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RESEARCH PAPER

Involvement of phospholipase D and phosphatidic acid in the light-dependent up-regulation of sorghum leaf phosphoenolpyruvate carboxylase-kinase

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Abstract

The photosynthetic phosphoenolpyruvate carboxylase (C_4-PEPC) is regulated by phosphorylation by a phosphoenolpyruvate carboxylase kinase (PEPC-k). In *Digitaria sanguinalis* mesophyll protoplasts, this light-mediated transduction cascade principally requires a phosphoinositide-specific phospholipase C (PI-PLC) and a Ca^{2+}-dependent step. The present study investigates the cascade components at the higher integrated level of *Sorghum bicolor* leaf discs and leaves. PEPC-k up-regulation required light and photosynthetic electron transport. However, the PI-PLC inhibitor U-73122 and inhibitors of calcium release from intracellular stores only partially blocked this process. Analysis of [^{32}P]phosphate-labelled phospholipids showed a light-dependent increase in phospholipase D (PLD) activity. Treatment of leaf discs with *n*-butanol, which decreases the formation of phosphatidic acid (PA) by PLD, led to the partial inhibition of the C_4-PEPC phosphorylation, suggesting the participation of PLD/PA in the signalling cascade. *PPCK1* gene expression was strictly light-dependent. Addition of neomycin or *n*-butanol decreased, and a combination of both inhibitors markedly reduced *PPCK1* expression and the concomitant rise in PEPC-k activity. The calcium/calmodulin antagonist W7 blocked the light-dependent up-regulation of PEPC-k, pointing to a Ca^{2+}-dependent protein kinase (CDPK) integrating both second messengers, calcium and PA, which were shown to increase the activity of sorghum CDPK.

Key words: Ca^{2+}-dependent protein kinase, phosphatidic acid, phosphoenolpyruvate carboxylase, phosphoenolpyruvate carboxylase kinase, phospholipase C, phospholipase D, *Sorghum bicolor*.

Introduction

Phosphoenolpyruvate carboxylase (PEPC: EC 4.1.1.31) is subject to reversible phosphorylation in plants. This is performed by a low-abundance, small molecular mass, calcium-independent Ser/Thr protein kinase (PEPC-k) that is encoded by a small multigene family in C_4 (Shenton *et al.*, 2006) and C_3 plants (Fontaine *et al.*, 2002; Marsh *et al.*, 2003; Sullivan *et al.*, 2004; Fukayama *et al.*, 2006). For instance, the genome of the C_4 plant maize contains at least four genes with distinct patterns of expression in different organs (Shenton *et al.*, 2006). It is a well-established fact that the PEPC-k form dedicated to C_4 PEPC phosphorylation in mesophyll cells (termed C_4 PEPC-k) is mainly regulated by light-dependent transcription and protein synthesis via a complex signal transduction cascade (Echevarría *et al.*, 1990;

Abbreviations: CDPK, Ca^{2+}-dependent protein kinase; DAG, diacylglycerol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; IP_3, inositol-1,4,5-trisphosphate; PA, phosphatidic acid; PEPC, phosphoenolpyruvate carboxylase; PEPC-k, phosphoenolpyruvate carboxylase kinase; PLC, phospholipase C; PLD, phospholipase D; qPCR, quantitative PCR; RT-PCR, reverse transcription-PCR.

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Chollet *et al.*, 1996; Echevarría and Vidal, 2003). Based on the use of selected pharmaceuticals, biochemical and cellular approaches, the existing model for the light-signalling pathway controlling the C_4 PEPC-k has been mainly derived from mesophyll-cell protoplasts of *Digitaria sanguinalis* (Giglioli-Guivarc'h *et al.*, 1996; Coursol *et al.*, 2000). Signalling components include (i) a photosynthetic metabolite (3-phosphoglyceric acid) formed in bundle sheath cells and circulating to the chloroplasts of mesophyll cells thereby causing cytosolic pH alkalinization, (ii) activation of a phosphoinositide-specific phospholipase C (PI-PLC; EC 3.1.4.11) resulting in a transient increase in inositol-1,4,5-trisphosphate (IP_3), (iii) calcium mobilization from vacuoles, and (iv) a Ca^{2+}-dependent protein kinase (CDPK) (Giglioli-Guivarc'h *et al.*, 1996; Coursol *et al.*, 2000; Osuna *et al.*, 2004). The synthesis of the leaf PEPC-k is rapid, being terminated after 1.5 h, and in subsequent darkness the enzyme activity decreased to basal levels.

However, while the protoplast has proven to be a valuable experimental system, it does not entirely reflect what occurs in the corresponding cells of the whole leaf, which possesses two distinct photosynthetic cell types (mesophyll and bundle sheath cells). Several steps of the transduction cascade remain elusive. These include the mechanisms underlying PI-PLC activation, the origin of calcium fluxes, the nature and regulation of the putative CDPK, and the regulation of PEPC-k gene expression. Finally, two PEPC-k genes have been reported to be expressed in sorghum leaves (Shenton *et al.*, 2006). Based on sequence and expression data, it was suggested that *PPCK1* encodes the C_4 kinase in mesophyll cells, whereas *PPCK2* is expressed in bundle sheath cells (which, in maize leaves, is up-regulated in the dark). A third sorghum *PPCK* gene (Sb06g22690) has recently been reported (Paterson *et al.*, 2009).

The aim of the present work was to characterize further the components of the signalling pathway controlling PEPC-k activity in the integrated system of the *Sorghum bicolor* leaf or leaf discs, and to analyse its effects on *PPCK1* gene expression. Using selected pharmaceuticals, it is shown that light-activated phospholipase D (PLD; EC 3.1.4.4.) and phosphatidic acid (PA) are new components of the cascade. In addition, the results suggest that this novel signalling pathway is branched to the previously identified PI-PLC pathway at the level of a CDPK leading to control *PPCK1* gene expression.

Materials and methods

Plant material and growth conditions

Sorghum plants (*Sorghum bicolor* L., Rhône-Poulenc, Seville, Spain) were grown hydroponically in 12/12 h light/dark cycles at 25 °C and 60% relative humidity and 20 °C and 70% relative humidity for each photoperiod, respectively. Light intensity was 350 μmol photons m^{-2} s^{-1} PAR. Plants were supplied with a nitrate-type nutrient solution.

Experiments were carried out on either whole leaves or excised leaf discs. For the former, fully expanded youngest leaves were excised and immediately transferred to a 3 ml cuvette containing 0.01 mM TRIS-HCl buffer, pH 8, and the indicated pharmaceuticals. Leaves were illuminated with 750 μmol photons PAR m^{-2} s^{-1} or kept in the dark for 2 h prior to the preparation of enzyme extracts. A 1 cm diameter cork borer was used to prepare leaf discs. Excised discs were vacuum-infiltrated (2 cycles of 5 min) with 0.1 M TRIS-HCl buffer, pH 8, 2 mM $NaHCO_3$, and then floated adaxial side up on plastic dishes, and illuminated or kept in the dark prior to enzyme extract preparation.

Enzyme extraction and analysis

Protein extracts were obtained by grinding 0.2 g fresh weight of leaf tissue in 1 ml of extraction buffer containing: 0.1 M TRIS-HCl pH 7.5, 20% (v/v) glycerol, 1 mM EDTA, 10 mM $MgCl_2$ and 14 mM β-mercaptoethanol. The homogenate was centrifuged at 15 000 *g* for 2 min and the supernatant was filtered through Sephadex G-25.

The determination of PEPC activity, the malate test, the *in vitro* phosphorylation assay, and SDS-PAGE has been described previously by Echevarría *et al.* (1990, 1994). PEPC activity was measured spectrophotometrically at the optimal pH of 8.0 using the NAD-MDH-coupled assay at 2.5 mM PEP. A single enzyme unit is defined as the amount of PEPC that catalyses the carboxylation of 1 μmol of phosphoenolpyruvate min^{-1} at pH 8 and 30 °C.

The phosphorylation state of PEPC was determined by the malate test (malate inhibition at the sub-optimal pH of 7.3) and expressed as the IC_{50}. A high IC_{50} is correlated to a high degree of PEPC phosphorylation.

The *in vitro* PEPC-k activity of sorghum leaves and leaf discs was measured in aliquots of desalted protein extracts (10 μg) that were incubated in a reaction medium containing 100 mM TRIS-HCl, pH 7.5, 20% (v/v) glycerol, 5 mM $MgCl_2$, 0.25 mM P^1P^5-di(adenosine-5')-pentaphosphate (adenylate kinase inhibitor), 1 mM EGTA and 0.2 units of nonphosphorylated sorghum PEPC. The phosphorylation reaction was initiated by the addition of 1 μCi of [γ-^{32}P]ATP (10 Ci $mmol^{-1}$) and incubated at 30 °C for 1 h. The reaction was stopped by boiling the samples for 3 min at 90 °C in the presence of dissociation buffer [100 mM TRIS-HCl, pH 8, 25% (v/v) glycerol, 1% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, and 0.05% (w/v) bromphenol blue]. The denatured proteins were separated by SDS-PAGE in a Miniprotean electrophoresis cell (Bio-Rad) and stained with Coomassie Brilliant Blue R-250. The gel was analysed with a phosphor imager (Fujix BAS 1000; Fuji).

Assay of CDPK activity

The CDPK-type protein kinase assay has been described previously (Osuna *et al.*, 2004). In summary, 0.2 g of leaves were ground thoroughly in a mortar with washed sand and 1 ml of buffer A [20 mM HEPES-KOH pH 7.4, 5% (v/v) glycerol, 10 mM EGTA, 2 mM EDTA, 14 mM β-mercaptoethanol, 1 mM phenylmethylsulphonyl fluoride, 1.8 mM benzamidine, 9 μM bestatin, 20 μM chymostatin, 5 μM E-64, 20 μM leupeptin, and 0.1 μg ml^{-1} okadaic acid]. The homogenate was centrifuged at 20 000 *g* for 15 min and proteins were precipitated by the addition of $(NH_4)_2SO_4$ to 60% saturation. Proteins were sedimented by centrifugation at 20 000 *g* for 10 min. The resulting pellet was resuspended in 100 μl buffer B (buffer A lacking EGTA and EDTA), filtered through Sephadex G25, and thereupon used as the desalted protein extract. The CDPK activity was assayed using the non-radioactive PepTag assay (Promega), using the manufacturer's recommended instructions. Assays (25 μl) were carried out in 20 mM HEPES-KOH pH 7.4, 1.3 mM $CaCl_2$, 1 mM DTT, 10 mM $MgCl_2$, 1 mM ATP, 1 mM phenylmethylsulphonyl fluoride, 5 μM E-64, 20 μM leupeptin, 1 μg ml^{-1} microcystin-LR, 0.1 μg ml^{-1} okadaic acid, 38 μM PepTag C1-Peptide (P-L-S-R-T-L-S-V-A-A-K), and an aliquot of the desalted protein extract from leaves (20 μg protein). The phosphorylation reaction was performed for 30 min at 30 °C and terminated by heating at 95 °C for 10 min. The phosphorylated-C1-peptides were separated from the non-phosphorylated ones by electrophoresis on a 0.8%

agarose gel at 100 V for 15 min and visualized with a UV transilluminator (260 nm).

Protein quantification

Protein concentrations were determined using the method of Bradford (1976) with BSA as the standard.

Lipid labelling and extraction

Individual leaf discs were labelled by floating them overnight on 100 μl 0.05% MES-KOH pH 5.8 with 10 μCi [^{32}P] orthophosphate (carrier free, 8500–9120 Ci mmol^{-1}) in 2 ml Eppendorf tubes. When indicated, 0.5% (v/v) *n*-butanol was added at the end of the overnight treatment, and the leaf discs were kept for 30 min in this solution prior to illumination for phosphatidyl-butanol formation and *in vivo* phospholipase D activity measurements. Reactions were terminated with 5% perchloric acid, leaf discs were vortexed for 10 min, transferred to a 0.4 ml solution containing $CHCl_3$:methanol:HCl (50:100:1, by vol.) and vortexed again for 10 min. To separate the different phases, 0.2 ml of 0.9% (w/v) NaCl and 0.4 ml $CHCl_3$ was added and the discs were vortexed briefly. A solution of $CHCl_3$:methanol:HCl (3:48:47, by vol.) was added to the lower phase ($CHCl_3$) and vortexed briefly. The lower phase ($CHCl_3$) was transferred to fresh tubes and dried in a Jouan evaporator centrifuge (type RC1010) connected to a CentriVap cold trap (Labconco). Dried lipids were dissolved in $CHCl_3$ and spotted onto silica TLC plates for separation and analysis as described below.

Analysis and quantification of radiolabelled phospholipids

Lipids were separated on Silica-60 TLC plates using either an alkaline solvent system ($CHCl_3$/methanol/25% NH_3/water, 90:70:4:16, by vol.) (alkaline TLC) or the upper organic phase of an ethyl acetate system (ethyl acetate/*iso*-octane/Hac/H_2O, 12:2:3:10, by vol.) (ethyl acetate TLC) as described by Munnik *et al.* (1995). Structural phospholipids were separated in the alkaline system, and the ethyl acetate separations were used to visualize and quantify PA and PtBut levels. Radioactivity was visualized and quantified by Storm phosphoimaging.

Assay of phospholipase D activity

In vivo phospholipase D activity was assayed by measuring phosphatidyl-butanol formation. A 30 min 0.5% (v/v) *n*-butanol pretreatment was applied to the leaf discs prior to illumination. Lipids were extracted as previously indicated. [^{32}P]phosphatidyl-butanol was separated from phosphatidic acid and the rest of the phospholipids using ethyl acetate TLC as described by Munnik *et al.* (1995).

RNA extraction

Total RNA was extracted from 100 mg of frozen, powdered leaves by the addition of Trizol (1 ml) (TRI reagent, Sigma) and centrifugation (10 min, 12 000 *g*, 4 °C). Chloroform (200 μl) was added to the supernatant and after centrifugation (15 min, 12 000 *g*, 4 °C), RNA was precipitated in 0.5 ml isopropanol (10 min, 4 °C), and recovered by centrifugation (10 min, 12 000 *g*, 4 °C). The pellet was washed with 70% ethanol, dried, and the RNA was dissolved in 20 μl sterile water. RNA concentrations were determined using a Qubit™ Fluorometer (Invitrogen).

RT-PCR experiments

Reverse transcription reactions were performed using 1 μg of purified total RNA. 1 μl ImProm-II™ Reverse Transcriptase (Promega) and a reaction buffer containing 0.5 mM dNTP, 6 mM $MgCl_2$, 20 U recombinant RNasin® ribonuclease inhibitor, and 0.5 μg oligo(dt)$_{15}$.

Subsequent RT-PCR reactions were performed in a final volume of 20 μl consisting of 1 μl of the reverse transcription product, 10 μM of the specific primers (see Supplementary Table S1 at *JXB* online) and 1 U of *Taq* DNA polymerase (Biotools). The amplified fragments were excised from the agarose gel and purified using a DNA extraction kit® (Roche), and sequenced (Newbiotechnic SA, Seville, Spain).

Quantitative PCR reactions (qPCR) were performed in a final volume of 20 μl consisting of 1 μl of the cDNA, 15 μM of the specific primers (see Supplementary Table S1 at *JXB* online), and 10 μl of FastStart SYBR Green Master Mix (Roche). PCR was conducted on the MiniOpticon™ Real-Time PCR Detection System (Bio-Rad), and the threshold cycles (C_t) were determined using Opticon Monitor™ analysis software for all treatments. To normalize the obtained values, 18S RNA was used as internal control in each sample.

Results

Leaf discs as a model to study the light-transduction cascade: involvement of electron transport and PI-PLC

The electron transport chain and the phosphoinositide cycle have previously been shown to be components of the PEPC-k cascade in mesophyll-cell protoplasts from *Digitaria sanguinalis* (Giglioli-Guivarc'h *et al.*, 1996; Coursol *et al.*, 2000). In the present work, the photosynthesis inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (Fig. 1) and the PI-PLC inhibitor U-73122 (Fig. 2A) were vacuum infiltrated in leaf discs before illumination. The inactive compound U-73343 was used as a control (Fig. 2C). Both DCMU and U-73122 were shown to reduce PEPC-k activity significantly in subsequent reconstituted phosphorylation assays. In addition, the time-course and amplitude of the rise in the kinase activity (Fig. 1) was similar to those found in *Digitaria* mesophyll protoplasts (Giglioli-Guivarc'h *et al.*, 1996) and leaves from the sorghum plant (Bakrim *et al.*, 1992). A similar inhibitory effect was produced by the water-soluble inhibitor of PLC activity neomycin. These results established the suitability of this experimental model in leaf discs.

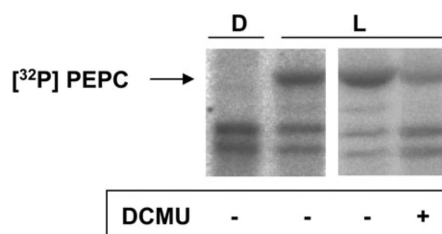

Fig. 1. Requirement of photosynthetic electron transport for the light-dependent up-regulation of PEPC-k activity of sorghum leaf discs. Leaf discs were vacuum-infiltrated in the presence or absence of 0.1 mM DCMU, and illuminated for 2 h (L) or kept in the dark (D). The *in vitro* PEPC-k activity was assayed using 15 μg of desalted protein extracts, in the presence of 1 μCi of [γ-^{32}P]ATP and 0.2 U exogenous purified C_4 PEPC, as described in the Materials and methods. Phosphorylated proteins were analysed by SDS-PAGE and autoradiography. Arrow shows the phosphorylated PEPC.

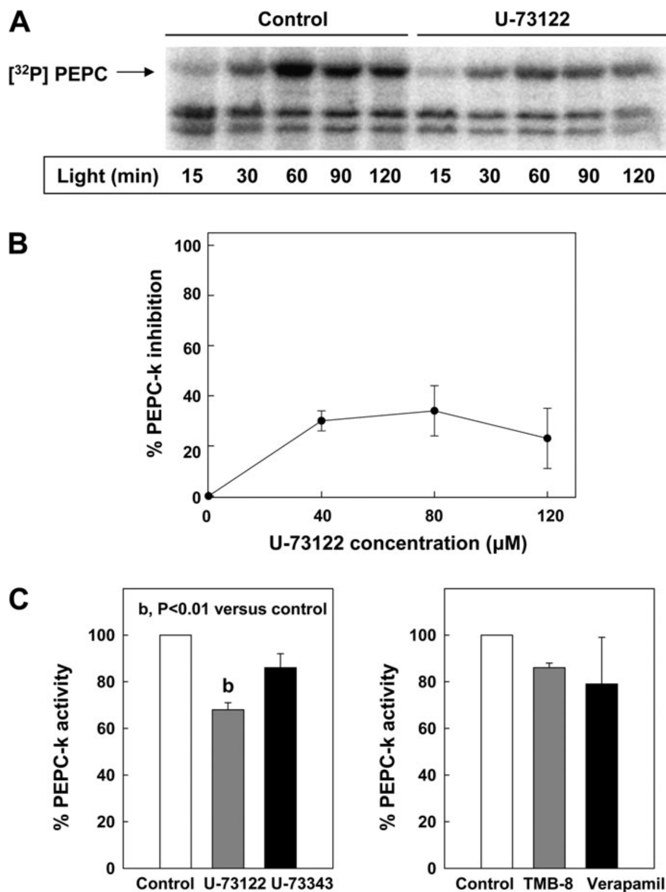

Fig. 2. Requirement PI-PLC activation for the light dependent up-regulation of PEPC-k activity of sorghum leaf discs. (A) Time-course of PEPC-k activity in response to light in the presence or absence of 40 µM U-73122. Leaf discs were vacuum-infiltrated in the presence or absence of 40 µM U-73122, and illuminated. The *in vitro* PEPC-k activity was assayed in aliquots (8 µg proteins) of desalted extracts, in the presence of 1 µCi of [γ-^{32}P]ATP and 0.15 U exogenous purified C_4 PEPC. Arrow shows the phosphorylated PEPC. (B) Dose–response for the inhibition of PEPC-k activity caused by U-73122 after 2 h of illumination. (C) Effect of the PI-PLC inhibitor U-73122 (n=6) and the calcium channel blockers TMB-8 and verapamil (n=2) on the PEPC-k activity of sorghum leaf discs. Leaf discs were vacuum-infiltrated in the presence or absence of 40 µM U-73122, 40 µM U-73343, 1 mM TMB-8, and 1 mM verapamil, and illuminated (2 h). Phosphorylated proteins were quantified by phosphoimaging (Fujix BAS 1000, Fuji, Tokyo). Data are means ±SE of independent experiments. Statistically significant difference (*t* test) is indicated in the graph.

The next step was to study further the light transduction cascade. However, in several assays, the PEPC-k response was consistently attenuated less by U-73122 in leaf discs (Fig. 2C) compared with mesophyll cell protoplasts (Coursol *et al.*, 2000); about 30% and 90% reduction compared with the controls, respectively. The effect of U-73122 could not be enhanced by increasing the concentration of the inhibitor. Dose–response curve showed that the maximum inhibition of PEPC-k activity was exerted by 40 µM U-73122, and the degree of inhibition kept unchanged from 40 µM to 120 µM (Fig. 2B). PI-PLC triggers a calcium flux through the tonoplast (inhibited by TMB-8) via a transient increase in its reaction product IP_3 (Coursol *et al.*, 2000). Previous results showed that this also occurs in leaf discs (Monreal *et al.*, 2007*b*), thereby supporting the present pharmacological data. Nevertheless, the inhibitory effect of the calcium channel inhibitors TMB-8 and verapamil on PEPC-k activity was limited (Fig. 2C); similar degrees of inhibition were produced by TMB-8 or verapamil concentrations ranging from 250 µM to 1 mM (not shown). Thus, it can be hypothesized that, in the leaf discs, PLC activation is not the sole signal controlling PEPC-k up-regulation.

PA and PLD signalling

The other reaction product of PI-PLC is diacylglycerol (DAG). In animal cells, DAG is required for protein kinase C activity. Plants, however, lack protein kinase C (Zonia and Munnik, 2006; Munnik and Testerink, 2009), and instead DAG is phosphorylated to PA, via a DAG kinase (Munnik, 2001). Indeed, PA has emerged as an important signalling component in plants (Testerink and Munnik, 2005). However, PA can also originate from PLD activity (Munnik *et al.*, 1995), which hydrolyses structural phospholipids, such as phosphatidylcholine and phosphatidylethanolamine, producing PA and a free-head group (Munnik *et al.*, 1995). A series of experiments were conducted to explore the occurrence of PA and other signalling compounds in the C_4 leaf and their possible involvement in the PEPC-k cascade: thin layer chromatography (TLC) phospholipid analysis (see Supplementary Fig. S1 at *JXB* online; Fig. 3), phosphorylation of PEPC and PEPC-k activity (Fig. 4), and *PPCK1* expression (Fig. 6).

Changes in the PA content of sorghum leaf discs in response to light were analysed in ^{32}P-labelled leaf discs using TLC. It was seen that light increased PA (see Supplementary Fig. S1 at *JXB* online; Fig. 3A), but PLD activity is not the only source of PA in plant cells. In the presence of *n*-butanol, PLD catalyses the formation of phosphatidyl-butanol by transferring the phosphatidyl group of its substrate to *n*-butanol instead of water; this reaction is specific to PLD and represents an index for the signal-dependent activation of PLD (Munnik, 2001). Light increased phosphatidyl-butanol production (see Supplementary Fig. S1B at *JXB* online; Fig. 3B), showing that PLD was activated in response to light, and that at least a fraction of the PA produced in the light might be a consequence of PLD activity.

Water and *n*-butanol compete as substrates for PLD, and some effect of this compound on PLD-mediated PA formation would be expected. Treatment of leaves with *n*-butanol reduced the light-dependent increase in C_4-PEPC phosphorylation (Fig. 4A) as judged by the malate test (IC_{50} values increase with the phosphorylation state of the enzyme; Echevarría *et al.*, 1994). PA by itself had no effect on PEPC phosphorylation, but when combined with *n*-butanol, PA reversed the inhibitory effect of this compound.

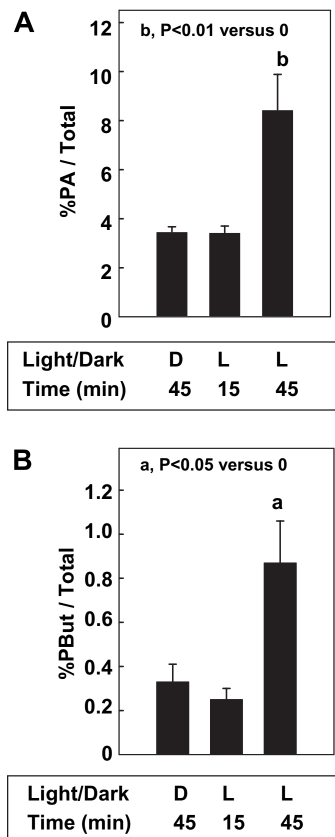

Fig. 3. Light-induced PLD activity in sorghum leaf discs. Discs were fed overnight with [^{32}P]orthophosphate (10 μCi disc^{-1}) and illuminated (15 min and 45 min) or kept in the dark (45 min), provided with a 30 min incubation with 0.5% (v/v) *n*-butanol pretreatment, and illuminated (15 min and 45 min) or kept in the dark (45 min). Phosphatidic acid (PA, means ±SE; *n*=8) and phosphatidyl-butanol (PBut, means ±SE; *n*=4) were quantified by using a phosphor imager. PA and PBut (*y*-axis) are expressed in % with respect to total ^{32}P on the TLC. Statistically significant differences (*t* test) are indicated in the graph.

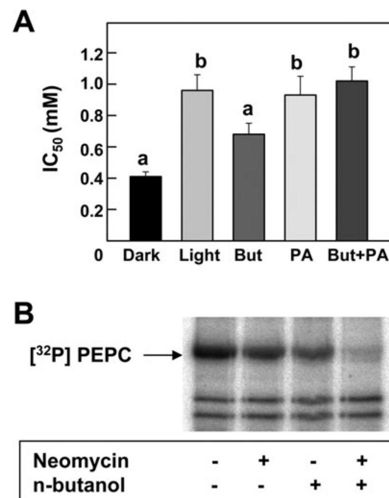

Fig. 4. Inhibition of PLC activity and of PLD-derived PA formation decreased PEPC-k activity. (A) Effect of PA and *n*-butanol on the phosphorylation state of sorghum PEPC. Excised leaves were placed in a 3 ml cuvette containing 0.01 mM TRIS-HCl buffer, pH 8, in the presence or absence of 1% *n*-butanol and/or 2 mM PA (8:0, water-soluble), and illuminated for 3 h. Malate test was measured in protein extracts, and mean values of the IC_{50} ±SE (mM malic acid) are presented (*n*=9). Statistically significant difference (*t* test) is indicated in the graph. a, $P<0.05$ versus light; b, $P<0.05$ versus But. (B) Effect of neomycin and *n*-butanol on the *in vitro* PEPC-k activity. Leaf discs (10 discs) were vacuum-infiltrated in the presence or absence of 1 mM neomycin and 1.5% *n*-butanol, and illuminated for 15 min. The *in vitro* PEPC-k activity was assayed using 10 μg of desalted protein extracts, in the presence of 1 μCi of [γ-^{32}P]ATP and 0.2 U exogenous purified C_4 PEPC. Phosphorylated proteins were analysed by SDS-PAGE and autoradiography. Arrow shows the phosphorylated PEPC.

These results indicate a link between PLD-dependent PA production in response to light and the *in vivo* phosphorylation of PEPC, which is known to be dependent on PEPC-k activity and its synthesis during the light period in C_4 plants (Echevarría *et al.*, 1990; Chollet *et al.*, 1996). In good agreement with this observation, *in vitro* measurements of PEPC-k activity showed that the light up-regulation of the kinase was decreased by *n*-butanol in leaf discs (Fig. 4B). However, although this observation was consistently obtained in several independent experiments, the amplitude of the effect was modest (as it was for C_4-PEPC phosphorylation). This could simply reflect the dual contribution of PI-PLC and PLD signalling pathways to the PEPC-k cascade. Similar to U-73122 (Fig. 2), the PI-PLC inhibitor neomycin (Gabev *et al.*, 1989) also exerted a weak inhibition of PEPC-k up-regulation in illuminated leaf discs (Fig. 4B). However, supplying the compounds together (*n*-butanol+neomycin) to the discs led to a severe reduction in the PEPC-k activity (Fig. 4B).

Thus, these results suggest that both the PLC- and PLD-signalling pathways control PEPC-k activity and PEPC phosphorylation in leaf discs. Moreover, since their effects are additive, they are not acting in sequence, but rather appear to converge at an, as yet unidentified, downstream step of the cascade.

The common step: involvement of a CDPK activity

A Ca^{2+}-dependent protein kinase (CDPK) is involved in the synthesis of PEPC-k in the C_4 plant *Digitaria sanguinalis* (Osuna *et al.*, 2004). The activity of this protein kinase can be measured using a commercial kit, which relies on the *in vitro* phosphorylation of a fluorescent, synthetic peptide substrate. Several phospholipids had little or no effect on the activity of this enzyme; however, the effect of PA on the *Digitaria sanguinalis* enzyme was not assayed (Osuna *et al.*, 2004).

Protein kinase activity in sorghum leaf discs was enhanced by calcium, inhibited by the calcium chelating agent EGTA and by the calcium/calmodulin antagonist W7 (Hidaka *et al.*, 1981) (Fig. 5A). Three phenomena were observed: (i) light rapidly and markedly increased this kinase activity in the sorghum leaf (Fig. 5B); (ii) supplying

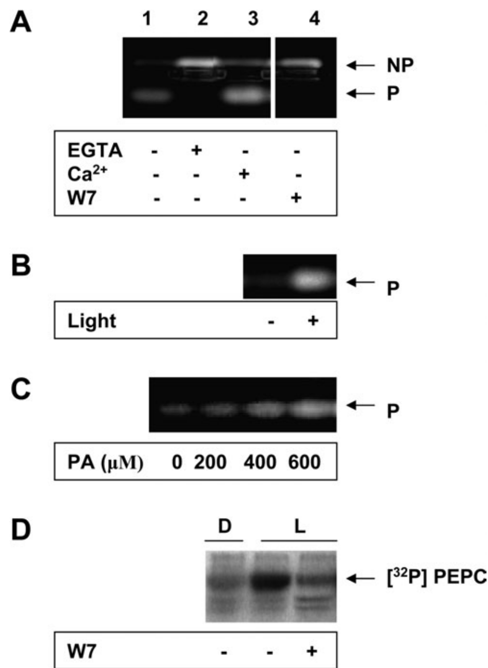

Fig. 5. Characterization of CDPK activity from sorghum leaves. (A) Excised leaves were illuminated for 3.5 h. CDPK activity was assayed using 20 μg of desalted leaf protein extracts, in the presence or absence of 2 mM EGTA, 2.3 mM $CaCl_2$, and 2 mM W7. (B) Excised leaves were illuminated (2 h) or kept in the dark and the enzyme activity was assayed in the presence of 1.3 mM $CaCl_2$. (C) Excised leaves were illuminated (2 h) and CDPK activity was measured in the presence of 1.3 mM $CaCl_2$ and increasing concentration of PA (8:0). NP, non-phosphorylated peptide; P, phosphorylated peptide. (D) W7 decreases the PEPC-k activity from sorghum leaves. Excised leaves were treated overnight in the presence or absence of 2 mM W7. Leaves were illuminated for 2 h. The *in vitro* PEPC-k activity was assayed using 20 μg of desalted protein extracts, in the presence of 1 μCi of [γ-^{32}P]ATP and 0.2 U exogenous purified C_4 PEPC. Phosphorylated proteins were analysed by SDS-PAGE and autoradiography. Arrow shows the phosphorylated PEPC.

PA to the reconstituted phosphorylation assay caused a concentration-dependent enhancement effect on the kinase activity (Fig. 5C); and (iii) in protein extracts from W7-fed leaves, the PEPC-k activity was maintained at the level of dark control (Fig. 5D). In conclusion, sorghum leaves contain a CDPK that is activated by both calcium and PA and is therefore a good candidate for the convergent integration of second messenger signals originating from PI-PLC and PLD.

PPCK1 expression

It is now a well-accepted fact that the light-dependent up-regulation of leaf PEPC-k is via an increase in the transcription rate of the corresponding gene (Nimmo, 2003). A small family of *PPCK* genes encode plant PEPC-k enzymes. Two *PPCK* (*PPCK1* and *PPCK2*) genes have been reported to occur in sorghum leaves; in maize the *PPCK1* orthologue (Zm*PPCK1*) is light-dependent and specifically expressed in mesophyll tissue, while the *PPCK2* orthologue is specifically expressed in the dark in bundle sheath tissue (Shenton *et al.*, 2006). The expression of sorghum *PPCK1* (DQ386731; Sb04g036570) was analysed in excised leaves and leaf discs from sorghum plants. In both cases a large increase in *PPCK1* transcripts was observed in illuminated tissues, as compared to the dark control (Fig. 6A, B). Using quantitative PCR (qPCR) it was found that the neomycin (PI-PLC inhibitor), or *n*-butanol (PLD-dependent PA production inhibitor), partially decreased the expression of *PPCK1* in illuminated leaf discs (Fig. 6C). Maximal inhibition was produced by the combination of the two inhibitors, confirming the notion of a convergent control of PEPC-k synthesis by PI-PLC and PLD signal transduction pathways, in agreement with the data of Fig 4. The calcium/calmodulin antagonist W7 completely blocked the light-dependent increase in *PPCK1* gene expression (Fig. 6D); thus, the W7-sensitive step appeared to be downstream of the confluence of PI-PLC and PLD signal transduction pathways.

The specificity of the suppression of *PPCK1* expression was studied by measuring the effect of the set of inhibitors used in this work on a control gene, i.e. the sorghum C_4-type PEPC gene (*PEPC1*). Table 1 shows that the inhibition was specifically exerted on *PPCK1* expression.

Discussion

Previous work on C_4 mesophyll protoplasts identified some of the major elements constituting the cascade that controls the light-induced up-regulation of PEPC-k activity and the phosphorylation of C_4 PEPC (Giglioli-Guivarc'h *et al.*, 1996; Coursol *et al.*, 2000; Osuna *et al.*, 2004). In the present paper, by making use of the more complex system of leaf and leaf discs, it is possible to draw a more complete picture of this highly multifaceted signalling system. The work demonstrates the addition of new cascade components, namely a PLD and its reaction product, the second messenger PA, and the connection of the PLD pathway to the previously identified PI-PLC pathway, presumably at the level of a calcium/PA activated kinase, and finally their combined effect on *PPCK1* gene expression and PEPC-k activity. High levels of PEPC-k activity could be a consequence of decreased degradation of protein. Both xenobiotic (lithium; Monreal *et al.*, 2007*b*) and physiological molecules (ABA; Monreal *et al.*, 2007*a*) can increase PEPC-k activity by decreasing its rate of degradation. For this reason, it was particularly important to assess that factors implied in changes of PEPC-k activity were in fact controlling *PPCK1* gene expression. Furthermore, two different *PPCK* genes have previously been reported to occur in sorghum leaves (Shenton *et al.*, 2006), and the existence of a third one has been revealed by recent sequencing of entire sorghum genome (Paterson *et al.*, 2009). The products of these three genes may contribute to overall PEPC-k activity.

Several compounds known to inhibit PLC activity and/or PLC signalling (U-73122, neomycin) impeded up-regulation of the PEPC-k activity, but the effect of these compounds

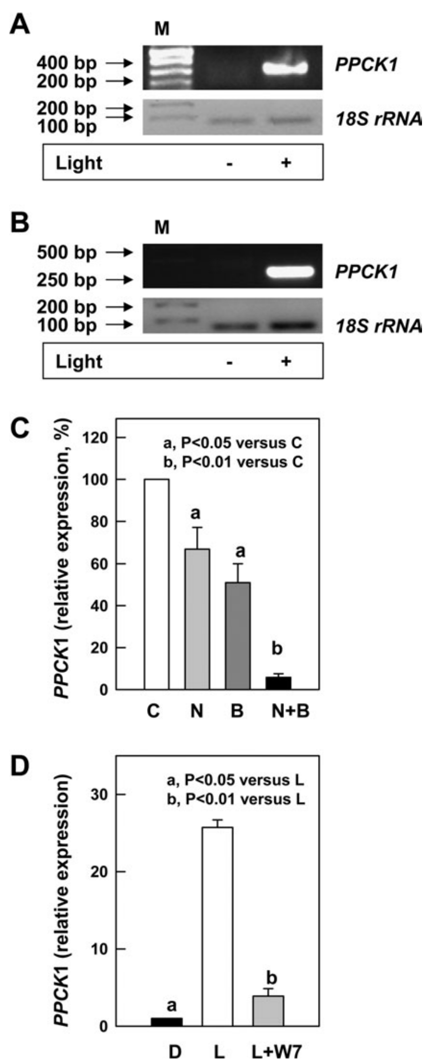

Fig. 6. Analysis of *PPCK1* expression. RT-PCR analysis of *PPCK1* gene. (A) Excised leaves from sorghum plants were illuminated (30 min) or kept in the dark. (B) Leaf discs (10 discs) were vacuum-infiltrated and illuminated (15 min) or kept in the dark. RT-PCR analysis of sorghum *PPCK1* mRNA was performed as indicated in the Materials and methods. *18S rRNA* was used as the endogenous control. M, molecular size marker. qPCR analysis of the relative *PPCK1* transcript levels. (C) Leaf discs (10 discs) were vacuum-infiltrated in the presence or absence (Control: C) of 1 mM neomycin (N), 1.5% *n*-butanol (B), or 1 mM neomycin and 1.5% *n*-butanol (N+B), and illuminated for 15 min. qPCR was used to analyse mRNA levels as described in the Materials and methods. The relative *PPCK1* transcript values were calculated based on the normalized 18S rRNA transcript level, which was amplified as a constitutive internal control. Each value (mean ±SE, four experiments and three replicates) represents the expression relative to control illuminated discs (100%). (D) Leaf discs (10 discs) were vacuum-infiltrated in the presence (L+W7) or absence of 2 mM W7, and illuminated 15 min (L) or kept at dark (D). Each value (mean ±SE, four experiments and three replicates) represents the expression relative to dark (denoted as 1).

was rather limited. This suggested that PLC activation is not the sole factor controlling PEPC-k up-regulation by light. The main finding of the present work concerns the involvement of a PLD and its reaction product PA in the cascade. A light signal is the primary event that leads to PLC activation and PEPC-k synthesis in C_4 mesophyll protoplasts (Giglioli-Guivarc'h *et al.*, 1996), and it is shown in this work that light increases PLD activity and PA production in sorghum leaf discs. Plants possess a multitudinous and diverse family of PLD genes (12 PLD family members in *Arabidopsis*) and the regulation of PLD activity by PIP_2, the α-subunit of heterotrimeric G-proteins and phosphorylation have been reported (Bargman and Munnik, 2006). In addition, most PLDs require calcium for their activity (Wang, 2001). The PLD substrate *n*-butanol has been used in this work (i) to measure PLD activity, and (ii) to decrease the formation of PA. *n*-Butanol has been used in several physiological contexts to demonstrate the implication of PLD in the process. For example, the inhibition by *n*-butanol of the induction of salicylic acid-responsive genes has been interpreted as PLD being a component of the salicylic acid signalling pathway (Krinke *et al.*, 2009).

Table 1. Effect of PLC inhibitors and *n*-butanol on *PPCK1* and *PEPC1* expression

Leaf discs (10 discs) were vacuum-infiltrated in the presence or absence of 1 mM neomycin; or 120 μM U-73122, combined with 1% *n*-butanol, and illuminated for 30 min. qPCR was used to analyse mRNA levels as described in the Materials and methods. Data are means ±SE of 3–6 independent experiments.

Treatment	*PPCK1* (relative expression, %)	*PEPC1* (relative expression, %)
Dark	1±0.3	76±11
Light	100	100
Light+neomycin+*n*-butanol	24±12	120±28
Light+U-73122+*n*-butanol	14±5	181±62

PA is a minor lipid (1–2% of total phospholipids) in eukaryotic cells. Most of it is involved in the biosynthesis of structural phospho- and glycolipids in the endoplasmic reticulum and plastids, where it is made by acylating glycerol 3-phosphate and lyso-PA. In addition to this well-established structural function, the role of PA as a second messenger has recently acquired greater relevance. PA can be generated by PLD in the plasma membrane when it is activated by a signal, such as hormone binding to specific receptors, pathogen elicitor, and multiple stress-related signals (Testerink and Munnik, 2005; Wang, 2005). PA can also be produced indirectly by the PI-PLC pathway; the second reaction product DAG is phosphorylated by a DAG kinase to produce PA (Munnik, 2001). The fact that the PI-PLC and PLD pathways may lead independently to a partial but substantial activation of *PPCK1* gene expression indicated that the cascade may function in the absence of the other pathway.

It is important to note that both PLC-dependent signalling and PLD-dependent PA formation need to be inhibited markedly to suppress the response to light (at both *PPCK1* gene expression and PEPC-k activity levels). This result suggests that (i) each enzyme activation can partially compensate for the lack of the other, but both are required for the

maximal level of PEPC-k synthesis; (ii) the two pathways are not sequentially organized, rather they are converging to a common step in the cascade permitting the integration and summing of individual inputs; and (iii) inhibition of the corresponding component (branching step) must lead to an extensive blocking of the response. In fact, the calcium/calmodulin antagonist W7 is a powerful inhibitor of the cascade in mesophyll protoplasts (Giglioli-Guivarc'h *et al.*, 1996; Coursol *et al.*, 2000). This was also observed in leaf discs and it suggested that the candidate enzyme must be activated by both calcium and PA. All these three points were perfectly exemplified when *PPCK1* gene expression was studied by qPCR. Neomycin (PI-PLC inhibitor) or *n*-butanol (PLD-mediated PA-formation inhibitor) caused a partial inhibitory effect of the gene expression but both drugs had to be combined to observe a marked and almost enhanced inhibition. When given to the discs, W7 was as powerful as the combined drugs as an inhibitor. *Sorghum* leaves contain a CDPK, which is activated by both calcium and PA, and therefore might be a good candidate for convergent integration of second messenger signals originating from PI-PLC (internal calcium and perhaps PA) and PLD (PA). It may represent the point of convergence of these two signalling pathways.

Calcium-dependent protein kinases (CDPKs) form a large family in plants (for instance the *Arabidopsis* genome contains not less than 34 genes encoding the sub-family of CDPK). These kinases are activated by calcium and regulated, positively or negatively, by phosphorylation (auto-phosphorylation or phosphorylation by another kinase, possibly by binding 14-3-3 proteins). In addition, CDPK moving to the nucleus in response to stresses have been described. Interestingly, cross-talk via CDPKs among different signalling pathways is thought to enable signal integration at different levels (Klimecka and Muszynska, 2007). Recently, phospholipid activation of CDPKs (e.g. the wound-responsive ZmCPK11 of maize leaves) has been reported to occur in plants (Szczegielniak *et al.*, 2005). CDPK is present in sorghum leaves and leaf discs. *In vitro*, it was strictly dependent on calcium and PA enhanced its activity, while W7 was a potent inhibitor. Interestingly, this enzyme activity was increased by light. The CDPKs are subjected to phosphorylation by other kinases, or to autophosphorylation that can activate the enzyme (Klimecka and Muszynska, 2007). Such a mechanism would account for the enhancing effect of light on the *Sorghum* leaf CDPK.

Finally, the cascade clearly involves nuclei in which the *PPCK1* gene expression is rapidly and highly activated by light. The simplest explanation of this is that a transcription factor (phosphorylated by cytosolic CDPK) enters the nucleoplasm to modulate the gene expression. Proteins regulating *Arabidopsis PPCK* gene expression have recently been reported to act during Pi starvation (BHLH32, negatively, TTG1, GL3, and EGL3, positively) (Chen *et al.*, 2007). Whether such factors may be implicated in the light-dependent control of *PPCK1* gene expression in the C_4 plant leaf remains to be investigated in order to clarify the mechanism underlying this important step of the cascade.

Conclusion

These results suggest the participation of additional elements controlling PEPC-k synthesis in sorghum leaves: PLD and PA. PLC and PLD signalling pathways could cross-talk at the level of a CDPK. Light, which is the primary signal that activates PEPC phosphorylation, also increased PLD and CDPK activity in sorghum leaves. These two elements are connected by PA, the product of the first enzyme and an activator of the second. The effects on PEPC-k activity were similarly exerted on *PPCK1* gene expression, thus showing that the enhanced PEPC-k activity was not due to a decreased rate of protein degradation. A model for the highly complex, light-dependent, up-regulation of PEPC-k synthesis and activity in sorghum leaves is shown in Supplementary Fig. S2 at *JXB* online.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Table S1. Primers used in RT-PCR and qPCR analyses.

Supplementary Fig. S1. TLC plates showing light-induced PLD activity in sorghum leaf discs.

Supplementary Fig. S2. Model for light-dependent up-regulation of PEPC-k synthesis and activity in sorghum leaves.

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