Coimmunopurification of Phosphorylated Bacterial- and Plant-Type Phosphoenolpyruvate Carboxylases with the Plastidial Pyruvate Dehydrogenase Complex from Developing Castor Oil Seeds

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The phosphoenolpyruvate carboxylase (PEPC) interactome of developing castor oil seed (COS; Ricinus communis) endosperm was assessed using coimmunopurification (co-IP) followed by proteomic analysis. Earlier studies suggested that immunologically unrelated 107-kD plant-type PEPCs (p107/PTPC) and 118-kD bacterial-type PEPCs (p118/BTPC) are subunits of an unusual 910-kD hetero-octameric class 2 PEPC complex of developing COS. The current results confirm that a tight physical interaction occurs between p118 and p107 because p118 quantitatively coimmunopurified with p107 following elution of COS extracts through an anti-p107-IgG immunoaffinity column. No PEPC activity or immunoreactive PEPC polypeptides were detected in the corresponding flow-through fractions. Although BTPCs lack the N-terminal phosphorylation motif characteristic of PTPCs, Pro-Q Diamond phosphoprotein staining, immunoblotting with phospho-serine (Ser)/threonine Akt substrate IgG, and phosphate-affinity PAGE established that coimmunopurified p118 was multiphosphorylated at unique Ser and/or threonine residues. Tandem mass spectrometric analysis of an endoproteinase Lys-C p118 peptide digest demonstrated that Ser-425 is subject to in vivo proline-directed phosphorylation. The co-IP of p118 with p107 did not appear to be influenced by their phosphorylation status. Because p118 phosphorylation was unchanged 48 h following elimination of photosynthate supply due to COS depodding, the signaling mechanisms responsible for photosynthate-dependent p107 phosphorylation differ from those controlling p118’s in vivo phosphorylation. A 110-kD PTPC coimmunopurified with p118 and p107 when depodded COS was used. The plastidial pyruvate dehydrogenase complex (PDCpl) was identified as a novel PEPC interactor. Thus, a putative metabolon involving PEPC and PDCpl could function to channel carbon from phosphoenolpyruvate to acetyl-coenzyme A and/or to recycle CO2 from PDCpl to PEPC.

Phosphoenolpyruvate (PEP) carboxylase (PEPC; E.C. 4.1.1.31) is a tightly regulated enzyme of vascular plants, green algae, and bacteria that catalyzes the irreversible β-carboxylation of PEP to yield oxaloacetate and inorganic phosphate (Pi). PEPC has been extensively studied with regard to its function in C3 and Crassulacean acid metabolism photosynthesis. It also plays pivotal metabolic roles in nonphotosynthetic and C4 photosynthetic cells, particularly the anaplerotic replenishment of tricarboxylic acid cycle intermediates consumed during biosynthesis and nitrogen assimilation. Most plant PEPCs are (1) homotetramers composed of identical subunits of approximately 100 to 110 kD; (2) allosteric enzymes whose activity is potently modulated by various metabolite effectors; and (3) also controlled by reversible phosphorylation at a highly conserved N-terminal seryl residue by a Ca2+-independent PEPC protein kinase and protein phosphatase type 2A (Chollet et al., 1996; Izui et al., 2004; Plaxton and Podesta, 2006). PEPC phosphorylation typically activates the enzyme by simultaneously reducing and increasing its sensitivity to L-malate inhibition and Glc-6-P activation, respectively.

In developing seeds, the partitioning of imported photosynthate between starch, storage lipid, and storage protein biosynthesis is of major agronomic concern. PEP metabolism via PEPC and pyruvate kinase (PK; E.C. 2.7.1.40) plays a prominent role in partitioning oil seed carbohydrates toward plastidic fatty acid biosynthesis versus the mitochondrial production of ATP and carbon skeletons needed for amino acid interconversion in support of storage protein biosynthesis.
PEPC Interactome of Developing Castor Oil Seeds

(Plaxton and Podestá, 2006; Andre et al., 2007; Junker et al., 2007). PEPC and PK activities are abundant in developing seeds and represent promising targets for metabolic engineering in this tissue (Sangwan et al., 1992; Turner et al., 2005; Weber et al., 2005). However, our understanding of the molecular and kinetic/ regulatory properties of seed PEPC and PK is relatively sparse. We recently described the biochemical properties of PEPC and cytosolic PK purified from the triglyceride-rich endosperm of developing castor oil seeds (COS; Ricinus communis; Blonde and Plaxton, 2003; Tripodi et al., 2005; Turner et al., 2005; Gennidakis et al., 2007). The combined results suggested that cytosolic pH and the allosteric effectors Glu and Asp are involved in the coordinate control of the glycolytic PEP branch point as mediated by cytosolic PK versus PEPC (Plaxton and Podestá, 2006). Our interest in COS PEPC was sparked by the discovery that exogenous L-malate supported maximal rates of fatty acid synthesis by purified leucoplasts from developing COS (Smith et al., 1992). Subsequent research revealed that t-malate import from the cytosol into the leucoplast stroma is catalyzed by a malate/Pi translocator within the COS leucoplast envelope (Eastmond et al., 1997).

While purifying PEPC from developing COS, low- and high-molecular-mass PEPC isoforms were resolved during Superdex 200 gel filtration that respectively exhibited striking physical and kinetic similarities to previously characterized green algal class 1 and class 2 PEPCs (Rivoal et al., 2001; Blonde and Plaxton, 2003). COS class 1 PEPC is a typical 410-kD homotetramer of 107-kD plant-type PEPC (PTPC) subunits (p107), whereas the native class 2 PEPC approximately 910-kD hetero-oligomeric complex appears to arise from an association between class 1 PEPC and an immunologically unrelated 118-kD bacterial-type PEPC (BTPC) polypeptide (p118; Blonde and Plaxton, 2003; Gennidakis et al., 2007). Characterization of the native class 2 PEPC has been hampered by p118’s extreme susceptibility to in vitro degradation to a 64-kD polypeptide (p64) by an endogenous thiol endopeptidase (Blonde and Plaxton, 2003; Gennidakis et al., 2007). The reversible in vivo seryl phosphorylation of the shared p107 subunit of class 1 and class 2 PEPCs in response to photosynthate supply indicated that PEPC (p107) protein kinase also contributes to the control of carbohydrate partitioning in developing COS at the level of the cytosolic PEP branch point (Tripodi et al., 2005; Murmu and Plaxton, 2007). Distinctive class 1 versus class 2 PEPC developmental profiles during COS filling, along with class 2 PEPC’s marked insensitivity to allosteric effectors relative to class 1 PEPC, led to the hypotheses that (1) class 1 and class 2 PEPCs respectively support carbon flux required for storage protein and storage lipid synthesis in developing COS; and (2) the p118 BTPC functions as a regulatory subunit of the class 2 PEPC complex (Blonde and Plaxton, 2003; Gennidakis et al., 2007). The existence of class 1 and class 2 PEPC isoforms within developing COS implies that they may have discrete metabolic functions in vivo and could be microcompartmented within the cytosol through unique posttranslational modifications and/or protein interactors.

This study exploits the availability of monospecific polyclonal antibodies raised against the native COS class 1 PEPC (Gennidakis et al., 2007) to investigate the PEPC interactome of developing COS using a coimmunopurification (co-IP) proteomics approach. Interactome studies via co-IP coupled with proteomic analysis can provide a robust in vitro depiction of protein-protein interactions prevailing in vivo (Howell et al., 2006). Our results indicate that a specific interaction between phosphorylated BTPC and PTPC polypeptides and the plastidial pyruvate dehydrogenase complex (PDCp) may exist in COS.

RESULTS AND DISCUSSION

Screening for PTPC-Interacting Protein Candidates from Developing COS

Protein-protein interactions of the COS p107 PTPC were assessed using co-IP, followed by immunoblotting and mass spectrometry (MS)-based proteomic analyses. The protein A-purified anti-p107-IgG specifically detected p107 on immunoblots of clarified COS extracts or coimmunopurified proteins eluting from the anti-p107-IgG column (Fig. 1B). Prior to co-IP, lysates from stage VII (full cotyledon) developing COS were precleared by eluting through a preimmune serum (pre-IS) protein column. No reduction in the total PEPC activity of clarified COS extracts occurred during this preclearing step. Nonbinding proteins were immediately absorbed onto a column containing covalently bound anti-p107-IgG. The vast majority of soluble proteins (>98%) eluted in the flow-through fractions during the phosphate-buffered saline (PBS) wash. However, 100% of total PEPC activity (approximately 19 units originating from 8 g of stage VII developing COS) was routinely eliminated from precleared COS lysates following their elution through the 3-mL anti-p107-IgG co-IP column. This implied that all of the class 1 and class 2 PEPCs present in developing COS extracts (Blonde and Plaxton, 2003; Tripodi et al., 2005; Gennidakis et al., 2007) were bound by the anti-p107-IgG column. Subsequent application of Gly-HCl (pH 2.8) buffer resulted in elution of p107 together with a number of putative p107 protein interactors as judged by SDS-PAGE, immunoblotting, and two-dimensional PAGE (2-DE; Figs. 1 and 2). In some instances, the washed co-IP column was eluted with Pierce’s gentle elution buffer (GEB) prior to the Gly-HCl (pH 2.8) buffer. As p107 interactors were eluted with 25% GEB, they appeared to be less tightly bound relative to p107, which was subsequently eluted following the application of the Gly-HCl (pH 2.8) buffer to the co-IP column (Fig. 3).

Initially, pH 3 to 10 immobilized pH gradient (IPG) strips were employed for isoelectric focusing (IEF) of
the coimmunopurified proteins prior to 2-D SDS-PAGE. Because all of the p107 interactors focused within the 4 to 7 pH range (data not shown), subsequent 2-DE was conducted using pH 4 to 7 IPG strips (Fig. 2A). Reproducible 1-D and 2-DE maps were obtained from at least three independent experiments (Supplemental Fig. S1; data not shown). SDS-PAGE provided optimal resolution of proteins having a molecular mass >90 kD (Fig. 1A), whereas 2-DE facilitated separation of about 20 lower molecular mass (approximately 35–90 kD) protein spots (Fig. 2A). Protein-staining bands and spots other than p107 were respectively excised from 1-D SDS-PAGE and 2-DE gels and identified using liquid chromatography (LC)-tandem mass spectrometry (MS/MS; Table I).

**BTPC Quantitatively Coimmunopurifies with PTPC from Developing COS**

Previous research suggested that the immunologically unrelated p107 PTPC and p118 BTPC (encoded by RcPpc3 and RcPpc4, respectively) are subunits of the native 910-kD class 2 PEPC heter-octameric complex of developing COS (Gennidakis et al., 2007). The p118 subunits are extremely prone to in vitro truncation to p64 (corresponding to p118’s C-terminal portion) by endogenous thiol endoprotease activity during incubation of clarified COS extracts on ice, or class 2 PEPC purification from developing COS (Blonde and Plaxton, 2003; Gennidakis et al., 2007). Interestingly, this study found that immunoreactive 118- and 64-kD BTPC polypeptides quantitatively coimmunopurified with p107 following elution of precleared control or 48-h depodded stage VII COS lysates through the anti-p107-IgG column (Fig. 1C). No immunoreactive BTPC polypeptides were detected in the corresponding co-IP flow-through fractions. The anti-(COS BTPC)-IgG immunoreactive 90-kD polypeptide (p90) observed on immunoblots of COS lysates and pooled co-IP flow-through fractions (Fig. 1C) arises from a nonspecific interaction of the anti-(COS BTPC) peptide antibody with a Suc synthase subunit (Gennidakis et al., 2007). LC-MS/MS confirmed that coimmunopurified p118 and p64 corresponded to full-length and truncated BTPC polypeptides, respectively (Table I; Fig. 4A). The 71-kD protein spot (p71) observed on the 2-DE gels (Fig. 2) was identified as a larger form of p64 (Table I; Fig. 4A), suggesting that in vitro p118 cleavage by endogenous endoproteases occurred at more than one peptide bond. Furthermore, coimmunopurified 50- and 46-kD polypeptides (p50 and p46) corresponded to truncated N-terminal portions of p118 (Table I; Fig. 4A). In contrast to p118, p71, and p64, p50 and p46 did not cross-react on immunoblots probed with the anti-(COS BTPC)-IgG (Fig. 1C). This was expected because this antibody was raised against a synthetic peptide matching a 12-amino acid sequence (Arg-782–Lys-793) in the vicinity of p118’s C terminus (Fig. 4A; Gennidakis et al., 2007). All tryptic peptides obtained by LC-MS/MS analysis of p118, p71, p64, p50, and p46 precisely corresponded with portions of p118’s deduced full-length sequence (Fig. 4A). Limited proteolytic degradation of p118 occurred during co-IP at room temperature; despite our use of conditions documented to negate its proteolysis during incubation of clarified COS extracts on ice (i.e. absence of thiol reducing agents, inclusion of ProteCEASE-100; Gennidakis et al., 2007). The aforementioned results are reminiscent of a recent study that employed anti-(Crassulacean acid metabolism PEPC)-IgG to immunoprecipitate PEPC activity from Arabidopsis (Arabidopsis thaliana) leaf extracts (Goussset-Dupont et al., 2005). Here, matrix-assisted
laser desorption time-of-flight (MALDI-TOF) MS analysis of tryptic peptides derived from the solubilized immunoprecipitate indicated the presence of all three Arabidopsis PTPC isozymes (AtPPC1–AtPPC3), in addition to the AtPPC4 BTPC (Gousset-Dupont et al., 2005).

Elimination of photosynthate supply to stage VII developing COS by incubating excised pods in the dark for 48 h appeared to have little impact on the interaction between p107 and BTPC polypeptides (Fig. 1, A and C). This agrees with previous findings suggesting that the relative amount of immunoreactive p107 or p118 polypeptides, or proportion of class 1 versus class 2 PEPC isoforms, is unaffected in 48-h depodded COS (Tripodi et al., 2005; Gennidakis et al., 2007). The influence of several detergents, as well as PEPP, pyruvate, and several COS PEPC allosteric effectors (Blonde and Plaxton, 2003), on p107-interacting proteins was also investigated. No differences in the protein profile occurred when stage VII COS extracts were subjected to co-IP in the presence of 2 mM PEP or pyruvate, 5 mM Glc-6-P or l-malate, 10 mM l-Asp, 0.1% (v/v) Triton X-100, or 0.3% (v/v) CHAPS (data not shown).

BTPC of Developing COS Is a Phosphoprotein

One-dimensional SDS-PAGE of coimmunopurified proteins from control and 48-h depodded stage VII COS was followed by Pro-Q Diamond phosphoprotein staining (ProQ-PPS) and imaging on a Typhoon fluorescence imager. Subsequent to ProQ-PPS staining, all gels were reincubated in SDS and stained with Sypro-Red to visualize total protein. Three phosphorylated proteins were detected following SDS-PAGE of control co-IP eluates: p118, p107, and p64 (Figs. 5A and 6A; Supplemental Fig. S2). ProQ-PPS staining of p107 was anticipated because its in vivo phosphorylation by a COS PEPC protein kinase has been well documented.

Figure 2. Two-dimensional PAGE and immunoblot analysis of coimmunopurified proteins from stage VII developing COS. A, Approximately 100 μg of coimmunopurified protein was subjected to 1-D IEF on 13-cm IPG strips (pH 4–7), followed by 2-D SDS-PAGE (12%). Reproducible Coomassie Blue R-250 staining p107 interactors were excised and analyzed by LC-MS/MS. Spot numbers correspond to Table I. A clarified COS extract and corresponding co-IP fractions from the anti-p107-IgG column were analyzed by SDS-PAGE and immunoblotting using 1,000-fold diluted rabbit IS raised against recombinant subunits of Arabidopsis PDCpl (E1αpl, E1βpl, E2pl, and E3pl) and a 100-fold diluted mouse monoclonal antibody raised against the recombinant E1α subunit of maize PDCmt (E1αmt). Loading of clarified extract, coimmunopurified proteins (Co-IP), and co-IP column flow-through fractions (Co-IP FT) was as follows: E1αpl and E1βpl immunoblots (5, 0.25, and 5 μg, respectively); E2pl and E3pl immunoblots (15, 1, and 15 μg, respectively); E1αmt Immunoblot (15, 5, and 15 μg, respectively).

Figure 3. Co-IP of PEPC (p107) interactors in developing COS. Clarified extracts from 8 g of endosperm tissue of stage VII COS were precleared through the pre-IS column prior to their elution through the anti-p107-IgG immunoaffinity column. Bound proteins were sequentially eluted with 25% GEB in HEPES-buffered saline (pH 7.4), followed by 100 mM Gly-HCl, pH 2.8 (Glycine). Concentrated eluates were analyzed by 10% SDS-PAGE (A; 5 μg protein/lane), and immunoblotting using anti-p107-IgG (B; 50 ng protein/lane) or anti-(COS BTPC peptide)-IgG (C; 500 ng protein/lane).
plants down-regulates this PEPC kinase and promotes in vivo p107 dephosphorylation (Fig. 5, A and D; Tripodi et al., 2005; Murmu and Plaxton, 2007). As p118’s phosphorylation status appeared to be unaffected by depodding (Fig. 5A), the signaling mechanisms responsible for photosynthetic-dependent p107 phosphorylation likely differ from those controlling in vivo p118 phosphorylation. In vivo dephosphorylation of p107 subunits via depodding rendered no effect on the co-IP of the class 2 PEPC. Similarly, in vitro dephosphorylation of p107 and p118 following pre-incubation of nondepodded COS extracts with exogenous alkaline phosphatase (alk-p’tase) exerted no obvious influence on the interaction between BTPC polypeptides and p107 (Fig. 5, B and E). The immuno-reactive BTPC p118 band disappeared on immunoblots of control co-IP column eluates when the clarified COS extracts were pre-incubated for 1 h at 30°C with alk-p’tase (Fig. 5E). However, this was attributed to p118’s enhanced proteolysis because (1) relative quantity of p64 present in the alk-p’tase pretreated co-IP eluate increased relative to the control extraction (Fig. 5B); (2) immuno-reactive anti-BTPC-IgG polypeptides were absent on immunoblots of the corresponding co-IP column flow-through fractions; and (3) incubation of a desalted COS endosperm extract in the absence of alk-p’tase for 1 h at 30°C resulted in the rapid p118 to p64 proteolysis (data not shown).

Further evidence for p118 phosphorylation was provided by phosphate-affinity PAGE of the BTPC-enriched co-IP eluates (Fig. 6A). The Phos-tag ligand is a polyacrylamide-bound dinuclear Mn® complex that reduces phosphoprotein mobility during SDS-PAGE relative to the corresponding dephosphoprotein (Kinosita et al., 2006). Phosphorylated class 1 PEPC purified from stage VII COS (Blonde and Plaxton, 2003; Crowley et al., 2005) provided a positive control for phosphate-affinity PAGE, while corroborating our previous estimate (Murmu and Plaxton, 2007) that approximately 50% of p107 is maximally phosphorylated by the COS PEPC kinase (Fig. 6B). By contrast, 100% of the communopurified p118 BTPC appeared to migrate as di- or monophosphorylated species upon phosphate-affinity PAGE (Fig. 6A). The fastest migrating dephospho-p118 was only detected in co-IP eluates that had been preincubated with A-p’tase for 30 min.

Sequential ProQ-PS and Sypro-Red staining in conjunction with densitometry revealed that p118 yields a relative phosphorylation signal approximately twice that of p107 throughout COS development (Student’s t test; P > 0.05; n = 3; Fig. 5, A and B; Supplemental Fig. S2). This corroborates the phosphate-affinity PAGE results of Figure 6 because it indicates that p118 possesses a higher stoichiometry of Pi incorporation relative to p107 that was reported to maximize at approximately 0.45 (Murmu and Plaxton, 2007). ProQ-PS staining of p118 or p64 as well as their cross-reaction with a commercially available phospho-Ser/Thr Akt substrate IgG was eliminated following incubation of BTPC-enriched 25% GEB co-IP eluates with exogenous A-p’tase (Fig. 7, A and C). Immunodetection of p118 and p64 by the phospho-Ser/Thr Akt substrate IgG indicated that p118 is phosphorylated on either a Ser or a Thr residue at a novel phosphorylation site localized in its C-terminal half. Phospho-Ser/Thr Akt substrate IgG specifically cross-reacts with phosphoproteins containing a phosphorylated Ser or Thr preceded by Lys/Arg at position −3. The deduced p118 sequence contains several Akt phosphorylation motifs in its C-terminal portion (Fig. 4A). Ser-879 is an attractive candidate because it is within a precise recognition motif for phosphorylation by plant SNF1-related protein kinase 1 (SnRK1), which comprises the phosphorylated Ser (or Thr), hydrophobic residues at positions −5 and +4, and basic residues at −3 and −6 (Halford, 2006). This consensus sequence is conserved in all other vascular plant BTPCs examined to date (Fig. 4B).

Ser-425 of COS BTPC Is Subjected to in Vivo Pro-Directed Phosphorylation

Identification of p118 phosphopeptides was attempted via electrospray ionization MS/MS and MALDI-quadrupole-TOF (qTOF) MS analyses of its tryptic and endoproteinase Lys-C peptide digests, respectively. Although a peak corresponding to a phospho-Ser-879-containing peptide was not detected, it is possible that a suppression effect could be occurring during phosphopeptide ionization and/or that there is substoichiometric phosphorylation of this particular site (Peck, 2006). However, MALDI-qTOF MS of a p118 Lys-C digest revealed several ion peak pairs exhibiting a mass difference of 80 D (= the loss of a phosphorylated group, HPO₄²⁻) that could possibly correspond to an unmodified peptide and its phosphorylated counterpart (Fig. 8A). All candidate phosphopeptides were subjected to MS/MS analysis to verify the identity. MS/MS measurements on the peptide ion at m/z 2,765.407 identified the p118/RePPC4 peptide sequence 413TTGNGSVANSSGSPRASFSSAQRK440 (calculated mass [MH]⁺: 2,765.393), whereas a parallel analysis performed on the low-abundance peak at m/z 2,845.340 demonstrated that this peptide was phosphorylated based on the characteristic neutral loss of 98 D (phosphoric acid, H₃PO₄) from the precursor ion. Manual inspection of a series of C-terminal fragments from this peptide revealed that the exact phosphorylation site corresponded to Ser-425 because an abnormal mass residue of 69 D (dehydroy-Ala) resulting from the dephosphorylated Ser was observed between the fragments y15 and y16 (Fig. 8B).

Ser-425 phosphorylation has been subsequently corroborated by immunoblot analysis of communopurified p118 using an anti-(phosphosite specific)-IgG against a 15-amino acid phospho-Ser-425-containing synthetic peptide (corresponding to Asn-416–Phe-430 of RePPC4’s deduced sequence; B. O’Leary and W.C. Plaxton, unpublished data). It is notable that Ser-425 is adjacent to a Pro residue at the position +1 (Fig. 4A) and is part of a seven-amino acid sequence of COS p118...
Table 1. Identification of p107-interacting protein candidates from 1-D SDS-PAGE (Fig. 1) and 2-DE (Fig. 2)

Proteins were identified via LC-MS/MS following p107 co-IP from stage VII developing COS.

<table>
<thead>
<tr>
<th>Band Letter</th>
<th>Theoretical kD/pI</th>
<th>Experimental kD/pI</th>
<th>Protein Identification</th>
<th>Accession No.</th>
<th>No. of Peptides</th>
<th>Coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>118.5/6.5</td>
<td>118/n.a.</td>
<td>PEPC (BTPC, full length, RcPPC4)</td>
<td>ABR29877</td>
<td>35</td>
<td>45%</td>
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<tr>
<td>B</td>
<td>110.6/6.1</td>
<td>110/n.a.</td>
<td>PEPC (BTPC) (p110, RcPPC3)</td>
<td>ABR29876</td>
<td>10</td>
<td>9%</td>
</tr>
<tr>
<td>1a</td>
<td>118.5/6.5</td>
<td>71/7.0</td>
<td>PEPC (BTPC, C-terminal portion, RcPPC4)</td>
<td>ABR29877</td>
<td>7</td>
<td>16%</td>
</tr>
<tr>
<td>1b</td>
<td>118.5/6.5</td>
<td>64/7.0</td>
<td>PEPC (BTPC, C-terminal portion, RcPPC4)</td>
<td>ABR29877</td>
<td>6</td>
<td>10%</td>
</tr>
<tr>
<td>C</td>
<td>118.5/6.5</td>
<td>64/n.a.</td>
<td>PEPC (BTPC, C-terminal portion, RcPPC4)</td>
<td>ABR29877</td>
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<td>4%</td>
</tr>
<tr>
<td>2</td>
<td>50.1/5.9</td>
<td>58/6.2</td>
<td>Dihydrolipoamide S-acetyltransferase</td>
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<td>22%</td>
</tr>
</tbody>
</table>

D 118.5/6.5 50/n.a. PEPC (BTPC, N-terminal portion, RcPPC4) ABR29877 4 6
3a 118.5/6.5 50/5.6 PEPC (BTPC, N-terminal portion, RcPPC4) ABR29877 3 4
3b 118.5/6.5 46/5.6 PEPC (BTPC, N-terminal portion, RcPPC4) ABR29877 3 4
4a 61.0/8.1 52/6.1 Lipoamide dehydrogenase (E3 subunit, PDCp) NP_566562 7 10
4b 61.0/8.1 51/6.2 Lipoamide dehydrogenase (E3 subunit, PDCp) NP_566562 6 8
4c 61.0/8.1 46/6.0 Lipoamide dehydrogenase (E3 subunit, PDCp) NP_566562 4 6
5 49.8/7.1 43/6.2 Putative dihydrolipoamide succinyl transferase CAB36337 3 4
6 38.2/5.8 41/5.4 Putative nitritase Q6H851 2 2
7a 47.6/5.8 41/5.4 Pyruvate dehydrogenase (E1a subunit, PDCp) NP_171617 4 10
7b 47.6/7.2 41/6.4 Pyruvate dehydrogenase (E1a subunit, PDCp) NP_171617 4 10
8 36.2/7.2 37/5.6 Pyruvate dehydrogenase (E1β subunit, PDCp) AAB66804 2 2
9 30.2/5.9 34/6.0 2S albumin 1707274A 6 25

*NCBI database. **n.a., Not applicable.

(Gly-424–Ser-431) that is absent in other deduced vascular plant BTPC sequences (i.e. Arabidopsis AtPPC4, soybean [Glycine max] GmPPC17, or rice [Oryza sativa] OsPPCb) reported to date (Gennidakis et al., 2007). Protein phosphorylation on a Ser or Thr residue immediately preceding a Pro (i.e. Pro-directed phosphorylation) is of great interest because it represents a major regulatory posttranslational event in many important eukaryotic cellular processes, such as cell proliferation and differentiation (Lu et al., 2002). Pro-directed kinases responsible for this type of phosphorylation are widespread in animals and plants and include cyclin-dependent kinases, mitogen-activated kinases, and glycogen synthase kinase-3 (Lu et al., 2002). Pro-directed phosphorylation can lead to pronounced conformational changes in the target protein because of the subsequent action of a peptidyl-prolyl isomerase that specifically binds to a phosphorylated Ser or Thr residue immediately preceding Pro and catalyzes cis/trans-isomerization of the peptide bond. The resulting conformational change (or so-called backbone switch) can profoundly influence target protein function, including modulation of its catalytic activity, phosphorylation status, protein-protein interactions, subcellular localization, and protein stability (Lu et al., 2002). Phosphorylation-specific peptidyl-prolyl isomerases have been documented in vascular plants and may fulfill similar functions as in other eukaryotes (Yao et al., 2001).

To the best of our knowledge, in vivo BTPC phosphorylation has not been described in vascular plants or green algae. However, the in vitro phosphorylation of the 130-kD BTPC polypeptide of a green algal class 2 PEPC complex by an endogenous protein kinase was reported (Rivoal et al., 2002). Work is in progress to determine (1) additional sites of p118 phosphorylation; (2) the influence of Ser-425 phosphorylation on COS p118 (including possible cis/trans-isomerization of the Ser-425/Pro-426 peptide bond by endogenous peptidyl-prolyl isomerase activity) and class 2 PEPC’s kinetic/regulatory and biological properties; and (3) the molecular and biochemical features of the as-yet uncharacterized BTPC protein kinases and protein phosphatases that mediate in vivo p118 (de)phosphorylation in developing COS. It will be particularly interesting to assess the roles that in vivo p118 phosphorylation may play in endosperm carbon metabolism and carbohydrate partitioning during COS development.

A Third PEPC Polypeptide (p110) Is Up-Regulated in Depodded COS

Co-IP was performed using depodded COS to assess the influence of in vivo p107 dephosphorylation (Tripodi et al., 2005; Murmu and Plaxton, 2007) on p107 interactors. However, this treatment reproducibly generated an additional 110-kD PTPC polypeptide (p110) in the resulting co-IP column eluate (Figs. 1A and 5B). The p110 cross-reacted with the anti-p107-IgG (Figs. 1B and 5C), generated no detectable ProQ-PPS fluorescence signal (Fig. 5A), and only occurred in co-IP eluates obtained from depodded COS. LC-MS/MS identified p110 as corresponding to a larger form of PEPC (PTPC) (p110, RcPPC3) in the resulting co-IP column eluate (Figs. 1A and 5B).
of co-IP eluates from depodded COS. The p107 of COS class 1 and class 2 PEPCs is missing five amino acid residues from its cDNA-deduced N terminus (Blonde and Plaxton, 2003; Gennidakis et al., 2007). This provides a rationale for the 3-kD size discrepancy between the p110 (full length) and p107 (N-terminal truncated) RcPPC3 polypeptides.

The presence of p110 in co-IP eluates from depodded COS implies either a potential interaction between p110 and p107 subunits to form a heterotetrameric native PEPC, as observed in banana (Musa cavendishii) fruit (Law and Plaxton, 1997), or a direct binding of p110 to the anti-p107-IgG on the immunoaffinity column. Direct interaction of p110 with the immobilized anti-p107-IgG is plausible because of its cross-reactivity with anti-p107-IgG on immunoblots (Figs. 1C and 5C).

Interestingly, a similarly immunoreactive p110 PTPC is synthesized de novo during the initial stages of COS germination (Sangwan et al., 1992). It was hypothesized that PEPC has a critical function during the early phase of COS germination to build up cellular levels of dicarboxylic acids required to spark the glyoxylate cycle and subsequent massive gluconeogenic conversion of stored fat into Suc (Sangwan et al., 1992).
accumulation of p110 in depodded developing COS may be attributed to the tissue’s possible transition to a gluconeogenic state in which the glyoxylate cycle becomes activated to mobilize energy from the large pools of storage triacylglycerides. Because depodding or prolonged darkness would result in a significant reduction in photosynthate delivery to the developing endosperm, it is conceivable that the carbon metabolism of depodded COS may be somewhat rearranged to parallel that of germinated COS. It will be of interest to establish the physiological and biochemical basis for p110’s up-regulation in depodded COS.

Plastid Isozyme of the PDC Co-IPs with PEPC from Developing COS

A remarkable observation of this study was the identification of the plastidial pyruvate dehydrogenase complex (PDC_{pl}) as a putative PEPC interactor in developing COS (Fig. 2; Table I). PDC catalyzes the irreversible decarboxylation of pyruvate into acetyl-CoA. Immunoblotting using monospecific antibodies raised against recombinant E1\alpha, E1\beta, E2, and E3 subunits of Arabidopsis PDC_{pl} verified the presence of all four PDC_{pl} subunits in co-IP eluates from stage III to IX COS (Fig. 2B; data not shown). PDC_{pl} subunits were substantially enriched relative to initial clarified COS extracts or co-IP column flow-through fractions (Fig. 2B). However, whereas class 1 and class 2 PEPC isoforms were quantitatively bound to the co-IP column (Fig. 1), immunoreactive PDC_{pl} subunits were detected on immunoblots of the pooled column flow-through fractions (Fig. 2B). It is possible that the PDC_{pl} partially dissociates during the co-IP procedure, causing some of its subunits to flow through the column. Native PDC_{pl} is difficult to purify because of subunit dissociation (Mooney et al., 2002). Although PEPC and PDC_{pl} are believed to be localized in different metabolic compartments, there are only two enzymatic steps between them (malate dehydrogenase and malic enzyme), and PEPC and PDC_{pl} have both been implicated in playing an important role in supporting fatty acid synthesis in developing COS (Reid et al., 1977; Sangwan et al., 1992; Smith et al., 1992). This raises the interesting question of whether PEPC and PDC_{pl} might interact in a multienzyme complex (metabolon) that could channel carbon from PEP to acetyl-CoA and/or recycle CO\textsubscript{2} from PDC_{pl} to PEPC in developing COS.

Plant cells are unique in containing two different PDC isozymes: mitochondrial PDC (PDC_{mt}) which links cytosolic glycolysis with the citric acid cycle, and PDC_{pl} which produces acetyl-CoA and NADH for plastidic fatty acid synthesis (Reid et al., 1977; Tovar-Mendez et al., 2003). PDC_{pl} was originally discovered in leucoplasts isolated from developing COS (Reid et al., 1977) and has since been found in all plants of Arabidopsis PDC_{pl} verified the presence of all four PDC_{pl} subunits in co-IP eluates from stage III to IX COS (Fig. 2B; data not shown). PDC_{pl} subunits were substantially enriched relative to initial clarified COS extracts or co-IP column flow-through fractions (Fig. 2B). However, whereas class 1 and class 2 PEPC isoforms were quantitatively bound to the co-IP column (Fig. 1), immunoreactive PDC_{pl} subunits were detected on immunoblots of the pooled column flow-through fractions (Fig. 2B). It is possible that the PDC_{pl} partially dissociates during the co-IP procedure, causing some of its subunits to flow through the column. Native PDC_{pl} is difficult to purify because of subunit dissociation (Mooney et al., 2002). Although PEPC and PDC_{pl} are believed to be localized in different metabolic compartments, there are only two enzymatic steps between them (malate dehydrogenase and malic enzyme), and PEPC and PDC_{pl} have both been implicated in playing an important role in supporting fatty acid synthesis in developing COS (Reid et al., 1977; Sangwan et al., 1992; Smith et al., 1992). This raises the interesting question of whether PEPC and PDC_{pl} might interact in a multienzyme complex (metabolon) that could channel carbon from PEP to acetyl-CoA and/or recycle CO\textsubscript{2} from PDC_{pl} to PEPC in developing COS.

![Diagram](image-url)
examined to date. The structure and control of PDC\textsubscript{pl} is quite different from that of PDC\textsubscript{mt}. For example, (1) the E1\textsubscript{a\textsubscript{pl}} and E1\textsubscript{b\textsubscript{pl}} subunits of PDC\textsubscript{pl} only exhibit about 30% amino acid sequence identity with their PDC\textsubscript{mt} counterparts (Johnston et al., 1997; Thelen et al., 1999); and (2) PDC\textsubscript{mt} is controlled by reversible phosphorylation, whereas PDC\textsubscript{pl} is not (Mooney et al., 2002; Tovar-Mendez et al., 2003). The structural distinctiveness of PDC\textsubscript{pl} and PDC\textsubscript{mt} allows their subunits to be discriminated by immunoblotting (Conner et al., 1996). Immunoblotting of clarified COS extracts with anti-(maize [\textit{Zea mays}] E1\textsubscript{a\textsubscript{mt}})-IgG revealed a single 43-kD immunoreactive band (Fig. 2B), which agrees with the molecular mass reported for plant E1\textsubscript{a\textsubscript{mt}} subunits (Thelen et al., 1999). Although immunoreactive E1\textsubscript{a\textsubscript{mt}} appeared to be abundant in clarified COS lysates and co-IP column flow-through fractions, no immunoreactive bands were apparent when an immunoblot of up to 5 \mu\text{g} of the co-IP column eluate was probed with the anti-(maize E1\textsubscript{a\textsubscript{mt}})-IgG (Fig. 2B).

A previous report indicated that PDC\textsubscript{pl} may not be exclusively plastidial in developing COS endosperm (Reid et al., 1977). In this study, the plastid marker enzyme acetyl-CoA carboxylase demonstrated 98% of its total activity to be leucoplast localized, whereas only 62% of total PDC activity was in the same fraction. Conversely, 2% and 38% of total acetyl-CoA carboxylase and PDC activity were respectively measured in the corresponding cytosolic fraction (Reid et al., 1977). Subcellular fractionation studies are in progress to establish PDC\textsubscript{pl}'s distribution in the leucoplast versus cytosol of the developing COS endosperm. Nevertheless, our findings support the hypothesis that a specific PEPC-PDC\textsubscript{pl} interaction may occur in developing COS. This interaction could facilitate CO\textsubscript{2} recycling from PDC to PEPC and/or the flux of glycolytic PEP to cytosolic acetyl-CoA required for the biosynthesis of isoprenoids, flavonoids, and malonated derivatives, in addition to the elongation of C16 and C18 fatty acids. Cytosolic acetyl-CoA is generally believed to be generated from citrate and CoA by ATP-citrate lyase (Fritsch and Beevers, 1979; Fatland et al., 2002). However, ATP-citrate lyase activity was barely detectable in developing COS extracts, whereas abundant activity was measured in endosperm extracts from germinated COS (Fritsch and Beevers, 1979). PDC\textsubscript{pl} could provide an alternative metabolic route for acetyl-CoA production within the developing COS cytosol. Further studies are required to determine whether the observed in vitro interaction between PEPC and PDC\textsubscript{pl} exists in vivo and, if so, the role it plays in carbohydrate partitioning and CO\textsubscript{2} recycling in developing COS.

Concluding Remarks

PEPC is an important enzyme situated at a critical branch point in primary plant metabolism that, through enzyme purification and biochemical analyses, was suggested to form a complex with the metabolically sequential malate dehydrogenase in C\textsubscript{4} leaves (Plaxton and Podesta\acute{e}, 2006), its own protein kinase in banana fruit (Law and Plaxton, 1997), as well as the BTPC leading to the formation of the unusual class 2 PEPC hetero-oligomer in unicellular green algae and developing COS (Rivoal et al., 2001; Blonde and Plaxton, 2003; Mamedov et al., 2005; Gennidakis et al., 2007; Moellering et al., 2007). This study employed a co-IP/proteomics approach to screen for class 1 PEPC-interacting proteins in developing COS. Surprising outcomes included the identification of PDC\textsubscript{pl} as a PEPC interactor and the up-regulation of a third PEPC polypeptide (p110) in depodded COS. Additional re-

Figure 8. Identification of a phosphorylation site in the coimmunopurified BTPC from stage VII developing COS. A, MALDI-\textit{qTOF} MS mapping of peptides obtained following a p118 digest with endoproteinase Lys-C. The inset shows an amplified profile of the m/z 2,700 to 3,000 region in which a pair of the peaks exhibited a mass difference of 80 D. B, MS/MS spectrum of the singly charged ion at m/z 2,845.3 obtained at low collision energy of 170 eV. The letters y and b denote C- and N-terminal fragment ions, respectively. Ser-425 is phosphorylated since an abnormal mass residue of 69 D (dehydro-Ala) resulting from the dephosphorylated Ser was observed between the fragments y15 and y16.
search is needed to determine whether a metabolon involving PEPC and PDC\textsubscript{pl} exists in vivo and whether PDC\textsubscript{pl} interacts with the class 1 and/or class 2 PEPC of developing COS. The discovery of a larger form of RcPPC\textsubscript{3} (p110) in co-IP eluates from 48-h depodded COS adds yet another layer of complexity to COS PEPC biochemistry. The in vivo function of p110 is unknown, although it has been hypothesized to be involved in the initial stages of COS germination (Sangwan et al., 1992).

Our results conclusively demonstrate that the p118 BTPC physically interacts with the p107 PTPC in developing COS, thus confirming preliminary evidence suggesting that these distinct PEPC polypeptides are subunits of an unusual 910-kD class 2 PEPC complex (Gennidakis et al., 2007). Formation of the high-molecular-mass p107/p118 hetero-octameric class 2 PEPC complex may be largely dictated by the relative amount of p118 being expressed in vivo. The use of ProQ-PPS, phospho-Ser/Thr Akt substrate IgG, and phosphate-affinity PAGE demonstrated that the coimmunopurified p118 BTPC was highly phosphorylated. MS/MS analysis of p118-derived peptides established Ser-425 as a novel Pro-directed phosphorylation site. (1) Biological functions of Ser-425 phosphorylation in COS BTPC, as well as additional p118 phosphorylation sites; (2) PEPC kinase-protein phosphorylation in COS BTPC, as well as additional p118 phosphorylation sites; and (4) catalytic and/or regulatory subunit functions for p118 within the class 2 PEPC hetero-oligomer.

**MATERIALS AND METHODS**

**Plant Material**

Castor bean (*Ricinus communis*) ‘Baker 296’ plants were cultivated in a greenhouse at 24°C and 70% humidity under natural light supplemented with 16 h of artificial light. Pods containing developing COS at various developmental stages were harvested at midday unless otherwise indicated. Stems containing intact pods of developing COS were also excised and placed in water in the dark at 48 h at 24°C. Endosperm (free of cotyledon) was rapidly dissected and routinely frozen in liquid N\textsubscript{2} and stored at −80°C until used.

**PEPC and Protein Assays**

PEPC was assayed at 340 nm using a Molecular Devices microplate spectrophotometer. Standard assay conditions were 100 mM HEPES-KOH, pH 8.0, 2.5 mM PEP, 5 mM NaHCO\textsubscript{3}, 5 mM MgCl\textsubscript{2}, 0.15 mM NADH, 10% (v/v) glycerol, 1 mM dithiothreitol (DTT), and 5 units mL\textsuperscript{−1} malate dehydrogenase (0.2 mL final volume). One unit of activity is defined as the amount of PEPC catalyzing the formation of 1 mmol oxaloacetate min\textsuperscript{−1} at 25°C. Protein concentrations were determined as previously described (Blonde and Plaxton, 2003) using bovine \gamma-globulin as the protein standard.

**Production of Anti-(COS p107)-IgG Immunoabsorbant**

The anti-p107-IgG present in 3 mL of rabbit IgS raised against the homogeneous and nonproteolyzed native class 1 PEPC from stage VII developing COS (Crowley et al., 2005; Gennidakis et al., 2007) was purified by chromatography on a column (2 mL) of ImmunoPure immobilized protein-A plus agarose (Pierce Chemicals) according to the manufacturer’s protocol. After eluting unbound serum proteins with PBS, anti-p107-IgG was eluted with 100 mM Gly-HCl, pH 2.8, and neutralized with 1 mM unbuffered Tris. A\textsubscript{280} absorbing fractions were concentrated to 1.5 mg mL\textsuperscript{−1} using an Amicon Ultra-15 centrifugal filter unit (10-kD cutoff) and dialyzed overnight against PBS. Dialyzed anti-p107-IgG as well as proteins in the corresponding pre-IS (3 mg each) were separately coupled to 3 mL of AminoLink plus gel (Pierce Chemicals) as stipulated by the manufacturer.

**Co-IP**

COS endosperm (8 g) was homogenized using a mortar and pestle in 2 volumes of ice-cold buffer A containing: 100 mM HEPES-KOH, pH 7.5, 1 mM EDTA, 1 mM EGTA, 15% (v/v) glycerol, 5 mM MgCl\textsubscript{2}, 100 mM KCl, 10 mM NaCl, 25 mM NaF, 1 mM Na\textsubscript{3}VO\textsubscript{4}, 0.1% (v/v) polyvinylpyrrolidone, 0.1% (v/v) Triton X-100, 2 mM 2,2’-dipyridyl disulfide, and 10 mM L\textsuperscript{−1} Protease\textsubscript{CEASE}-100 (G-BioSciences). Homogenates were centrifuged at 4°C and 17,000g for 10 min, and the supernatant fluid recentrifuged for 5 min and filtered through a layer of Miracloth (Calbiochem). Clarified extracts were preclared at 25°C by eluting at 0.5 mL min\textsuperscript{−1} through the pre-IS protein-coupled AminoLink column (1 × 3.8 cm). Unbound proteins were immediately absorbed at 0.5 mL min\textsuperscript{−1} onto the anti-p107-IgG co-IP column (1 × 3.8 cm) that had been pre-equilibrated with PBS. Flow-through fractions were collected by eluting the column at 1 mL min\textsuperscript{−1} with PBS containing 10 mM L\textsuperscript{−1} Protease\textsubscript{CEASE}-100 until the A\textsubscript{280} decreased to baseline, and bound proteins eluted at 0.5 mL min\textsuperscript{−1} with 100 mM Gly-HCl, pH 2.8, and neutralized (1-mL fractions collected into 0.1 mL of unbuffered 1 M Tris). A\textsubscript{280} absorbing fractions were concentrated to 1 mg mL\textsuperscript{−1} as above and analyzed for their polypeptide composition by 1-D SDS-PAGE, 2-DE, and immunoblotting. In some instances, the co-IP column was eluted stepwise with increasing concentrations of Immunopure Ab/Ag GEB (Pierce Chemicals) prior to elution with the acidic Gly buffer.

**Phosphatase Treatments**

Clarified extracts were prepared in buffer A (adjusted to pH 8.2) ± 25 mM NaF and 1 mM sodium orthovanadate, and respectively incubated at 30°C for 1 h ± 60 units mL\textsuperscript{−1} calf intestinal alk-p’tase type VII-S (Sigma Chemical Co.). Prior to co-IP, alk-p’tase activity was quenched with 25 mM NaF and 1 mM Na\textsubscript{3}VO\textsubscript{4}, followed by preclaring of the extracts with the pre-IS column. Phosphatase treatment of BTPC-enriched co-IP eluates (obtained after elution of the anti-p107-IgG co-IP column with 25% [v/v] GEB in HEPES-buffered saline, pH 7.4) was performed using λ-p’tase (New England Biolabs). An aliquot of the co-IP concentrate (25 μg in 50 μL) was incubated ±8,000 units mL\textsuperscript{−1} of λ-p’tase in the presence of 50 mM Tris-HCl (pH 7.5), 2 mM MnCl\textsubscript{2}, 100 mM NaCl, 2 mM DTT, 0.1 mM EGTA, and 0.01% [v/v] Brij 35 for up to 30 min at 30°C.

**SDS-PAGE and Immunoblotting**

SDS-PAGE was performed using Bio-Rad Protein III minigel (200 V, 50 min) or Protein II xi (50 mA, 6 h) gels as previously described (Blonde and Plaxton, 2003). Gels were stained for total protein using either Coomassie Blue R-250 or Sypro-Red, or for phosphoproteins using ProQ-PPS (Molecular Probes). Sypro-Red and ProQ-PPS stained gels were scanned using a Typhoon 8600 fluorescence imager and relative band intensities quantified using ImageQuant software, version 5.0 (GE Healthcare). ProQ-PPS stained and imaged gels were incubated overnight in 0.05% (v/v) SDS at 24°C prior to visualization of total proteins using Sypro-Red. Polypeptide molecular mass estimates by SDS-PAGE were as previously described (Blonde and Plaxton, 2003). Phosphatase-affinity PAGE was conducted at 25 V for 5 h with the Bio-Rad minigel system described above except that the resolving gel (7.5% acrylamide) contained 75 μM Phos-tag acrylamide (www.phos-tag.com) and 150 μM MnCl\textsubscript{2} (Kinschecka et al., 2006).

For immunoblotting, SDS-PAGE minigels were electroblotted onto polyvinylidene difluoride membranes (Blonde and Plaxton, 2003) and probed using antibodies described in the relevant figure legends. Mn\textsuperscript{2+} present following phosphatase-affinity PAGE was removed prior to electroblotting by incubating the gels for 10 min in transfer buffer containing 1 mM EDTA, and then for 10 min in transfer buffer lacking EDTA. Immunoreactive polypeptides were visualized using an alk-p’tase conjugated secondary antibody and chromogenic detection (Blonde and Plaxton, 2003). Immunological specificities were confirmed by performing immunoblots in which rabbit pre-IS was substituted for the various antibodies. For anti-p107 phosphorylation site-
specific) IgG (p107 APS-IgG) immunoblots, nonphosphorylated p107 was used to block any nonspecific antibodies raised against the nonphosphorylated sequence (Tripodi et al., 2005). All immunoblot results were replicated a minimum of three times, with representative results shown in the various figures.

2-DE

Protein samples were precipitated using a 2-DE clean-up kit (GE Healthcare). Following centrifugation, pellets were resuspended in 50 mL of rehydration buffer (6 M urea, 2 M thiourea, 4% [w/v] CHAPS, 0.5% [v/v] IPG buffer [pH 4–7; GE Healthcare], 50 mM DTT, and a trace of bromphenol blue), and proteins quantified using the 2-DE Quant kit (GE Healthcare). IEF was carried out at 20°C using IEF strips (pH 4–7, 13 cm) and an Ettan IEFphor II system (GE Healthcare). One hundred micrograms of protein in 250 mL of rehydration buffer was loaded onto the IEF tray and passive rehydration carried out overnight under a layer of mineral oil. IEF was performed using stepwise increases in voltage and running times: 250 V for 15 min, then 1,000 V for 6 h, and finally, 8,000 V for a total of 80,000 Vh. Focused strips were equilibrated for 15 min in 50 mL Tris-HCl, pH 8.8, containing 6 mL urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 1% (w/v) DTT, followed by incubation for 15 min in the same buffer lacking DTT, but containing 2.5% (w/v) iodoacetamide. Equilibrated IEF strips were placed on 12% SDS gels and sealed with a 3% (w/v) agarose solution. SDS-PAGE was carried out at 10 mA for 1 h and 50 mA for 5 h using the Bio-Rad Protein X II cell.

LC-MS/MS Analysis

Coomassie Blue R-250-stained poly peptides were excised from SDS or 2-DE gels and digested with Promega sequencing grade trypsin (6 ng mL−1) in 50 mM NH4HCO3 for 5 h at 37°C using a Micromass massPREP robotic protein-handling system. Peptides were gel extracted with a 1% formic acid/2% acetonitrile solution, followed by two extractions with 50% acetonitrile. All three extractions were pooled and evaporated to 20 mL using a Thermoflask Speedvac concentrator (model: SPD221P). Capillary scale HPLC-MS/MS was performed on a Waters CapLC XE chromatography system connected to a Waters qTOF Global mass spectrometer (Protein Function Discovery Facility, Queen’s University) or, for p118/RcPPC4, an Agilent MSD ion trap XCT (Southern Alberta Mass Spectrometry Centre, University of Calgary), equipped with a nanoelectrospray ion source. LC Packings C18 PepMap 100 columns (75 μm × 15 cm, particle size 3 μm, pore size 100 Å; Dionex) were used for enhanced peptide separation. Column elution was performed using a 5% to 80% acetonitrile/0.1% formic acid gradient over 35 min at a flow rate of 4 μL min−1, with a downstream postcolumn T-split to provide a 200-nL min−1 flow rate to the electrospray inlet. Each mass spectrometer was operated in data-dependent mode to automatically switch between MS and MS/MS acquisition. Parent ions were scanned in the range of m/z 400 to 2,000, with a switching threshold for MS/MS selection of 10 counts s−1 and a charge state recognition filter to select only +2 and +3 charged species. MS/MS ions were scanned in the range of m/z 50 to 2,200. All MS/MS spectra files were combined into a single file, smoothed, and centroided using Waters ProteinLynx. Smoothing made use of Savitzky-Golay mode, while centroiding was only performed on the top 80% of a given peak.

Identification of a p118/BTPC Phosphorylation Site by MS

Following reduction in the presence of 10 mM DTT (in 100 mM NH4HCO3) at 56°C for 1 h, immoniumpurified p118 was alkylated with an equal volume of 55 mM iodoacetamide at 37°C for another 45 min. The sample was then dialyzed against 1 mM NH4HCO3 and further concentrated using a SpeedVac centrifuge. The p118 protein was incubated with 50 ng endoprotease Lys-C (Roche Diagnostic Corp.) in 25 mM ammonium bicarbonate (pH 7.6) at 37°C for 4 h. The resulting peptides were deposited on a MALDI target by mixing with an equal volume (0.5 μL) of 2.5-dihydroxybenzoic acid matrix (100 mg mL−1 in 50% acetonitrile). MALDI data were acquired using an Applied Biosystems/MDS Sciex QStar XL (qTOF) mass spectrometer (Department of Chemistry, Queen’s University) equipped with an eMALDI II source and a nitrogen laser operating at 337 nm. Following MALDI-qTOF MS mapping, the phosphopeptide sequence was determined by MS/MS measurements using argon as the collision gas.

Database Searching and Protein Sequence Analysis

All peptide fingerprinting masses were searched by MS-Fit against the National Center for Biotechnology Information (NCBI) database using ProteinProspector at the University of California, San Francisco, Web site (http://prospector.ucsf.edu), whereas the MS/MS ions search on each tandem mass spectrum was performed through Mascot search engine (MatrixScience, http://www.matrixscience.com) using the NCBI nonredundant database (NCBInr released 9/22/2007; containing 5,507,867 protein sequences). These searches take into account up to one missed tryptic cleavage site and the modifications of S/T/Y phosphorylation, carbamidomethylation, Asn and Gln deamidation to aspartic acid and Glu, as well as N-terminal pyrogulatation and Met oxidation. The mass tolerance between calculated and observed masses used for database search was considered at the range of ±100 ppm for MS peaks and ±20 D for MS/MS fragment ions.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Replicate 2-DE gels of concentrated co-IP eluates obtained following anti-p107-IgG immunoaffinity chromatography of independently prepared precleared stage VII COS lysates.

Supplemental Figure S2. Profiles of relative p118 and p107 phosphorylation during COS development.

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LITERATURE CITED


PEPC Interactome of Developing Castor Oil Seeds


phosphoenolpyruvate carboxylase from leaves and mesophyll cell protoplasts of Arabidopsis thaliana. Plant Sci 169: 1096–1101


