Plant Physiol. (1992) 99, 445–449
0032-0889/92/99/0445/05/$01.00/0

Received for publication October 31, 1991
Accepted December 13, 1991

Phospho*enol*pyruvate Carboxylase Activity and Concentration in the Endosperm of Developing and Germinating Castor Oil Seeds[1]

Rajender S. Sangwan, Neelam Singh, and William C. Plaxton*

Department of Biology, Queen's University, Kingston, Ontario, Canada K7L 3N6

ABSTRACT

Monospecific polyclonal antibodies against maize leaf phospho*enol*pyruvate carboxylase (PEPC, EC 4.1.1.31) were utilized to examine the subunit composition and developmental profile of endosperm PEPC in developing and germinating castor oil seeds (*Ricinus communis* L. cv Baker 296). PEPC from developing endosperm consists of a single type of 100-kilodalton subunit, whereas the enzyme from 2- to 5-day germinated endosperm appears to contain equal proportions of immunologically related 103- and 108-kilodalton subunits. The maximal activity of PEPC in developing endosperms (2.67 micromoles oxaloacetate produced per minute per gram fresh weight) is approximately 20-fold and threefold greater than that of fully mature (dry seed) and germinating endosperms, respectively. The most significant increase in the activity and concentration of endosperm PEPC occurs during the middle cotyledon to full cotyledon stage of seed development; this period coincides with the most active phase of storage oil accumulation by ripening castor oil seeds. The data are compatible with the recent proposal (RG Smith, DA Gauthier, DT Dennis, DH Turpin [1992] Plant Physiol 1233–1238) that PEPC plays a fundamental role *in vivo* in the cytosolic production of an important substrate (malate) for fatty acid biosynthesis by developing castor oil seed leucoplasts. Immediately following seed imbibition, PEPC activity and concentration increase in parallel, with the greatest levels attained by the third day of germination. It is suggested that during this early phase of seed germination PEPC has a critical function to build up cellular dicarboxylic acid pools required to initiate significant activities of both the tricarboxylic acid and glyoxylate cycles.

PEPC[2] is a ubiquitous plant cytosolic enzyme that catalyzes the irreversible β-carboxylation of PEP to yield oxaloacetate and Pi. This enzyme is particularly abundant in the mesophyll cells of C_4 and CAM leaves where it participates in photosynthesis by catalyzing the initial fixation of atmospheric CO_2. C_4 leaf PEPC has been well characterized with respect to its physical and kinetic properties, regulation of gene expression, and structure-function relationships (1, 8, 9, 16). By contrast, the properties and functions for the enzyme from C_3 plants and nonphotosynthetic tissues of C_4 and CAM plants are less well understood. Proposed roles for the C_3 enzyme are diverse and include (a) regulation of cellular pH and cation balance (12), (b) production of dicarboxylic acids used as respiratory substrates by bacteroids of legume root nodules (21), (c) the anaplerotic replenishment of TCA cycle intermediates consumed in biosynthesis (12, 20), and (d) providing both an ADP-independent "bypass" to pyruvate kinase and Pi-recycling during nutritional Pi deprivation (24).

Smith and coworkers (23) recently demonstrated that malate supports very high rates of fatty acid synthesis by isolated leukoplasts prepared from developing COS. Based on the measurement of leucoplast-localized $NADP^+$-malic enzyme, they proposed that malate may be a key carbon source for fatty acid synthesis *in vivo*. It was suggested (23) that PEPC, in concert with cytosolic malate dehydrogenase, would convert PEP (derived from imported sucrose via cytosolic glycolysis) into malate which is then imported into the leucoplast. In the present investigation, we examined PEPC subunit structure and developmental profiles for PEPC activity and concentration in developing and germinating COS. To our knowledge, this is the first investigation of an oil seed PEPC. The results suggest that the synthesis of PEPC isoforms is highly regulated in COS and that this regulation follows a preset developmental pattern.

MATERIALS AND METHODS

Chemicals and Plant Material

PEP (tricyclohexylammonium salt), DTT, 5-Br-4-Cl-3-indoyl phosphate, Bis-Tris propane, and nitroblue tetrazolium were purchased from Research Organics. NADH was from Boehringer Mannheim, and SDS, acrylamide, and Tris base were from Schwartz/Mann Biotech. Prestained molecular mass standards, protein assay reagent, and ammonium persulfate were from Bio-Rad. Other biochemicals, coupling enzymes, and alkaline phosphatase-conjugated goat anti-(rabbit immunoglobulin G) immunoglobulin G were from Sigma. Poly(vinylidene difluoride) membranes (Immobilon P) were obtained from Millipore. All other reagents were of analytical grade and were supplied by BDH Chemicals. All solutions were prepared with Milli-Q-processed water.

Castor seeds (*Ricinus communis* L., var Baker 296) were germinated at 30°C and 80% RH in light-proof incubators, and endosperms were harvested as described in ref. 17. Plants were grown in a greenhouse in natural light, supplemented with 16 h of fluorescent light. Endosperm was collected at

[1] Supported by the Natural Sciences and Engineering Research Council of Canada (NSERC).

[2] Abbreviations: PEPC, phospho*enol*pyruvate carboxylase; COS, castor oil seed endosperm; PEP, phospho*enol*pyruvate.

different stages of seed development, the exact stage being determined as described by Greenwood and Bewley (7). Harvested tissues were frozen quickly in liquid N_2 and stored at −80°C until used. All observations reported represent the average of three independent determinations and are reproducible to within ±10% SE.

Enzyme Extraction

Tissue was homogenized (1:2, w/v) in ice-cold 25 mM KPi (pH 7.0) containing 1 mM EDTA, 1 mM EGTA, 25 mM NaF, 5 mM thiourea, 0.1% (v/v) Triton X-100, 20% (v/v) glycerol, 5 mM malate, 10 mM $MgCl_2$, 4% (w/v) PEG-8000, 2 mM DTT, 2 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL chymostatin, and 1% (w/v) insoluble polyvinylpolypyrrolidone using a Polytron. Homogenates were centrifuged at 4°C for 40 min at 30,000*g*. Supernatants were further clarified by centrifugation for 10 min at 16,000*g* and 4°C using an Eppendorf microcentrifuge.

PEPC Assay

The PEPC reaction was coupled with the malate dehydrogenase reaction and assayed at 30°C by monitoring NADH oxidation at 340 nm using a Gilford recording spectrophotometer. The assay mixture contained 50 mM Bis-Tris propane-HCl (pH 8.0), 2.5 mM PEP, 5 mM $MgCl_2$, 5 mM $KHCO_3$, 15% (v/v) glycerol, 2 mM DTT, 0.15 mM NADH, and 5 units malate dehydrogenase in a final volume of 1.0 mL. Assays were initiated by the addition of enzyme extract. One unit of enzyme activity is defined as the amount of PEPC resulting in the oxidation of 1 μmol NADH·min^{-1} at 30°C. All assays were conducted in duplicate, optimized with respect to pH and substrate concentration, and corrected for any contaminating NADH oxidase activity. Activity in all assays was proportional to the amount of extract added and remained linear with respect to time.

Western Blotting

Western blotting was performed as described previously (17) except that a polyvinylidene difluoride membrane was used in place of nitrocellulose. Rabbit anti-(maize leaf PEPC) immune serum was diluted 200-fold in blocking buffer. Immunoreactive polypeptides were visualized using an alkaline phosphatase-conjugated secondary antibody (17). Immunological specificities were confirmed by performing western

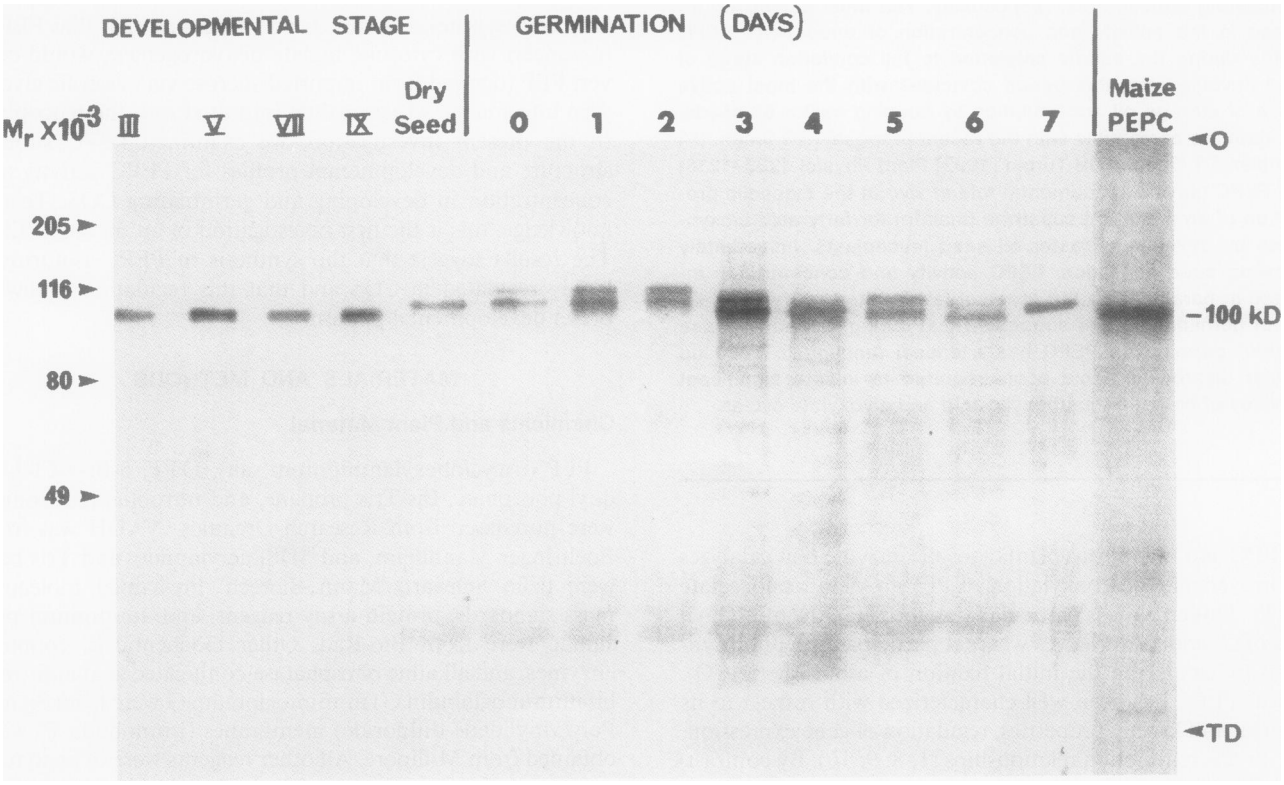

Figure 1. Immunological detection of castor oil seed and maize leaf PEPCs. Extracts were subjected to SDS-PAGE and blot transferred to a polyvinylidene difluoride membrane. Western analysis was performed using a 1:200 dilution of rabbit anti-(maize leaf PEPC) immune serum. Immunoreactive polypeptides were visualized using an alkaline phosphatase-conjugated secondary antibody as described in "Materials and Methods"; phosphatase staining was for 5 min at 30°C. Lanes (Developmental Stage) "III," "V," "VII," and "IX" correspond to the heart-shaped embryo, midcotyledon, full cotyledon, and maturation stages of endosperm development, respectively (7) (0.5 μg protein per lane). Lane "Dry Seed" designates a fully mature castor oil seed (30 μg protein). All other lanes contain 15 μg of protein, except lane "Maize PEPC," which contains 70 ng of protein of partially purified maize leaf PEPC (Sigma, specific activity 3.8 units·mg protein^{-1}). O, Origin; TD, tracker dye front.

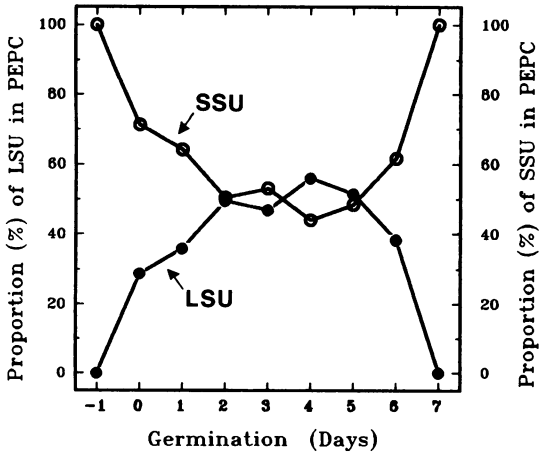

Figure 2. Relative proportion of the 108-kD or large subunit (LSU) *versus* the 103-kD or small subunit (SSU) of PEPC in COS at various stages of germination.

blots in which rabbit preimmune serum was substituted for the anti-(PEPC) immune serum.

Quantification of PEPC in crude extracts was performed by analysis of the immunoreactive bands using a LKB Ultroscan XL enhanced laser densitometer and GelScan XL software (version 2.1). Serial dilutions of maize leaf PEPC were used as the calibration standard. Immunoquantification of COS crude extracts was linear in the concentration range of 0.5 to 40 μg of protein. Molecular masses of immunoreactive polypeptides were estimated by comparing the mobility of the various antigenic polypeptides with that of the following prestained standard proteins: myosin (205 kD), β-galactosidase (116.5 kD), BSA (80 kD), and ovalbumin (49.5 kD).

Protein Determination

Protein was determined by the method of Bradford (4) using bovine γ-globulin as standard.

RESULTS

Immunodetection of COS PEPC

To visualize PEPC antigens of developing and germinating COS, western blots of the respective developmental time-course extracts were probed with antibodies against maize leaf PEPC (Fig. 1). Western blots of extracts from developing COS uniformly revealed a single immunoreactive polypeptide that comigrated with the 100-kD subunit of authentic maize leaf PEPC. A slightly larger (approximately 103 kD) immunoreactive polypeptide was detected on blots of fully mature (dry) COS and 0- to 7-d-old germinated COS (Fig. 1). Interestingly, an antigenic polypeptide having a molecular mass of about 108 kD gradually appeared during the first 2 d of seed germination, when it constituted approximately 50% of PEPC protein (Figs. 1 and 2). The 108-kD immunoreactive polypeptide persisted up to the sixth day of seed germination but was absent on a western blot of an extract from 7-d-old (senescent) germinated COS.

To ascertain whether the various immunoreactive polypeptides that were observed arose following tissue extraction via proteolytic degradation of PEPC by endogenous proteases (18, 25), stage V developing COS and 3-d-old germinated COS were extracted under totally denaturing conditions in the presence of 10% (v/v) TCA (26). TCA-precipitated proteins were solubilized and analyzed by western blotting; the pattern and intensity of immunoreactive bands did not differ from those observed on blots of the respective extracts shown in Figure 1. Also, western blots of developing or germinated COS extracts prepared in the absence of the protease inhibitors PMSF, leupeptin, and chymostatin and incubated at room temperature for up to 24 h did not show any progressive alteration in banding pattern or relative intensities of the immunoreactive polypeptides seen in Figure 1.

PEPC Activity and Concentration in Developing and Germinating COS

Developmental profiles for COS PEPC activity and concentration are shown in Figure 3. The large increase in PEPC activity during COS development and early seedling growth correlates well with an increase in PEPC concentration. Likewise, the large reduction in COS PEPC activity that accompanies seed maturation and the later stages of seed germination coincides with a marked reduction in the concentration of PEPC. The enzyme was considerably more abundant in developing *versus* germinating COS (Fig. 3). Maximal PEPC activity of about 2.7 units·g fresh weight^{-1} (or 0.05 units·mg protein^{-1}) was obtained in stage VII (or full cotyledon stage) developing COS where the enzyme constituted approximately 1.0% of the total soluble protein. PEPC activity and concentration was low in fully mature (dry) COS. Upon seed imbibition, both the activity and amount of PEPC immediately increased, with maximal levels attained by day 3 and a significant decline occurring by day 5 (Fig. 3).

DISCUSSION

The anti-(maize leaf PEPC) immune serum used in the present study appears to be monospecific for PEPC from developing and germinating COS (Fig. 1). The same immune serum was previously shown to specifically cross-react with partially purified PEPC from soybean root nodules (21). These

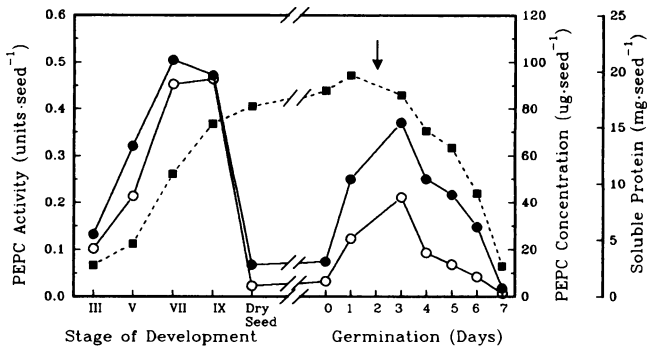

Figure 3. Developmental profiles of soluble protein (■), PEPC activity (●), and concentration (○) in endosperm of developing and germinating castor oil seeds. The arrow indicates the emergence of the radicle.

immunological data indicate a high degree of structural similarity between the C_4- and C_3-type PEPCs. Similar to the enzyme from a range of other C_3, C_4, and CAM plants, PEPC from developing and fully mature COS appears to be composed of a single type of subunit having a molecular mass of approximately 100 kD (Fig. 1). In contrast, following imbibition of fully mature seeds, a larger antigenic polypeptide gradually appears, such that after 2 d of germination the enzyme seems to be composed of equal proportions of immunologically related polypeptides having molecular masses of about 108 and 103 kD, respectively (Figs. 1B and 2). To the best of our knowledge, this is the first evidence suggesting that a plant PEPC might be composed of related, but nonidentical, subunits. These findings are remarkably reminiscent of the relationship between the immunologically related but structurally and kinetically distinct cytosolic pyruvate kinase isoforms of developing *versus* germinating COS; the homotetrameric developing COS enzyme is composed of a single 56-kD subunit, whereas the heterotetrameric germinating COS cytosolic pyruvate kinase contains equal proportions of closely related, but nonidentical, 57- and 56-kD subunits (17). Tissue-specific isoforms of COS PEPC and cytosolic pyruvate kinase may reflect the differing metabolic requirements of tissues whose carbon metabolism is dominated by either sucrose conversion to oil (developing COS) or oil conversion to sucrose (germinating COS).

Role of PEPC during COS Development

The pattern of changes in PEPC activity and amount during COS development are similar to previously reported profiles for other enzymes of oil seed carbon metabolism including phosphoglyceromutase and leucoplast pyruvate kinase (3, 19, 22). Significantly, the developmental period during which PEPC activity and concentration shows the largest increase is coincident with the onset of the most active phase of storage lipid accumulation by developing COS (22). The maximal PEPC activity of about 2.7 units·g fresh weight^{-1} that was measured in full cotyledonary stage developing COS is in the same range as that reported for mature leaves of the CAM plant *Bryophyllum fedtschenkoi* (14). Developing *Brassica napus* (canola) seeds and microspore-derived (cultured) embryos also contain relatively high PEPC activity (1.3 and 0.9 units·g fresh weight^{-1}, respectively) during their most active phase of oil synthesis (R.S. Sangwan, N. Singh, and W.C. Plaxton, unpublished data).

Of pertinence are the recent studies of Smith *et al.* (23) who have demonstrated that malate, compared to pyruvate and acetate, supports the highest rates of fatty acid synthesis by intact leucoplasts isolated from developing COS and that its metabolism (via leucoplast-localized NADP-malic enzyme and pyruvate dehydrogenase) is capable of providing all the reductant required for carbon incorporation into fatty acids. The findings of the present study are in accord with their proposal (23) that PEPC, in concert with cytosolic malate dehydrogenase, may convert PEP (derived from imported sucrose via cytosolic glycolysis) into malate, which is then utilized by the leucoplast for fatty acid biosynthesis. Consistent with this hypothesis are the high rates of dark CO_2 fixation exhibited by intact developing COS (2). It has also been established that the glycolytic pathway of developing oil seed leucoplasts probably plays an important role in generating carbon skeletons and/or energy required for long-chain fatty acid biosynthesis (19, 22). Elucidation of the relative contributions *in vivo* of leucoplast glycolysis *versus* the "PEPC-malate dehydrogenase-malic enzyme" pathway for generating precursors required for oil seed fatty acid synthesis is a fundamental area for future research.

Role of PEPC during COS Germination

More than 50% of the dry weight of mature COS is storage lipid, and the most striking metabolic event occurring following seed germination is the massive conversion of these reserve triacylglycerols into sucrose, which is absorbed by the cotyledons of the growing seedling. In castor seedlings growing at 30°C, this process starts after the third day of germination and becomes maximal by day 5 (6). The various glyoxylate cycle and gluconeogenic enzymes that are required for this metabolism are absent in fully mature (dry) COS but appear to be synthesized *de novo* following several days of germination and generally attain maximal activities on approximately day 5 (6, 15). This developmental pattern differs from that observed for PEPC in the present study, in which (a) low but measurable levels of the enzyme are present in dry COS and (b) upon seed imbibition, additional PEPC is immediately synthesized *de novo*, with maximal levels attained by day 3 and a significant decline occurring by day 5 (Fig. 3). These data suggest that PEPC may fulfill a critical metabolic function early in the germination process. This could initially involve building up cellular pools of the C_4 acids (*i.e.* oxaloacetate and malate) needed to "trigger" significant TCA and glyoxylate cycle activity. A subsequent role for the enzyme may be to catalyze the anaplerotic replenishment of TCA cycle intermediates depleted in anabolism. A similar function has been ascribed to the PEPC of germinating maize and sorghum seeds (10, 13). The increase in the PEP concentration of germinating COS at day 4 onward (11) may be at least partially attributed to the observed decline in PEPC levels during this period.

ACKNOWLEDGMENT

We wish to thank Dr. Raymond Chollet of the University of Nebraska for the generous gift of antibodies against maize leaf PEPC.

LITERATURE CITED

1. **Andreo CS, Gonzalez DH, Iglesias AA** (1987) Higher plant phospho*enol*pyruvate carboxylase. Structure and regulation. FEBS Letts **213:** 1–8
2. **Benedict CR, Beevers H** (1961) Formation of sucrose from malate in germinating castor beans. I. Conversion of malate to phosphoenol-pyruvate. Plant Physiol **36:** 540–544
3. **Botha FC, Dennis DT** (1986) Phosphoglyceromutase activity and concentration in the endosperm of developing and germinating *Ricinus communis* seeds. Can J Bot **65:** 1908–1912
4. **Bradford MM** (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254
5. **Doucet JP, Trifaró JM** (1988) A discontinuous and highly porous sodium dodecyl sulfate-polyacrylamide slab gel system of high resolution. Anal Biochem **168:** 265–271
6. **Gerhardt BP, Beevers H** (1970) Developmental studies on glyoxysomes in *Ricinus* endosperm. J Cell Biol **44:** 94–102

7. **Greenwood JS, Bewley JD** (1991) Seed development in *Ricinus communis* (castor bean). I. Descriptive morphology. Can J Bot **60:** 1751–1760
8. **Hudspeth R, Glackin CA, Bonner J, Grula JW** (1986) Genomic and cDNA clones for maize phosphoenolpyruvate carboxylase and pyruvate orthophosphate dikinase: expression of different gene-family members in leaves and roots. Proc Natl Acad Sci USA **83:** 2884–2888
9. **Jiao J-A, Chollet R** (1991) Posttranslational regulation of phospho*enol*pyruvate carboxylase in C_4 and crassulacean acid metabolism plants. Plant Physiol **95:** 981–985
10. **Khayat E, Dumbroff EB, Glick BR** (1991) The synthesis of phospho*enol*pyruvate carboxylase in imbibing sorghum seeds. Biochem Cell Physiol **69:** 141–145
11. **Kobr MJ, Beevers H** (1971) Gluconeogenesis in the castor bean endosperm. I. Changes in glycolytic intermediates. Plant Physiol **47:** 48–52
12. **Latzko E, Kelly GJ** (1983) The many-faceted function of phospho*enol*pyruvate carboxylase in C_3 plants. Physiol Veg **21:** 805–815
13. **Lebolová S, Strakošová A, Vojtěchová M** (1991) Regulation of the activity of phospho*enol*pyruvate carboxylase isolated from germinating maize (*Zea mays* L.) seeds by some metabolites. Biol Plant **33:** 66–74
14. **Nimmo GA, Nimmo HG, Hamilton ID, Fewson CA, Wilkins MB** (1986) Purification of the phosphorylated night form and dephosphorylated day form of phosphoenolpyruvate carboxylase from *Bryophyllum fedtschenkoi*. Biochem J **239:** 213–220
15. **Nishimura M, Beevers H** (1981) Isoenzymes of sugar phosphate metabolism in endosperm of germinating castor beans. Plant Physiol **67:** 1255–1258
16. **O'Leary MH** (1982) Phosphoenolpyruvate carboxylase: an enzymologist's view. Annu Rev Plant Physiol **33:** 279–315
17. **Plaxton WC** (1989) Molecular and immunological characterization of plastid and cytosolic pyruvate kinase isozymes from castor-oil-plant endosperm and leaf. Eur J Biochem **181:** 443–451
18. **Plaxton WC** (1991) Leucoplast pyruvate kinase from developing castor oil seeds. Characterization of the enzyme's degradation by a cysteine endopeptidase. Plant Physiol **97:** 1334–1338
19. **Sangwan RS, Gauthier DA, Turpin DH, Pomeroy MK, Plaxton WC** (1992) Canola seed and microspore embryo pyruvate kinase isozymes: developmental profiles and subunit composition. Planta (in press)
20. **Schuller KA, Plaxton WC, Turpin DH** (1990) Regulation of phospho*enol*pyruvate carboxylase from the green alga *Selenastrum minutum*. Properties associated with replenishment of tricarboxylic acid cycle intermediates during ammonium assimilation. Plant Physiol **93:** 1303–1311
21. **Schuller KA, Turpin DH, Plaxton WC** (1990) Metabolite regulation of partially purified soybean nodule phospho*enol*pyruvate carboxylase. Plant Physiol **94:** 1429–1435
22. **Simcox PD, Garland W, DeLuca V, Canvin DT, Dennis DT** (1979) Respiratory pathways and fat synthesis in the developing castor oil seed. Can J Bot **57:** 1008–1014
23. **Smith RG, Gauthier DA, Dennis DT, Turpin DH** (1992) Malate- and pyruvate-dependent fatty acid synthesis in leukoplasts from developing castor endosperm. Plant Physiol **99:** 1233–1238
24. **Theodorou ME, Elfrifi IR, Turpin DH, Plaxton WC** (1991) Effects of phosphorus limitation on respiratory metabolism in the green alga *Selenastrum minutum*. Plant Physiol **95:** 1089–1095
25. **Tully RE, Beevers H** (1978) Proteases and peptidases of castor bean endosperm. Enzyme characterization during germination. Plant Physiol **62:** 746–750
26. **Wu FS, Wang MY** (1984) Extraction of proteins for sodium dodecyl sulfate-polyacrylamide gel electrophoresis from protease rich plant tissues. Anal Biochem **139:** 100–103