same buffer for 2 h before use in phosphorylation assays. The purification procedure was essentially the same when aleurone protoplasts were used

as the source of PEPC-PK. Extracted proteins in buffer B (0.7 mg of protein per 250 μL) were loaded onto a BDA column equilibrated with buffer C (500 μL of packed gel). In this case, the high-salt-eluted proteins were desalted by gel filtration through Sephadex G-25 (1.4 mL of deposited gel) equilibrated with buffer D. The final volume of the preparation was 200 μL.

In Vitro Phosphorylation Assays

An aliquot (45 μL) of BDA-purified aleurone-endosperm PEPC-PK from protoplasts or 20 μL of BDA PEPC-PK from de-embryonated seeds (in buffer D) was incubated with 6 μg of recombinant, nonphosphorylated sorghum C_4 PEPC (Pacquit et al., 1993). The assay mixture (70 μL) consisted of 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 50 μM $CaCl_2$, 20% (v/v) glycerol, 1 mM DTT, 1 μM microcystin-LR, and 8 μM [γ-^{32}P]ATP (3 μCi). After the sample was incubated for 1 h at 30°C in the presence or absence of 1 mM EGTA, the assays were made 1% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, and 0.002% (w/v) bromphenol (final concentrations) and heated for 2 min at 90°C. The samples were then subjected to SDS-PAGE (10% [w/v] acrylamide) and autoradiography (Echevarría et al., 1990).

For PEPC-phosphorylation assays, proteins were partially purified from crude extracts by $(NH_4)_2SO_4$ precipitation as described above. The malate sensitivity of PEPC was recorded in the absence or presence of 1 mM L-malate before and after incubation of the extract in the presence or absence of either 2.5 mM ATP or 2.5 mM ATP plus 2.5 mM EGTA for up to 1 h at 30°C.

In Situ ^{32}P Labeling and Immunoprecipitation

Four de-embryonated seed halves were soaked in 200 μL of distilled water and 200 μCi of [^{32}P]Pi in the absence (control) or presence of various inhibitors (CHX, W7, W5, or TMB-8). After 36 h of imbibition at room temperature, about one-half of the solution was absorbed by the seeds. The seeds were washed to remove nonabsorbed [^{32}P]Pi, and proteins were extracted as described above in 400 μL of buffer E containing 100 mM Tris-HCl, pH 7.5, 20% (v/v) glycerol, 1 mM EDTA, 10 mM $MgCl_2$, 10 μg mL^{-1} chymostatin, 10 μg mL^{-1} leupeptin, 1 mM PMSF, 14 mM 2-mercaptoethanol, 50 mM KF, and 1 mM nonradioactive ATP (the addition of ATP was to minimize in vitro ^{32}P phosphorylation). The homogenate was centrifuged at 12,000*g* for 2 min.

One-hundred microliters of the clarified supernatant fraction (0.06 unit of PEPC, which is about 225 μg of total protein) was immediately mixed with SDS sample buffer (1% [w/v] SDS, 20% [v/v] glycerol, 10% [v/v] 2-mercaptoethanol, and 0.002% [w/v] bromphenol blue, final concentrations). In parallel, 140 μL of the supernatant fluid (0.084 unit of PEPC) was incubated with the appropriate amount of purified polyclonal C_4 PEPC IgG from sorghum (34 μg of protein) for 1 h on ice. Protein A-Sepharose beads were added to the incubated sample to 4% (w/v) and vortexed briefly with a 5-min interval (Osuna et al., 1996). The beaded immunocomplexes were sedimented by centrifugation at 12,000*g* for 5 min, washed once with buffer containing 0.5 M Tris-HCl, pH 8.0, 1.5 M NaCl, and 1% (v/v) Triton X-100, and once with 0.1 M Tris-HCl alone. The pellet was resuspended in SDS sample buffer, heated for 10 min at 90°C, and centrifuged for 5 min at 12,000*g* at room temperature.

Both denatured preparations were analyzed by SDS-PAGE (10% [w/v] acrylamide). Proteins in the gels were either stained with Coomassie blue (total proteins) or transferred to a nitrocellulose membrane (immunoprecipitated PEPC); both samples were autoradiographed (3 d at −80°C) or analyzed with a phosphor imager (Fujix BAS 1000, Fuji, Tokyo, Japan), and the seed PEPC was immunocharacterized as described below.

Seed PEPC Immunocharacterization and Electrophoresis

The samples were subjected to SDS-PAGE (8% or 10% [w/v] acrylamide, according to the method of Laemmli, 1970) for 14 h at 85 V and room temperature in an electrophoresis cell (Bio-Rad). For western-blot experiments, proteins were electroblotted onto a nitrocellulose membrane (N-8017, Sigma) at 10 V (3 mA cm^{-2}) for 75 min in a semidry transfer-blot apparatus (Bio-Rad). Protein bands were immunochemically labeled by overnight incubation of the membrane at room temperature in 20 mL of TBS (20 mM Tris-HCl and 0.15 M NaCl, pH 7.5) containing protein A-purified polyclonal C_4 PEPC IgG (28 μg of protein). Subsequent detection was with a peroxidase assay using affinity-purified goat anti-rabbit IgG horseradish peroxidase conjugate.

Determination of PEPC Activity and Apparent Phosphorylation State

PEPC activity was recorded spectrophotometrically at 30°C at optimal (8.0) and suboptimal (7.3 and 7.1) pH values using the NAD-malate dehydrogenase-coupled assay (at 2.5 mM PEP) described by Echevarría et al. (1994). Assays were initiated by addition of an aliquot of crude seed extract. An enzyme unit was defined as the amount of PEPC that catalyzed the carboxylation of 1 μmol PEP min^{-1} at pH 8.0 and 30°C. In the APS-IgG-binding assay, crude extracts (25 μL, 0.02 unit of PEPC) were incubated with 5 μL (10 μg of protein) of affinity-purified APS-IgG for 5 min at 0°C, and then activity was recorded at pH 7.1. The apparent phosphorylation state of PEPC was estimated by using the velocity test, which is the PEPC activity at pH 8.0 divided by the PEPC activity at pH 7.1 (Echevarría et al., 1994; Osuna et al., 1996); the APS-IgG test, which is the PEPC activity at pH 7.1 plus a saturating amount of the APS-IgG divided by the PEPC activity at pH 7.1 (Pacquit et al., 1995; Osuna et al., 1996); and the malate sensitivity test, which is the PEPC activity at pH 7.3 in the presence or absence of 1 mM L-malate (Bakrim et al., 1992). Protein was assayed by a sensitive dye-binding method (Bradford, 1976) using BSA as a standard.

RESULTS

Characterization of PEPC in Protein Extracts from Dry and Germinating Barley Seeds

Barley seeds were soaked for up to 4 d, and proteins from aleurone endosperm were extracted at different times, resolved by SDS-PAGE, and analyzed by western blotting (Fig. 1A). Polyclonal IgGs (raised against C_4 PEPC from sorghum) immunodecorated a constitutive 103-kD polypeptide in both dry and soaked seeds, and a newly formed 108-kD polypeptide was detected after 1 d of imbibition (Fig. 1A, lane 1) and was present for up to 4 d (Fig. 1A, lanes 2 and 4). No bands were detected with preimmune serum, and sorghum C_4-PEPC IgG immunoprecipitated all of the PEPC activity from the barley seed extract (data not shown). We previously obtained similar results for wheat seed PEPC using the same sorghum C_4-PEPC IgG and preimmune serum (González et al., 1998). During this imbibition period PEPC activity did not change significantly when expressed on a per-seed basis, whereas its specific activity on a total-soluble-protein basis showed an approximately 3-fold increase (Fig. 1B); this was likely due to the mobilization of proteins from aleurone-endosperm tissues (Osuna et al., 1996).

In Situ Phosphorylation of Barley Seed PEPC

The in situ phosphorylation of PEPC during imbibition was established after the seeds were fed for 36 h with

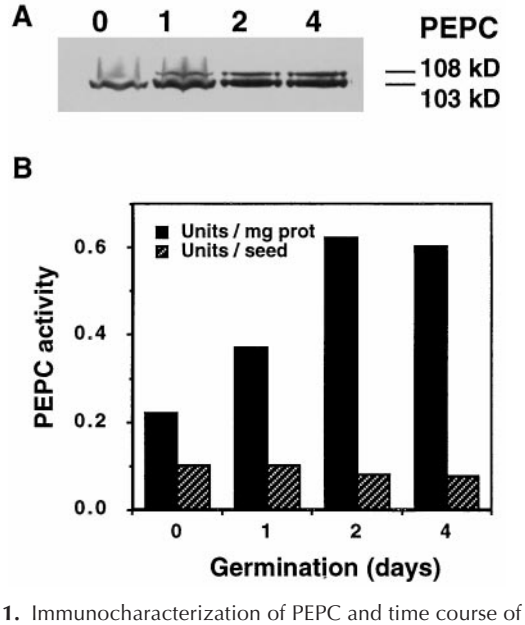

Figure 1. Immunocharacterization of PEPC and time course of PEPC activity changes during germination of barley seeds. A, At the indicated times, soaked whole seeds were de-embryonated and soluble protein from crude extracts (34 μg) was resolved by SDS-PAGE, transferred onto nitrocellulose, and probed with polyclonal anti-C_4 PEPC IgGs (28 μg per 15 mL of incubation medium). Immunolabeled proteins were detected by a horseradish peroxidase assay. B, Equivalent preparations were assayed for PEPC activity at optimal pH (8.0) and [PEP] (2.5 mM). Results are the means of six experiments, which did not vary by more than 10%.

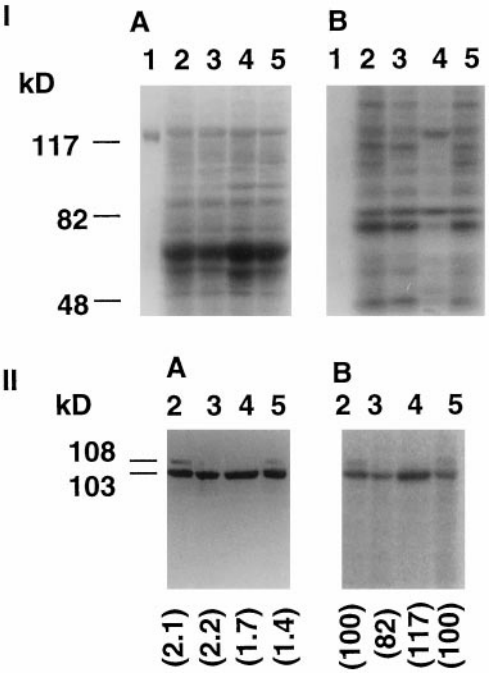

Figure 2. Effects of various pharmacological reagents on in situ protein phosphorylation in germinating barley seeds. Four de-embryonated seed halves were soaked in 200 μL of distilled water and 200 μCi of [^{32}P]Pi, in the absence or presence of 25 μM CHX, 0.5 mM W7, or 0.5 mM TMB-8 for 36 h. Following these treatments soluble protein extracts were prepared and resolved by SDS-PAGE (10% acrylamide) and visualized by Coomassie blue staining (I, A) and autoradiography (I, B). Alternatively, the PEPC was immunoprecipitated, electrophoresed, and transferred onto nitrocellulose for staining (II, A) and autoradiography (II, B). Lanes 1, Purified, recombinant sorghum C_4 PEPC (5 μg); lanes 2, control seeds; lanes 3, CHX-treated seeds; lanes 4, W7-treated seeds; and lanes 5, TMB-8 treated seeds. Numbers in parentheses represent the apparent phosphorylation state of PEPC from aleurone endosperm of soaked de-embryonated seed halves in the presence of the corresponding pharmacological reagents and in the control seeds using the velocity test (A) and relative quantification (radioactive PEPC band expressed as a percentage of the control) of the radiolabeled 103-kD PEPC subunit using an image analyzer (B).

[^{32}P]Pi and subsequent immunoprecipitation of the enzyme from crude extracts. Both the 103- and 108-kD polypeptides were radiolabeled (Fig. 2II, B, lane 2). Consistent with this preliminary observation, malate inhibition of PEPC decreased from 80% to 20% in protein extracts partially purified by $(NH_4)_2SO_4$ precipitation, indicating that the protein becomes highly phosphorylated following seed imbibition. However, in many instances (Nimmo et al., 1986; Rajagopalan et al., 1994; Chollet et al., 1996), PEPC has been reported to be highly sensitive to partial proteolytic degradation in crude extracts despite the presence of proteinase inhibitors. Since germinating seeds contain high levels of proteolytic enzymes, and truncation of the N-terminal phosphorylation domain causes the enzyme's functional and regulatory properties to be modified, we devised two distinct, more reliable procedures to estimate the apparent phosphorylation status of PEPC in seed extracts.

Using sorghum recombinant C_4 PEPC (Echevarría et al., 1994) and wheat seed C_3 PEPC (Osuna et al., 1996), we showed previously that the determination of the activity ratio at pH 8.0/7.1 was a sensitive and accurate way to estimate the degree of the enzyme's apparent phosphorylation state in rapidly prepared protein extracts from the corresponding organ. Indeed, phosphorylation induced a large increase in PEPC activity when measured at suboptimal pH and [PEP] values (7.1 and 2.5 mM, respectively), whereas there was little change at optimal pH (8.0). For the purified sorghum C_4 enzyme extensively phosphorylated in vitro in the presence of the catalytic subunit of mammalian cAMP-dependent PK, the phosphorylation-dependent stimulation of activity was found to be in the range of 5.5- to 7-fold at pH 7.1 and approximately 1.35-fold at pH 8.0 (Echevarría et al., 1994). Dry and imbibed barley seeds were collected and the soluble protein from aleurone-endosperm was extracted. The activity ratio of PEPC decreased from 6.5 in dry seeds to 2.8 after 1 d of imbibition (Table I). A similar trend was also observed (from 6.3 to 2.2) after 1 d of imbibition of de-embryonated seeds (Table I). For sorghum C_4 PEPC, this result suggests that the constitutive 103-kD PEPC subunit is mainly in a nonphosphorylated state in the dry seed and undergoes phosphorylation during imbibition, along with the newly formed 108-kD subunit.

A second procedure was based on the use of APS-IgG, which in previous work with sorghum (Pacquit et al., 1995) and wheat (Osuna et al., 1996) was shown to mimic the effect of phosphorylation on the regulatory Ser. As a reference, upon rapid binding of a saturating amount of the antibody to the nonphosphorylated enzyme, C_4 PEPC activity at pH 7.1 increased approximately 7- to 9-fold, whereas the fully phosphorylated species, which binds the antibody equally well (Pacquit et al., 1995), responded relatively weakly to this treatment (1.5-fold increase). In addition, because of the rapidity of the assay (the APS-IgG effect is almost instantaneous) and the protection of PEPC by the bound antibodies against possible proteolytic degradation of the corresponding N-terminal domain, this test ensures that the increase in the measured PEPC activity at pH 7.1 is due to an N-terminally intact form of the enzyme.

In practice, a saturating amount of affinity-purified APS-IgG (or preimmune serum in the controls) was added to rapidly prepared protein extracts from dry or soaking seeds. PEPC activity was measured spectrophotometrically in parallel cuvettes at pH 7.1, and the experiment was completed within minutes. The results depicted in Table I confirm those of the malate sensitivity and velocity tests and are also consistent with extensive phosphorylation of PEPC during germination of barley seeds. The fact that PEPC phosphorylation showed a similar trend in the aleurone endosperm from both soaked whole or de-embryonated seeds (Table I) shows that GAs produced in the embryo were not necessary to trigger this regulatory mechanism.

Characterization of Barley Seed PEPC-PK and a Pharmacological Study of the Signal Transduction Chain Leading to the Phosphorylation of PEPC

In a second set of experiments we addressed the question of whether the upstream signaling components of the cascade leading to the phosphorylation of PEPC, as identified in C_4 mesophyll protoplasts (Pierre et al., 1992; Giglioli-Guivarc'h et al., 1996), are also key elements in seeds. Protoplasts were prepared from aleurone cell layers dissected from seeds after 3 d of imbibition. In crude protein extracts from these protoplasts, the 103- and 108-kD PEPC subunits were immunodetected by western blotting (Fig. 3I, lanes 1 and 2). Based on the velocity and APS-IgG tests, aleurone-protoplast PEPC was found to be highly phosphorylated (data not shown). Extracted proteins were also subjected to chromatography on BDA according to the classical procedure described by Jiao and Chollet (1989),

Table I. *Time course of changes in the apparent phosphorylation state of barley seed PEPC during germination estimated by using the malate sensitivity, velocity, and APS-IgG tests on whole seeds and on de-embryonated seed halves*

At the indicated time after imbibition of whole or de-embryonated half seeds, proteins from aleurone endospem were rapidly extracted and PEPC activity was determined at different pH values and a [PEP] of 2.5 (or 0.5) mM. Values are the average of two independent experiments, which did not vary by more than 15%.

Imbibition Time	PEPC Activity				
	Malate Sensitivity Test[a]	Velocity Test		APS-IgG Test[b]	
		Whole	Half	Whole	Half
d	*% inhibition*			*ratio*	
0 (dry seeds)	80	6.5	6.3	5.5	5.5
1	20	2.8	2.2	2.6	1.6
2	–	1.8	–	–	–
4	–	1.8	–	–	–

[a] Whole seeds were assayed at pH 7.3 and 0.5 mM PEP. L-Malate sensitivity is expressed as a percentage of inhibition of PEPC activity in the absence of inhibitor. [b] Determination of PEPC activity in the presence of APS-IgG. Crude extract (25 μL, 0.02 unit of PEPC) was preincubated with 10 μg of affinity-purified APS-IgG for 5 min at 0°C, and the minus APS-IgG control was performed under the same conditions with an equivalent amount of preimmune serum.

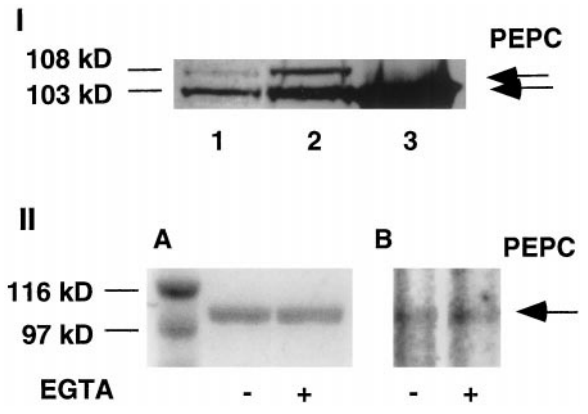

Figure 3. Immunocharacterization of PEPC and identification of PEPC-PK in protein extracts from barley aleurone protoplasts. Protoplasts were isolated from aleurone layers dissected from seeds after 3 d of imbibition. I, PEPC immunoblots. Lane 1, Proteins from aleurone protoplasts (1.4 milliunits of PEPC, 6 μg of protein); lane 2, proteins from aleurone layers (6.6 milliunits of PEPC, 24 μg of protein); lane 3, purified, recombinant C_4 PEPC from sorghum (15 milliunits of PEPC, 54 μg of protein). Proteins were resolved by SDS-PAGE (8% acrylamide) and then transferred onto nitrocellulose membranes. II, In vitro phosphorylation assays performed in the presence of exogenous, immunopurified C_4 PEPC from sorghum (0.2 unit of PEPC), BDA-purified proteins from aleurone protoplasts (45 μL), and the other components of the reconstituted phosphorylation reaction in the presence (+) or absence (−) of 1 mM EGTA. Proteins were resolved by SDS-PAGE (10% acrylamide) and autoradiographed. A, Coomassie blue-stained gel. B, Corresponding autoradiograph. The unmarked lane in A corresponds to the stained protein standards.

which, in previous experiments on various plant materials, partially purifies the Ca^{2+}-independent PEPC-PK (Chollet et al., 1996).

BDA-purified protoplast proteins were assayed in reconstituted phosphorylation reactions in the presence of nonphosphorylated, recombinant sorghum C_4 PEPC as a target and other components of the phosphorylation reaction. The autoradiograph of proteins subsequently resolved by SDS-PAGE shows that PEPC was radiolabeled in vitro by a BDA-purified PK whose activity was insensitive to the presence of the Ca^{2+} chelator EGTA (Fig. 3II, B). Therefore, a homolog of the PEPC-PK identified in other plant systems was present in aleurone cell protoplasts from germinating barley seeds.

Since protoplasts prepared from aleurone layers after 3 d of imbibition had a phosphorylated PEPC, we used de-embryonated seeds to investigate the possible regulatory cascade leading to the control of PEPC-PK activity and PEPC phosphorylation in situ. De-embryonated seeds were soaked for 24 h, during which time the phosphorylation of PEPC occurred (Table I) in the absence or presence of one of the following components: propionic or acetic acid (to lower pHc); TMB-8, a tonoplast-directed Ca^{2+}-channel blocker; W7, a calmodulin antagonist; CHX, a cytoplasmic protein-synthesis inhibitor (Giglioli-Guivarc'h et al., 1996); and W5, a less-active analog of W7 (Hidaka et al., 1981). However, none of these pharmacological reagents had a significant effect on PEPC phosphorylation in situ, as judged by the velocity test (data shown in Fig. 2II, A) and the APS-IgG test (data not shown). Incubation with propionic or acetic acid also did not affect PEPC phosphorylation. The values from the velocity test were 1.98 and 2.10, respectively.

A related phosphorylation experiment was carried out to ensure that the pharmacological reagents were taken up by the seeds and to examine in more detail the effects of these drugs in vivo. After the seeds were soaked for 36 h in the presence of [^{32}P]Pi and CHX, W7, W5, or TMB-8, soluble-protein extracts and immunoprecipitated PEPC were analyzed by SDS-PAGE. The various drugs did not change the protein patterns qualitatively, as judged by Coomassie blue staining of the gel, although some quantitative alterations were seen (Fig. 2I, A). This was also true for the radiolabeled protein patterns in the corresponding autoradiograph (Fig. 2I, B) with the exception of those with CHX (Fig. 2I, B, lane 3) and, more markedly, those with W7 (Fig. 2I, B, lane 4). In the latter case, this calmodulin antagonist inhibited the phosphorylation of many proteins, most of those detected in the control (Fig. 2I, B, lane 2).

The radiolabel in the major, 103-kD PEPC subunit was analyzed in more detail by PEPC immunoprecipitation and found not to be modified by TMB-8 (Fig. 2II, B, lane 5) and to be either slightly decreased or increased by CHX and W7, respectively (Fig. 2II, B, lanes 3 and 4). These latter changes could be at least partially accounted for by corresponding variations in the amount of immunoprecipitated PEPC (Fig. 2II, A, lanes 3, 4). The radiolabel in the minor, 108-kD subunit was detectable in the control and TMB-8-treated seeds (Fig. 2II, B, lanes 2 and 5) but was essentially absent in the CHX- and W7-treated samples (Fig. 2II, B, lanes 3 and 4). Both compounds blocked the accumulation of the 108-kD subunit (Fig. 2II, A, lanes 3 and 4), suggesting that a Ca^{2+}-/calmodulin-dependent event might be involved in the accumulation of this inducible PEPC polypeptide. W5 did not significantly affect either the radiolabeled protein pattern or the accumulation of the 108-kD PEPC polypeptide (data not shown). Therefore, the drugs were taken up by the seeds in sufficient amounts to permit specific alterations to be exerted on a variety of proteins, the major 103-kD PEPC polypeptide underwent modest changes at best in terms of its phosphorylation state (Fig. 2II, B).

These results and those of the related velocity test (Fig. 2II, A, numbers in parentheses) and APS-IgG test (data not shown) suggest that the transduction chain controlling PEPC-PK activity in germinating barley seeds is either at variance with that in C_4 mesophyll protoplasts and leaves or is only weakly active at the developmental stage examined. Assuming that this latter possibility is valid, then PEPC-PK activity had to be already present in the dry seed to account for the observed in situ phosphorylation of PEPC during early imbibition. To test this possibility, protein extracts from dry and 24-h soaked, de-embryonated seeds were subjected to BDA chromatography. SDS-PAGE separation of proteins from reconstituted phosphorylation assays containing an aliquot of this protein fraction and a nonphosphorylated, purified C_4 PEPC from sorghum

showed that the Ca^{2+}-independent PEPC-PK was even more active in dry seeds than in soaked samples (Fig. 4B).

The addition of ATP to an aliquot of a crude $(NH_4)_2SO_4$ fraction (0%–60% saturation) obtained from de-embryonated dry seeds led to a markedly decreased malate sensitivity of endogenous PEPC from 80% to 16%, and the Ca^{2+} chelator EGTA had no significant effect on this change (Fig. 5). Consistent with previous results from in situ radiolabeling experiments, this latter observation shows that essentially no Ca^{2+}-dependent PEPC-PK activities are present in seeds. Therefore, we conclude that dry barley seeds contain a slow turnover, Ca^{2+}-independent PEPC-PK that starts phosphorylating PEPC in the aleurone tissue upon imbibition and maintains a high phosphorylation state of the target enzyme during subsequent germination to achieve efficient protection against feedback inhibition by L-malate.

DISCUSSION

PEPC specific activity was shown to increase during the germination of barley seeds, whereas activity expressed on a per-seed basis remained almost constant. Western analysis revealed that anti-sorghum C_4 PEPC polyclonal antibodies detected two main polypeptides with molecular masses of 103 and 108 kD. These results are reminiscent of wheat and castor seed PEPC, for which an inducible (108-kD) PEPC subunit was immunocharacterized during the same stage of germination (Sangwan et al., 1992; Osuna et al., 1996).

Malate sensitivity and velocity tests suggested that PEPC is phosphorylated in vivo in soaking seeds. The APS-IgG test (Pacquit et al., 1995) confirmed these results and also ensured both the presence of the N-terminal phosphorylation domain and the intactness of the PEPC N terminus in seed extracts (Nimmo et al., 1986; Rajagopalan et al., 1994). The major 103-kD and the minor 108-kD PEPC polypeptides were also shown to be radiolabeled in situ. These collective data not only established phosphorylation of PEPC as a mechanism that regulates the activity of the cereal grain enzyme in vivo (Osuna et al., 1996) but also

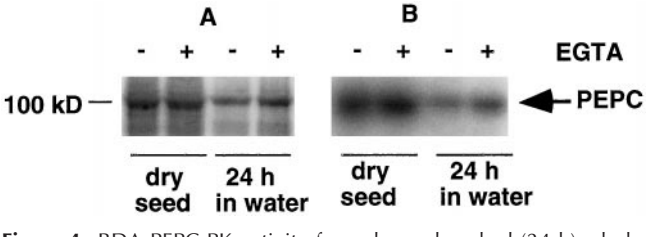

Figure 4. BDA PEPC-PK activity from dry and soaked (24 h) whole seeds. BDA PEPC-PK from aleurone endosperm was isolated chromatographically, and in vitro phosphorylation assays were performed in the presence of exogenous, immunopurified C_4 PEPC from sorghum (0.2 unit of PEPC), BDA-purified proteins from aleurone (20 μL), and the other components of the reconstituted phosphorylation reaction in the presence (+) or absence (−) of 1 mM EGTA. Radiolabeled proteins were resolved by SDS-PAGE (10% acrylamide) and detected by autoradiography. A, Coomassie blue-stained gel. B, Corresponding autoradiograph.

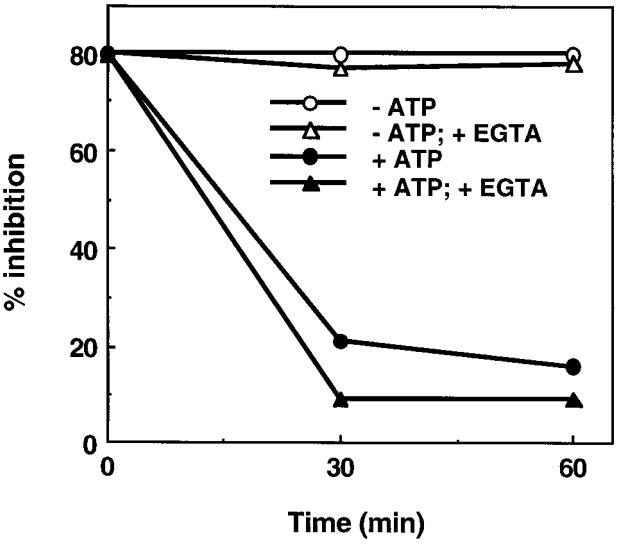

Figure 5. ATP-dependent changes in malate sensitivity of PEPC in protein extracts from aleurone endosperm of dry seeds. $(NH_4)_2SO_4$-precipitated proteins in crude extracts from aleurone-endosperm tissues from 15 de-embryonated seeds were sedimented by centrifugation at 45,000*g* for 5 min and then resuspended in 400 μL of buffer A (3.1 mg protein mL^{-1}). The protein preparation was clarified by centrifugation at 45,000*g* for 5 min. ATP (2.5 mM), EGTA (2.5 mM), or ATP plus EGTA were added to aliquots and incubation was performed at 30°C for up to 60 min. Malate sensitivity of PEPC was recorded in the absence or presence of 1 mM L-malate (expressed as the percentage of inhibition) before and after incubation of the extract. Values are the averages of two independent experiments, which did not vary by more than 15%.

validated the aleurone endosperm of barley seed as a potential model system to undertake the study of the requisite PEPC-PK and the presumed signal transduction pathway.

Barley aleurone cells have been the focus of recent studies of signaling in plants (Gilroy and Jones, 1992; Gilroy, 1996; Schuurink et al., 1996; Swanson and Jones, 1996). These cells possess a three-cell aleurone layer, unlike wheat, and the preparation of protoplasts has been described (Gilroy and Jones, 1992; Heimovaara-Dijkstra et al., 1994). In this study we report the presence of a Ca^{2+}-independent PEPC-PK activity partially purified on BDA using aleurone-cell protoplasts and aleurone-endosperm tissue of dry and germinating barley seeds (Figs. 3II, B, and 4B). This seed PK was similar to the PEPC-PK already described in various plant systems, e.g. C_4 (maize, sorghum, and hairy crabgrass), CAM (*Mesembryanthemum* and *Bryophyllum*), and C_3 (wheat and tobacco) leaves; sorghum, hairy crabgrass, and barley mesophyll protoplasts; and soybean root nodules (Chollet et al., 1996; Vidal and Chollet, 1997; Zhang and Chollet, 1997). This Ca^{2+}-independent PEPC-PK appears to be unique in that its activity was not modulated directly by second messengers or by phosphorylation/dephosphorylation processes but, rather, through rapid changes in its apparent turnover rate (Carter et al., 1991; Jiao et al., 1991; Chollet et al., 1996; Hartwell et al., 1996; Vidal and Chollet, 1997).

It has been proposed that a light-modulated signal transduction cascade involving alkalinization of pHc, an in-

crease in cytosolic [Ca^{2+}] and the activity of a Ca^{2+}-dependent PK, and protein synthesis are required for up-regulation of the Ca^{2+}-independent PEPC-PK in C_4 mesophyll protoplasts (Giglioli-Guivarc'h et al., 1996). In contrast, our results show that Ca^{2+}-independent PEPC-PK activity is already present in dry seeds (Fig. 4B) and that CHX and other pharmacological reagents (TMB-8, W7, and weak acids) that block the above-mentioned steps of the cascade in C_4 mesophyll-cell protoplasts did not significantly impair the in situ phosphorylation of PEPC in germinating seeds (Fig. 2II and data not shown for weak acids). Our results allow us to conclude that this Ca^{2+}-independent PEPC-PK activity is not inducible by a C_4-like signaling mechanism in the aleurone endosperm of barley during germination. The fact that PEPC phosphorylation occurs in the aleurone endosperm from both soaked whole and de-embryonated seeds (Table I) shows that GAs produced by the embryo were not necessary to trigger this regulatory mechanism. A consistent working hypothesis to account for these observations is that the activation of the putative transduction chain and the up-regulation of PEPC-PK have already occurred during seed maturation, when PEPC activity and malate content show a tremendous increase in barley pericarp, testa, aleurone, and starchy endosperm tissues (Macnicol and Jacobsen, 1992). It has also been reported that ABA induces an intracellular pH increase, possibly because of the activation of plasma membrane H^+ pumps in barley aleurone (Heimovaara-Dijkstra et al., 1994).

In reconstituted assays a variety of PKs (the catalytic subunits of mammalian cAMP-dependent PKs [Terada et al., 1990], plant Ca^{2+}-dependent PKs [Echevarría et al., 1988; Ogawa et al., 1992], and Ca^{2+}-independent PEPC-PKs [Li and Chollet, 1993]) are able to specifically phosphorylate the target PEPC in its N-terminal domain (Chollet et al., 1996; Vidal et al., 1996; Vidal and Chollet, 1997). This casts doubt about the nature and identification of the physiological PK. Data from the present work show that the in vivo effect of the calmodulin antagonist W7 was a marked decrease in the radiolabeling of many soluble seed proteins (Fig. 2I, B, lane 4) but not the phosphorylation state of PEPC (Fig. 2I, B, and 2II, B, lane 4). In contrast, W5, a less-active analog of W7, affected neither the radiolabeled protein pattern nor PEPC phosphorylation (data not shown). Since W7 is known to act on a broad spectrum of Ca^{2+}-/calmodulin-dependent PKs and Ca^{2+}-dependent, calmodulin-like PKs (Roberts and Harmon, 1992; Abo-El-Saad and Wu, 1995), our results rule out the possibility that members of these classes of protein-Ser/Thr kinases are involved in the in situ phosphorylation of barley seed PEPC. This pharmacology-based observation provides strong support for the view that the Ca^{2+}-independent PEPC-PK identified to date in a variety of plant systems, including seeds (Figs. 3II, B, and 4B), is the best candidate for the physiological PEPC-PK (Li and Chollet, 1993; Chollet et al., 1996; Hartwell et al., 1996; Vidal et al., 1996; Vidal and Chollet, 1997; Zhang and Chollet, 1997). Our results also demonstrate that the accumulation of the inducible, 108-kD PEPC subunit during germination was blocked by both CHX and the calmodulin antagonist W7 (Fig. 2II, A, lanes 3 and 4) but not by W5 (data not shown), suggesting that a Ca^{2+}-/calmodulin-dependent event(s) is involved in the synthesis of this PEPC polypeptide during germination.

Ironically, a relatively high PEPC-PK activity is found in dry seeds along with the nonphosphorylated form of its target PEPC (Table I; Fig. 4B). It has been reported that the in vitro phosphorylation of C_4 PEPC is inhibited markedly by L-malate via a target (i.e. substrate) effect (Wang and Chollet, 1993; Echevarría et al., 1994). Since malate levels are high at late stages of seed maturation and in dry seeds (Macnicol and Jacobsen, 1992; Drozdowicz and Jones, 1995), this could explain why the PEPC-phosphorylation state is low but PEPC-PK is present and fully active (Fig. 4B). Alternatively, a high PEPC phosphatase activity during late stages of seed maturation could account for the observed anomaly between PEPC-PK activity and the apparent phosphorylation state of PEPC (Duff and Chollet., 1995; Smith et al., 1996). This could be the case in barley seeds, because an increase in okadaic-acid-sensitive protein phosphatases is detected in aleurone cells during maturation in relation to the ABA response (Kuo et al., 1996).

Finally, several roles have been proposed for L-malate production via PEPC during seed maturation: the acidification of the endosperm (Mikola and Virtanen, 1980; Macnicol and Jacobsen, 1992; Heimovaara-Dijkstra et al., 1994; Drozdowicz and Jones, 1995), the anaplerotic replenishment of citric-acid-cycle intermediates to sustain amino acid synthesis and protein filling of seeds (González et al., 1998; Macnicol and Raymond, 1998), and fatty acid synthesis in fat-rich seeds (Sangwan et al., 1992). It has been firmly established that the carbon flux through PEP by the PEPC branch is 3 to 5 times greater than that through the pyruvate kinase branch in aleurone from maturing barley seeds (Macnicol and Raymond, 1998). Malate is accumulated in the endosperm during maturation (Macnicol and Jacobsen, 1992), whereas its concentration markedly decreases during germination (Drozdowicz and Jones, 1995).

Our results show that the PEPC capacity and apparent phosphorylation state are enhanced within the first 24 h of imbibition in aleurone endosperm (Fig. 1; Table I), indicating that the enzyme maintains sustained activity. This PEPC activity could account for a continued synthesis of malate to be released in the endosperm, contributing to its acidification (Mikola and Virtanen, 1980; Heimovaara-Dijkstra et al., 1994), or to be efficiently transported to the developing embryo (Drozdowicz and Jones, 1995). Whenever high levels of L-malate are produced, PEPC needs enhanced protection against this feedback inhibitor for the carboxylation reaction to proceed efficiently in this adverse condition (Chollet at al., 1996). Ours results show that this was the case in the germinating barley grain (Table I), and this has recently been shown to occur in guard cells during stomatal opening as well (Zhang et al., 1994; Du et al., 1997).

ACKNOWLEDGMENTS

The authors thank Marion and Phil Isle for correcting the manuscript. The photographic work of M.J. Cubas is appreciated.

Received June 12, 1998; accepted October 29, 1998.

LITERATURE CITED

Abo-El-Saad M, Wu R (1995) A rice membrane calcium-dependent protein kinase is induced by gibberellin. Plant Physiol **108:** 787–793

Andreo CS, Gonzales DH, Iglesias AA (1987) Higher plant phospho*enol*pyruvate carboxylase. FEBS Lett **213:** 1–8

Bakrim N, Echevarría C, Cretín C, Arrio-Dupont M, Pierre JN, Vidal J, Chollet R, Gadal P (1992) Regulatory phosphorylation of *Sorghum* leaf phospho*enol*pyruvate carboxylase: identification of the protein-serine kinase and some elements of the signal-transduction cascade. Eur J Biochem **204:** 821–830

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Carter PJ, Nimmo HG, Fewson CA, Wilkins MB (1991) Circadian rhythms in the activity of a plant protein kinase. EMBO J **10:** 2063–2068

Chollet R, Vidal J, O'Leary MH (1996) Phospho*enol*pyruvate carboxylase: a ubiquitous, highly regulated enzyme in plants. Annu Rev Plant Physiol Plant Mol Biol **47:** 273–298

Drozdowicz YM, Jones RL (1995) Hormonal regulation of organic and phosphoric acid release by barley aleurone layers and scutella. Plant Physiol **108:** 769–776

Du Z, Karthik A, Outlaw WH Jr (1997) In vivo phosphorylation of phosphoenolpyruvate carboxylase in guard cells of *Vicia faba* L. is enhanced by fusicoccin and suppressed by abscisic acid. Arch Biochem Biophys **337:** 345–350

Duff SMG, Chollet R (1995) In vivo regulation of wheat-leaf phospho*enol*pyruvate carboxylase by reversible phosphorylation. Plant Physiol **107:** 775–782

Echevarría C, Pacquit V, Bakrim N, Osuna L, Delgado B, Arrio-Dupont M, Vidal J (1994) The effect of pH on the covalent and metabolic control of C_4 phospho*enol*pyruvate carboxylase from *Sorghum* leaf. Arch Biochem Biophys **355:** 425–430

Echevarría C, Vidal J, Jiao J-A, Chollet R (1990) Reversible light activation of the phospho*enol*pyruvate carboxylase protein-serine kinase in maize leaves. FEBS Lett **275:** 25–28

Echevarría C, Vidal J, Le Maréchal P, Brulfert J, Ranjeva R, Gadal P (1988) The phosphorylation of *Sorghum* leaf phospho*enol*pyruvate carboxylase is a Ca^{2+}-calmodulin dependent process. Biochem Biophys Res Commun **155:** 835–840

Giglioli-Guivarc'h N, Pierre J-N, Brown S, Chollet R, Vidal J, Gadal P (1996) The light-dependent transduction pathway controlling the regulatory phosphorylation of C_4 phospho*enol*pyruvate carboxylase in protoplasts from *Digitaria sanguinalis.* Plant Cell **8:** 573–586

Gilroy S (1996) Signal transduction in barley aleurone protoplasts is calcium dependent and independent. Plant Cell **8:** 2193–2209

Gilroy S, Jones RL (1992) Gibberellic acid and abscisic acid coordinately regulate cytoplasmic calcium and secretory activity in barley aleurone protoplast. Proc Natl Acad Sci USA **89:** 3591–3595

González MC, Osuna L, Echevarría C, Vidal J, Cejudo J (1998) Expression and localization of phospho*enol*pyruvate carboxylase in developing and germinating wheat grains. Plant Physiol **116:** 1249–1258

Hartwell J, Smith LH, Wilkins MB, Jenkins GI, Nimmo HG (1996) Higher plant phospho*enol*pyruvate carboxylase kinase is regulated at the level of translatable mRNA in response to light or a circadian rhythm. Plant J **10:** 1071–1078

Heimovaara-Dijkstra S, Heistek JC, Wang M (1994) Counteractive effects of ABA and GA_3 on extracellular and intracellular pH and malate in barley aleurone. Plant Physiol **106:** 359–365

Hidaka H, Sasaki Y, Tanaka T, Endo T, Ohno S, Fujii Y, Nagata T (1981) N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide, a calmodulin antagonist, inhibits cell proliferation. Proc Natl Acad Sci USA **78:** 4354–4357

Jiao J-A, Chollet R (1989) Regulatory seryl-phosphorylation of C_4 phospho*enol*pyruvate carboxylase by a soluble protein kinase from maize leaves. Arch Biochem Biophys **269:** 526–535

Jiao J-A, Echevarría C, Vidal J, Chollet R (1991) Protein turnover as a component in the light/dark regulation of phospho*enol*-pyruvate carboxylase protein-serine kinase activity in C_4 plants. Proc Natl Acad Sci USA **88:** 2712–2715

Kuo A, Cappelluti S, Cervantes-Cervantes M, Rodriguez M, Bush DS (1996) Okadaic acid, a protein phosphatase inhibitor, blocks calcium changes, gene expression, and cell death induced by gibberellin in wheat aleurone cells. Plant Cell **8:** 259–269

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Law RD, Plaxton WC (1997) Regulatory phosphorylation of banana fruit phospho*enol*pyruvate carboxylase by a copurifying phospho*enol*pyruvate carboxylase-kinase. Eur J Biochem **247:** 642–651

Li B, Chollet R (1993) Resolution and identification of C_4 phospho*enol*pyruvate-carboxylase protein-kinase polypeptides and their reversible light activation in maize leaves. Arch Biochem Biophys **307:** 416–419

Macnicol PK, Jacobsen JV (1992) Endosperm acidification and related metabolic changes in the developing barley grain. Plant Physiol **98:** 1098–1104

Macnicol PK, Raymond P (1998) Role of phosphoenolpyruvate carboxylase in malate production by the developing barley aleurone layer. Physiol Plant **103:** 132–138

Mikola J, Virtanen M (1980) Secretion of L-malic acid by barley aleurone layers (abstract no. 783). Plant Physiol **65:** S-142

Nimmo GA, Nimmo HG, Hamilton ID, Fewson CA, Wilkins MB (1986) Purification of the phosphorylated night form and dephosphorylated day form of phosphoenolpyruvate carboxylase from *Bryophyllum fedtschenkoi.* Biochem J **239:** 213–220

Ogawa N, Okumura S, Izui K (1992) A Ca^{2+}-dependent protein kinase phosphorylates phospho*enol*pyruvate carboxylase in maize. FEBS Lett **302:** 86–88

Osuna L, González MC, Cejudo FJ, Vidal J, Echevarría C (1996) In vivo and in vitro phosphorylation of the phospho*enol*pyruvate carboxylase from wheat seeds during germination. Plant Physiol **111:** 551–558

Pacquit V, Giglioli N, Crétin C, Pierre JN, Vidal J, Echevarría C (1995) Regulatory phosphorylation of C_4 phospho*enol*pyruvate carboxylase from *Sorghum*: an immunological study using specific anti-phosphorylation site-antibodies. Photosynth Res **43:** 283–288

Pacquit V, Santi S, Cretin C, Bui VL, Vidal J, Gadal P (1993) Production and properties of recombinant C3-type phospho*enol*-pyruvate carboxylase from *Sorghum vulgare*: *in vitro* phosphorylation by leaf and root PyrPC protein serine kinase. Biochem Biophys Res Commun **197:** 1415–1423

Pierre J-N, Pacquit V, Vidal J, Gadal P (1992) Regulatory phosphorylation of phospho*enol*pyruvate carboxylase in protoplasts from *Sorghum* mesophyll cells and the role of pH and Ca^{2+} as possible components of the light-transduction pathway. Eur J Biochem **210:** 531–537

Rajagopalan AV, Devi MT, Raghavendra AS (1994) Molecular biology of C_4 phospho*enol*pyruvate carboxylase: structure, regulation and genetic engineering. Photosynth Res **39:** 115–135

Roberts DM, Harmon AC (1992) Calcium-modulated proteins: target of intracellular calcium signals in higher plants. Annu Rev Plant Physiol Plant Mol Biol **43:** 375–414

Sangwan RS, Singh N, Plaxton WC (1992) Phospho*enol*pyruvate carboxylase activity and concentration in the endosperm of developing and germinating castor oil seeds. Plant Physiol **99:** 445–449

Schuller KA, Werner D (1993) Phosphorylation of soybean (*Glycine max* L.) nodule phospho*enol*pyruvate carboxylase in vitro decreases its sensitivity to inhibition by L-malate. Plant Physiol **101:** 1267–1273

Schuurink RC, Chan PV, Jones RL (1996) Modulation of calmodulin mRNA and protein levels in barley aleurone. Plant Physiol **111:** 371–380

Smith LH, Lillo C, Nimmo HG, Wilkins MB (1996) Light regulation of phospho*enol*-pyruvate carboxylase in barley mesophyll protoplasts is modulated by protein synthesis and calcium, and

not necessarily correlated with phosphoenolpyruvate carboxylase kinase activity. Planta **200:** 174–180

Swanson SJ, Jones RJ (1996) Gibberellic acid induces vacuolar acidification in barley aleurone. Plant Cell **8:** 2211–2221

Terada K, Kai T, Okuno S, Fujisawa H, Izui K (1990) Maize leaf phospho*enol*pyruvate carboxylase: phosphorylation of Ser^{15} with a mammalian cyclic AMP-dependent protein kinase diminishes sensitivity to inhibition by malate. FEBS Lett **259:** 241–244

Vidal J, Chollet R (1997) Regulatory phosphorylation of C_4 phospho*enol*pyruvate carboxylase. Trends Plant Sci **2:** 230–237

Vidal J, Godbillon P, Gadal P (1981) Recovery of active highly purified phospho*enol*pyruvate carboxylase from specific immunoadsorbent column. FEBS Lett **118:** 31–34

Vidal J, Pierre JN, Echevarría C (1996) The regulatory phosphorylation of the phospho*enol*pyruvate carboxylase: a cardinal event in C_4 photosynthesis. *In* DPS Verma, ed, Signal Transduction in Plant Growth and Development. Springer-Verlag, New York, pp 141–166

Wang YH, Chollet R (1993) Partial purification and characterization of phospho*enol*pyruvate carboxylase protein-serine kinase from illuminated maize leaves. Arch Biochem Biophys **304:** 496–502

Zhang XQ, Chollet R (1997) Phospho*enol*pyruvate carboxylase protein kinase from soybean root nodules: partial purification, characterization, and up/down-regulation by photosynthate supply from the shoots. Arch Biochem Biophys **343:** 260–268

Zhang XQ, Li B, Chollet R (1995) In vitro regulatory phosphorylation of soybean nodule phospho*enol*pyruvate carboxylase. Plant Physiol **108:** 1561–1568

Zhang XQ, Outlaw WH Jr, Chollet R (1994) Lessened malate inhibition of guard-cell phospho*enol*pyruvate carboxylase velocity during stomatal opening. FEBS Lett **352:** 45–48