Regulation of Phosphoenolpyruvate Carboxylase in Higher Plants

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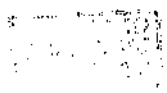

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The abbreviations used in this thesis are described in full below:

AAT, aspartate aminotransferase; ADP, adenosine diphosphate; AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; ATP, adenosine triphosphate; [γ-^{32}P]ATP, radioactively labelled adenosine triphosphate; BAC, bacterial artificial chromosome; bp, base pair; Ca^{2+}, calcium ion, CAM, crassulacean acid metabolism; CaMK, calmodulin-dependent protein kinase; cAMP, cyclic adenosine monophosphate; cDNA, complementary deoxyribonucleic acid; CDK, cyclin-dependent kinase; CDPK, calcium-dependent protein kinase (or calmodulin-like domain protein kinase); CK, casein kinase; CO_2, carbon dioxide; DCDP, 3,3-dichloro-2-dihydroxyphosphinoylmethyl-2-propenoate; DHAP, dihydroxyacetone phosphate; DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; DMDP, 3,3-dimethyl-2-dihydroxyphosphinoylmethyl-2-propenoate; DMO, 5,5-dimethyl-2,4oxazolidinedione; DNA, deoxyribonucleic acid; DTT, dithiothreitol; EDTA, ethylene diamine tetra-acetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)N, N, N', N'-tetra acetic acid; EST, expressed sequence tag; gln, glutamine; glu, glutamate; GOGAT, glutamine 2-oxoglutarate aminotransferase (glutamate synthase); G3P, glyceraldehyde 3-phosphate; GS, glutamine synthetase; GSK, glycogen synthase kinase; GTP, guanosine triphosphate; HCl, hydrochloric acid; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; KAPP, kinase associated protein phosphatase; kb, kilobase; K_i, inhibition constant; kDa, kilodalton; KNO_3, potassium nitrate; K^+, potassium ion; LRR, leucine-rich repeat; MAPK, MAPKK or MAPKKK, mitogen activated protein kinase, kinase kinase or kinase kinase kinase; MDH, malate dehydrogenase; ME, malic enzyme; [^{35}S]Met, radioactively labelled methionine; mM, millimolar; mRNA, messenger ribonucleic acid; MSMO, Murashige & Skoog Minimal Organics; NAD^+/NADH, nicotinamide adenine dinucleotide oxidized/reduced form; $NADP^+$/NADPH, nicotinamide adenine dinucleotide phosphate oxidized/reduced form; NH_4Cl, ammonium chloride; NH_4NO_3, ammonium nitrate; NIP, nitrate reductase inhibitor protein; NO_3^- nitrate; NR, nitrate reductase; OAA, oxaloacetate; O_2, oxygen; PCO, photosynthetic carbon oxidation; PCR, polymerase chain reaction; PEP, phosphoenolpyruvate; PEPc, phosphoenolpyruvate carboxylase; 3-PGA, 3-

phosphoglycerate; P_i, inorganic phosphate; PPDK, pyruvate, phosphate dikinase; PP_i, inorganic phosphate; PP1, 2A, 2B or 2C, protein phosphatase type-1, -2A, -2B or -2C; RLK, receptor-like protein kinase; RNA, ribonucleic acid; RNase, ribonuclease; RPP, reductive pentose phosphate; RT, reverse transcription; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SNF-1, sucrose non-fermenting protein kinase-1; SPS, sucrose phosphate synthase; SRK, S-locus receptor kinase; TP, triose phosphate; μCi, microcurie; UDP, uridine diphosphate; μE, microEinstein; μM, micromolar; UTR, untranslated region.

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Abstract

The regulation of higher plant phosphoenolpyruvate carboxylase (PEPc) has been the focus of intensive research for many years. In CAM, C_4 and C_3 species the enzyme's sensitivity to feedback inhibition by malate is modulated by reversible phosphorylation of a serine residue by a calcium-independent PEPc kinase. This kinase is controlled by a circadian oscillator in CAM plants whereas in C_4 and C_3 plants the activity is induced by light. In all cases, PEPc kinase activity is regulated by a process involving *de novo* protein synthesis as opposed to a phosphorylation cascade or a second messenger. Nitrate has also been implicated in the regulation of PEPc from C_3 species and the involvement of cytosolic alkalinization and calcium ion movements has been demonstrated in the signalling pathway by which light triggers phosphorylation of PEPc in C_4 plants.

The overall objective of this present research was to investigate the physiological roles of the phosphorylation of PEPc and the signalling pathways that bring this about. In the first part of the work, the involvement of cytosolic pH in the regulation of PEPc was studied in the CAM plant *K. fedtschenkoi*. An increase in cytosolic pH had no affect on the malate sensitivity of PEPc or the PEPc kinase activity. In contrast, treatment of leaf disks with 3 mM acetic acid to reduce cytosolic pH prevented the nocturnal increase in the apparent K_i of PEPc for malate, PEPc kinase activity and PEPc kinase translatable mRNA. The effect was not metabolic but was due to cytosolic acidification. Measurement of the malate content of leaf disks revealed that acetic acid treatment prevented the decarboxylation of malate. It is proposed that when the cytosolic malate concentration is above a certain threshold at the end of a day period, PEPc kinase activity is inhibited during the following night and PEPc remains in a dephosphorylated, inactive form. The effect of acid treatment was slow, requiring 9-12 hours, and was therefore not considered to be involved in CAM PEPc signal transduction.

In the second part of this research aspects of PEPc regulation were studied in the model C_3 species *A. thaliana*. The first full-length cDNA for PEPc from *A. thaliana* was cloned and sequenced. Northern analysis of poly A^+ RNA from different *A. thaliana* plant tissues suggested that the cDNA encoded a root isoform of PEPc. The specific activity and malate sensitivity of PEPc were measured in a photomixotrophic cell culture of *A. thaliana* were measured. The phosphatase inhibitor, cantharidin, decreased the malate sensitivity of PEPc from the cell culture but light did not have any effect on the malate

sensitivity of PEPc from the cell culture or *A. thaliana* plant tissue. Using Northern analysis of poly A^+ RNA transcripts for PEPc and PEPc kinase were detected in the cell culture. PEPc transcripts were detected in root and flower/bud tissue using a conserved PEPc probe and PEPc kinase transcripts were detected in several different tissues of *A. thaliana*. The effects of sucrose and nitrate on the transcript levels of PEPc and PEPc kinase in *A. thaliana* cell culture were studied. Transcript levels of PEPc and PEPc kinase were higher in 1% sucrose compared with 3% sucrose but no apparent trend was evident in the effect of nitrate on either PEPc or PEPc kinase. Therefore, it can be concluded that PEPc kinase is present in both *A. thaliana* tissue and cell culture and that the malate sensitivity of PEPc from the cell culture is regulated by reversible phosphorylation. The potential use of the *A. thaliana* cell culture for studies of the regulation of PEPc in C_3 plants has been demonstrated.

e.g. PFK, pyrophosphate dependent phosphofructokinase
F 2,6-BP activates glycolysis
PPi activates gluconeogenesis

e.g. ferredoxin, ferredoxin thioredoxin reductase and thioredoxin
Ford et al., 1987 – 'protein modulase', p_1-p_2 interactions thought to be involved, ambiguous interactions

Chapter One

INTRODUCTION

1.1 The regulation of plant enzyme activity

Plants and animals respond in different ways to their environments. The plant lifestyle, characterized by a lack of movement and the consequent inability to search for food or move away from threatening or adverse situations, necessitates a plasticity of metabolism, growth and development. This requires that external signals are recognised and used to stimulate internal responses; however, the mechanisms of signal transduction are still relatively poorly understood. At one level (e.g. gene expression or proteolysis) of these signalling pathways, changes are relatively slow. However, in contrast to this coarse control, another type of metabolic control exists to effect rapid (i.e. seconds to minutes) and generally energetically inexpensive responses within the plant to external stimuli. This fine control modulates the activity of pre-existing enzyme molecules, and thus the rate of flux through the various metabolic pathways, to meet the requirements of individual cells in response to perceived changes in the plant's environment. Thus, this regulation of enzyme activity is important in the overall metabolic profile of the plant.

The fine control of enzyme regulation can be brought about in different ways, which, it must be emphasized, often operate in concert. One type of fine control is a change in the concentration of a reaction substrate. This is particularly significant for enzymes which show sigmoidal substrate saturation kinetics. Enzyme activity can also be controlled by changes in intracellular pH, the extent to which this is important for each enzyme being determined by how broad or narrow the pH activity profile of that enzyme. Regulation of enzyme activity can also involve reversible associations of subunits within a multimeric protein, normally induced by an effector molecule to increase or decrease activity, or association of enzymes within a metabolic pathway. In the latter example, micro-compartmentation of enzymes has been proposed as a mechanism facilitating the direct transfer of intermediates between sequential enzymes thus increasing metabolic flux. This enzyme association can also result in the alteration of the kinetic properties of enzymes, presumably by inducing conformational changes in proteins during interactions between different enzymes.

In comparison with the mechanisms of fine metabolic control just mentioned, much more is known about allosteric and covalent regulation of enzyme activity. Allosteric effectors can

e.g. ferredoxin, ferredoxin thioredoxin reductase and thioredoxin shuttle electrons from e.t.c. to selected target enzymes, thioredoxin f, m and h. This regulation appears to be of much greater significance in photosynthetic organisms. ⟶

alter enzyme activity by binding to a site distinct from the active site and eliciting a precise change in the enzyme's conformation. This conformational change can then either promote or hinder the interaction of the enzyme with its substrate to affect metabolic flux through a pathway. Covalent modification usually operates in conjunction with allosteric regulation, the covalent modification causing a conformational change in the enzyme which affects its substrate binding and can enhance or reduce the affect of allosteric molecules. Reversible covalent modification is the major mechanism of enzyme regulation used by higher eukaryotes to coordinate the appropriate response to external stimuli. Despite there being over one hundred different types of post-translational protein modification known to occur *in vivo*, very few are thought to be important in enzyme regulation and of these dithiol-sulfide interconversion and reversible phosphorylation are the principal mechanisms of enzyme regulation by covalent modification in higher plants.

Reversible phosphorylation is of particular relevance to the topic of this research as it is a major mechanism of regulating phosphoenolpyruvate carboxylase (PEPc). However, as already mentioned, the different mechanisms of fine control are not mutually exclusive and this concept is illustrated in the research findings described in this thesis. But, before reviewing what is already known about PEPc in higher plants, it will be useful to first consider more thoroughly the process of reversible phosphorylation and the enzymes involved in this method of covalently modifying proteins.

1.2 Protein phosphorylation in plants

The reversible phosphorylation of enzyme protein is a very efficient mechanism of regulation which can yield an enhanced sensitivity of response to a given level of effectors. In some cases, several enzymes in a single or related pathways can be controlled by phosphorylation, by the same or different kinases or phosphatases. Moreover, some enzymes can be phosphorylated at several sites, which may have different effects on activity. Thus phosphorylation can control single pathways or the coordination of pathways in response to different signals.

The phosphorylation state of an enzyme is determined by the balance of kinase and phosphatase activity acting on the protein. The donor for phosphorylation is most often ATP, but can also be GTP, ADP or phosphoenolpyruvate which all have high phosphate group transfer potentials. Dephosphorylation is generally a simple hydrolysis reaction catalyzed by a

protein phosphatase, yielding orthophosphate (P_i). The steady state activity of the target enzyme is adjusted by positive and/or negative effectors influencing the rates of phosphorylation and dephosphorylation. A notable exception to this form of regulation by phosphorylation/dephosphorylation is the chloroplastic enzyme pyruvate, phosphate dikinase (PPDK) which is active when phosphorylated on a catalytic histidine residue and inactive when phosphorylated on a regulatory threonine residue (Roeske and Chollet, 1989; Smith et al., 1994). There is some evidence for lipid-activated kinases (Nanmori et al., 1994; Xing et al., 1996: Subramaniam et al., 1997) although no role has been assigned to them, and a chloroplast kinase responds to redox state in its regulation of the light-harvesting chlorophyll a/b-binding complex (Allen, 1995), but so far, Ca^{2+} is the best characterized second messenger that can affect protein phosphorylation in plants (for review see Hardie, 1999).

1.2.1 Plant protein kinases

It has been estimated that 2-3% of the eukaryotic genome codes for protein kinases. In response to a large and diverse number of signals, these enzymes can phosphorylate proteins on serine, threonine, tyrosine and histidine residues, usually to effect conformational changes in the substrate protein thus altering its properties. Almost all eukaryotic protein kinases contain a kinase catalytic domain of some 260 residues with alternating regions of high and low conservation (Hanks and Hunter, 1995). There are twelve conserved regions, referred to as subdomains, and within these subdomains are to be found some invariant residues which have been shown by crystal structure determinations to be important in catalysis and/or overall structure (for a review, see Wei et al., 1994). Plant protein kinases have been categorized into families based on the eukaryotic protein kinase phylogenetic classification scheme (see Hanks and Hunter, 1995 and Stone and Walker, 1995) and phylogenetic analysis of kinase domains from 89 *A. thaliana* protein kinase sequences (Hardie, 1999). This latter analysis revealed that most of the *A. thaliana* sequences clustered together into about 12 major subfamilies which could be used as a basis for discussing the structure and function of all known plant protein kinases. Those subfamilies for which the most biochemical information is available and which are similar to the conventional protein kinases are outlined below.

The largest well-defined subfamily of the plant protein kinases is the calcium-dependent protein kinases (CDPKs). These are placed in the CaMK group by Hanks and Hunter (1995). However, these differ quite significantly from mammalian members of this family by virtue of

their apparent independence of the calcium-binding protein calmodulin. The DNA sequence of the first plant CDPK clone from soybean (Harmon et al., 1987), was found to encode a kinase catalytic domain followed by a C-terminal domain with 39% identity to calmodulin, with the four "EF-hand" Ca^{2+}-binding motifs of calmodulin being conserved. Hence, these plant kinases are also refered to as calmodulin-like domain protein kinases. Several isoforms of CDPK have been found in many plant species, including *A. thaliana*, their existence potentially enabling both specificity and flexibility in the response to different Ca^{2+}-elevating stimuli. CDPKs have a junction region between the two domains mentioned above, which is thought to function in a regulatory manner similar to the autoinhibitory region of the calmodulin-dependent protein kinases (CaMK), its effect being relieved upon binding of calcium to the protein. Reversible inactivation of nitrate reductase by phosphorylation has been demonstrated using a CDPK from spinach leaves (Douglas et al., 1998) and sucrose synthase can be phosphorylated by CDPK activity from maize leaf and soybean root nodules (Huber et al., 1996; Zhang and Chollet, 1997). However, there are a few members of this subfamily whose activity is either not independent of calmodulin or varies in their requirement for calcium.

The plant SNF1-like kinases are homologs of the *S. cerevisiae* SNF1 serine/threonine protein kinase required for carbon catabolite derepression. The SNF1/AMPK related protein kinase 1 (SnRK1) subgroup exists as large multimeric complexes regulated by phosphorylation by an upstream kinase kinase. A SnRK1 from spinach has recently been shown to be regulated by AMP in a manner similar to the mammalian SNF1 homologs (Sugden et al., 1999). SnRK1 kinases are also proposed to be activated by stresses like starvation for a carbon source and have similar *in vitro* substrate specificity, phosphorylating and thus inactivating the key regulatory enzymes of isoprenoid and sucrose synthesis and nitrogen assimilation, HMG-CoA reductase, sucrose synthase and nitrate reductase, respectively. No *in vivo* substrates have yet been confirmed for the SnRK1 kinases. Members of the other plant SNF1-like kinase subgroup, SnRK2, contain kinase domains related to those of Snf1 and AMPK, but the C-terminal domains are unrelated and the functions and biochemical properties of these kinases remain unknown.

The receptor-like kinase (RLK) subfamily share many structural features with the mammalian receptor-linked kinases (single transmembrane helix, C-terminal cytoplasmic kinase domain) but so far have no identifiable ligands. However, many interesting members belong to the group including the *Xa21* gene in rice responsible for resistance to some strains of bacterial

pathogen, the leucine-rich repeat (LRR) group which is thought to be involved in protein-protein interactions and the *S* locus receptor-like kinase (SRK) involved in the genetically well-characterized phenomenon of self-incompatability in *Brassicaceae*. Autophosphorylation occurs on the kinase domains of most RLKs, reminiscent of the better characterized receptor-linked kinases whereby autophosphorylation facilitates the binding of signalling complexes to the kinase domain of the receptor thus initiating the intracellular transduction of a signal.

The next four subfamilies of plant kinases to be discussed are classed together in the CMGC group according to Hanks and Hunter (1995) and are the mitogen activated protein kinases, the cyclin-dependent kinases, glycogen synthase kinase-3 homologs and the casein kinase II family.

Mitogen-activated protein kinases (MAPK) are serine/threonine kinases that are activated by dual phosphorylation on threonine and tyrosine residues by MAPK kinase (MAPKK). The phosphorylation motif recognised by MAPKK is threonine-X-tyrosine in the activation loop of MAPK. MAPKK is an unusual type of kinase in that it phosphorylates serine, threonine and tyrosine residues. MAPKK is phosphorylated by MAPKK kinase (MAPKKK) and thus by forming these phosphorylation cascades, MAP kinase and associated kinases are able to link extracellular signals to intracellular events such as cell division, gene expression and control of growth, through phosphorylation of yet other molecules like transcription factors. Many plant MAPK homologs have been cloned and identified and have been shown to be activated by plant hormones and environmental stimuli (touch, cold shock, wounding, pathogen infection). The ATMPK1 and ATMPK2 homologs from *A. thaliana* were able to phosphorylate an artificial MAPK substrate (myelin basic protein, MBP), and this kinase activity was enhanced upon addition of purified *Xenopus* MAPKK. A putative MAPKK activity also accumulated in response to auxin treatment, stimulating phosphorylation of both the MAPK and its MBP kinase activity (Mizoguchi et al., 1984). Although the activity of MAPKs can be observed in various plant species and in response to various external signals, no plant MAPK cascade has yet been mapped out in any detail.

Cyclin-dependent kinases (CDK) form complexes with cyclins and/or inhibitory subunits to control progression through the cell cycle in eukaryotes. CDKs have a conserved amino acid motif, PSTAIRE, involved in the interaction with cyclin. Full activation of CDKs requires both binding of the appropriate cyclin and phosphorylation on a threonine residue in the activation loop. Phosphorylation of CDK on a tyrosine residue in the ATP-binding loop (P

Glycogen is a readily mobilized storage form of glucose, GS adds UDP-glucose to nonreducing ends of glycogen chains, glycogen phosphorylase cleaves glucose 1-P from glycogen, insulin activates glycogen synthase by the dephosphorylation of glycogen synthase, GSK-3 is involved in the multisite phosphorylation of IRS-1 phosphatase activity acting on glycogen synthase

loop) inhibits the kinase function in some mammalian systems. It would seem that an individual CDK may have multiple roles, determined by the cyclin to which it is bound and suggested by the greater number of cyclins than CDKs. Homologs of both cyclin and CDK have been found in many different plant species and direct evidence of CDK involvement in cell cycle regulation is emerging e.g. expression of a dominant negative mutant of *A. thaliana* Cdc2 kinase in tobacco plants disrupted the cell division *in vitro* of leaf mesophyll protoplasts derived from the transgenic plants (Hemerley et al., 1998).

Mammalian glycogen synthase kinase-3 (GSK3) is implicated in hormonal responses, phosphorylating and inactivating glycogen synthase. It is part of a signal transduction pathway downstream of the insulin and insulin-like growth factor-1 receptors and is inactivated through phosphorylation by protein kinase B on a serine residue in response to stimulation of the pathway. The phosphorylation requirement of a specific tyrosine residue for maximal enzyme activity highlights in GSK3 both the importance of protein phosphorylation in the regulation of many signalling pathways and the compexity of regulating different kinase activites acting onthe same substrate. Autophosphorylation on serine, threonine and tyrosine residues occurs with GSK3 homologs in *A. thaliana*. Several GSK3 homologs have been identified in plants, and together with mammalian GSK3 have good sequence similarity with the *shaggy* gene from *Drosophila melanogaster* which encodes a protein kinase involved in a pathway controlling development. Therefore, the different isoforms of the plant GSK3/*shaggy* homologs which show tissue-specific expression patterns have been assumed to contribute to control of plant development although little evidence exists to confirm this.

Casein kinase I and II homologs have been identified in plants. Similar to the mammalian enzymes, both CKI and CKII phosphorylate serine or threonine residues in the context of nearby acidic side chains and phosphorylate many different substrates *in vitro*. Although an *in vivo* function has yet to be characterized for the plant enzymes, and they appear to lack regulation *in vivo*, *Arabidopsis* plant extracts contain a CK2-like activity that affects the formation of a DNA-protein complex containing the transcription factor CCA1 (circadian clock-associated 1). *In vitro* studies showed that CCA1 is able to specifically interact with different subunits of CK2 and that recombinant CK2 can phosphorylate CCA1 (Sugano et al., 1998).

Thus, it is apparent that many plant protein kinases are found ubiquitously in other eukaryotes (e.g. SNF1, MAPK and CDK) whilst other kinases are conspicuously absent from plants, such as the cyclic nucleotide-dependent protein kinases and conventional protein tyrosine

kinases. Conversely, higher plants have unique protein kinases distinct from those found in most eukaryotes (e.g. RLKs and CDPKs). Intriguingly, in plants, the protein kinases implicated in the earlier stages of signaling pathways are unique but the different signals often then converge into pathways that use more highly conserved protein kinases that are universal in eukaryotes. In consideration of the many environmental and developmental signals to which plants must respond, it is perhaps understandable why these differences in the early steps of signal transduction exist.

Although somewhat lagging behind mammalian protein kinase research, the knowledge of plant protein kinases has increased dramatically in the last decade. However, much of this knowledge is limited to the amino acid sequence of several hundred plant protein kinases available on database, the result of much cloning by sequence homology. Other molecular approaches like the yeast two-hybrid screen and interactional cloning, complemented by biochemical analysis, are required to learn more about the function and regulation of individual kinases.

1.2.2 Plant protein phosphatases

Protein phosphatase activity has been detected in different subcellular compartments of plants (Smith and Walker, 1996). They have been classed into three major groups which are biochemically almost indistinguishable from the mammalian type-1, -2A and -2C protein/serine phosphatases, classed according to their specificities for the substrate phosphorylase kinase (MacKintosh and Cohen, 1989). Some less well characterized plant protein phosphatases may represent novel enzymes that are unique to plants.

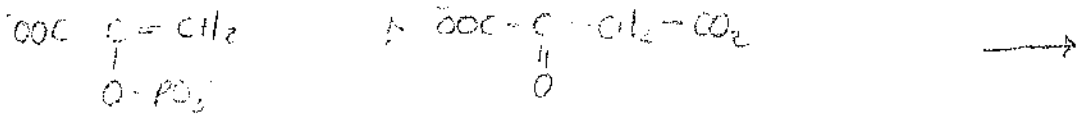
Mammalian and fungal PP1 enzyme is a complex of a catalytic subunit and one or more regulatory subunits thought to define specific functions of the catalytic activity of PP1 *in vivo* by controlling the subcellular location and substrate specificity of the enzyme complex. An interesting mammalian example is the regulatory subunit I-2 which is phosphorylated by GSK-3 in the presence of ATP-Mg and is hypothesized to then act as a chaperone that binds and activates newly synthesized PP1 catalytic subunits (Smith and Walker, 1996). At present, very little is known about the structure, function or physiological substrates of PP1 activity in plants, however similarities in biochemical properties and subcellular distribution with animal PP1 may give some hints as to what identification and characterization of PP1 regulatory subunits will reveal.

The PP2 subgroups are based on differences in subunit composition, divalent cation requirements and substrate specificites. PP2A is a heterotrimer of a catalytic subunit and two distinct regulatory subunits and does not require divalent cations for activity. PP2B, which has not been identified in plants, is a heterodimer with a catalytic subunit and a regulatory subunit which contains an EF-hand motif able to bind calcium for its activation. PP2C is a monomer requiring magnesium for activity.

Weiner and Weiner (1997) suggested that plants have more genes for the regulatory and catalytic subunits of PP2A than animals, thus implying that PP2A-type enzymes have some distinct regulatory properties and functions in plants. They demonstrated that the PP2A activity which activates sucrose phosphate synthase (SPS) in spinach leaves after illumination, is regulated by an interconversion between the trimeric (high activity) and dimeric (low activity) forms of the enzyme. Although the exact signal for this interesting mode of regulation was unknown, protein synthesis was shown to be involved in the trimer formation which it is proposed occurs in response to perceived changes in the metabolic phosphate and sugar status in the cytoplasm. PP2A is also the major enzyme responsible for inactivating the cytosolic enzyme quinate dehydrogenase (QDH) (MacKintosh et al., 1991) and is responsible for the dephosphorylation of PEPc, SPS and NR, three key metabolic enzymes, discussed in section 1.8 in the context of coordination of carbon and nitrogen metabolism.

Use of protein phosphatase inhibitors has helped reveal the involvement of protein phosphatases in plant signal transduction. Some of these inhibitors are natural toxins such as cyclosporin A and the marine toxin okadaic acid which are readily taken up by animal and plant cells. Differences in the potency of inhibitors for protein phosphatases has been utilized in several studies. For example, Sheen (1993) used okadaic acid to investigate the role of phosphatase activity in the greening of etiolated maize leaves in response to light. 1 μM okadaic acid blocked the transcription of light-induced genes from the C_4 *ppdk* (pyruvate phosphate dikinase) promoter and the *rbcS* ((Rubisco) promoter but was ineffective at 100 nM. Okadaic acid inhibits PP2A ($I_{0.5}$= 0.1-1.0 nM) more potently than PP1 ($I_{0.5}$=10-100 nM) (MacKintosh and MacKintosh, 1994). Thus, this evidence might suggest that PP1, and not PP2A, activity is necessary for the light-induction of *ppdk* and *rbcS* in etiolated maize leaves.

Although physiological substrates for PP2C have not been identified in plants, its activity has been detected in carrot cells, pea leaves and wheat leaves (MacKintosh et al., 1991). An interesting example of a plant PP2C involved in plant signalling is kinase-associated protein



ΔG value across reaction is irreversible when large and negative

phosphatase (KAPP). Isolated from *A. thaliana* by interactional cloning, KAPP interacts with the autophosphorylated form of the catalytic domain of RLK5 and consists of an N-terminal signal anchor, a kinase interaction domain and a C-terminal PP2C domain (Stone et al., 1994). The structure of KAPP and its interaction with RLK5 suggests that KAPP may control early steps in the RLK5 signalling pathway which participates in self-incompatability, defense responses and plant development.

A paucity of knowledge regarding the physiological substrates and regulatory subunit role of plant protein phosphatases will need to be overcome in order to further knowledge of the biological processes that these enzymes help regulate. Use of inhibitors has so far, and will hopefully continue, to help in this task.

1.3 Phosphoenolpyruvate carboxylase in higher plants

1.3.1 The reaction catalyzed by PEPc

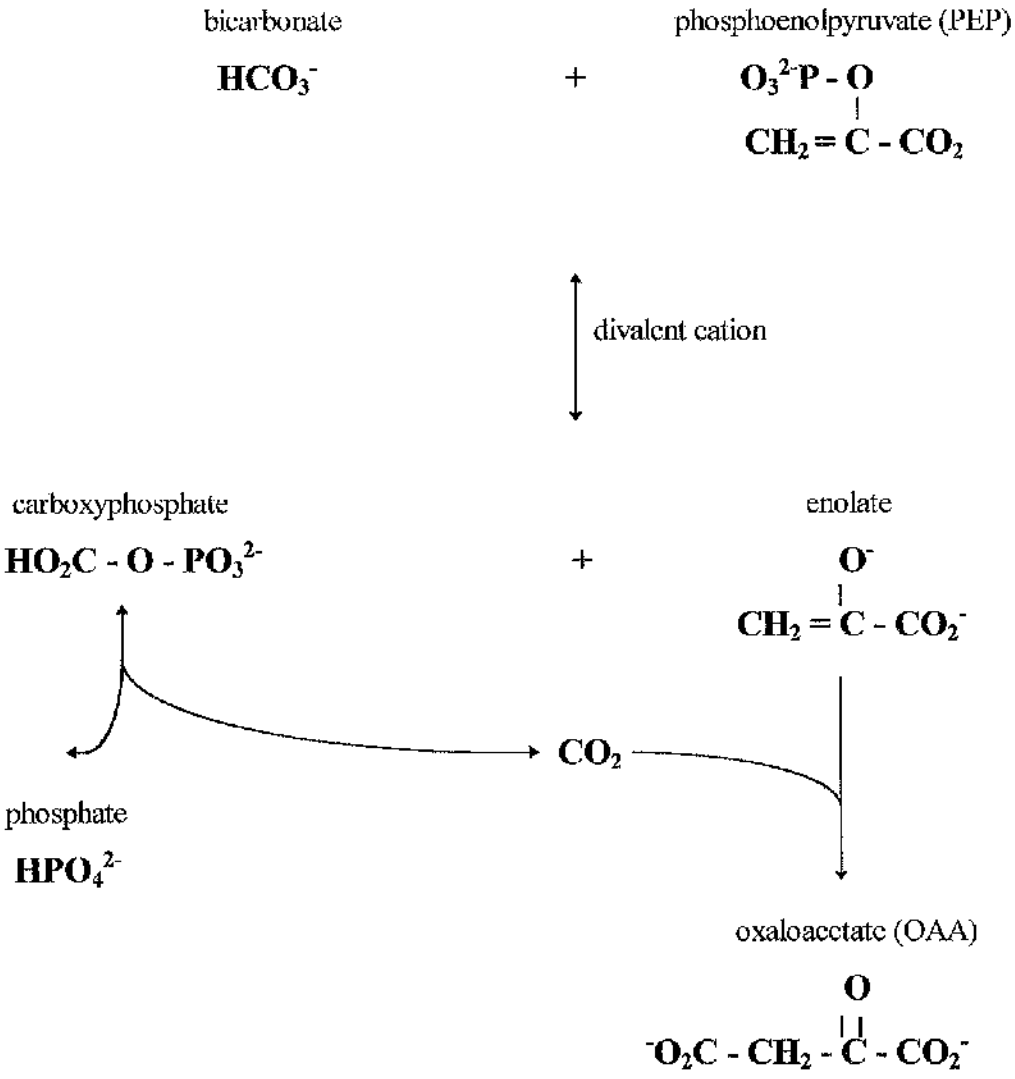
PEPc (EC 4.1.1.31) is found in the cell cytosol of all higher plants and catalyzes the carboxylation of PEP to OAA. The substrate is bicarbonate (HCO_3^-) rather than CO_2, and a divalent cation is required for activity, Mg^{2+} fulfilling this role *in vivo* (Andreo et al., 1987). The reaction is very exergonic, having a ΔG value of -6 to -8 kcal/mol. Experimental evidence (Andreo et al., 1987 and references therein) indicates that the reaction mechanism occurs in two steps (Figure 1.1). The first step is reversible and consists of the formation of carboxyphosphate and the enolate of pyruvate from the substrates. The second step is thought to be irreversible (Chollet et al., 1996) and combines the CO_2 and enolate with the concomitant release of OAA and P_i. The OAA is then rapidly reduced into malate by NADP-malate dehydrogenase (MDH, EC. 1.1.1.37) or transaminated into aspartate by aspartate aminotransferase (AAT, EC 2.6.1.1).

1.3.2 The regulation of PEPc

The activity of plant PEPc is subject to allosteric control by a variety of positive and negative metabolite effectors, especially when assayed at sub-optimal pH values that approximate that of the cytosol. Malate is a strong inhibitor of PEPc and glucose 6-phosphate activates the activity of the enzyme (Doncaster et al., 1987; Wedding et al., 1990; O'Leary et al., 1982). The sensitivity of PEPc to control by these allosteric effectors is determined by the phosphorylation state of the PEPc protein. When assayed at pH 7.3 and 1 mM pyruvate,

Figure 1.1 The reaction catalyzed by PEPc

Mechanism of the carboxylation of PEP catalyzed by PEPc. The figure was modified from Vidal and Chollet (1997).



recombinant PEPc from *Sorghum* had an apparent K_i for malate of 0.17 mM and 1.2 mM, and a K_a for glucose 6-phosphate of 1.3 mM and 0.28 mM, for the dephosphorylated and phosphorylated forms of PEPc respectively (Duff et al., 1995). Therefore, although changes in the cytosolic levels of these opposing allosteric effectors and H^+ likely contribute to the overall regulation of PEPc activity *in vivo*, research over the past decade has focused primarily on the reversible phosphorylation which fine tunes the response of the enzyme to allosteric control.

PEPc has been shown to be phosphorylated at the N-terminal on one or more serine residues in many different plant species (Budde and Chollet, 1986; Nimmo et al., 1986; Jiao and Chollet, 1988; Nimmo et al., 1987; Van Quy et al., 1991; Duff and Chollet, 1995; Zhang et al., 1995). Phosphorylation of the enzyme is catalyzed by a highly regulated protein kinase refered to as PEPc kinase (section 1.8) and dephosphorylation by a typical mammalian-type protein phosphatase type 2A (e.g.; Carter et al., 1990; Echevarria et al., 1990; Jiao et al., 1991; McNaughton et al., 1991; Jiao and Chollet, 1992). PEPc is "activated" *in vivo* when it is phosphorylated but this activation does not result in an increase in the V_{max} of the enzyme but manifests itself by a decrease in sensitivity to feedback inhibition by malate (Nimmo et al., 1984; Nimmo et al., 1987). The serine residue that becomes phosphorylated resides in a plant-invariant motif SIDAQ that is absent in the bacterial and cyanobacterial primary structures deduced to date. Thus, this reversible means of fine tuning the activity and allosteric properties of PEPc is apparently unique to the plant enzyme (Chollet et al., 1996).

It has been proposed that dimer-tetramer interconversion (Wu and Wedding, 1985; Wu et al., 1990; Willeford and Wedding, 1992) and the redox state of certain critical cysteine residues (Iglesias and Andreo, 1984; Chardot and Wedding, 1992) may be involved in the regulation of PEPc activity of L-malate sensitivity but these are based wholly on *in vitro* observations and there has been no evidence *in vivo* to support the involvement of either aggregation state changes or the five plant-invariant cysteines in PEPc regulation.

1.3.3 The function of PEPc

The physiological functions of PEPc in higher plants are diverse. The most extensively studied function of the enzyme is the initial fixation of atmospheric CO_2 during CAM and C_4 photosynthesis described in sections 1.5 and 1.6. This important function of PEPc activity concentrates CO_2 at the stromal location of Rubisco activity and, in so doing, favours the

carboxylase activity of Rubisco over its oxygenase activity thus minimizing the loss of energy through photorespiration. Other specialized and more general functions of PEPc are discussed in section 1.7.1.

1.3.4 The structure of PEPc

The PEPc protein is thought to be a homotetramer with an approximately 110 kD subunit. Isoforms of the enzyme have been characterized from both photosynthetic and non-photosynthetic tissues of various plants (for a review see Lepiniec et al., 1994 and Toh et al., 1994). These isoforms can be distinguished by their chromatographic, immunological and kinetic properties. Consistent with the enzyme's functional diversity (section 1.3.3), small multigene families have been found which consist of at least three to four members coding for different forms of PEPc. For example, *Sorghum* has three genes for PEPc which encode for the C_4-photosynthetic isoform, the root form and the C_3- like housekeeping form (Lepiniec et al., 1993). PEPc gene families have also been found in plant species performing CAM and C_3 photosynthesis (Lepiniec et al., 1994; Newman et al., 1994).

PEPc amino acid sequences have been deduced from many different plant species. When aligned, these sequences have been found to contain many highly conserved residues and motifs which have been proposed to be involved in the active site and/or regulation of the enzyme (Lepiniec et al., 1994; Toh et al., 1994). Subsequent site-directed mutagenesis and chemical modification studies have confirmed the involvement of many of these identified sites in such roles. In general, the N-terminal half of the PEPc subunit contains the regulatory sites (Jiao and Chollet, 1990; Jiao et al., 1990; Terada et al., 1990; Toh et al., 1994), for example, the plant invariant phosphorylation motif, and the C-terminal half contains most of the residues implicated in the active site (Andreo et al., 1987; Jiao et al., 1990; Rajagopalan et al., 1994; Terada et al., 1992).

Site-directed mutagenesis studies of the phosphorylation motif of a recombinant sorghum C_4-PEPc demonstrated that the introduction of negative charge into this PEPc domain, by changing the target serine residue to an aspartate (S8D), is sufficient to mimic the regulatory effects of phosphorylation (Wang et al., 1992). Although the S8D form of the enzyme could not be phosphorylated by the C_4 leaf kinase, it was malate-insensitive like the phosphorylated form of the enzyme *in planta* (Duff et al., 1995; Wang et al., 1992). However, when the

serine residue was changed to a cysteine (S8C), the enzyme was neither able to be phosphorylated nor insensitive to inhibition by malate (Wang et al., 1992).

Site-directed mutagenesis studies have also suggested that histidine residues play important roles in the regulatory and catalytic functions of the enzyme e.g. the histidine residue of the amino acid motif VfTAHPT (no. 215-221, the residues noted with capital letters being absolutely conserved, Lepiniec et al., 1993) is thought to be essential for the second partial reaction of PEPc (Terada et al., 1992), whilst chemical modification studies have identified an invariant lysine residue in the highly conserved sequence QqVMvGYSDSgKDaG as essential for catalysis (Jiao et al., 1990). Cysteine residues have also been identified in chemical modification studies as important for the catalytic activity of PEPc but no invariant site occupied by cysteine residues has been found in PEPc sequence alignments. Therefore, cysteine residues in PEPcs may not be involved in catalysis although some may be located near the active sites.

The three-dimensional structure of PEPc from *E. coli* has recently been determined (Kai et al., 1999) and this, together with further mutagenesis studies of conserved sites in the PEPc sequence, should advance knowledge of the quaternary structure of plant PEPc and its relationship to the function of the enzyme.

Due to the sufficient number of PEPc amino acid sequences deduced, it has been possible to study the phylogeny of the different PEPc isoforms. The information resulting from these analyses has led to the proposal of a molecular evolutionary pathway of PEPc which seeks to explain the conservation of residues and motifs between the different isoforms. Considering the plants enzymes only, it has been inferred from these phylogenetic trees that all PEPc sequences diverged from a common ancestral gene (Chollet et al., 1996 and references therein). The PEPc sequences from C_4 monocots like *Sorghum* and maize can be grouped and distinguished from the sequences of the other isoforms in these species and various CAM and C_3 isoforms (Lepiniec et al., 1994), whilst the sequence from the C_4 dicot *Flaveria trinervia* is more closely related to C_3 isoforms of the enzyme than to the C_4 monocot sequences. Further accumulation of PEPc sequence data and more elaborate computational analysis will be required before a clearer and more complete explanation of the origin of the different PEPc isoforms can be presented.

1.4 Photosynthesis and photorespiration

1.4.1 The C_3 pathway of photosyntheis

As mentioned in section 1.3.1, PEPc catalyzes the initial fixation of atmospheric CO_2 in C_4 photosynthesis and CAM, additional specialised photosynthetic pathways developed by certain species in adaptation of their environments. This function of PEPc and its regulation in this role will be discussed in more depth in following sections. However, it will first be useful to briefly recap on the reactions involved in photosynthesis by plants .

Photosynthesis is the process by which green plants use the sun's energy to build up carbohydrate reserves. This carbohydrate is then transported around the plant to tissues where it is either stored or broken down to release the energy it contains to fuel various reactions and processes essential for the plants' survival. It is also the means whereby plants acquire CO_2 from the air for the assimilation of carbon into the various molecules found in the plants. The fundamental pathway of photosynthesis is the same in all plants and is referred to as the C_3 pathway. It derives its name from the three-carbon compound 3-phosphoglycerate (3-PGA) that is produced, but is also known as the reductive pentose phosphate (RPP) or Calvin-Benson cycle (after two of its co-discoverers).

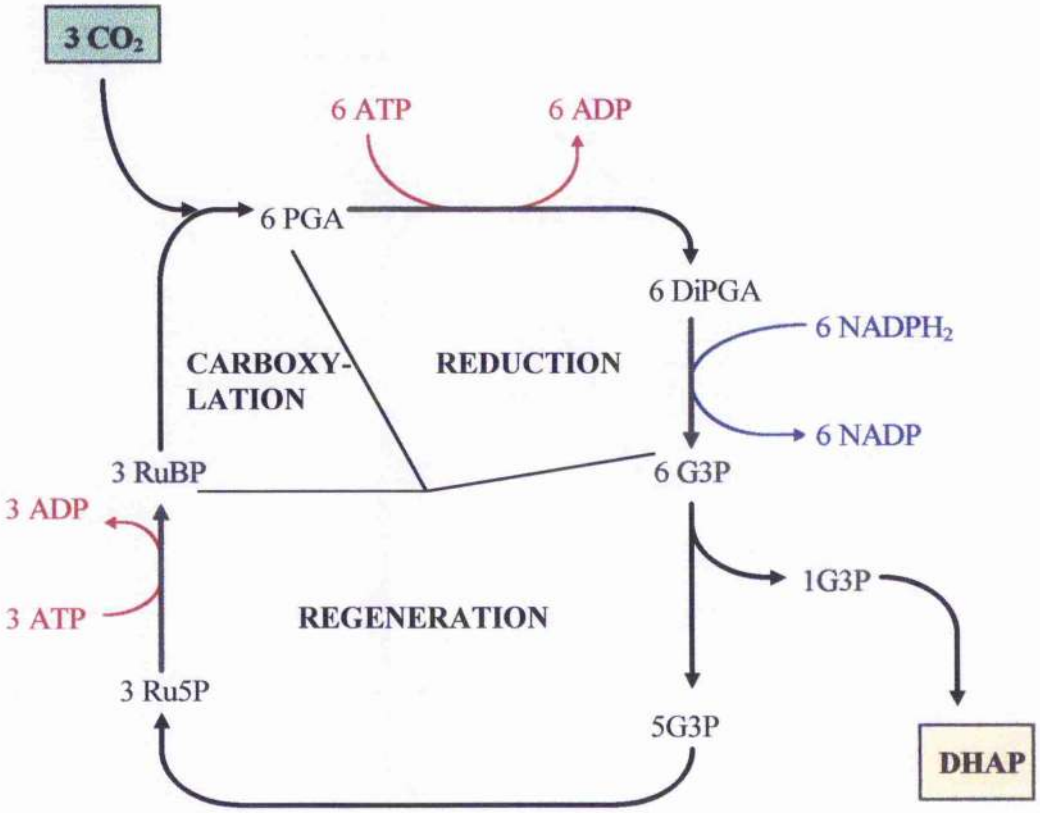
The stromal enzyme Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase, catalyzes the fixation of atmospheric CO_2 and ribulose 1,5-bisphosphate into a short-lived six-carbon intermediate that is is broken down to yield two molecules of three-carbon 3-PGA. Three turns of the RPP cycle use three molecules of CO_2 to produce six glyceraldehyde 3-phosphate (G3P) molecules, of which five are used to regenerate ribulose 1,5-bisphosphate and the sixth results in the net synthesis of one molecule of the triose phosphate, dihydoxyacetone-phosphate (DHAP). This DHAP is then converted into starch in the chloroplast or passes out of the chloroplast into the cytosol where it is used to synthesize sucrose. Thus, the overall pathway comprises three phases: carboxylation of ribulose 1,5-bisphosphate, reduction of 3-PGA using ATP and NADPH produced by the light reactions of photosynthesis, and regeneration of ribulose 1,5-bisphosphate. The individual reactions and the enzymes involved in the pathway are detailed in Figure 1.2.

1.4.2 Photorespiration

Photorespiration is the light-dependent evolution of CO_2 and O_2 uptake which occurs in green leaves of C_3 plants performing photosynthesis. It appears not to have an essential function in

Figure 1.2 The reductive pentose phosphate pathway (RPP) cycle

A simplified diagram of the RPP cycle is outlined below, showing the carboxylation, reduction and regeneration phases and the synthesis of one net molecule of triose phosphate (DHAP) from three molecules of carbon dioxide. For clarity, various intermediate steps have been omitted in the regeneration phase as have the enzymes involved in the cycle. Abbreviations: RuBP, ribulose 1,5-bisphosphate; PGA, 3-phosphoglycerate; DiPGA, 1,3-bisphosphoglycerate; Ru5P, ribulose 5-phosphate; G3P, glyceraldehyde 3-phosphate. Figure is modified from Macdonald, FD and Buchanan, BB (1997).



the life of the plant but is a consequence of the oxygenase activity of Rubisco. CO_2 and O_2 are competitive substrates for the enzyme, their addition to ribulose 1,5-bisphosphate yielding two molecules of 3-PGA via the C_3 pathway, and a molecule of 3-PGA and a molecule of phosphoglycolate via the photosynthetic carbon oxidation (PCO) cycle, respectively (Figure 1.3). The PCO cycle involves the coordinated activity of reactions in the chloroplast, peroxisome and mitochondrion. For every four carbons (two molecules) of phosphoglycolate metabolized in the PCO cycle, one carbon is released as CO_2 from the decarboxylation of glycine by glycine dehydrogenase in the mitochondrion. This reaction also produces ammonia (NH_3) which is reassimilated by glutamine synthetase (GS) followed by glutamate synthase in the chloroplast. Glutamate synthase is also known as glutamine:oxoglutarate aminotransferase (GOGAT) and the system is refered to as the GS/GOGAT system. The other three carbons remaining from the original two molecules of phosphoglycolate are returned to the PCR cycle in the chloroplast as 3-PGA.

Increases in temperature increase the ratio of photorespiration to photosynthesis in C_3 leaves by favouring the oxygenation of ribulose 1,5-bisphosphate by Rubisco and increasing the solubility of O_2 and decreasing the solubility of CO_2. This means that in C_3 plants, the CO_2 compensation point (the CO_2 concentration at which release of CO_2 by photoresiration is equal to uptake of CO_2 by photosynthesis) increases with temperature so that the loss of CO_2 becomes greater than its assimilation. Therefore, certain plants which grow in hot climates have developed supplementary pathways for fixing atmospheric CO_2 thus enabling them to concentrate CO_2 at the site of Rubisco and minimize photorespiration. These pathways are known as Crassulacean Acid Metabolism (CAM) and the C_4 or Hatch-Slack pathway and the enzyme PEPc is central to their specialized biochemistry.

1.5 PEPc and Crassulacean Acid Metabolism

1.5.1 The role of PEPc in CAM

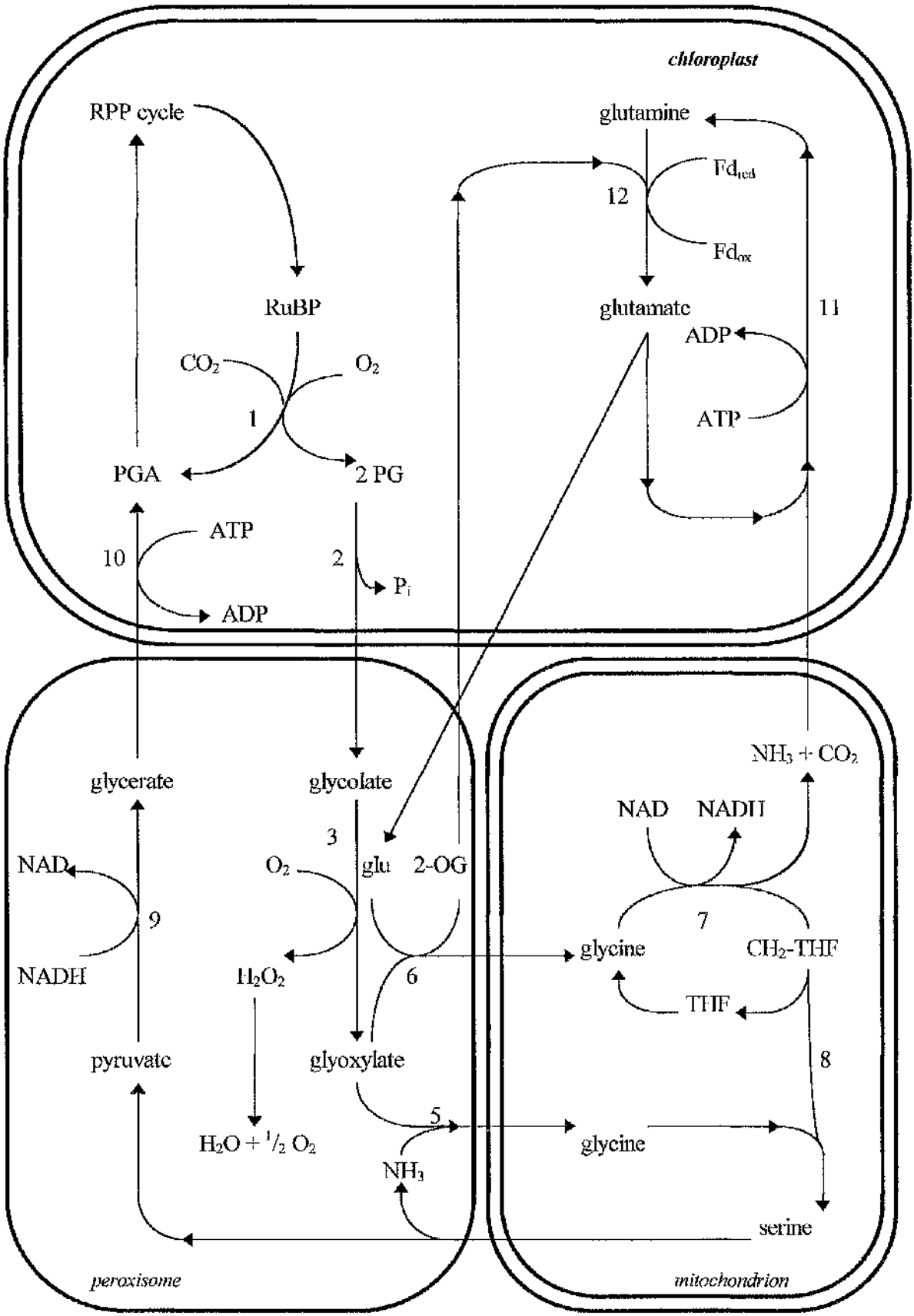
Crassulacean Acid Metabolism is performed in addition to C_3 photosynthesis in plants that live in arid environments. Three features are central to CAM physiology. Firstly, the stomata open at night for gas exchange thus minimizing water loss through evaporation by keeping the stomata closed during the day. Secondly, atmospheric CO_2 is fixed by PEPc when the stomata are open i.e. at night, and stored as malic acid in the cell vacuole. Thirdly, the malic acid diffuses out of the vacuole the following day and is decarboxylated by malic enzyme or

Figure 1.3 Photosynthetic carbon oxidation (PCO) cycle

Abbreviations: Fd(red or ox), ferredoxin (reduced or oxidized); glu, glutamate; 2-OG, 2-oxoglutarate; 2-PG, 2-phosphoglycolate; PGA, 3-phosphoglycerate; RPP, reductive pentose phosphate; RuBP, ribulose 1,5-bisphosphate; THF, tetrahydrofolic acid. The numbers represent the enzymes which catalyze each step and are as follows:

1. Rubisco (ribulose 1,5-bisphosphate carboxylase/oxygenase)
2. phosphoglycolate phosphatase
3. glycolate oxidase
4. catalase
5. serine:glyoxylate aminotransferase
6. glutamate:glyoxylate aminotransferase
7. glycine decarboxylase
8. serine hydroxymethyltransferase
9. hydroxypyruvate reductase
10. glycerate kinase
11. glutamine synthetase
12. glutamate synthase

Figure modified from Canvin, DT and Salon, C (1997).


chloroplast
RPP cycle
RuBP
CO_2
O_2
1
PGA
2 PG
ATP
10
ADP
2
P_i
glutamine
Fd_{red}
12
Fd_{ox}
glutamate
11
ADP
ATP
glycerate
glycolate
3
NAD
9
NADH
O_2
glu
2-OG
H_2O_2
6
pyruvate
glyoxylate
$H_2O + {}^1/_2\ O_2$
5
NH_3
$NH_3 + CO_2$
NAD
NADH
7
glycine
CH_2-THF
THF
8
glycine
serine
peroxisome
mitochondrion

PEP carboxykinase (PEPCK), depending on the type of CAM plant. The CO_2 released is then fixed by Rubisco in the chloroplast in the normal C_3 photosynthetic pathway. Therefore, the primary and secondary CO_2 fixation reactions are temporally compartmented, fixation by PEPc occuring at night and fixation by Rubisco occuring during the day respectively. This compartmentation is represented diagrammatically in Figure 1.4. Therefore, CAM concentrates CO_2 in the leaf to a level sufficient to inhibit the oxygenase activity of Rubisco and minimize the loss of energy and assimilation products (gln) that occurs as a consequence of photorespiration. CO_2 fixation during CAM is characterized by four distinct phases:

1) a marked increase in CO_2 assimilation catalyzed by PEPc during the night which begins to decline towards the end of the night due to feedback inhibition of PEPc activity by the high levels of malate being accumulated

2) a brief burst of CO_2 fixation into both C_4 and C_3 compounds as illumination activates Rubisco whilst PEPc is still active

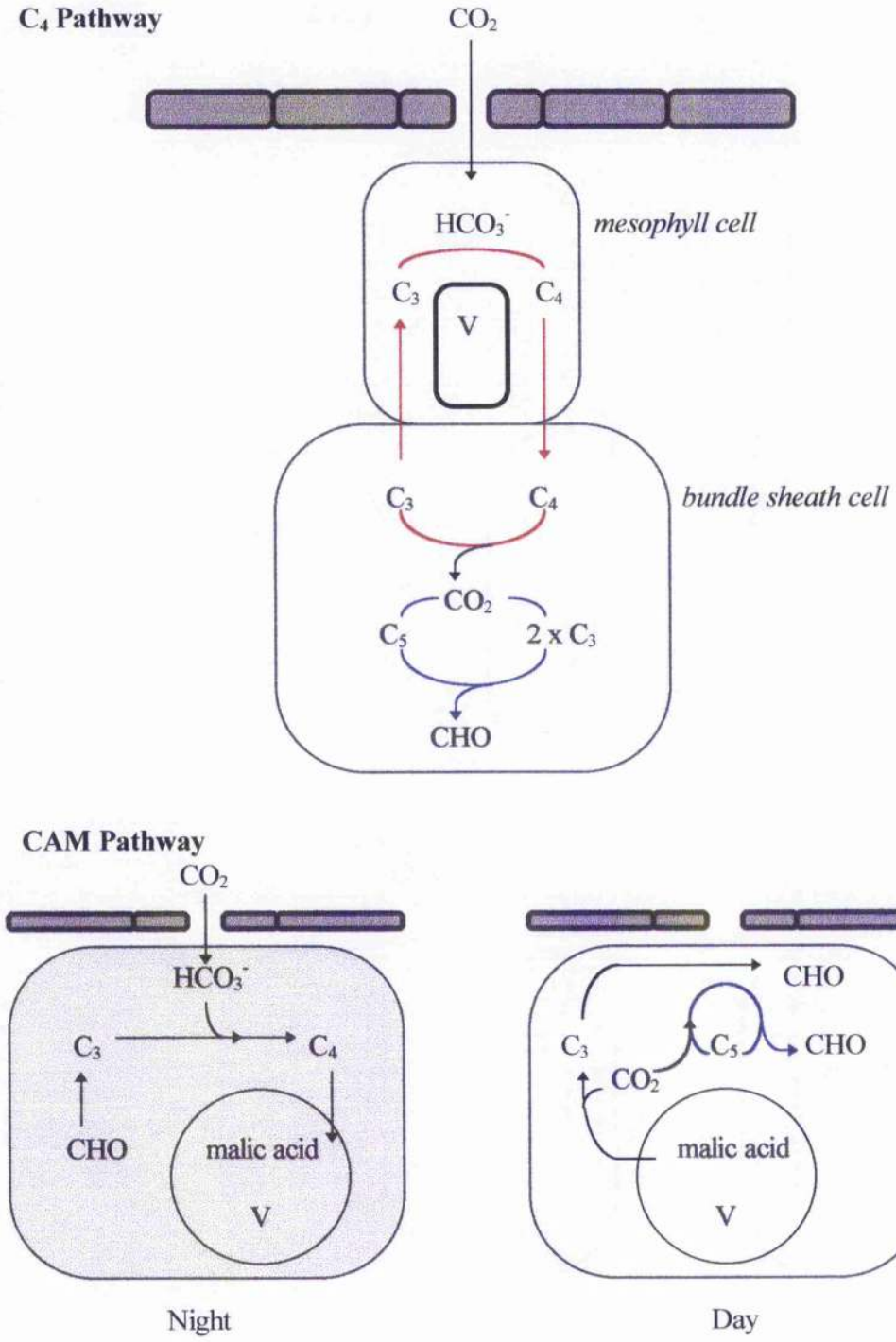
3) net CO_2 uptake reaches a minimum during the day as stomata are closed, vacuolar malic acid is decarboxylated and the CO_2 released is refixed by Rubisco

4) atmospheric CO_2 uptake then begins to increase due to the activity of Rubisco at the end of the day as stomata open, and PEPc activity may also be contributing towards the end of this phase. As the day ends Rubisco activity ceases and PEPc activity continues to fix atmospheric CO_2 as the cycle returns to phase 1 at the onset of night.

An interesting feature of CAM is the rhythm of CO_2 assimilation which has been studied extensively in the CAM plant *Kalanchoë (Bryophyllum) fedtschenkoi*. Leaves detached from plants at the end of a day period and kept in continuous darkness and a stream of CO_2-free air at 15°C, exhibited a circadian rhythm of CO_2 assimilation for 3-4 days (Wilkins, 1959; Wilkins, 1962). The rhythm is characterized by peaks in CO_2 output attributed to inactivity of PEPc and consequent loss of respired CO_2, and troughs attributed to PEPc refixing respired CO_2, the only CO_2 available. PEPc activity is inhibited by the accumulation of malate that it synthesizes (peaks) until the malate is pumped into the vacuole and PEPc activity is resumed (trough). The plateau in CO_2 output at the end of 3 or 4 days has been explained by a vacuolar maximum capacity for malate (Wilkins, 1984) which when reached results in accumulation of newly synthesized malate in the cytoplasm and inhibition of any further fixation of respired CO_2 by PEPc. An alternative explanation is that the rhythm dies out because the energy reserves of the leaves have been used up (Nimmo et al., 1987). The

Figure 1.4 Compartmentation of CO_2 fixation reactions in C_4 and CAM species

The diagram below shows the spatial compartmentation and temporal compartmentation of the CO2 fixation reactions catalyzed by PEPc and Rubisco.The C_4 cycle is indicated by red lines (—) and the Calvin cycle is indicated by blue lines (—). Vacuoles are denoted by a V. Figure modified from Leegood et al. (1997).



rhythm can be reinitiated however, by a 4 hour exposure of the leaves to light which causes the malate to diffuse out of the vacuole into the cytoplasm where it is decarboxylated giving rise to a large peak in CO_2 output (Wilkins, 1992).

Detached *K. fedtschenkoi* leaves kept in continuous light and a stream of normal air at any temperature, below 31°C, exhibit a circadian rhythm of CO_2 assimilation as opposed to output, which lasts for at least 10 days and which has a shorter time period between peak and trough (Anderson and Wilkins, 1989; Wilkins, 1984; Wilkins, 1992). The persistence of the rhythm can be explained by malate moving readily from the vacuole to the cytoplasm which prevents any accumulation of malate in the cytoplasm when the vacuole reaches its maximum malate capacity. Malate synthesized during the first period of PEPc activity inhibits the enzyme and then is decarboxylated sooner than in continuous darkness (hence the shorter rhythm) thus allowing PEPc to become active again repeating the cycle with a low leaf malate content. CO_2 released from decarboxylation of malate while PEPc is still inactive is either refixed by Rubisco or escapes from the leaf.

1.5.2 The regulation of PEPc in the CAM plant *K. fedtschenkoi*

Early work on CAM PEPc suggested that it was regulated simply by changes in cytosolic malate concentration. However, further studies indicated that the regulation was more complex. For example, it was reported that PEPc from the CAM species *Mesembryanthemum crystallinum* is more sensitive to inhibition by malate (i.e. less active under physiological conditions) during the day than at night (Winter, 1982). Biochemical investigation of PEPc in *K. fedtschenkoi* revealed that the enzyme was subject to a striking diel regulation *in vivo* that paralleled the changes in CO_2 assimilation. PEPc enzyme assayed in desalted extracts of *K. fedtschenkoi* leaves taken from the middle of a night period had an apparent K_i for L-malate of 3.0 mM and PEPc assayed from desalted day extracts has an apparent K_i for L-malate of 0.3 mM (Nimmo et al., 1984). Thus, a significant change in the sensitivity of PEPc to L-malate and consequently the activity of PEPc, was demonstrated under near-physiological assay conditions but without accompanying changes in V_{max} or PEPc amount (Nimmo et al., 1984). Inactivation of the enzyme during the day would avoid the futile cycling of CO_2 fixation by PEPc.

Immunological confirmation that the amount of PEPc protein did not change throughout the period studied (Nimmo et al., 1986) suggested that PEPc was regulated by some form of

post-translational modification. $^{32}P_i$ was fed to detached leaves for 72 hours and then extracts from the middle of the day and night were made and the PEPc immunoprecipitated. The day and night PEPc samples were then analyzed for incorporation of the radioactivity by SDS-PAGE and autoradiography. Only PEPc from the night extract contained $^{32}P_i$ (Nimmo et al., 1984) and two-dimensional thin-layer electrophoresis revealed that the phosphate group was bound to a serine residue (Nimmo et al., 1986). As suggested by these results, purification of the day and night forms of *K. fedtschenkoi* PEPc and the ability to alter the apparent K_i for L-malate of the night form to that of the day form by removal of the phosphate group by alkaline phosphatase *in vitro* (Nimmo et al., 1986), confirmed that the diel variation in the malate sensitivity of PEPc was regulated by post-translational phosphorylation of the PEPc protein.

This result led to two further developments in the understanding of the regulation of PEPc in *K. fedtschenkoi*. First, Nimmo et al. (1987) investigated the malate sensitivity of PEPc extracted from leaves kept in continuous darkness and CO_2-free air at 15°C and found the PEPc to exhibit a persistent circadian rhythm of interconversion between the malate sensitive and malate insensitive forms. This rhythm paralleled that of CO_2 output previously observed by Wilkins (1959; 1962). However, the rhythm was not observed in leaves kept in continuous darkness and normal air at 15°C (Nimmo et al., 1987). Second, a protein phosphatase type-2A (PP2A) activity was identified in *K. fedtschenkoi* and found to be capable of dephosphorylating the phosphorylated "night" form of PEPc (Carter et al., 1990). However, no diurnal variation occurred in the specific activity of the PP2A over a 24 hour period (Carter et al., 1991).

Thus attention began to focus on a kinase activity in *K. fedtschenkoi* leaves capable of phosphorylating PEPc as the major regulatory component of PEPc malate sensitivity. A time course of this PEPc kinase activity showed that its presence at a high level throughout the middle of a 16 hour night period period and at a virtually undetectable level throughout the 8 hour day period correlated with the high and low apparent K_i values of PEPc for L-malate, respectively (Carter et al., 1991). The kinase activity appeared 4-5 hours after the beginning of the night period and disappeared 2-3 hours before the end of the night period, exactly in parallel with the changes in the malate sensitivity of PEPc. Moreover, kinase activity oscillated in constant conditions. Therefore, it had been demonstrated that kinase activity is responsible for the diurnal and circadian rhythm of PEPc phosphorylation. Treatment of

detached *K. fedtschenkoi* leaves with the protein synthesis inhibitors cycloheximide and puromycin blocked the nocturnal appearance of kinase activity. They also blocked both the circadian rhythm in CO_2 output and the interconversion of PEPc between the phosphorylated malate-insensitive and dephosphorylated malate-sensitive forms observed under constant darkness and CO_2-free air (Carter et al., 1991). This suggested that PEPc kinase is regulated directly by protein synthesis, controlled by a circadian oscillator. An alternative explanation was that the inhibitors were preventing the synthesis of another protein that activated PEPc kinase. Further studies using the RNA synthesis inhibitors rifampicin and actinomycin D prevented the appearance of PEPc kinase activity in the middle of the night period following the inhibitor treatment (Nimmo et al., 1993) and the malate sensitivity of PEPc in these night extracts was typical of the dephosphorylated form of the enzyme. This suggested that both protein synthesis and RNA synthesis were involved in the circadian control of PEPc activity by PEPc kinase in *K. fedtschenkoi*.

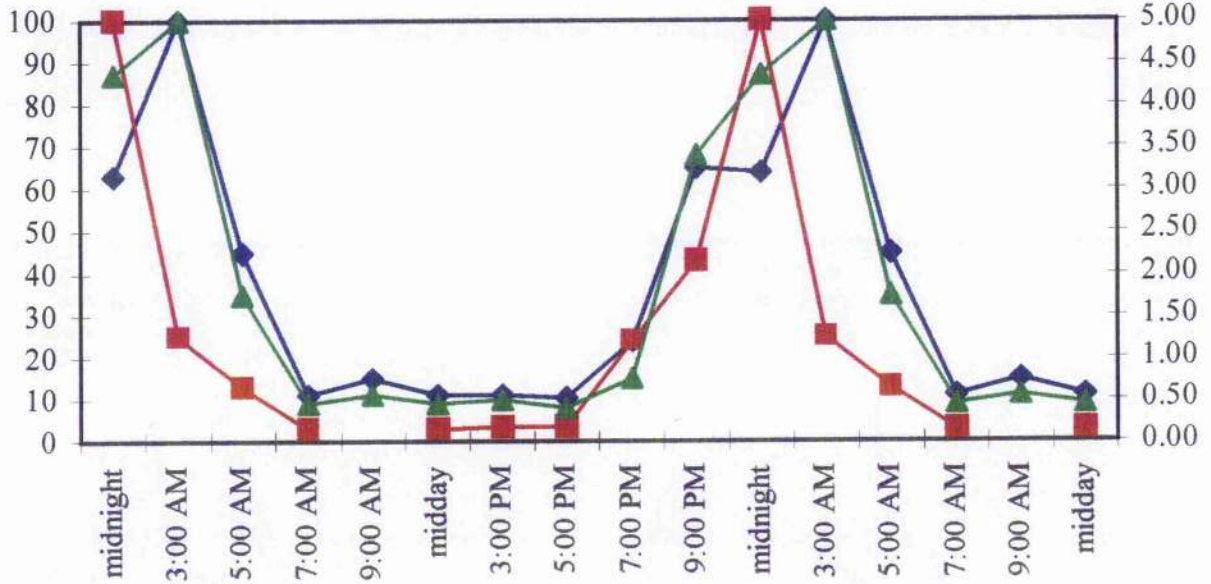
The development of a combined *in vitro* translation and kinase assay (Hartwell et al., 1996) allowed the quantification of translatable mRNA for the PEPc kinase protein. Total RNA was isolated from leaves taken at various points throughout the normal diurnal cycle of *K. fedtschenkoi* and translated *in vitro*. The translation products were then assayed for PEPc kinase activity. Extracts from leaves taken at the same points were also assayed directly for kinase activity and the apparent K_i of PEPc for L-malate. All three sets of data are shown together in Figure 1.5. The translatable PEPc kinase mRNA, PEPc kinase activity and apparent K_i of PEPc for L-malate exhibit the same diurnal pattern. This excellent correlation between the three makes it unlikely that the effect of protein synthesis inhibitors is to prevent synthesis of a protein that activated the kinase (Carter et al., 1991).

This apparent regulation of PEPc kinase by protein synthesis was an important and exciting finding as protein kinases in animal systems are typically regulated by a second messenger, a metabolite or a phosphorylation cascade (section 1.2.1) without net synthesis/degradation of the kinase. The changes in the level of translatable PEPc kinase mRNA may be due to changes in transcription or mRNA turnover. Also worth considering is the possibility that PEPc kinase synthesis is controlled by variations in the translatability of the kinase mRNA, rather than its amount. However, the nature of the assay for kinase translatable mRNA makes this unlikely but not impossible. Hartwell et al. (1996) also demonstrated that the appearance of kinase translatable mRNA can be prevented by not only the RNA synthesis inhibitor

Figure 1.5 Diurnal variation in PEPc kinase protein, translatable mRNA and PEPc K_i for L-malate.

The data from the experiment are shown in the graph opposite: % PEPc kinase protein (◆); % PEPc kinase translatable mRNA (■); PEPc K_i for L-malate (▲). Figure taken from Hartwell et al. (1996).

Diurnal variation in PEPc kinase protein, translatable mRNA and PEPc K_i for L-malate



cordycepin, but by treatment with the protein synthesis inhibitor puromycin, thus implying that there is a requirement for protein synthesis at the level of appearance of the PEPc kinase translatable mRNA as well as at the translation of the mRNA into kinase protein (Carter et al., 1991).

The correlation between the rhythms of PEPc kinase translatable mRNA, kinase activity and apparent K_i was observed during all inhibitor treatments and incubation conditions already discussed (Nimmo et al., 1987; Carter et al., 1991). In addition, Carter et al. (1995) showed that the circadian disappearance of PEPc kinase activity could be accelerated by high temperature and delayed by low temperature. Temperature also affected the amount of translatable kinase mRNA (Hartwell et al., 1996), the effect paralleling that of the kinase activity (Carter et al., 1995). When applied during troughs of CO_2 output i.e. when PEPc kinase is active and PEPc is phosphorylated, high temperature was also shown capable of resetting the phase of the circadian rhythm of CO_2 metabolism in *K. fedtschenkoi* (Wilkins, 1992).

One final facet of the regulation of PEPc kinase in *K. fedtschenkoi* to be mentioned is its calcium-independence. Some plants contain a CDPK capable of phosphorylating PEPc with low activity, but the role of this is not clear (Ogawa et al., 1998; Nhiri et al., 1998) Neither the PEPc kinase assayed directly from *K. fedtschenkoi* leaf extracts (Carter et al., 1991) nor the *in vitro* translated activity of PEPc kinase require Ca^{2+} (Hartwell et al., 1996) and it is generally accepted that the kinase important for the regulation of PEPc is calcium-independent.

From the work described above, a picture is emerging of a tightly coordinated regulation of PEPc in *K. fedtschenkoi*, the principal features being circadian control of a PEPc kinase at the level of its translatable mRNA and activity. This present knowledge of the regulation of PEPc in *K. fedtschenkoi* is outlined in Table 1.1. The phosphorylation and circadian rhythms of PEPc activity have been observed in several other CAM species as has PEPc kinase activity (for a review see Nimmo, 1998).

1.6 PEPc and C_4 photosynthesis

1.6.1 The role of PEPc in C_4 photosynthesis

Many plants that grow in climates with high temperature and light intensity perform C_4 photosynthesis. Like CAM, C_4 photosynthesis serves to concentrate CO_2 at the stromal site

Table 1.1 Comparison of the regulation of PEPc in CAM, C_4 and C_3 species

The table below is a summary and comparison of the regulation of PEPc in the three photosynthetic classes, CAM, C_4 and C_3. The different components involved are listed on the left-hand column along with some of the relevant references. For brevity, not all the significant experimental data is given but a more complete coverage of research developments is given in the text of Chapter 1.

	CAM	C_4	C_3
malate inhibition (Doncaster and Leegood, 1987; Wedding et al., 1990)	yes	yes	yes
malate sensitivity altered by reversible phosphorylation by Ca^{2+}-independent PEPc kinase (Carter et al., 1991; Jiao and Chollet, 1989; Duff and Chollet, 1995)	yes; under circadian control	yes; phosphorylation state increases in response to illumination	yes; phosphorylation state increases in response to illumination
***de novo* protein synthesis involved** (Hartwell et al., 1996; Bakrim et al., 1992)	yes; two protein synthesis steps involved - production of PEPc kinase mRNA and translation of PEPc kinase protein	yes; one protein synthesis step involved in the production of PEPc kinase activity	yes; one protein synthesis step involved in the production of PEPc kinase activity

transcriptional regulation involved (Carter et al., 1991; Hartwell et al., 1996; Nimmo et al., 1993; Giglioli-Guivarc'h et al., 1996)	yes; RNA synthesis inhibitors block circadian increase in kinase activity; increase in translatable PEPc kinase mRNA levels parallels that of kinase activity	yes; conflicting data on the effect of RNA synthesis inhibitors on the increase in kinase activity; increase in translatable PEPc kinase mRNA levels parallels that of kinase activity	yes; RNA synthesis inhibitors prevent increase in PEPc kinase activity; increase in translatable PEPc kinase mRNA levels parallels that of kinase activity
Ca^{2+}/calmodulin interaction (Hartwell et al., 1996; Pierre et al., 1992; Giglioli-Guivarc'h et al., 1996; Smith et al., 1996)	yes; e.g. Ca^{2+}/calmodulin antagonist prevented increase in PEPc kinase translatable mRNA and kinase activity	yes; e.g. Ca^{2+}/calmodulin antagonist prevented increase in PEPc kinase translatable mRNA and kinase activity	yes; addition of Ca^{2+} inhibits PEPc kinase activity but depletion increased K_i of PEPc for malate without increasing PEPc kinase activity
N-metabolism (Van Quy et al., 1991; Duff and Chollet, 1995)	?	?	yes; nitrate enhances PEPc kinase activity
cytosolic pH (Pierre et al., 1992; Giglioli-Guivarc'h et al., 1996)	?	yes; weak bases increase cytosolic pH and phosphorylation state of PEPc	?
photosynthetic electron transport (McNaughton et al., 1991; Giglioli-Guivarc'h et al., 1996; Duff and Chollet, 1995)	?	yes; pre-treatment with inhibitors blocked light-induction of PEPc kinase activity	yes; pre-treatment with inhibitors blocked light-induction of PEPc kinase activity

of Rubisco activity. In contrast to CAM, the two CO_2 fixation events catalyzed by PEPc and Rubisco are separated spatially as opposed to temporally. This spatial compartmentation is facilitated by the specialized "Kranz" leaf anatomy displayed by C_4 plants (Figure 1.4). The CO_2 fixation by phosphoenolpyruvate carboxylase (PEPc) occurs in the mesophyll cells of the C_4 leaf. The cytosolic PEPc combines atmospheric CO_2 with phosphoenolpyruvate (PEP) to form the four-carbon compound oxaloacetate (OAA), hence the name C_4 photosynthesis. The OAA is converted into either malate by malate dehydrogenase (MDH) or aspartate by aspartate aminotransferase (AAT) and passes into the bundle sheath cells which form the inner ring of the characteristic Kranz anatomy. In these cells the malate and aspartate is decarboxylated, depending on the plant, by NADP- or NAD- malic enzyme or PEP carboxykinase to provide CO_2 for Rubisco. The three-carbon acid remaining (pyruvate or PEP) returns to the mesophyll cells where it is used to capture another molecule of atmospheric CO_2. The CO_2 released in the bundle sheath cells raises the local concentration of the molecule to a level that out-competes the oxygen. The Rubisco located in the stroma of the bundle sheath cell chloroplasts is thus able to carboxylate ribulose 1,5-bisphosphate. The C_4 pathway which concentrates CO_2 thus reducing oxygenation of ribulose 1,5-bisphosphate and subsequent production of phosphoglycolate, costs energy. However, at high light intensity this extra energy cost is not a serious disadvantage because energy availability is not limiting. It is therefore seen that the C_4 pathway, by means of concentrating CO_2 at the site of Rubisco activity, is able to minimize the wasteful process of photorespiration.

1.6.2 The regulation of PEPc in C_4 plants

PEPc activity in C_4 plants is regulated by metabolites as it is in CAM species (O'Leary et al., 1982; Doncaster and Leegood, 1987). Nimmo et al. (1987) showed changes in the phosphorylation state and kinetic properties of PEPc in C_4 maize leaf in response to light and dark. The apparent K_i of PEPc for L-malate was found to be higher in extracts of leaves which had been illuminated than of leaves which had been kept in darkness. The conversion between the two forms of the enzyme took about 1 hour from light/dark transition. Phosphorylation of PEPc on one or more serine residues was shown by pre-labelling leaves with $^{32}P_i$ and the sample with the highest apparent K_i for malate also had the greatest ^{32}P incorporation. Phosphorylation of PEPc on a serine residue *in vitro* was then performed using

purified dark dephosphorylated PEPc from maize, a partially purified protein kinase from maize and [γ-^{32}P]ATP (Jiao and Chollet, 1989). Phosphorylation of PEPc was accompanied by a decrease in its malate sensitivity thus establishing a direct correlation between the kinetic properties and phosphorylation of the enzyme. Illumination of maize leaves with white, blue or red light (600 $\mu E/m^2/s$) for 1 hour gave similar, approximately 2.5 fold, increases in the apparent K_i of PEPc for malate (McNaughton et al., 1991). This suggested that the activation of PEPc kinase was not controlled by phytochrome or the blue-light photoreceptor. The appearance of kinase activity correlated well with the timing of changes in the kinetic properties of PEPc observed on illumination of intact maize leaves but protein phosphatase type-2A activity from maize leaves was not affected by light. Thus, as with studies investigating the regulation of PEPc in CAM, the focus of regulatory control of C_4 PEPc was PEPc kinase.

Cycloheximide, a cytoplasmic protein synthesis inhibitor, prevented the *in vivo* light activation of PEPc kinase activity in the C_4 species maize, *Sorghum* and *Digitaria sanguinalis* (Jiao et al., 1991; Bakrim et al., 1992; Pierre et al., 1992). This finding implicated *de novo* protein synthesis of PEPc kinase or a secondary component in the reversible light activation of PEPc in C_4 plants by phosphorylation, as had been previously shown in CAM plants (section 1.5.2) and kinase assays of *in vitro* translated RNA from maize leaves showed that the PEPc kinase mRNA increased in response to light (Hartwell et al., 1996).

The ability of the photosynthesis inhibitor 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) to block the light-induced increase in the apparent K_i of PEPc for malate in maize (McNaughton et al., 1991) was consistent with other studies which suggested that photosynthetic electron flow was involved, either directly or indirectly, in the mechanism of light-activation *in vivo* (Samaras et al., 1988; Jiao and Chollet, 1992). Detached maize leaves were treated with various photosynthesis inhibitors before being given a light or dark treatment. Both the light activation of PEPc kinase activity and the subsequent phosphorylation of PEPc were prevented by inhibiting photosystem I and photosystem II (with methylviologen and DCMU or isocil, repectively)-directed electron transport, and the Calvin Cycle (with DL-glyceraldehyde) (Jiao and Chollet, 1992). The same effect was observed in *Sorghum* and the use of the ATP synthesis inhibitor gramicidin demonstrated a requirement of ATP synthesis for the light induction of the C_4 PEPc kinase in *Sorghum* (Bakrim et al., 1992). These inhibitor studies led to the proposal that the Calvin Cycle

supplies the mesophyll cells with a putative signal (e.g. phosphorylated metabolite, amino acid) that interacts with the cytoplasmic protein synthesis event (Jiao et al., 1991) to effect the light activation of PEPc kinase and the concomitant phosphorylation of PEPc. The Calvin Cycle would also supply high levels of known positive effectors of PEPc e.g. triose phosphate and glucose 6-phosphate to the mesophyll cells. The effect of such regulation would be desensitization of PEPc in the mesophyll cells to the high levels of malate which are required to sustain rapid diffusion from the mesophyll to the bundle sheath cells during high rates of C_4 photosynthesis. Development of this idea by treating mesophyll cells from the C_4 species *Digitaria sanguinalis* with 3-phosphoglycerate or pyruvate in the dark led to significant increases in PEPc kinase activity and decreases in the malate sensitivity of PEPc, thus providing direct evidence that bundle sheath-derived C_4 pathway intermediates are involved in the activation of PEPc in the C_4 mesophyll cytosol (Duff et al., 1996).

Studies using *Sorghum* mesophyll cell protoplasts (Pierre et al., 1992) demonstrated that the malate sensitivity of PEPc was decreased by increasing the cytosolic pH using the weak bases NH_4Cl and methylamine. This suggested that cytosolic alkalinization mediated the light induction of PEPc phosphorylation. Consistent with this hypothesis is the finding of Yin et al. (1990) that cytosolic alkalinization occurs during photosynthesis in C_3 plants. This was attributed to two components: protonated phosphoglycerate transferred into chloroplasts and pumping of protons into the vacuole. Studies using the *D. sanguinalis* mesophyll cell protoplasts also indicated an involvement for calcium in the light-transduction pathway. The *in situ* light-dependent stimulation of PEPc kinase activity and phosphorylation of PEPc was inhibited by incubation of protoplasts using the Ca^{2+}-chelating agent EGTA and the Ca^{2+}-selective ionophore A23187 (Pierre et al., 1992). These results are internally consistent as cytosolic alkalinization would mobilize Ca^{2+} from intracellular stores, but other reports showed that PEPc kinase activity assayed from C_4 species was calcium-independent (Jiao et al., 1989; Hartwell et al., 1996). These results therefore suggested that Ca^{2+} was involved at a step upstream of the PEPc kinase in the C_4 light signal transduction pathway. In contrast, the findings of another group were that the PEPc kinase from maize leaves is calcium-dependent (Ogawa et al., 1992). Two types of protein kinase, one requiring and one not requiring Ca^{2+} for activity, were then shown to phosphorylate a recombinant C_4-type Sorghum PEPc at the specific serine residue (Li and Chollet, 1993). The calcium-independent kinase has been highly purified from maize leaves and its activity *in vivo* is light-dependent and sensitive to

inhibitors of photosynthesis and cytosolic protein synthesis (Li and Chollet, 1993; Wang and Chollet, 1993). These properties make the calcium-independent kinase the most physiologically likely candidate for regulating PEPc. Giglioli-Guivarc'h et al. (1996) built upon the previous work using *D. sanguinalis* protoplasts (Pierre et al., 1992) and proposed that a highly regulated cross-talk occurs between the two photosynthetic cell types of the C_4 leaf. Fluorescence imaging confirmed the involvement of cytosolic pH in the control of PEPc kinase activity and PEPc phosphorylation via light and calcium mobilization from vacuoles. The photosynthesis-related metabolite 3-PGA was able to mimic the effect of weak bases in contributing to cytosolic alkalinization suggesting that 3-PGA may be transported from the bundle-sheath cells to the mesophyll cells in an intact C_4 leaf as part of the light-induction of PEPc kinase. The various inhibitor treatments used in previous studies were repeated and confirmed the involvement of electron transport (DCMU), ATP synthesis (gramicidin) and cytosolic protein synthesis (cycloheximide) in the C_4 PEPc signal transduction pathway (Jiao et al., 1991; Bakrim et al., 1992). However, the RNA polymerase II inhibitors α-amanitin and actinomycin D had no effect on the light plus weak base-dependent phosphorylation of PEPc *in situ*. They proposed that the light induction of PEPc kinase did not require RNA synthesis. This is in direct contrast to the findings of Hartwell et al. (1999) who showed a light-dependent increase in maize PEPc kinase mRNA which was blocked by RNA synthesis inhibitors. Calcium/calmodulin antagonists were also investigated and from the results obtained, Giglioi-Guivarc'h et al. (1996) hypothesized that the signalling pathway is perhaps multicyclic, involving an upstream calcium/calmodulin-dependent kinase and the calcium-independent, light-activated PEPc kinase (Figure 1.6).

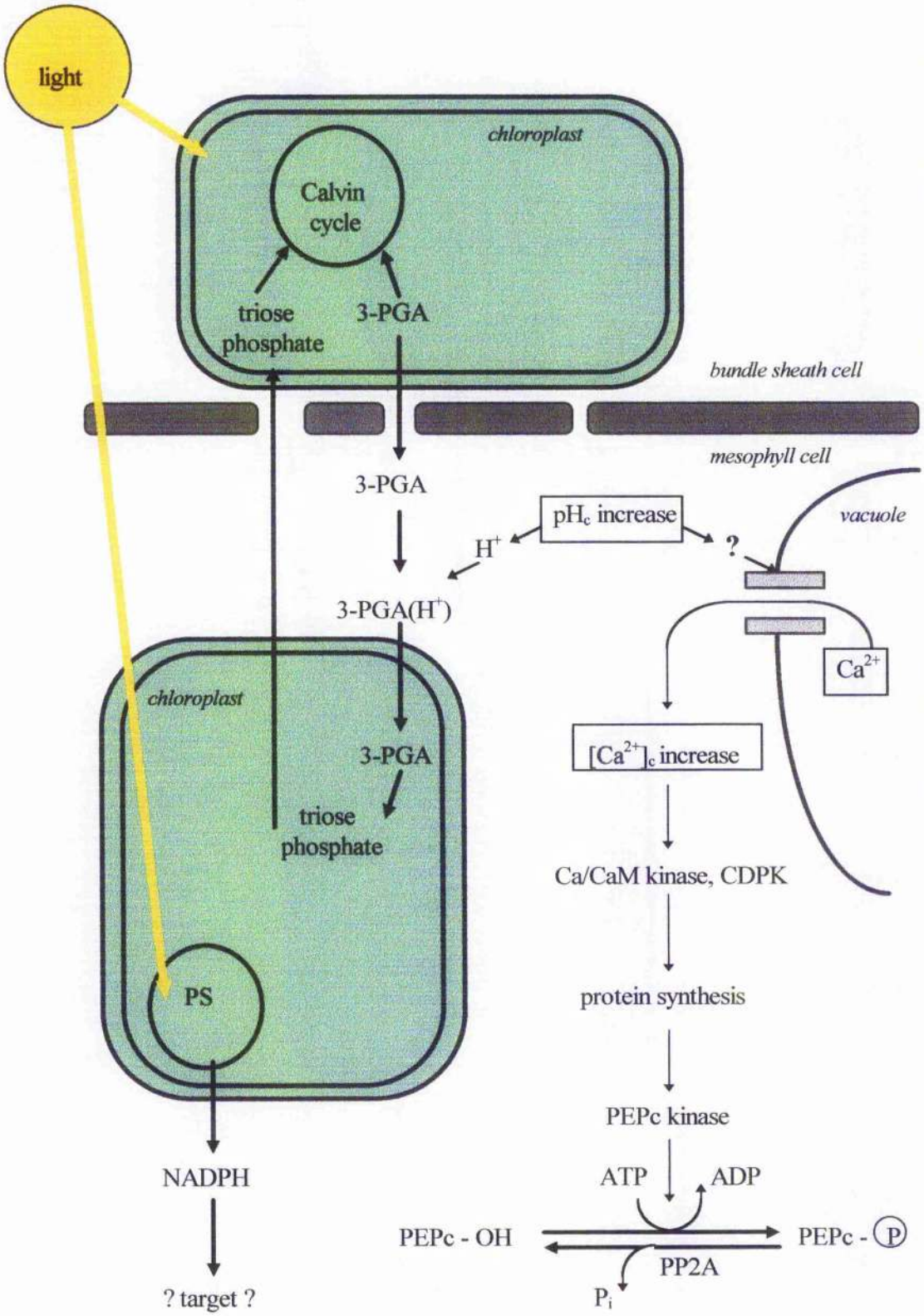
1.7 PEPc and C_3 photosynthesis

1.7.1 The role of PEPc in C_3 plants

In the leaves of C_3 plants and non-photosynthetic tissues the role of PEPc is anaplerotic, topping up intermediates from the TCA cycle that are removed for biosynthesis, particularly amino acid biosynthesis and nitrogen assimilation (Melzer and O'Leary, 1987; Van Quy et al., 1991a). In general, this necessitates stimulation of PEPc in processes that require intensive plant cell division and organogenesis e.g. germination (Lepiniec et al., 1994).

Figure 1.6 C_4 PEPc transduction pathway

Abbreviations: PS, photosystem; $[Ca^{2+}]_c$, cytosolic calcium concentration. Figure modified from Giglioli-Guivarc'h et al. (1996).



There are also a variety of what are termed "housekeeping" functions in which PEPc activity is involved (Lepiniec et al., 1993). These include maintenance of pH, electroneutrality and ionic balance, recapture of respiratory CO_2 and transfer of reducing power (NADH).

Specialized functions of PEPc other than those described in section 1.5.1 and 1.6.1 include N_2 fixation in legume root nodules (Deroche and Carrayol, 1988; Schuller et al., 1990; Schuller and Werner, 1993) and an involvement in stomatal guard cell movements (Tarczynski and Outlaw, 1993; Zhang et al., 1994). In symbiotic dinitrogen fixation, the plant cytosolic PEPc provides both energy, in the form of C_4 acids, for bacteroid metabolism and carbon skeletons for ammonia assimilation.

1.7.2 The regulation of PEPc in C_3 plants

By comparison with the regulation of the C_4 and CAM isoforms of PEPc, relatively little is known about the regulation of the non-photosynthetic isoforms of PEPc. However, many aspects of PEPc regulation in C_4 and CAM species is common to C_3 species.

The light activation of PEPc activity in a C_3 species was first demonstrated in wheat (Van Quy et al., 1991). Comparison studies showed that the light activation of PEPc activity in C_3 species is only 30 % of that found in C_4 species (Rajagopalan et al., 1993). The K_i of PEPc for malate increased about 3-fold upon illumination in C_4 species, but increased only slightly in C_3 species (Gupta et al., 1994). The reversible phosphorylation of PEPc responsible for the change of malate sensitivity of PEPc in C_3 species was shown to affect the activity and allosteric properties of the enzyme to a much lesser extent than in C_4 species (Gupta et al., 1994). Consistent with these findings, the level of translatable PEPc kinase mRNA from the C_3 species barley increased only about two-fold in response to light compared with an approximately 7-fold light increase in C_4 maize (Hartwell et al., 1996). Interestingly, whilst Matsuoka and Hata (1987) reported increased levels of PEPc protein in C_3 rice in response to light, they also observed a reduction in levels of a second type of PEPc protein in response to the same light signal. Rajagopalan et al. (1993) proposed that the low level of light-induced alkalinization of cell cytoplasm in C_3 species may limit light activation of PEPc. They reported that the light-dependent alkalinization of cell sap is three times higher in C_4 plants than in C_3 plants, but information on changes in the cytosol of mesophyll cells *in vivo* cannot be derived from crude leaf extract.

The first increase in the phosphorylation state of PEPc from a C_3 species in response to light was demonstrated using ^{32}P labelling studies and okadaic acid and was enhanced by feeding the nitrogen-deficient leaves 40 mM nitrate before illumination (Van Quy et al., 1991). This treatment also altered the malate sensitivity of PEPc such that the $I_{0.5}$ (malate) was 1.45 mM, 1.7 mM and 3.4 mM for the enzyme from dark control, illuminated low nitrate and illuminated high nitrate leaves respectively. In contrast, Duff and Chollet (1995) reported little short-term effect of nitrate feeding on the activity and malate sensitivity of C_3 PEPc in either illuminated or darkened wheat leaves detached from low-N-grown plants. However, the light-activation of PEPc kinase activity was enhanced by nitrate, or more specifically glutamine levels (Wang and Chollet, 1993; Duff and Chollet, 1995). Nitrate was suggested to modulate the activity of cytosolic protein kinases (PEPc kinase and SPS kinase) resulting in the diversion of photosynthetic carbon flow from sucrose synthesis to amino acid biosynthesis (Champigny and Foyer, 1992; Huber et al., 1994): the resupply of nitrate to N-deficient wheat leaves caused an increase in the production of amino acids and a short-term inhibition of sucrose synthesis (Champigny et al., 1992; Van Quy et al., 1991b). Treatment of leaves with mannose depletes the pool of inorganic phosphate through the production of mannose 6-phosphate and thereby inhibits ATP synthesis. Thus by blocking kinase reactions, mannose was able to inhibit the NO_3^--activation of PEPc and inactivation of sucrose phosphate synthase (SPS) (Van Quy and Champigny, 1992). Treatment of leaves with the phosphatase inhibitor okadaic acid increased the PEPc activation and SPS inactivation by light and nitrate (Van Quy and Champigny, 1992) suggesting that as with CAM and C_4 species, the phosphorylation state of PEPc is controlled by the activity of PEPc kinase rather than phosphatase activity.

The feeding of detached N-depleted wheat leaves with various nitrogen-containing compounds and with nitrate plus various inhibitors of enzymes involved in nitrogen metabolism (NR, GS, GOGAT, AAT- see page x for abbreviations) led to the proposal that glutamine and not nitrate was the most likely effector controlling the nitrogen-dependent PEPc activation, via the modulation of PEPc kinase and PEPc gene expression by the glutamine:glutamate ratio (Manh et al., 1993). A similar conclusion was drawn from inhibition of GS in barley leaves using phosphoinothricine (Diaz et al., 1996; Diaz et al., 1995). Specific inhibitors of glutamine synthetase have also been shown to block the light activation of tobacco leaf PEPc kinase under both photorespiratory and non-photorespiratory

conditions (Li et al., 1996). This striking effect is partially and specifically reversed by exogenous glutamine. Doubt was later cast as to the regulatory role of glutamine proposed by Manh et al.(1993) when no direct effect of glutamine was observed on the *in vitro* activity of the wheat leaf PEPc kinase activity assayed using a [γ-^{32}P]ATP-based *in vitro* kinase assay (Duff and Chollet, 1995). However, the wheat PEPc kinase activity assayed by Duff and Chollet (1995) was calcium-independent (Wang and Chollet, 1993; Li et al., 1996) whereas the PEPc kinase activity assay used by Manh et al. (1993) had not included the Ca^{2+}-chelating agent EGTA and so it is possible that the glutamine-enhanced PEPc kinase activity is different from the calcium-independent PEPc kinase activity detected by others. It is equally possible that glutamine might facilitate expression of the calcium-independent PEPc kinase.

Protein synthesis inhibitors and photosynthesis inhibitors were able to prevent the increase in PEPc kinase activity in response to light plus nitrate and light alone in C_3 species (Duff and Chollet, 1995; Li et al., 1996; Smith et al., 1996), similar to the effects of these compounds observed in C_4 illuminated plants without nitrate feeding (Jiao et al., 1991; Bakrim et al., 1992; Pierre et al., 1992; McNaughton et al., 1991; Jiao and Chollet, 1992). Studies using barley mesophyll protoplasts have confirmed the presence of a light-activated, cycloheximide-sensitive kinase but also indicated the presence of a constitutive, cycloheximide-insensitive kinase (Smith et al., 1996).

In contrast to the general trend in results indicating the central importance of phosphorylation in the regulation of PEPc, a few studies using barley and pea tissues have raised the possibility that metabolite regulation of C_3-leaf PEPc is more important than post-translational regulation. Kromer et al. (1996a; 1996b) demonstrated the light-activation of barley leaf PEPc measured as an increase in phosphorylation state and a decrease in malate sensitivity but showed that physiological concentrations of glucose 6-phosphate were able to overcome the malate inhibition. They then proposed that feedback inhibition by glutamate and aspartate was perhaps more physiologically significant because these metabolites were to be found at higher concentrations *in vivo* than malate. Leport et al. (1996) then used protein kinase and phosphatase inhibitors to show that PEPc from pea leaf and root is controlled allosterically by changes in the relative concentrations of PEP, malate and glucose 6-phosphate and not by phosphorylation. However, the validity of any such interpretation is questionable because no attempts were made to determine the effect of light or nitrate on the *in vivo* phosphorylation status of PEPc or PEPc kinase activity.

Studies using barley leaf mesophyll protoplasts can be seen as the C_3 PEPc equivalent to the *D. sanguinalis* protoplasts studies of C_4 PEPc carried out by Giglioli-Guivarc'h et al. (1996) which brought together and confirmed much of the previous data in an attempt to construct a model of the C_4 PEPc signal transduction pathway. Smith et al. (1996) carried out a series of experiments using barley leaf protoplasts and different inhibitors and chemicals used previously in other studies and were able to piece together the following biochemical scenario of PEPc regulation in the C_3 species. Two different PEPc kinase activities are present in the barley protoplasts: one is a basal activity, slowly turned over and present in the dark and the other is light-activated and rapidly turned over, similar to the PEPc kinase activity described in other C_3 species. The light-activation of PEPc kinase activity occurred in the presence or absence of nitrogen and effected the typical changes in allosteric properties of PEPc in response to activation e.g. decrease in malate sensitivity. The light-activated kinase activity was not prevented by treatment with DCMU (although light-activation of nitrate reductase in the same system was affected (Lillo et al., 1996) , indicating that the light-activation of barley leaf protoplast PEPc kinase does not require photosynthesis. Reduction in the intracellular calcium concentration using EGTA and the calcium ionophore A23187 enhanced the light-mediated reduction in the malate sensitivity of PEPc but did not affect PEPc kinase activity assayed *in vitro*.

Reversible phosphorylation of various other nonphotosynthetic isoforms of PEPc have been reported. Schuller et al. (1990) found PEPc from root nodules to be regulated by various metabolites including malate, glucose 6-phosphate and aspartate and *in vitro* studies showed the presence of PEPc kinase activity in soybean root nodules which is capable of effecting a change in the malate sensitivity of PEPc by phosphorylating it (Schuller and Werner, 1993). Studies with $^{32}P_i$ demonstrated the reversible phosphorylation of PEPc in soybean root nodules *in vivo* (Zhang et al., 1995) and complementary changes in PEPc activity and/or malate sensitivity under near-physiological assay conditions (i.e. low pH, low [PEP] relative to K_m). It would seem that translocation of sucrose from the leaves to the nodule is the signal regulating PEPc activity. In the light, when the supply of photosynthate from the phloem is available to the nodule, PEPc kinase activity is high and the malate sensitivity of PEPc is low. If this supply of photosynthate is disrupted by exposure to darkness, decapitation (shoot removal) or stem girdling, PEPc kinase activity decreases and the PEPc is sensitive to malate inhibition (Wadham et al., 1996; Zhang and Chollet, 1997; Zhang et al., 1995). PEPc kinase

has been partially purified from soybean root nodules and is calcium-independent (Zhang and Chollet., 1997).

PEPc is a key enzyme in stomatal movements in most plants by providing malate ($malate^{2-}$) which is accumulated by the stomatal guard cells to maintain intracellular pH and ion balance during the proton extrusion and K^+ uptake associated with the changes in stomatal aperture (Du et al., 1997). PEPc from *Vicia faba* guard cells microdissected from stomata was significantly inhibited by 400 μM malate in the dark but not during opening (Zhang et al., 1994). Further investigation of guard cell PEPc regulation demonstrated that this change in the malate sensitivity of PEPc is correlated with an increase in PEPc phosphorylation state which can be promoted by guard cell opening resulting from treatment with fusicoccin, or suppressed by guard cell closure resulting from treatment with abscisic acid (Du et al., 1997). Osuna et al. (1996) demonstrated that the components required for the covalent regulation of PEPc are present in wheat seeds and that phosphorylation occurs *in vivo* during germination. There was a significant (50%) increase in PEPc specific activity and indication of the enzyme's stability during the germinative and post-germinative periods in wheat. Anti-phosphorylation site antibodies which cross-reacted with both the phosphorylated and non-phosphorylated forms of the enzyme were shown to enhance PEPc activity and cause an increase in the IC_{50} for L-malate. They also facilitated the identification of phosphorylatable seed PEPc subunits by specifically inhibiting their phosphorylation *in vitro*. A partial inhibitory effect of EGTA on PEPc phosphorylation in desalted crude wheat seed extracts was observed suggestive of calcium-dependency.

Various *in vitro* studies have established that PEPc kinase activity is present in many different C_3 tissues (Duff and Chollet, 1995; Wang and Chollet, 1993; Pacquit et al., 1993), and have demonstrated this kinase's similarity to the C_4 and CAM enzymes with respect to its Ca^{2+} independency, chromatographic properties, and catalytic subunit(s). However, the results of many studies have also begun to reveal the existence of differentiated light-signal transduction pathways in illuminated C_3 and C_4 leaves which could be beneficial for the post-translational regulation of PEPc to fulfil the distinct anaplerotic and photosynthetic functions of the respective isoforms. The present knowledge of PEPc regulation in C_3 species is summarized and compared with that of CAM and C_4 isoforms of PEPc in Table 1.1.

1.8 Molecular properties of phosphoenolpyruvate carboxylase (PEPc) kinase

The regulation of PEPc kinase activity has been described in the previous sections. The molecular properties of the enzyme will now be described.

McNaughton et al. (1991) reported that PEPc kinase activity from maize leaves was inhibited by malate but that Ca^{2+}, calmodulin, EGTA and trifluoperazine had no significant effect on the activity of PEPc kinase, nor did PEP, glucose 6-phosphate, glycerol 3-phosphate and fructose 2,6-bisphosphate. Another study reported inhibition by glucose 6-phosphate in addition to malate (Jiao and Chollet, 1991).

The extremely low abundance PEPc kinase has been partially purified from a few species (Jiao et al., 1989; Carter et al., 1991; Wang et al., 1993). Maize PEPc kinase was purified about 4000-fold and, as isolated, catalyzes neither autophosphorylation nor the phosphorylation of heterologous substrates e.g. casein (Jiao and Chollet, 1989; Li and Chollet, 1993; Li and Chollet, 1994; Wang and Chollet, 1993). In contrast, all plant PEPc isoforms examined serve as substrates for the kinase *in vitro* (Li and Chollet, 1994; Wang and Chollet, 1993; Wang et al., 1992) and although some preference for the corresponding PEPc kinase has been reported (Li, B, Zhang, XG and Chollet, R, unpublished data quoted in Chollet et al., 1996), *K. fedtschenkoi* PEPc has been used as a substrate for the PEPc kinases of *K. fedtschenkoi*, maize, barley and sugarcane (Nimmo, HG, personal communication).

SDS-PAGE and a subsequent *in situ* renaturation method was used to identify two Ca^{2+}-independent protein kinase polypeptides (~30/32 kDa and ~37/39 kDa) capable of phosphorylating PEPc from C_4 maize, C_3 tobacco and the CAM species *Mesembryanthemum crystallinum in vitro* exclusively at the target serine residue (Li and Chollet, 1993; Li et al., 1996; Li and Chollet, 1994). One Ca^{2+}-dependent (~57 kDa) protein kinase polypeptide capable of phosphorylating maize PEPc was also identified (Li and Chollet, 1993). Only the Ca^{2+}-independent, 30- to 39-kD PEPc kinase has been shown to be light-dark (C_4) or day-night (CAM) regulated *in vivo* (Li and Chollet, 1993; Li and Chollet, 1994).

1.9 The coordinate regulation of carbon and nitrogen metabolism in plants by protein phosphorylation

As mentioned previously (section 1.7.2), nitrate has been proposed to regulate the activity of cytosolic kinases thus controlling the flow of photosynthetic carbon between sucrose synthesis and amino acid biosynthesis (Champigny and Foyer, 1992) As PEPc kinase and its

sucrose synthesis

UDP-glucose + fructose 6-phosphate $\xrightarrow{SPS}$ sucrose 6-phosphate + UDP

sucrose breakdown

sucrose + UTP $\xrightarrow{SS}$ UDP-glucose + fructose

substrate has already been discussed in this context, this section will focus on the other two enzymes refered to, nitrate reductase (NR) kinase and sucrose phosphate synthase (SPS) kinase, and their substrates.

Nitrate reductase (NR) catalyzes the reduction of nitrate to nitrite in the assimilation of nitrogen in plants. Sucrose phosphate synthase (SPS) is involved in sucrose synthesis and catalyzes the conversion of UDP-glucose and fructose 6-phosphate to sucrose 6-phosphate which is then rapidly dephosphorylated by sucrose synthase to form sucrose. Both enzymes are light-activated and regulated by reversible phosphorylation, phosphorylation causing their deactivation. Phosphorylation of SPS by SPS kinase occurs at multiple sites although phosphorylation of a specific serine residue is thought to be essential for deactivating the enzyme (Huber et al., 1995). Okadaic acid (a phosphatase inhibitor) has been shown to decrease SPS activity and mannose (which sequesters P_i) has been shown to prevent SPS deactivation (Van Quy et al., 1992). Phosphorylation of SPS increases its sensitivity to inhibition by P_i and decreases it sensitivity to activation by glucose 6-phosphate. Phosphorylation of NR is slightly more complex as it has no effect on the activity of NR but rather phosphorylation of a specific serine residue enables NR to interact with an inhibitor protein called NIP, with a conformational change in the N-terminal region of NR induced by phosphorylation suggested to be the mechanism involved (Douglas et al., 1995; Huber et al., 1995; MacKintosh et al., 1995). Purification of NIP revealed that it was a member of the 14-3-3 family of proteins (Moorhead et al., 1997) which have been shown to participate in many diverse signalling pathways. Their exact mode of action is unknown although they have been shown to function as adapter molecules bringing specific target proteins together or to particular locations, and as solubility factors and enzyme inhibitors (Ferl, 1996). No allosteric effectors are known for NR

Three peaks of SPS and/or NR kinase activity have been identified after anion-exchange chromatography of spinach leaf extracts (McMichael et al., 1995). The first peak of kinase activity is capable of phosphorylating both SPS and NR and is itself regulated by phosphorylation (Pauline Douglas and Carol MacKintosh, unpublished, quoted in MacKintosh, 1997) and a divalent cation. The second peak of kinase activity is calcium dependent and acts only on NR and the third peak of kinase activity acts only on SPS. Like activity 1, activity 3 is regulated by phosphorylation and was demonstrated to be a CDPK (Douglas et al., 1997). SnRK1 kinase activity (plant homolog of SNF1) has been reported to

inactivate NR and SPS activity *in vitro* (Hardie et al., 1997), suggesting that NR kinase and SPS kinase are closely related members of the SNF1 related protein kinase family.

The "dual specificity" of kinase activity 1 (McMichael, 1995) which phosphorylates both NR and SPS would provide a mechanism for coordinately regulating NR and SPS, whilst kinase activity 2 and 3 enable NR and SPS to be regulated independently where their response to a particular signal is different. Huber et al. (1995) reported that nitrate enhances the progressive inactivation of SPS (phosphorylation) and activation of NR (dephosphorylation) in response to accumulation of the end-products of photosynthesis. This therefore would be an example of where kinase activity 3 would act alone.

When the effects of nitrate on PEPc, NR and SPS are considered together, the results are consistent with the hypothesis suggested by Champigny and Foyer (1992). The common requirements of nitrate and carbon dioxide metabolism in terms of reducing power, ATP and carbon skeletons also make the coordinate regulation of PEPc, NR and SPS quite logical.

A simplified scheme of the regulation of PEPc, NR and SPS in C_3 leaves is shown in Figure 1.7. In addition to the regulatory phosphorylation and allosteric effectors discussed already, several other factors known to be involved in the regulation of these enzymes are shown in the diagram thus indicating the complexity of the signalling pathways involved.

1.10 Project objectives

The preceding discussion has provided a brief overview of the function and regulation of PEPc in higher plants. A summary and comparison of the emerging PEPc regulatory pathways in the three photosynthetic classes is given in Table 1.1. The overall objective of this research was to investigate the physiological roles of the phosphorylation of PEPc and the signalling pathways that bring this about.

The regulation of PEPc in CAM and C_4 species have certain common features like the calcium-independency of the principal kinase activity phosphorylating PEPc and the involvement of *de novo* protein synthesis of PEPc kinase. However, a major component of the C_4 PEPc signal transduction pathway is cytosolic alkalinization. Little was known about the regulatory involvement of pH in the CAM PEPc signalling pathway so the objective of the first part of this research was to investigate the involvement of cytosolic pH in the regulation of PEPc in the CAM species *K.fedtschenkoi* (Figure 1.8).

Figure 1.7 Coordinate regulation of PEPc, SPS and NR in C_3 leaves

In the diagram opposite, the factors enclosed in blue shaded areas e.g. anoxia, NO_3^- override the photosynthesis signals to promote the activation or inactivation of the enzymes as indicated. Abbreviations: PK, protein kinase; PP, protein phosphatase; NIP, nitrate reductase inhibitor protein protein; C, carbon; N, nitrogen. Figure modified from MacKintosh, C (1997).

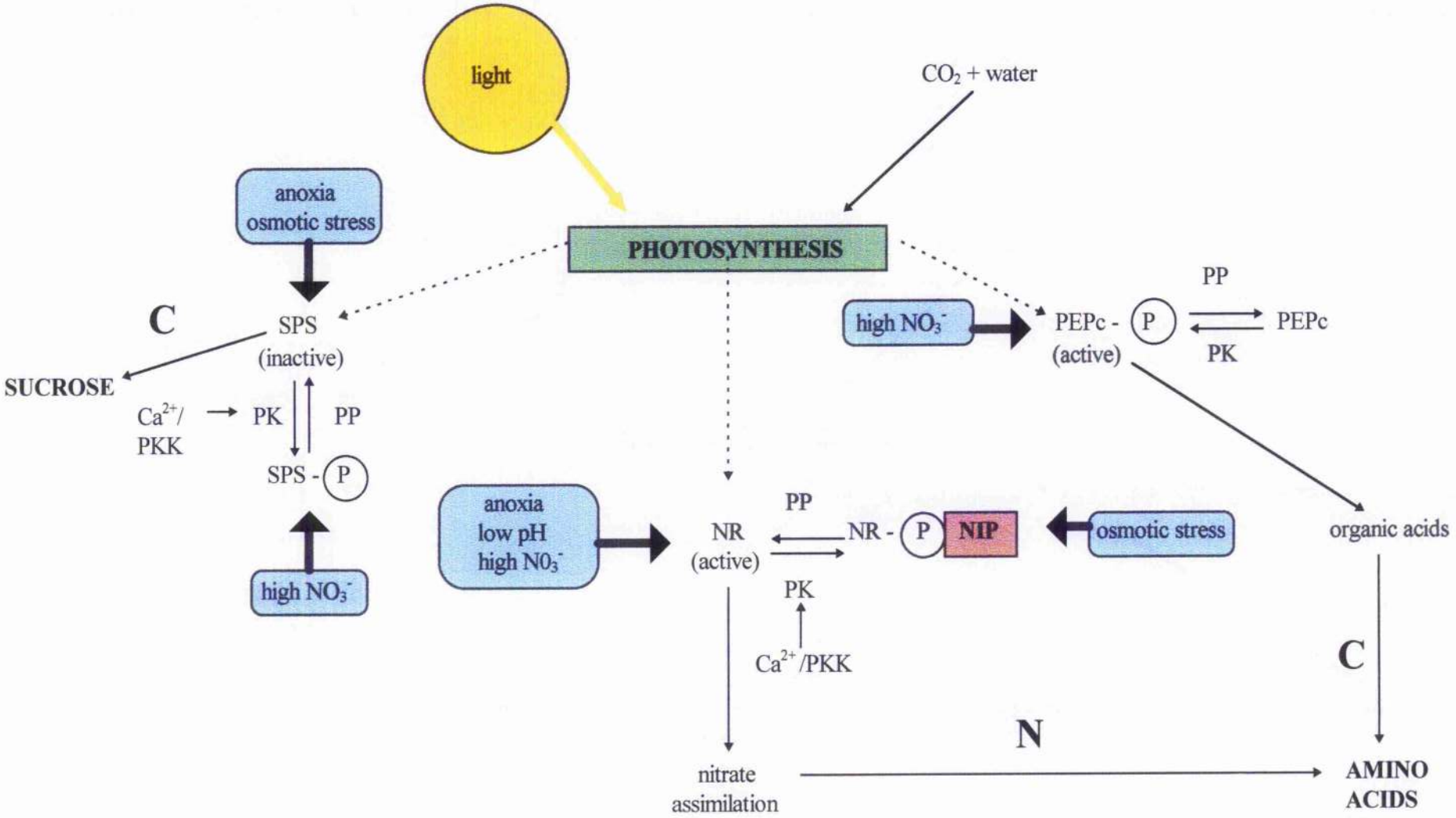
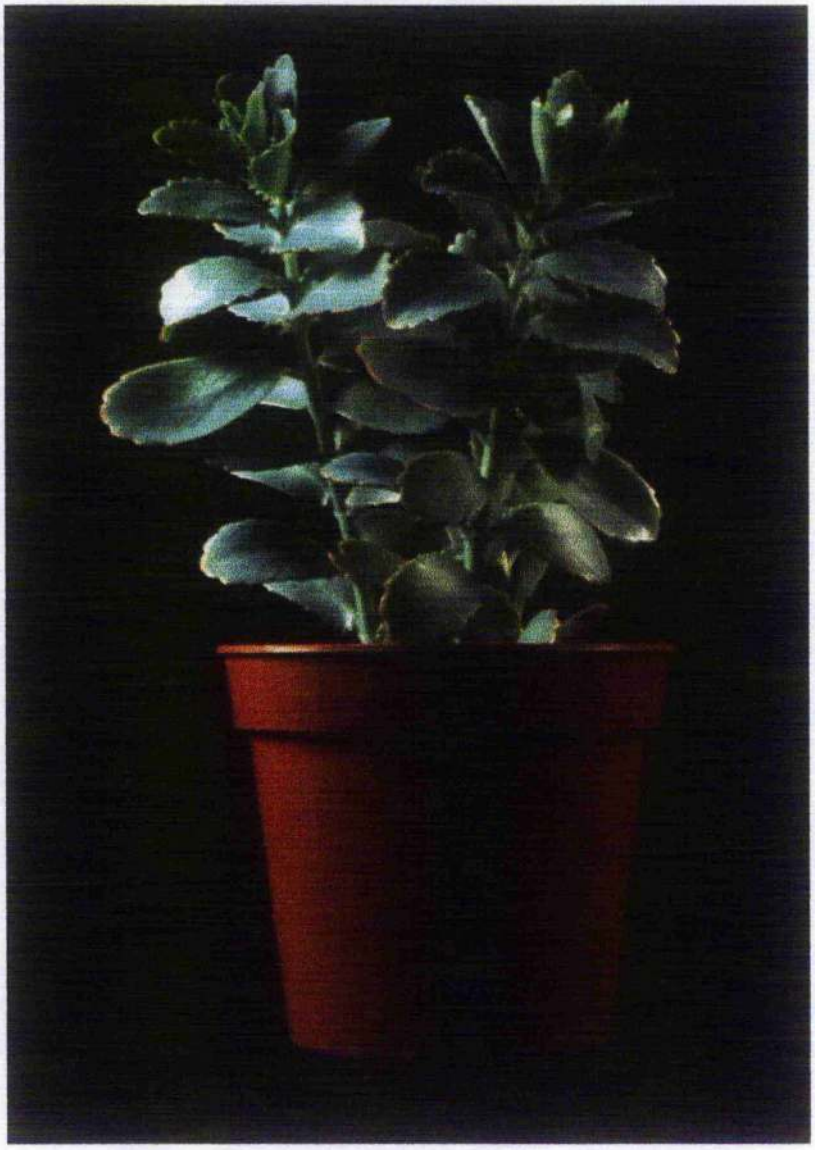

light
CO_2 + water
anoxia
osmotic stress
PHOTOSYNTHESIS
C
SPS
(inactive)
SUCROSE
Ca^{2+}/
PKK
PK
PP
SPS - P
high NO_3^-
high NO_3^-
PP
PEPc - P
(active)
PEPc
PK
anoxia
low pH
high NO_3^-
NR
(active)
PP
NR - P
NIP
osmotic stress
PK
Ca^{2+}/PKK
organic acids
C
N
nitrate
assimilation
AMINO
ACIDS

Figure 1.8 **Photograph of *Kalanchoë (Bryophyllum) fedtschenkoi***



The objective of the second part of this research was to discover to what extent the mechanisms and signalling involved in regulating PEPc in CAM and C_4 species are the same for C_3 species. Plant tissue (Figure 1.9) and a photomixotrophic cell suspension culture of *Arabidopsis thaliana* (Figure 1.10) were used as experimental systems for this purpose.

Figure 1.9 **Photograph of *Arabidopsis thaliana* var. Landsberg erecta**

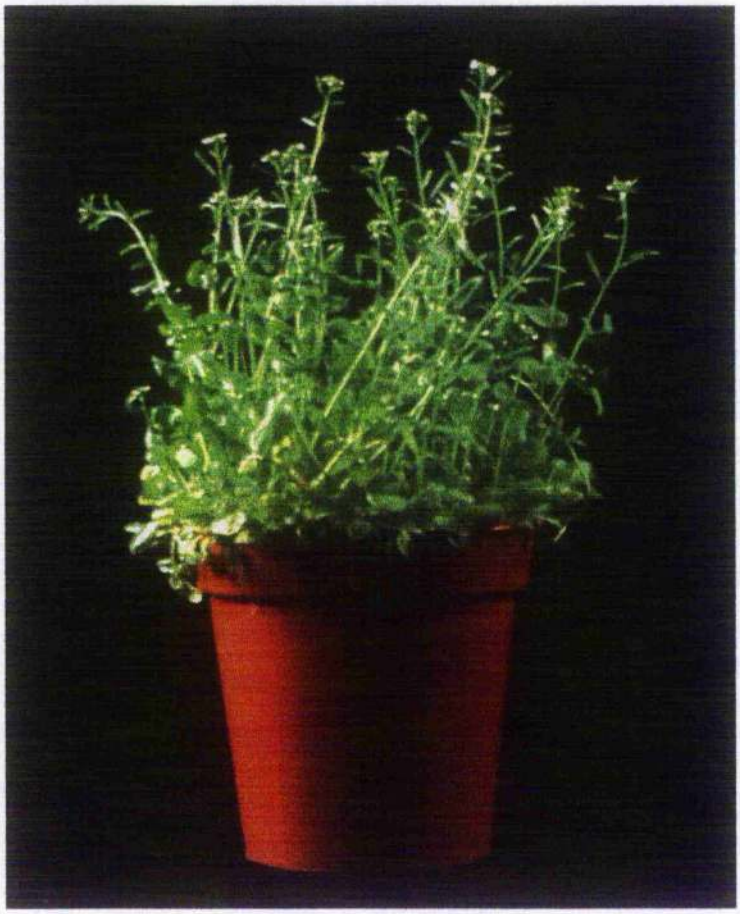


Figure 1.10 Photograph of *Arabidopsis thaliana* photomixotrophic cell suspension culture

Chapter Two

MATERIALS AND METHODS

2.1 MATERIALS AND MATERIAL SOURCES

[γ-^{32}P]ATP (triethylammonium salt, 3000 Ci/mmol), [α-^{32}P]dCTP (3000 Ci/mmol), Hybond™-N nylon membrane, rabbit reticulocyte lysate *in vitro* translation kit, T7 Sequenase version 2.0 DNA sequencing kit, the Rediprime and Decaprime DNA labelling kits and Redivue™ ^{35}S-methionine (1000 Ci/mmol) were obtained from Amersham International, Bucks., U.K.

Antipain hydrochloride, benzamidine hydrochloride, Bis-Tris, bovine serum albumin (BSA), bromophenol blue, cantharidin, casein acid hydrolysate C-9386, casein enzymatic hydrolysate C-0626, chymostatin, Coomassie brilliant blue G250, diethyl pyrocarbonate (DEPC), formaldehyde, formamide, hexadecyltrimethylammonium bromide (CTAB), leupeptin (hemisulphate salt), lithium chloride, L-malate (disodium salt), octanol, mineral oil, M_r marker proteins for SDS/polyacrylamide gel electrophoresis, MSMO salts, polyvinylpolypyrrolidone (PVPP or insoluble PVP), propionic acid, silver nitrate, sodium azide, N,N,N',N'-tetramethylethylenediane (TEMED) and Tween 20 were obtained from the Sigma Chemical Co. (London), Poole, Dorset, UK.

ATP (disodium salt), dithiothreitol (DTT), nicotinamide adenine dinucleotide reduced form (NADH, disodium salt), phosphoenolpyruvate (monosodium salt), and pig heart malate dehydrogenase were from Boehringer Corp. (London) Ltd., Lewes, Sussex, UK.

Sephadex G-25 (medium) and Sephadex G50 were from Pharmacia, Milton Keynes, Bucks., U.K.

Acrylamide monomer, ammonium persulphate, ethanol, glacial acetic acid, glycine, hydrogen peroxide, 2-mercaptoethanol, methanol, N',N' methylenebisacrylamide, potassium dihydrogen orthophosphate, sodium dodecyl sulphate (SDS) and trichloracetic acid were 'AnalaR' grade materials from BDH Chemicals, Poole, Dorset, U.K.

37.5:1 acrylamide:bis-acrylamide solution was from Bio-Rad Laboratories (England) Ltd., Bramley, Kent, U.K.

Acetone, ammonium sulphate, boric acid, chloroform, disodium hydrogen phosphate, ethylene diamine tetra-acetic acid (EDTA, disodium salt), Hepes, hydrochloric acid,

magnesium chloride, magnesium sulphate, Optiflow Safe 1 scintillation fluid, potassium chloride, sodium acetate, sodium chloride, sodium dodecyl sulphate, sodium hydrogen carbonate, sodium hydroxide and sucrose were from Fisher Scientific UK Ltd., Loughborough, Leics., LE11 5RG, UK.

Agarose (electrophoresis grade) and 1 kb DNA ladder were from Gibco BRL Life Technologies Ltd., Paisley, Scotland.

Agar and yeast extract were from Merck, Darmstadt, Germany.

Okadaic acid was from Moana Bioproducts.

AMV reverse transcriptase, Poly Atract mRNA isolation system, RNA size markers (0.28 - 6.6 kb), Taq DNA polymerase and all DNA restriction enzymes were from Promega (U.K.) Ltd., Southampton, U.K.

T7 mMessage Machine *in vitro* transcription kit was from Ambion Inc., AMS Biotechnology (U.K.) Ltd., U.K.

QIA-quick gel extraction kit and QIA-prep spin plasmid miniprep kit were from Qiagen Ltd., Crawley, West Sussex, U.K.

All other reagents used were of the highest grade commercially available.

2.2 EXPERIMENTAL TISSUES

2.2.1 Culture material

An *Arabidopsis thaliana* photomixotrophic suspension culture was established (May and Leaver, 1993). Cells were grown under continuous low intensity white light (about 25 $\mu mol/m^2/s$) at 20°C with constant shaking at 110 rpm. Subculturing was performed after 7 days by transferring 20 ml of culture to 180 ml fresh sterile medium in a 500 ml conical flask. The culture medium consisted of:

3% sucrose; 0.5 mg/L Naphthalene Acetic Acid; 0.05 mg/L Kinetin; 1x Murashige & Skoog Minimal Organic (MSMO) salts.

MSMO salts contain:

1650.0 mg/L ammonium nitrate; 6.2 mg/L boric acid; 332.2 mg/L calcium chloride anhydrous; 0.025 mg/L cobalt chloride.$6H_2O$; 0.025 mg/L cupric sulphate.$5H_2O$; 27.8 mg/L ferrous sulphate.$7H_2O$; 180.7 mg/L magnesium sulphate; 16.9 mg/L manganese sulphate.H_2O; 0.25 mg/L molybdic acid (sodium salt).$2H_2O$; 0.83 mg/l potassium iodide;

1900.0 mg/L potassium nitrate; 170.0 mg/L potassium phosphate monobasic; 8.6 mg/L zinc sulphate.$7H_2O$; 100.0 mg/L myo-inositol; 0.4 mg/L thiamine/HCl.

The medium was then adjusted to pH 5.8 with 0.2 M KOH and sterilised by autoclaving.

2.2.2 Plant material

(i) Seeds of wild type *A. thaliana* var. Columbia or Landsberg erecta were spread out onto watered soil in plant pots, covered with clingfilm to prevent moisture loss and left to cold-imbibe at 4°C in the dark for 3 days. The pots were then transferred to 16 hour light/8 hour dark conditions at approximately 70 μmoles/m^2/s white light and 21°C and 18°C in the light and dark respectively. Other pots were put under constant light conditions. Plants were watered every 2 to 3 days.

Rosette leaves, bracts, bolts, flowers and buds were taken from 5-week old plants for the Northern blot analysis of PEPc and PEPc kinase gene expression in mature tissue. The rosette leaf tissue included all rosette leaves removed from a few individuals of approximately the same size and development, whereas only the first bracts from a group of similarly sized plants were taken. Only primay bolts of approximately 15 cm were harvested. These were excised approximately 1 cm from the base and the bracts, secondary bolts, and top of the bolt 1 cm below the first silique were removed. Completely green siliques of no less than 1.5 cm were excised from plants 6-8 weeks old. Suitable root tissue was obtained by spreading sterilized seed on 1.5% agar plates containing 0.5x MS salts and 20 mM sucrose. The seeds were cold-imbibed at 4°C in the dark for 3 days then grown for 10 days under constant illumination (~20 μmoles/m^2/s) at 20°C. The seedlings were then transferred into glass conical flasks (six seedlings per flask) containing sterile *A. thaliana* root culture medium (see section 2.4) and shaken at 80 rpm under the same growth conditions as previously. After 14 days the seedlings had grown extensive root systems (Czako, M. and Marton, L., in ATCG Arabidopsis: The Compleat Guide. AFRC PMB Arabidopsis Programme).

(ii) *Kalanchoë (Bryophyllum) fedtschenkoi* Hamet et Perrier was propagated by cuttings from the original stock used in previous studies (Wilkins, 1959; Wilkins, 1960). Cuttings were grown in a glasshouse under a 16 hour photoperiod, maintained throughout the year using mercury-vapour lamps. Four to six month old plants were transferred to either of two controlled environment growth chambers. In one chamber the 8 hour photoperiod

was from 0800 h - 1600 h and in the other the photoperiod was 1600 h - 2400 h (reverse-phase). Light was provided by white fluorescent tubes and twelve 100W tungsten lamps, giving a radiant fluence rate of 20 $W/m^2/s$. The temperature was 28°C during the photoperiod and 15°C in the dark. Plants were watered every 4 to 5 days. Plants were allowed to adjust to growth chamber conditions for at least 7 days prior to use. All experiments were carried out using leaf material from between nodes six and ten.

2.3 GENERAL BIOCHEMICAL METHODS

2.3.1 pH calibrations

All pH calibarations were performed using a Russel pH probe connected to an EDT Instruments RE 357 Microprocessor pH meter. The pH of all buffers and solutions was adjusted at their working temperature (i.e. 4°C or room temperature).

2.3.2 Glassware and plastics

Glassware and plastics were washed in hot water containing the detergent Pyroneg, rinsed in clean water and dried in ovens.

2.3.3 Spectrophotometric assays

All spectrophotometric assays were carried out in semi-micro quartz cuvettes or plastic disposable cuvettes (1 cm path length, 1 ml volume) using either a Gilford Instrument 2600 spectrophotometer or a Philips PU 8700 series UV/Vis scanning spectrophotometer for the determination of DNA/RNA solution concentrations and enzyme assays.

2.3.4 Centrifugation

Beckman J2-HS and J2-21 centrifuges were used with the appropriate Beckman centrifuge rotors for all centrifuge steps. Centrufugation of small volume samples (<1.5 ml) was carried out in an MSE Microcentaur microfuge.

2.3.5 Micropipetting

(0.5 µl - 1 ml) All micropipetting was performed using adjustable Finnpipette micropipettes.

2.3.6 Chromatographic materials

Sephadex G25 and G50 were swollen and packed according to the manufacturer's protocols. Sephadex G25 was stored in 0.05 % (w/v) azide.

2.4 BUFFERS AND SOLUTIONS

***A. thaliana* extraction buffer** at pH 8.0 consisted of: 100 mM Tris/HCl; 2 mM EDTA; 10 mM malate; 2% (w/v) polyethylene glycol 20,000; 1 mM DTT; 1 mM benzamidine hydrochloride.

***A. thaliana* root culture medium** at pH 5.8 contained per litre: 4.3 g MS salts; 3 ml 6% (w/v) KH_2PO_4; 200 mg myo-inositol; 2 ml Vitamix stock; 30 g sucrose.

***A. thaliana* RNA extraction buffer** consisted of: 25 mM Tris/HCl, pH 8.0; 25 mM EDTA; 75 mM NaCl; 1% SDS; 7.8% β-mercaptoethanol.

Bradford reagent consisted of: 100 mg Coomassie Brilliant Blue (G250); 50 ml 95% ethanol; 100 ml 85% (w/v) phosphoric acid.

CTAB RNA extraction buffer consisted of: 2% CTAB; 2% PVP 40; 100 mM Tris/HCl, pH 8.0; 25 mM EDTA; 2 mM NaCl; 0.5 g/l spermidine/HCl; 2% β-mercaptoethanol. The prepared solution was autoclaved and the 2% β-mercaptoethanol then added.

6x DNA loading buffer consisted of: 30% (v/v) glycerol; 0.25% (w/v) xylene cyanol; 0.25% (w/v) bromophenol blue.

***K. fedtschenkoi* desalting buffer** at pH 7.5 consisted of: 100 mM Tris/HCl; 1 mM benzamidine hydrochloride; 1 mM DTT; 2 mM EDTA; 10 mM L-malate.

Laemmli sample buffer consisted of: 50 mM Tris/HCl; 1% (w/v) SDS; 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue; 1% (v/v) 2-mercaptoethanol.

Lauri Bertrani (LB) medium contained per litre: 10 g Bactotryptone; 5 g bacto yeast extract; 10 g NaCl adjusted to pH 7.0 with 5 M NaOH.

10x Mops buffer at pH 7.0 and 8.0 consisted of: 0.2 M Mops; 50 mM sodium acetate; 10 mM EDTA.

MS salts consisted of the same ingredients as MSMO (section 2.2.1) without ammonium nitrate or potassium nitrate and with 2.0 mg/L glycine, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine-HCl and 0.1 mg/L thiamine.

NZYCM agar contained per litre: 1 g Casamino acids (casein hydrolysate Σ type C9386); 5 g NaCl; 2 g $MgSO_4.7H_2O$; 5 g yeast extract; 10 g NZ amine (casein hydrolysate Σ type C0626); 15 g agar (7 g in top agar).

Phosphorylation buffer at pH 8.0 consisted of: 100 mM Tris/HCl; 5 mM $MgCl_2$; 1 mM EDTA; 5% glycerol; 1 mM DTT.

RNA sample buffer consisted of: 72 µg/ml ethidium bromide; 2x Mops at pH 8.0; 6 % formaldehyde; 70 % formamide. All manipulations using the buffer were carried out in a fume cupboard.

SDS-PAGE running buffer consisted of: 3 g/L Tris; 14.4 g/L glycine; 0.1% SDS.

SM buffer contained per litre: 5.8 g NaCl; 2 g $MgSO_4.7H_2O$; 5 ml 1 M Tris/HCl, pH 7.5; 5 ml 2% gelatin.

Sonication buffer at pH 7.4 consisted of: 50 mM Hepes/KOH; 0.45 M sorbitol and 1 mM $CaCl_2$.

20x SSC at pH 7.0 consisted of: 3 M NaCl, 0.3 M trisodium citrate, pH 7.0.

0.5x TBE buffer at pH 8.0 consisted of: 45 mM Tris-borate; 1 mM EDTA.

TE at pH 8.0 consisted of: 10 mM Tris/HCl, 1 mM EDTA.

Vitamix (500x stock solution) contained per 100 ml: 500 mg thiamine pyrophosphate (vitamin B1); 50 mg pyridoxal phosphate (vitamin B6); 100 mg glycine; 50 mg nicotinic acid; 25 mg folic acid; 50 mg biotin (vitamin H).

2.5 METHODS FOR THE EXTRACTION OF ENZYME ACTIVITIES

2.5.1 *A. thaliana* cell culture

20 ml of cell culture was harvested and centrifuged in a Beckman for 5 min and 1,000 rpm. The pelleted cells were then resuspended in 1 ml of sonication buffer.

Aliquots (1 ml) of the cell suspension were sonicated for 30 s at an amplitude of 5 microns using the Sanyo Soniprep and spun for 2 min at 13,000 rpm in a microfuge. The supernatant was then spun for a further 20 min at 30,000 rpm using a Sorvall OTD ultracentrifuge. The resulting supernatant was precipitated with 75% ammonium sulphate on ice for 30 min. The material was then spun for a further 2 min at 13,000 rpm and the pellet resuspended in 50 µl of sonication buffer.

2.5.2 *A. thaliana* leaf extracts

1 g of *A. thaliana* leaves was collected from plants which had flowered and then had their bolts removed. The leaves were ground up in a mortar and pestle at 4°C with some sand, a few drops of octanol, 1 ml/g extraction buffer, and 5% (w/v) $NaHCO_3$.

Extracts were desalted as described in section 2.5.4.

A 1 ml volume of desalted extract was then taken to 75% saturation with ammonium sulphate and left on ice for 30 min to precipitate. Finally the extract was spun at 13,000 rpm for 2 min and the pellet resuspended in 100 µl of phosphorylation buffer (see section 2.4).

2.5.3 *K. fedtschenkoi* leaves and leaf disks

K. fedtschenkoi leaves and leaf disks were ground up as described in section 2.5.2. The extracts were desalted as described in section 2.5.4 and assayed directly.

2.5.4 Desalting crude extracts

Crude extracts from *A. thaliana* leaves and *K. fedtschenkoi* leaves and disks were spun at 4°C, 13,000 rpm for 5 min. The resulting supernatant was then loaded onto a 10 ml G25 Sephadex column using a glass pasteur pipette and desalted by pumping buffer through the column using a peristaltic pump. Phosphorylation buffer was used to pre-equilibrate and desalt the *A. thaliana* extracts (section 2.4).

Fractions of the desalted extract were collected as approximate 500 µl volumes in 1.5 ml microcentrifuge tubes. Fractions were assayed for phosphoenolpyruvate carboxylase (PEPc) activity (section 2.6.1) and the fractions containing the peak of activity pooled.

K. fedtschenkoi leaves and disks were desalted in the *K. fedtschenkoi* desalting buffer (see section 2.4).

Fractions of the desalted extract were collected as 1 ml volumes in glass test tubes using a BIORAD model 2110 fraction collector, assayed for PEPc activity and the fractions containing the peak of activity pooled.

2.6 ASSAY PROCEDURES

All assays were repeated, in duplicate at least, unless otherwise stated. PEPc activities given are the mean of two assays that agree to within 15% and PEPc kinase activity data is representative of duplicate experiments.

2.6.1 Determination of PEPc activity and the apparent K_i of PEPc for L-malate

PEPc assay reaction mix was added to the volume of cell extract being assayed to give a total reaction volume of 1 ml in a 1 ml plastic cuvette. The cuvette was then inverted five times to mix. The oxidation of nicotinamide adenine dinucleotide (NADH) by the coupling enzyme malate dehydrogenase (MDH) was monitored as the decrease in the A_{340} at 25°C. This decrease was proportional to the PEPc concentration in the enzyme sample (Nimmo et al., 1984). The difference in rate of change of absorbance was attributed to PEPc activity. Contaminating NADH oxidase activity was accounted for by measuring the rate of change of absorbance in the absence of PEP.

PEPc activity assay mixture for *A. thaliana* at pH 7.6 (adapted from Van Quy et al., 1991) consisted of:

50 mM Tris/HCl; 10 mM $MgCl_2$; 0.3 mM PEP; 20 mM $NaHCO_3$; 0.2 mM NADH; 5 mM DTT; 5 units NAD-linked MDH.

PEPc activity assay mixture for *K. fedtschenkoi* at pH 7.8 consisted of:

50 mM Tris/HCl; 10 mM $MgCl_2$; 2 mM PEP; 10 mM $NaHCO_3$; 0.2 mM NADH; 5 units NAD-linked MDH.

The L-malate sensitivity of PEPc was determined by measuring the activity of the enzyme in the presence of different concentrations of L-malate. The PEPc activity was plotted against L-malate concentration as a percentage of the activity in the absence of any inhibitor. The apparent K_i for malate of PEPc could then be read from this graph.

2.6.2 PEPc kinase activity

A volume of desalted extract equivalent to 0.001-0.003U PEPc was used in reactions to assay extracts for PEPc kinase activity. PEPc kinase reaction mixture at pH 7.8 consisted of:

50 mM Tris/HCl; 1 mM benzamidine hydrochloride; 10 μg antipain/ml; 10 μg leupeptin/ml; 5 nM okadaic acid; 0.03 units purified, dephosphorylated PEPc from *K. fedtschenkoi*; 5 mM $MgCl_2$; 1 μCi [γ-^{32}P] ATP; 0.1 mM ATP.

The reaction mixture was assembled in 1.5 ml microcentrifuge tubes on ice and the ATP mix (0.4 μCi/nmol) was added last, initiating the reaction. The total reaction volume was 25 μl. All reactions were then transferred to a 30°C water bath and incubated for 30 min. The reactions were stopped by removing all the tubes to an ice bath and adding 10 μl Laemmli sample buffer (section 2.4) before boiling for 4 min to denature the samples. The denatured samples were then run on an 8% SDS/polyacrylamide gel, stained, destained overnight and the gel dried down (see section 2.7). The gel was then either phosphorimaged or autoradiographed as preferred (see section 2.7.3) to determine the incorporation of ^{32}P into the major PEPc band for each sample. (*K. fedtschenkoi* PEPc contains two related subunits with molecular mass values of 112 000 and 123 000 in a ratio of some 10:1 (Nimmo et al., 1986)). This incorporation is a measure of the PEPc kinase activity for each sample.

For kinase assays on products of *in vitro* translation, 5 μl of each translation reaction was used. The kinase reactions were stopped by addition of 10 μl of rabbit anti-*K. fedtschenkoi* PEPc antiserum. The PEPc was immunoprecipitated on ice for 1 hour. The samples were spun for 5 min at 13,000 rpm in a microfuge at room temperature and the supernatant removed. The pellet was washed in 200 μl of 2 M NaCl / 2 mM EDTA and spun for another 5 min. The pellet was then resuspended in 10 μl of Laemmli sample buffer and boiled for 4 min to denature protein.

Partially purified maize PEPc kinase (a gift from Dr. GA Nimmo) was used as a control in all PEPc kinase activity assays.

2.6.3 Malate Dehydrogenase (MDH) activity

MDH activity of samples was measured by the rate of decrease in the A_{340} at 25°C due to oxidation of NADH. The total reaction volume of assay reaction mix and sample was 1 ml. The cuvette was inverted five times to mix. The assay mix, pH 7.0, consisted of:
0.15 mM NADH; 1.0 mM OAA; 50 mM NaH_2PO_4/Na_2HPO_4.

2.7 GEL ELECTROPHORESIS TECHNIQUES

2.7.1 SDS/polyacrylamide gel electrophoresis

Proteins were separated by discontinuous SDS-PAGE according to the method originally outlined by Laemmli (1970). All SDS-PAGE gels contained an 8% polyacrylamide

separating gel and a 3% stacking gel. Samples were denatured by the addition of one fifth volume of Laemmli sample buffer (section 2.4) to achieve a final concentration of 1x, and heating in a boiling water bath for 4 minutes. Samples were then loaded on to the gel and electrophoresed at 60 - 70 mA for 2 - 2.5 hours until the tracking dye had left the bottom of the separating gel.

2.7.2 Coomassie Staining of SDS/polyacrylamide gels

Gels were stained for protein in the following solution for 30 min at 37°C:
0.1% Coomassie Brilliant Blue G250; 50% (v/v) methanol; 10% (v/v) acetic acid.
The gels were then destained at 37°C for 30 min, the destain changed and left overnight. The destain consisted of:
10% (v/v) methanol; 10% (v/v) acetic acid.

2.7.3 Drying, autoradiography and phosphorimaging of gels

Destained gels were dried onto Whatman 3 MM chromatography paper using a Biorad Laboratories Slab Gel Drier model 1125 connected to an Aquavac Junior multi-purpose vacuum unit (Uniscience Ltd., 12-14 Ann's Crescent, London SW18 2LS, UK.). Gels which had been dried were autoradiographed using Fuji RX X-ray film with an intensifying screen. Autoradiography, which although less rapid than phosphorimaging results in a higher resolution image, was carried out at -80°C before developing the film using a Kodak X-OMAT Processor Model ME-3. The period of exposure was usually between 3 hours and five days. Radioactive gels were also routinely phosphorimaged using a Fuji Bio-Imaging Analyser (Fuji Photo Film Co. Ltd., Japan). Exposed plates were developed automatically by the Fuji Bio-Imaging Analyser and the images were captured onto a Macintosh Quadra 650 computer running Mac-Bas software (Fuji Photo Film Co. Ltd., Japan). Phosphorimages were analyzed by quantifying the intensity of each band. These intensities were then expressed as a percentage or relative intensity of the most intense band.

2.7.4 Agarose gel electrophoresis of nucleic acids

A 1% agarose gel was prepared by adding 1 g of agarose to 100 ml of 0.5x TBE buffer (see section 2.4).

The gel solution was boiled until all the agarose had dissolved. When the solution was sufficiently cool to pour, 1 µl of 10 mg/ml ethidium bromide was added and the solution poured into the gel apparatus. Approximately 20 µl of sample was loaded into each well with an appropriate amount of 6x DNA loading buffer (see section 2.4).

Marker DNA was run on agarose gels to estimate the size of the DNA that was being analyzed. The 1 kb ladder was most frequently used. This contained marker bands ranging in size between 75 bp and 12 kb.

Gels were run at 80 mA until the required resolution of DNA fragments was achieved. Ethidium bromide stained bands were visualised on a UV transilluminator and recorded with a polaroid camera.

2.7.5 Denaturing agarose gel electrophoresis of RNA

This method ensures accurate separation of RNA according to size as the denaturing conditions remove secondary structures that can cause RNA to run at aberrant molecular weights in non-denaturing agarose gels.

Denaturing agarose gels for the electrophoresis of RNA were made by dissolving 1 g of agarose in 100 ml of water in the microwave. When the agarose had cooled to about 60°C, 10% (v/v) formaldehyde and 1x Mops buffer at pH 8.0 (see section 2.4) were added to the cooling solution. The gel was then poured and allowed to set for 45 min. All manipulations were carried out in a fume cupboard.

Equal volumes of RNA sample and RNA sample buffer (see section 2.4) were mixed and heated to 65°C for 2.5 min and then snap cooled on ice. This denatured any secondary structures.

The sample was then loaded onto the gel and run out at 100 mV for 2-4 hours in 1x Mops at pH 7.0.

2.8 NORTHERN AND SOUTHERN BLOTTING

2.8.1 Capillary transfer of nucleic acids onto nylon membranes

DNA and RNA fragments were separated by gel electrophoresis as described in sections 2.7.4 and 2.7.5 respectively.

Prior to blotting of DNA, the nucleic acid was denatured by soaking the agarose gel in 3 volumes of denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 45 min and then

neutralised by soaking in neutralising solution (1 M Tris/HCl (pH 8.0), 1.5 M NaCl) for 45 min. The gel was rinsed in 2x SSC (see section 2.4) before blotting. A support was prepared onto which was placed a wick of Whatman 3 MM paper which was soaked in 20x SSC (see section 2.4). The gel was placed on top of the wick with the well side of the gel face down, ensuring that there were no air bubbles between the wick and the gel. A piece of Hybond-N™ nylon membrane and two pieces of Whatman 3 MM paper were cut to the size of the gel, soaked in 2x SSC and placed on top of the gel in the order given. The immediate area of the wick surrounding the gel was covered with parafilm to prevent a short circuit and a large quantity of paper towels folded to the approximate size of the gel was then placed on top of the 3 MM paper. A glass plate was placed on top of the stack of towels and a weight of approximately 500 g was placed on top. The blot was left overnight for the capillary transfer of the DNA from the agarose gel onto the nylon membrane to occur. The blot was then disassembled and the membrane dipped briefly in distilled water to remove any excess salt deposits. DNA was then fixed to the membrane using the UVP CL-1000 ultraviolet crosslinker at 120,000 $\mu J/cm^2$.

Capillary transfer of RNA onto a nitrocellulose membrane was performed as for Southern blotting but the denaturation step prior to setting up the transfer was unnecessary.

2.8.2 Random priming of cDNAs to synthesize radioactive probes

cDNA fragments were radiolabelled for use as probes on blots by the method of random priming using the Klenow fragment of DNA polymerase I. Approximately 25 ng of DNA was labelled with the Decaprime or *redi*prime DNA labelling system (Amersham, UK) according to the manufacturer's protocol, using 50 µCi of Redivue [α-^{32}P]dCTP (Amersham, UK). Once synthesized, probes were denatured by heating to 95-100°C for 5 min followed by chilling on ice before adding to the hybridization solution as described in section 2.8.4.

2.8.3 Separation of labelled DNA from un-incorporated radionucleotides using spin columns

This was achieved by constructing a mini Sephadex G50 column in a 0.5 ml microcentrifuge tube sitting inside a larger 1.5 ml microcentrifuge tube. A small hole was

made in the bottom of the smaller eppendorf using a small bore needle and some sterile washed glass beads packed into the bottom of the tube. G50 Sephadex in TE, pH 8.0 (see section 2.4) was then added to the top of the tube and spun until dry. The probe was then loaded onto the column and spun through. The un-incorporated radionucleotides became trapped in the Sephadex and the larger molecules of radiolabelled DNA passed through the column and were collected in the bigger 1.5 ml tube in which the spin column was located.

The incorporation into and the specific radioactivity of cDNA probes were determined by measuring the ^{32}P-radioactivity of the probe before and after passing through the spin column. 1 µl samples were diluted in 3 ml of scintillation fluid and counted in a scintillation counter.

2.8.4 Hybridization of radiolabelled DNA probes to Northern and Southern blots

All hybridization steps were performed in an hybridization oven (Techne Hybridizer HB-1D). Blots were incubated at 55°C for 1-2 hours in 20 ml pre-hybridization solution which consisted of:

0.5 M Na_2HPO_4, pH 7.2; 7 % SDS; 10 mg/ml BSA.

The radioactive DNA probe was then added to the pre-hybridization solution and the blot left to hybridize overnight at 55°C except where otherwise stated.

The hybridization solution was then poured off and stored at 4°C for future use. Blots were routinely washed in 2x SSC/1% SDS at 55°C for 10 min. This was followed by a number of washes at higher stringency (depending on the radioactivity of the blot) until the signal to background ratio was acceptable.

Washed filters were heat sealed in plastic and autoradiographed or phosphorimaged as preferred.

2.8.5 Stripping Hybond-N membranes of radioactive nucleotides

After probing, Hybond-N membranes were stripped of radioactive nucleotides by washing in boiling 0.1% SDS until radioactivity could no longer be detected by a Geiger counter.

2.9 DNA EXPERIMENTS

All experiments using DNA were carried out only once unless otherwise stated.

2.9.1 Prevention of degradation of DNA samples by DNase contamination

All solutions used in the preparation or manipulation of DNA samples were autoclaved. Tips and plasticware were autoclaved and only handled with gloved hands. On occasion sterile filter tips were used as extra protection against contamination of samples and reagents.

2.9.2 Isolation of plasmid DNA

Plasmid DNA was isolated using the QIAGEN QIAprep Spin Plasmid Miniprep kit (Qiagen, U.K.) following the manufacturer's instructions.

2.9.3 Determination of nucleic acid concentration and quality

100 µl of water in a quartz cuvette was used to record a baseline scan on a Philips spectrophotometer between 240 nm and 300 nm. 1-2 µl of nucleic acid sample was added to the cuvette and the sample scanned in the range mentioned. The ratio of absorbance at 260 nm to 280 nm indicates the purity of the nucleic acid (a ratio of 1.8 or greater is desirable). An absorbance at 260 nm (A_{260}) of 1 was taken to indicate the following concentrations:

Form of nucleic acid	Concentration (µg/ml)
Double stranded DNA	50
Single stranded DNA/RNA	40
Oligonucleotides	20

2.9.4 Digestion of DNA with restriction endonucleases

The DNA to be digested was prepared in a solution as follows:

1x reaction buffer

approximately 2 units of restriction enzyme

1x BSA

2 μg DNA

sterile distilled water.

The reactions were incubated at 37°C for 1-24 hours and the reactions were monitored where sufficient DNA was available by running an aliquot of the reaction on an agarose gel against uncut DNA.

2.9.5 Polymerase Chain Reaction (PCR)

The reactions were assembled on ice in a total volume of 25 μl and overlaid with some mineral oil. Reactions contained template DNA (approximately 20-200 ng) and the following components:

1x reaction buffer; 2.5 mM $MgCl_2$; 0.5 mM dNTP mix; 0.5 μM primer 1; 0.5 μM primer 2; 1 unit Taq polymerase.

The annealing temperature for each PCR was decided upon from the melting temperature of the primers calculated using the following equation:

Annealing temperature (°C) = n[G + C] + n[A + T] - 5 where n is the total number of each purine/pyrimidine base pair.

The PCR protocol used was as follows:

Denaturation - 95°C for 1 min
Annealing - X°C for 30 s
Extension - 72°C for 2 min
} 30 cycles

PCR products were separated on a 1% agarose gel and the ethidium bromide stained bands visualised using an UV-illuminator.

2.9.6 Sequencing of DNA

The nucleotide sequence of both cDNA strands was determined by the dideoxynucleotide chain-termination method. The first approximately 300 bp of 5'- and 3'- sequence were determined using the T7 Sequenase Version 2.0 DNA Polymerase

Sequencing kit (see protocol for instructions). Subsequent rounds of sequencing were carried out in collaboration with the Molecular Biology Sequencing Unit, IBLS, University of Glasgow, Glasgow G12 8QQ using automated sequencing.

2.9.7 Design of primers

Specific primers for PEPc and PEPc kinase from *A. thaliana* and all primers used for the sequencing of the *A. thaliana* PEPc clone were designed using GeneJockey II software. The conserved kinase primers were degenerate primers designed to the conserved domains I and VIII of *K. fedtschenkoi* and *A. thaliana* PEPc kinase sequences. The sequences for the conserved PEPc primers and *A. thaliana* α-tubulin primers were obtained from Honda et al., (1996) and Jackson et al., (1995), respectively. All primers were synthesized by Cruachem Ltd., Todd Campus, West of Scotland Science Park, Acre Road, Glasgow G20 0UA.

The alignment of the different primers to the appropriate cDNA is shown diagrammatically in Figure 2.1. The fragment size amplified and the annealing temperatue used are also given in this figure.

2.10 RNA EXPERIMENTS

All experiments using RNA were carried out only once unless otherwise stated.

2.10.1 Prevention of RNA sample degradation by RNase contamination

All solutions used in the preparation or manipulation of RNA samples were made with DEPC-treated water. Tips and plasticware were autoclaved and handled only with gloved hands. On occasion sterile filter tips were used as extra protection against contamination of samples and reagents.

2.10.2 Isolation of RNA from plant tissue and cell culture

(i) *A. thaliana* cell culture and plant tissue. Cell culture samples were filtered through Whatman 3 MM paper using Buchner apparatus under vacuum to remove liquid medium from the samples. The cells were then weighed and packaged in aluminium foil before being snap-frozen and stored at -80°C until required.

Figure 2.1 Alignment of primers for *A. thaliana* PEPc, PEPc kinase and α-tubulin

The primer sequences for each cDNA sequence are given below along with the size of fragment amplified and the annealing temperature. The alignment site for each primer is shown diagrammatically opposite as a red arrow (sense primer → ; antisense primer ←).

***A. thaliana* PEPc-specific primers:**

fragment size 174 bp; annealing temperature 55°C

sense 5'- GGCAAGCAGGAGGTCATGATCGG -3'

antisense 5'- GAAGTACTCGACAAATCGAGGCTC -3'

Conserved PEPc primers:

fragment size 404 bp; annealing temperature 55°C

5'- GGCAAGCAGGAGGTCATGATCGG -3'

5'- GAAGTACTCGACAAATCGAGGCTC -3'

***A. thaliana* α-tubulin-specific primers:**

fragment size 501 bp; annealing temperature 55°C

5'- TTTCTTTAGCGACTGGTGC -3'

5'- TCAACCTGTTCAAGTTTGTG -3'

Conserved PEPc kinase primers:

fragment size 468 bp; annealing temperature 55°C

5'- TGCGAGGAGATCGGCGKG -3'

5'- ACCTCCGGCGCCACGTARTAC -3'

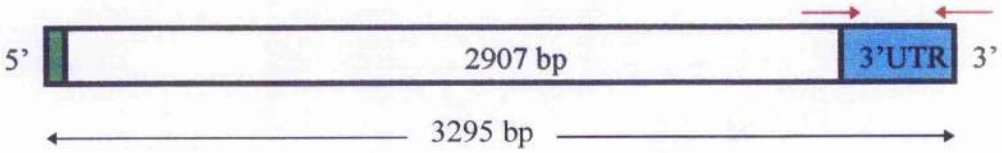
***A. thaliana* PEPc kinase-specific primers:**

fragment size 501 bp; annealing temperature 55°C

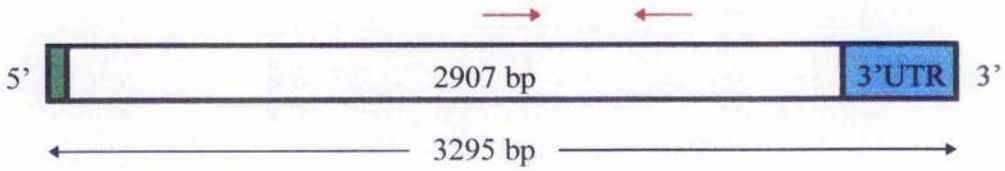
5'- ATGGCTCTCTTGTCCTATCACC -3'

5'- CTTTAGCCATAGATGAAACCCC -3'

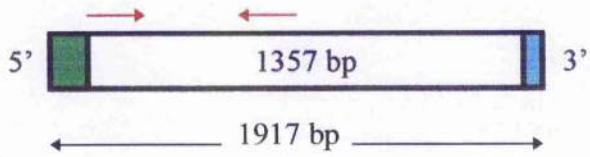
A. thaliana PEPc with *A. thaliana* PEPc-specific primers



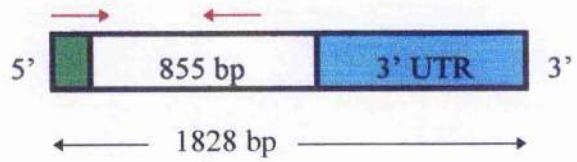
A. thaliana PEPc with conserved PEPc primers



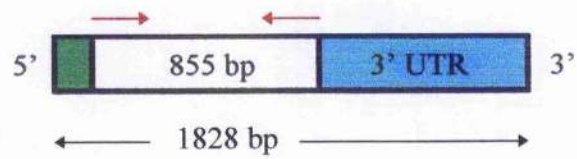
A. thaliana α-tubulin with *A. thaliana* α-tubulin-specific primers



A. thaliana PEPc kinase with conserved PEPc kinase primers



A. thaliana PEPc kinase with *A. thaliana* PEPc kinase-specific primers



Cell culture and *A. thaliana* tissue were ground up in a chilled mortar and pestle using liquid nitrogen. 900 µl of RNA extraction buffer was then added (see section 2.4).

Each sample was ground in the buffer until it was like fine sand. 900 µl of phenol:chloroform:isoamyl alcohol (PIC) in a ratio of 25:24:1 was then added and the sample ground very fast. Tissue was kept frozen throughout the extraction by repeated additions of liquid nitrogen to the mortar and pestle. Each sample was then transferred to a microfuge tube, allowed to thaw at room temperature and spun for 10 min at 13,000 rpm at 4°C. The aqueous layer was then removed and put into a new eppendorf. 1 volume of PIC was added, the sample was vortexed and then spun again. This was repeated until a clean interface between the organic and aqueous phase was obtained. The aqueous layer was removed to a new tube, 1 volume of chloroform was added and the sample was spun. The aqueous phase was again removed to a new tube and 10 M LiCl added to a final concentration of 2 M. The sample was mixed very quickly by inversion to prevent DNA precipitation and then left overnight at 4°C.

Precipitated RNA was collected by centrifugation at 13,000 rpm for 15 min at 4°C and the supernatant was carefully removed. The pellet was washed with 500 µl 2 M LiCl and then vortexed to lift the pellet. The sample was spun again and the supernatant was removed completely. The pellet was then resuspended in 500 µl DEPC-treated water. 0.1 volume of 3 M sodium acetate, pH 5.5 was added and the sample was vortexed. 2.5 volumes of ethanol was then added and vortexed and the sample was left on ice for 15 min. The sample was then spun for 15 min and the supernatant was removed. The pellet was washed with 70% ethanol, the sample was spun for 5 min and the supernatant was removed. The pellet was air-dried by inverting the tube on paper towel for 10-15 min. The dried pellet was resuspended in DEPC-treated water and its purity and concentration determined by measuring the absorbance at 260-280 nm.

Samples were frozen at -80°C until required.

(ii) *K. fedtschenkoi* leaves and disks. 2-3 g of tissue was ground up in a mortar and pestle using liquid nitrogen. The ground tissue was then added to a tube containing 10 ml of CTAB extraction buffer (see section 2.4) in a water bath at 65°C and the tube was inverted to mix. The lid of the tube was previously punctured using a syringe needle. An equal volume of chloroform (i.e. 10ml) was then added to the tube which was spun at

9000 rpm for 10 min at 4°C in a Beckman centrifuge to extract the RNA. The supernatant was then removed and another 10 ml of chloroform was added to the tube which was centrifuged at 5000 rpm for 10 min. The supernatant was again removed and 3 ml of 10 M LiCl was added. The RNA was then left to precipate overnight at 4°C.

After centrifugation at 9000 rpm for 20 min at 4°C the resulting pellet was dissolved in 500 µl sterile TE, pH 7.5 (see section 2.4) and transferred to a microfuge tube. The RNA was extracted with 500 µl of chloroform and microfuged at 13,000 rpm for 10 min at 4°C. The top aqueous layer was then removed to a clean tube and 50 µl of 3 M sodium acetate added. The tube was then filled with ice-cold 100% ethanol and left to precipate on ice for 1 hour. The RNA was then pelleted by spinning at 13,000 rpm for 20 min in a microfuge at 4°C and the pellet dried using a Savant Speed Vac® Plus SC110A. The pellet was then allowed to dissolve in a small volume of DEPC-treated water (e.g. 25 µl).

2.10.3 Isolation of poly A^+ RNA

Poly A^+ RNA was obtained from isolated total RNA using Promega's Poly Atract system (Promega, U.K.) following the manufacturer's instructions.

2.10.4 *In vitro* translation of RNA samples

The rabbit reticulocyte lysate system (Amersham, U.K.) possessed no detectable PEPc kinase activity and was used for the *in vitro* translation of RNA samples. 100-200 µg/ml of total RNA were translated according to the manufacturer's protocols using Redivue™ [^{35}S] - methionine as the labelled amino acid. Incubations were for 45 min at 30°C. The incorporation of [^{35}S] - methionine into protein was measured by precipitation with trichloroacetic acid according to the maufacturer's instructions to allow standardisation of the subsequent PEPc kinase assays.

RNA isolated from *K. fedtschenkoi* leaves in the middle of the night by Dr. J Hartwell was used as a control in all *in vitro* translation-kinase assays.

2.10.5 RT-PCR

RNA samples were reverse-transcribed using Promega's AMV reverse transcriptase. 0.5 μg primer / μg RNA in a total volume of ≤ 15 μl in water was heated to 70°C for 5 minutes to denature any secondary structure and then chilled on ice for 5 minutes. The reverse transcription reaction components were then added to the annealed primer/template in the order below:

AMV RT 1x reaction buffer; 10 mM dNTP mix; 40 units RNasin® ribonuclease inhibitor; 4 mM sodium pyrophosphatase (prewarmed to 42°C); 30 units AMV RT.

The reaction mix was incubated at 42°C for 60 minutes and then put on ice. 5 μl of reverse transcription products were then put into a 50 μl PCR containing:

1x reaction buffer; 2.5 mM $MgCl_2$; 0.5 mM dNTP mix; 0.5 μM primer 1; 0.5 μM primer 2; 1 unit Taq polymerase.

The reactions were assembled on ice and overlayed with some mineral oil. The reaction conditions used were as follows:

95°C for 2 minutes;

55°C for 30 s, 72°C for 1 minute and 95°C for 30 s for 30 cycles;

55°C for 1 minute;

72°C for 5 minutes.

The PCR products were then run out on a 1% agarose gel and the ethidium bromide-stained bands visualised using an UV-illuminator.

2.10.6 *In vitro* transcription of *A. thaliana* PEPc kinase cDNA

In vitro transcription of *A. thaliana* PEPc kinase cDNA was carried out using T7 mMessage Machine according to the manufacturer's instructions. The kit requires that linear DNA be used as the template for the transcription reaction and so plasmid pZL1 that contained the kinase cDNA was linearized by digestion with *Hind* III (section 2.9.4).

2.11 CLONING TECHNIQUES

2.11.1 Preparation of plating bacteria

A stab of plating cells *E.coli* strain Y1090ZL was obtained from the Arabidopsis Biological Resource Center (ABRC), Ohio, USA and streaked out on NZYCM agar (see section 2.4).

The solution was calibrated to pH 7.5 before dissolving the agar. A single colony was then used to inoculate 5 ml of sterile Lauri Bertrani (LB) medium (see section 2.4) in a sterile universal and grown at 37°C and 250 rpm in a rotary shaker.

When this "starter culture" had developed a cloudy appearance after approximately 2-3 hours, 100 µl was removed and added to 100 ml of sterile LB medium supplemented with 0.2% maltose in a sterile 250 ml glass conical flask. This was grown up under the same conditions until a A_{600} of about 0.4 was reached. The cells were then centrifuged at 4000 g for 10 min at room temperature. The supernatant was discarded and the pellet was resuspended and washed in sterile 0.01 M $MgSO_4$ by centrifuging at 4000 g for 10 min at room temperature. The pelleted cells were again resuspended in 0.01 M $MgSO_4$ to a A_{600} of 2.0. The cells were stored at 4°C and used within 3 days. Approximately 200 µl of cells were added to 4 ml top NZY agar for plating out the bacteriophage in which the cDNA of the λPRL2 library is packaged.

2.11.2 Titration of λPRL2 cDNA library and λZIPlox recombinants

An *A. thaliana* cDNA library (λPRL2) was obtained from the Arabidopsis Biological Resource Center (ABRC), Ohio, USA. The given titre of the λPRL2 library was 10^8/ml. In order to confirm this, a few microlitres of the library were taken and serial dilutions were made in SM buffer (see section 2.4).

These dilutions were then incubated at 37°C for 20 min with 100 µl of Y1090ZL plating cells before being added to 4 ml of top agar (48°C) in a sterile 15 ml Falcon tube, vortexed briefly and immediately poured onto 20 ml of hardened, pre-warmed NZYCM bottom agar supplemented with 0.1 M $MgSO_4$ and 0.2% maltose in a 90 mm petri dish. The plate was swirled gently to allow the formation of a level top agarose layer and then left to stand on a flat surface at room temperature. When the top agar had hardened, the

Petri dishes were inverted and incubated at 37°C for 12-16 hours. The number of plaques that formed in the bacterial lawn were counted and found to be much lower than the value estimated from the titre quoted above.

2.11.3 Preparation of *A. thaliana* ESTs

A PEPc EST (identified as in Chapter 4) was obtained from ABRC as a stab. A loopful of the stab was streaked onto an LB plate containing 100 µg/ml ampicillin and 10 mM $MgCl_2$. A colony was then used to inoculate 5 ml of LB broth containing the required ampicillin selection and the broth was grown overnight at 37°C and 250 rpm. Plasmid was isolated from the bacteria as described in section 2.9.2.

2.11.4 Immobilization of λZIPlox recombinants onto nitrocellulose membrane

The method used was based on that of Benton & Davies (1977). Plates were prepared as outlined above (2.11.2). As the titre of the library was much lower than that specified, the entire library was plated out on six NZY agar plates in 140 mm diameter petri dishes with the Y1090ZL cells. 90 mm diameter plates were used for all subsequent rounds of screening. When the Y1090ZL bacteria had formed a lawn on the NZY agar the plates were chilled for 2 hours at 4°C. Using forceps and wearing gloves, 90 mm diameter nitrocellulose circles were then placed carefully onto the top agar of each plate for 2 min. A needle and some ink were used to prick through the filters and the agar for orientation. Duplicate membranes were allowed to transfer for 4 min. The nitrocellulose membranes were then denatured after lifting by submerging in 1.5 M NaCl and 0.5 M NaOH for 2 min. The membranes were then neutralized for 5 min in 1.5 M NaCl, 0.5 M Tris/HCl (pH 8.0). Finally the membranes were rinsed for 30 s maximum in 0.2 M Tris/HCl (pH 7.5) and 2x SSC buffer solution. After briefly blotting the membranes on Whatman 3 MM paper the DNA was crosslinked to the membranes using a UVP CL-1000 Ultraviolet Crosslinker to deliver 120,000 µJ/cm^2 of UV energy for about 30 s.

2.11.5 Hybridization using radiolabelled PEPc probe

Hybridization using radiolabelled PEPc probe was carried out in a shaking water bath at 63°C, the calculated hybridization temperature for the 500 bp PEPc probe. Just enough

pre-hybridisation solution was used to cover the filter(s) in the bottom of a plastic box. The pre-hybridization solution contained:

6x SSC (see section 2.4); 20 mM Na_2HPO_4; 0.4% SDS; 5x Denhardt's reagent; 500 µg/ml denatured, sonicated salmon sperm DNA.

After hybridisation, the filters were washed at low stringency in 6x SSC and 0.1% SDS and shaken carefully at room temperature for 5 min. The wash was then repeated with fresh solution for a further 20 min at 63°C.

2.11.6 Isolation of putative positive phage

The plates were aligned with the autoradiographs and plaques containing putative positives were identified. The plaques were then cored out of the agar plates using the end of either a sterile pasteur pipette or yellow tip and put into a sterile eppendorf containing 500 µl of SM (see section 2.4) buffer. The phage were allowed to elute into the buffer overnight at 4°C.

2.11.7 *In vivo* excision of recombinant pKP42

Putative positive phage were eluted into SM buffer as described (2.11.6). The phage were then vortexed vigorously for 10 s and incubated at room temperature for 5 min. 50 µl, 150 µl and 300 µl of the supernatant from the eluted phage was mixed with 100 µl of DH10B(ZIP) cells in a sterile eppendorf and incubated at room temperature for a further 5 min. (The DH10B(ZIP) cells had been prepared as described in 2.11.1 for the plating cells Y1090ZL). The entire mixture was spread onto a NZYCM plate containing 10 mM $MgCl_2$ and 100 µg/ml ampicillin. The plate was then inverted and incubated overnight at 37°C. Any colonies that appeared contained recombinant pKP42 plasmid which conferred resistance to ampicillin.

2.12 BRADFORD ASSAY FOR PROTEIN DETERMINATION

The A_{595} of duplicate BSA standards of 2, 4, 8, 12, 16 and 25 µg of protein were used to construct a calibration curve for determining the protein concentration of samples. BSA standards and protein samples were made up to a volume of 25 µl in plastic cuvettes and

975 µl of dilute Bradford reagent (see section 2.4) was then added to each cuvette which was subsequently inverted five times to mix the contents.

3 parts of reagent were diluted with 17 parts of water immediately before use and filtered through Whatman 3 MM paper.

2.13 GAS EXCHANGE MEASUREMENTS

For experiments on *K. fedtschenkoi*, rates of net CO_2 assimilation were continuously on the same leaf over 24-72 hours. The leaves were enclosed in porometer heads which tracked the environmental conditions in the growth chamber with gas exchange parameters measured using an open infra-red gas exchange system (H. Walz, GmbH Effeltrich, Germany) with Binos gas analyser. The temperature, light and air-supply conditions are as described in Chapter 3. Gas exchange parameters were calculated using DIAGAS software supplied by Walz.

2.14 MEASUREMENT OF TOTAL LEAF MALATE CONTENT

A crude extract of *K. fedtschenkoi* leaf disks was prepared by crushing 7 disks in a garlic press. The malate content of the leaf disk extract was determined enzymatically using malate dehydrogenase in a spectrophotometric assay at 340 nm by monitoring the oxidation of NADH. All leaf malate content measurements were carried out only once.

Chapter Three

THE REGULATION OF PHOSPHOENOLPYRUVATE CARBOXYLASE FROM *KALANCHOË FEDTSCHENKOI* BY PHOSPHORYLATION

3.1 INTRODUCTION

The malate sensitivity of PEPc from the CAM species *Kalanchoë (Bryophyllum) fedtschenkoi* is controlled by a circadian oscillator (Nimmo et al., 1984; Nimmo et al., 1987). Rhythmic changes are also seen in PEPc kinase activity and translatable mRNA (Carter et al., 1990; Hartwell et al., 1996). Protein synthesis inhibitors block the circadian appearance of PEPc kinase activity and translatable mRNA in *K. fedtschenkoi* (Carter et al., 1991; Hartwell et al., 1996) implying that two protein synthesis steps exist between the circadian clock and the appearance of the kinase protein. The RNA synthesis inhibitor, cordycepin, also blocked the nocturnal increase in PEPc kinase mRNA in *K. fedtschenkoi* (Hartwell et al., 1996). However, at present nothing else is known about the transduction pathway in CAM species between the circadian oscillator and the PEPc kinase RNA.

In both C_4 and C_3 species, light activates PEPc kinase activity via increasing the levels of the kinase mRNA (Hartwell et al., 1996). In C_4 plants, the light-induced change in PEPc activity has been linked to the light-mediated cytosolic alkalinization and subsequent release of Ca^{2+} from the vacuole (Giglioli-Guivarc'h et al., 1996; Pierre et al., 1992). In C_3 barley leaf protoplasts kept in darkness, PEPc was activated by treatment with both acid and a weak base (Lillo et al., 1996).

In consideration of the evident involvement of pH in the regulation of PEPc from C_4 and C_3 species, the effect of cytosolic pH on CAM PEPc was investigated in an attempt to elucidate more of the CAM signal transduction pathway affecting PEPc activity. The pH work in C_4 and C_3 species had used protoplasts but previous work had shown that protoplasts from the CAM species *K. fedtschenkoi* were too fragile for use (P.J. Carter, unpublished), presumably due to the large size of the CAM leaf vacuole. Disks cut from the leaves of *K. fedtschenkoi* were therefore used as the experimental system for this particular research.

3.2 RESULTS

3.2.1 Cytosolic alkalinization has no affect on the malate sensitivity of PEPc and PEPc kinase in *K. fedtschenkoi* leaf disks

Disks of diameter 1.5 cm were cut from *K. fedtschenkoi* leaves in the middle of the day of an 8-hour day (28°C)/16-hour night (15°C) growth regime (section 2.2.2(ii)). The disks were incubated in 90 mm petri dishes containing distilled water and transferred to fresh water after 1 and 2 hours incubation. A previous experiment had shown that when *K. fedtschenkoi* disks were incubated in distilled water over a period of 2 hours, the conductivity of the water increased suggesting that malate and other cell contents had diffused out of the damaged cells into the incubation solution. Transferring the disks into a petri dish containing fresh distilled water ("washing") after 15, 45 75 and 120 minutes helped to reduce the conductivity of the incubation solution. All control disks in subsequent experiments were therefore washed for 2 x 1 hours. As judged by enzymatic assay, no malate is released after these washes.

At the beginning of the dark period the disks were transferred either to fresh water or to ammonium chloride (NH_4Cl) (10 mM, 20 mM or 50 mM). Samples were then taken after 0, 3, 6 and 8 hours of darkness. Fourteen disks were used for each NH_4Cl concentration and incubation time. Disks were blotted dry, and desalted extracts made (section 2.5). The PEPc extracted from disks incubated in water had an apparent K_i for L-malate of 0.4 mM (section 2.6.1) at the beginning of the dark treatment which rose steadily to a value of 1.4 mM at the end of the 8 hour period. The NH_4Cl did not have any affect on the increase of the apparent K_i of PEPc for malate compared with controls.

Thus when assessed by the malate sensitivity of PEPc, there is no evidence that NH_4Cl increases the level of PEPc kinase activity in CAM species as it has been reported to do in C_4 species. Therefore, the effect of *lowering* the cytosolic pH was investigated.

3.2.2 Cytosolic acidification prevents the nocturnal increase in the apparent K_i of PEPc for L-malate and PEPc kinase activity in *K. fedtschenkoi* leaf disks

Leaf disks were prepared from *K. fedtschenkoi* just before the light phase began at which time PEPc is dephosphorylated (Nimmo et al., 1984; Carter et al., 1991). Disks were incubated in 90 mm petri dishes containing water, 0.1 mM acetic acid, 1.0 mM acetic acid and 3.0 mM acetic acid for 16 hours, that is, until the middle of the following dark

phase. Fourteen disks were again used for each condition of this experiment and all subsequent experiments using leaf disks. PEPc activity was then extracted from the disks, assayed and the malate sensitivity determined. The activity of PEPc kinase was measured in the same extracts.

Increasing the concentration of acetic acid had the effect of lowering the apparent K_i of PEPc in the *K. fedtschenkoi* leaf disk extracts (Figure 3.1A). This suggested that the PEPc was in a low-phosphorylated state after this treatment i.e. that the acid treatment prevented the usual nocturnal phosphorylation of PEPc. In almost all other situations tested to date, the phosphorylation state of PEPc has reflected the activity of PEPc kinase. Disk extracts were therefore assayed for PEPc kinase activity (section 2.6.2). Figure 3.1B demonstrates that there was indeed a decrease in kinase activity in the acid-treated disks, consistent with the increased sensitivity of the enzyme to feedback inhibition by malate.

This preliminary experiment showed that acetic acid treatment reduced the rise in PEPc kinase activity in *K. fedtschenkoi* disks at night and consequently reduced the apparent K_i of PEPc for malate. The effects of acid treatment of disks were therefore examined in more detail.

3.2.3 Optimisation of the incubation conditions

The apparent K_i values observed for PEPc extracted from the *K. fedtschenkoi* disks in Figure 3.1 were low compared to those previously seen in leaves at similar time points, e.g. 3 mM in leaves in the middle of the night (Nimmo et al., 1984) compared to about 1.5 mM in disks. It was therefore decided to compare leaf and disk samples from the same plants.

Intact leaves were detached at the start of the photoperiod and allowed to take up water through the petiole. Disks were cut from the same plants at the same time and incubated in water. Samples were taken every 2 hours for 10 hours into the dark period. The apparent K_i values obtained for the leaves and disks at 10 hours were approximately 2.35 mM and 1.43 mM respectively (Figure 3.2A). This result confirms that the PEPc in disks does become phosphorylated in the dark but presumably to a lower extent than in leaves.

Figure 3.1 The effect of acetic acid on the malate sensitivity of PEPc and PEPc kinase activity of *K. fedtschenkoi* leaf disks

Leaf disks of *K. fedtschenkoi* were incubated in different concentrations of acetic acid from the beginning of the day period to the middle of the night period, a total of 16 hours. Desalted extracts of disks were made and assayed for the malate sensitivity of PEPc and PEPc kinase activity.

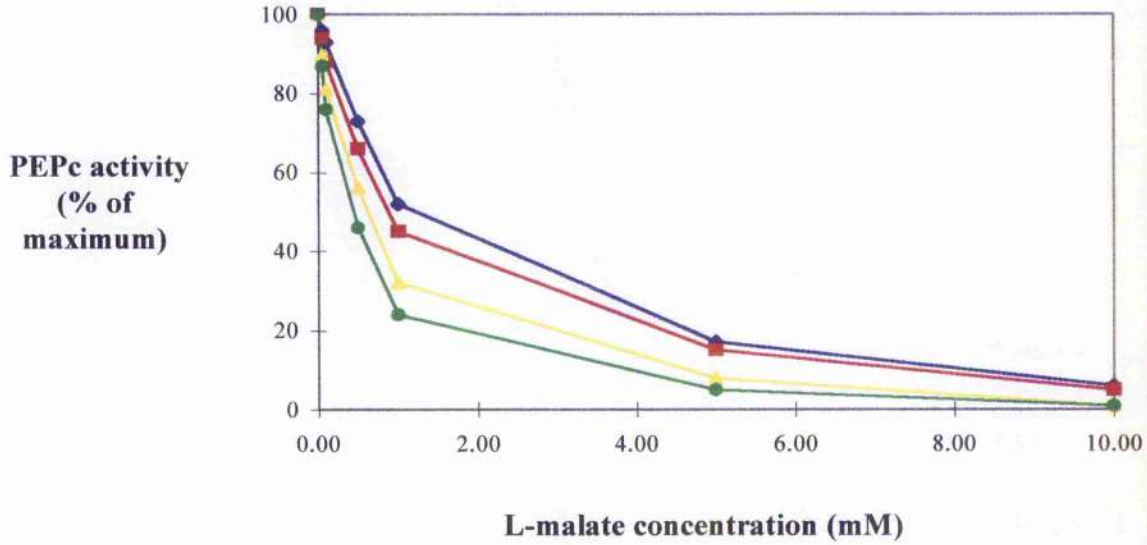
(A) Graph used to determine the apparent K_i of PEPc for L-malate (i.e. L-malate concentration at 50% maximum PEPc activity).

The different treatments are represented thus: water (◆); 0.1 mM acetic acid (■); 1.0 mM acetic acid (▲); 3.0 mM acetic acid (●).

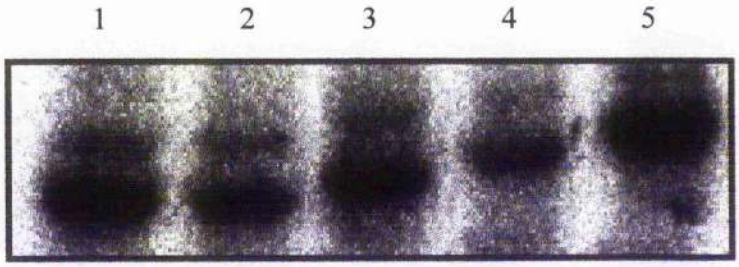
(B) Phosphorimage showing the PEPc kinase activity in desalted extracts from different disk samples, expressed as a percentage of the value obtained with the kinase control:
Lane 1 - water
Lane 2 - 0.1 mM acetic acid
Lane 3 - 1.0 mM acetic acid
Lane 4 - 3.0 mM acetic acid
Lane 5 - kinase control (partially purified maize PEPc kinase)

A

The effect of acetic acid on the malate sensitivity of PEPc from *K. fedtschenkoi* leaf disks



B



%	83	44	57	32	100

Figure 3.2 The malate sensitivity of PEPc and PEPc kinase activity of *K. fedtschenkoi* leaves and leaf disks at night

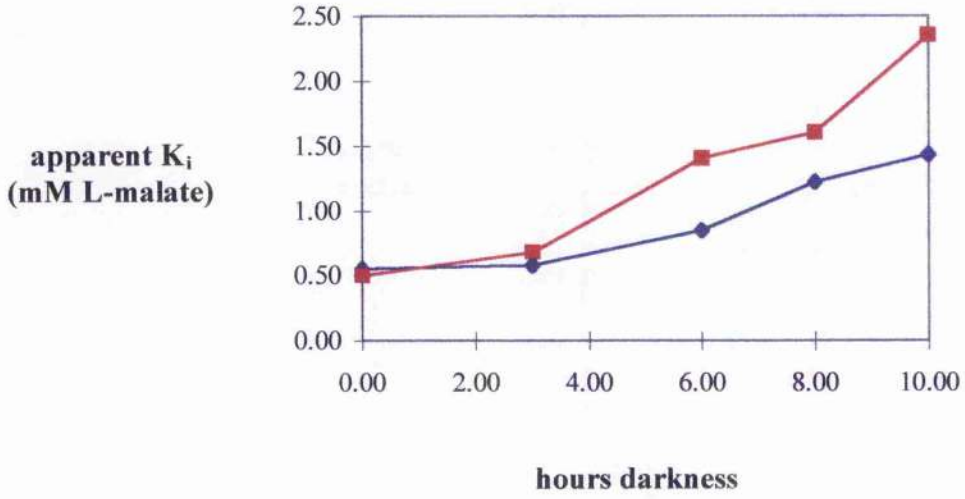
Intact leaves and leaf disks were taken from *K. fedtschenkoi* plants at the beginning of the day. Leaves were allowed to take up water through the petiole and disks were incubated in water. Samples were taken at different times during the night. Desalted extracts of leaves and disks were assayed for the malate sensitivity of PEPc and PEPc kinase activity.

(A) Graph showing the apparent K_i of PEPc for L-malate in disks (◆) and leaves (■) throughout a night period time course.

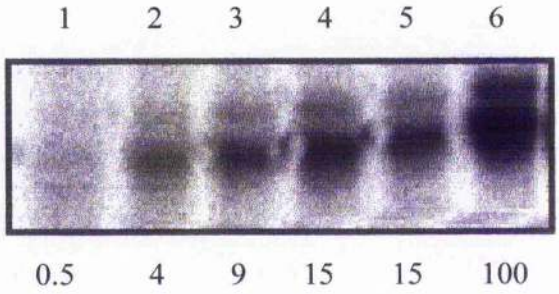
(B) Phosphorimage showing the PEPc kinase activity in desalted extracts from leaves (i) and disks (ii), expressed as a percentage of the value obtained with kinase control:
Lane 1 - 0 hours
Lane 2 - 3 hours
Lane 3 - 6 hours
Lane 4 - 8 hours
Lane 5 - 10 hours
Lane 6 - kinase control (partially purified maize PEPc kinase)

A

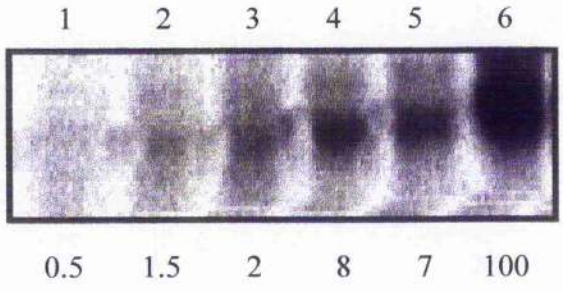
A comparison of the malate sensitivity of PEPc from *K. fedtschenkoi* disks and leaves at night



B(i)



B(ii)



The PEPc kinase activity in leaves and disks increased in a manner consistent with the observed changes in malate sensitivity (Figure 3.2B).

Thus there appeared to be a significant difference between *K. fedtschenkoi* leaves and disks in the apparent K_i values of PEPc for L-malate and PEPc kinase activities. In a further experiment disks were shaken gently (approximately 75 rpm) during incubation with no lids covering the petri dishes to ensure adequate aeration of the incubation solution. Leaves and disks were again taken at the beginning of the light period. Samples were taken for the first 8 hours of the dark period. Although there was a slight delay in the increase of apparent K_i from the disk samples, by 8 hours darkness the disks shaken gently in water and the detached leaves had apparent K_i values for PEPc of 2.2 mM and 2.65 mM L-malate respectively (Figure 3.3). Shaking the disks during incubation appeared to allow the apparent K_i of PEPc for L-malate to increase quite significantly, to a level comparable to that of leaves. All subsequent experiments therefore involved the aeration of disk incubation medium in this way.

3.2.4 Acetic acid affects PEPc via the acidification of cytosolic pH

It is possible that the effect seen with acetic acid is actually metabolic i.e. derived from the metabolism of acetate, rather than a consequence of reduction in cytosolic pH. Lipid soluble weak acids/bases can be used to manipulate cytosolic pH whereas strong acids, which are fully dissociated, are not lipid permeable and so are unable to alter the cytosolic pH. To investigate the nature of the effect of acetic acid on PEPc, disks were cut at the beginning of the day and put into water, 3 mM acetic acid, 3 mM propionic acid or approximately 1 mM HCl. Acetic acid and propionic acid are both weak acids but their uptake would affect metabolites in different ways. HCl is a strong acid. The pH of each was approximately 3.70. The samples were incubated until the middle of the following night when desalted extracts were made and the malate sensitivity of PEPc assayed. Figure 3.4A shows the malate sensitivities obtained.

It can be seen that 3 mM propionic acid and 3 mM acetic acid both decrease the dark apparent Ki value as previously observed with acetic acid. However, 1 mM HCl has no affect on the malate sensitivity of PEPc when compared to the control.

Figure 3.3 The effect of shaking on the malate sensitivity of PEPc from *K. fedtschenkoi* disks and leaves at night

Intact leaves and leaf disks were taken from *K. fedtschenkoi* plants at the beginning of the day. Leaves were allowed to take up water through the petiole and disks were shaken gently in water and left uncovered to facilitate aeration. Samples were taken at different times during the night and desalted extracts assayed for the malate sensitivity of PEPc.

The graph below shows the apparent K_i of PEPc for L-malate in disks (◆) and leaves (■) throughout a night period time course.

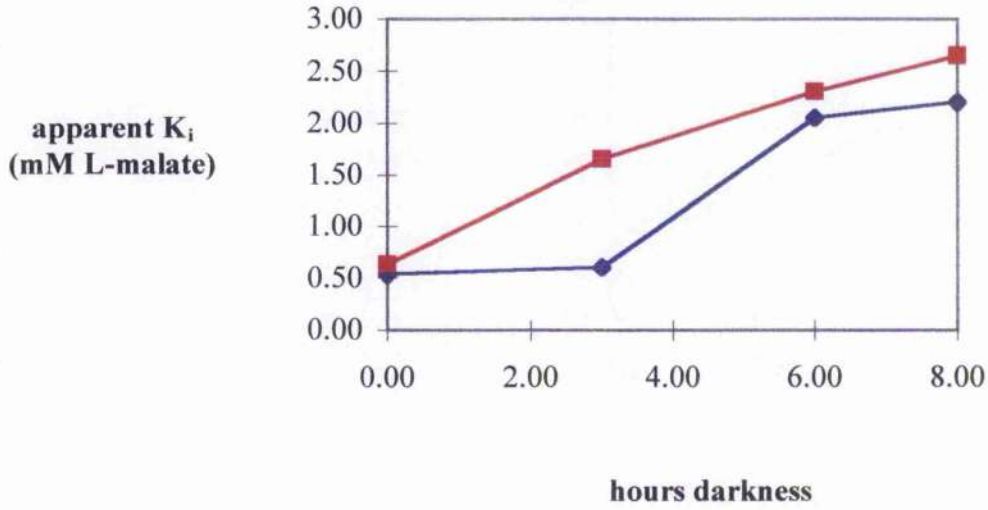


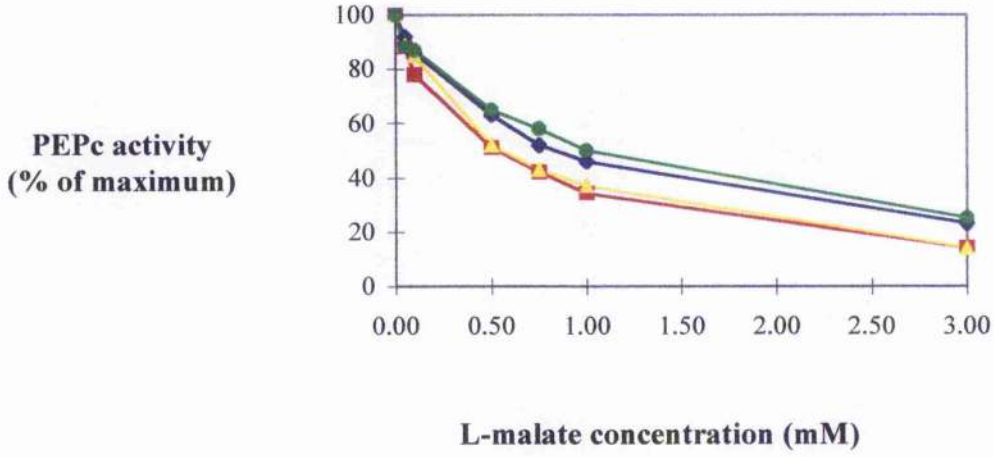
Figure 3.4 The effect of different acids on the malate sensitivity of PEPc from *K. fedtschenkoi* disks

Leaf disks were cut from *K. fedtschenkoi* plants at the beginning of the day period and incubated in different acid solutions until the middle of the following night period. Desalted extracts of the disks were made and assayed for the malate sensitivity of PEPc and PEPc kinase activity.

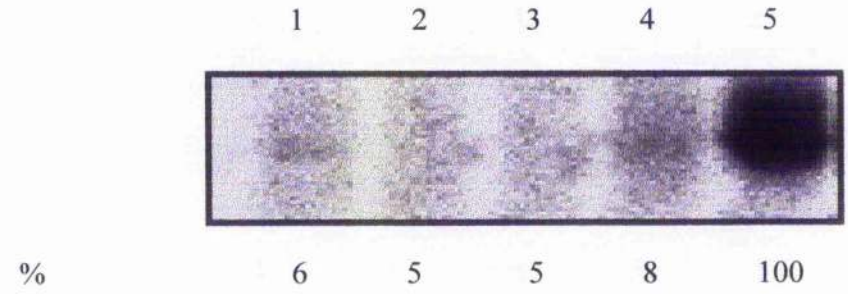
(A) Graph used to determine the apparent K_i of PEPc for L-malate i.e. L-malate concentration at 50% PEPc activity. The different treatments are represented thus: water (◆); 3 mM acetic acid (■); 3 mM propionic acid ( ); 1 mM hydrochloric acid (●).

(B) Phosphorimage showing the PEPc kinase activity in desalted extracts from different disk samples, expressed as a percentage of the value obtained with the kinase control:
Lane 1 - water
Lane 2 - 3 mM acetic acid
Lane 3 - 3 mM propionic acid
Lane 4 - 1 mM hydrochloric acid
Lane 5 - kinase control (partially purified maize PEPc kinase)

A **The effect of different acids on the malate sensitivity of PEPc from *K. fedtschenkoi* leaf disks**



B



The samples were also assayed for PEPc kinase activity (Figure 3.4B). Although in this experiment the PEPc kinase activities are low, the results suggest that PEPc kinase is still present in the control and HCl- treated disks but reduced in the acetic acid- and propionic acid-treated disks. These results therefore suggest that the effect of acetic acid is not metabolic but rather is due to cytosolic acidification.

3.2.5 Time course of the effect of 3 mM acetic acid

In the work described above, treatment of leaf disks with 3 mM acetic acid over the course of a normal day prevented the usual rise in apparent K_i for malate (i.e. phosphorylation) of PEPc in the following night. To assess the duration of treatment with acetic acid that was required to give this effect, a time course experiment was performed. Disks were cut as the light period was beginning and put into either water or 3 mM acetic acid. At midday, 3 pm and 6 pm disks were transferred from the water into 3 mM acetic acid. All samples were shaken until the middle of the night when disks were removed from their incubation medium, blotted dry and snap frozen. The PEPc activity was then extracted from the disks, assayed and the malate sensitivity determined. Figure 3.5A shows that the apparent K_i value decreased with increasing length of 3 mM acetic acid treatment. However, even treatment with acetic acid from midday onwards had much less effect on the malate sensitivity of PEPc than treatment from 8 am onwards. Extracts were assayed for PEPc kinase activity and the results were consistent with the apparent K_i values obtained (Figure 3.5B). It can be seen that when disks were transferred to acetic acid at 6 pm, i.e. 2 hours after the dark period had begun, the kinase activity extracted is actually greater than that extracted from the control (Figure 3.5B; lanes 1 and 9). RNA was also extracted from the disks (section 2.10.2(ii)) and this was translated *in vitro* (section 2.10.4). Translation products were then assayed for kinase activity and results corrected for the efficiency of each *in vitro* translation as determined by [^{35}S]methionine incorporation. The control RNA (Figure 3.5 IC lane 1) translated much less efficiently than the other samples which explains the apparent incongruity between the value given for the % kinase activity and the actual intensity of the band apparent from the phosphorimage (compare with Figure 3.5 IIC). The RNA isolation from the intact leaf at midnight (Figure 3.5 IC lane 7) was carried out by Dr J. Hartwell

Figure 3.5 The effect of acetic acid treatment length on the malate sensitivity of PEPc, PEPc kinase activity and level of PEPc kinase translatable mRNA

Leaf disks were cut from *K. fedtschenkoi* plants at the beginning of the day period and put into either water or 3 mM acetic acid. At midday, 3 pm and 6 pm some disks were transferred from the control (water) incubation into 3 mM acetic acid. All samples were incubated until the middle of the night. Desalted extracts of the disks were assayed for the malate sensitivity of PEPc and PEPc kinase activity. RNA extracted from the disks was translated *in vitro* and assayed for PEPc kinase activity. Data from two separate experiments are shown in Figure 3.5I and 3.5II.

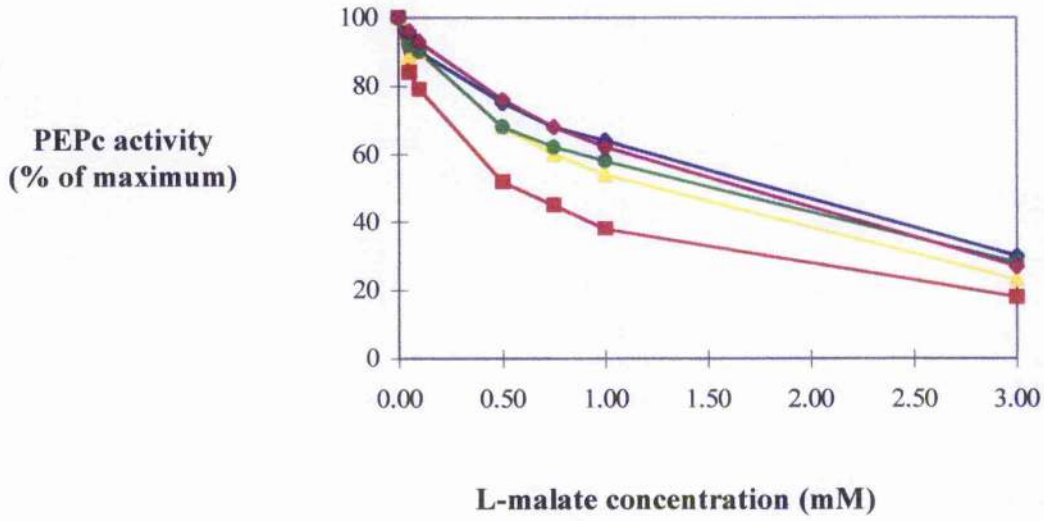
(A) Graph used to determine the apparent K_i of PEPc for L-malate i.e. L-malate concentration at 50% PEPc activity. The different treatments are represented thus: control 8 am (◆); acetic acid 8 am (■); acetic acid midday (▲); acetic acid 3 pm (●); acetic acid 6 pm (◆).

(B) Autoradiograph showing the PEPc kinase activity in desalted extracts from different disk samples, expressed as a percentage of the highest activity (lane 9) disregarding the control samples (lanes 11 - 13):
Lane 1 - control 8 am
Lane 2 - " without PEPc
Lane 3 - acetic acid 8 am
Lane 4 - " without PEPc
Lane 5 - acetic acid midday
Lane 6 - " without PEPc
Lane 7 - acetic acid 3 pm
Lane 8 - " without PEPc
Lane 9 - acetic acid 6 pm
Lane 10 - " without PEPc
Lane 11 - mid-dark leaf
Lane 12 - " without PEPc
Lane 13 - kinase control (partially purified maize PEPc kinase)

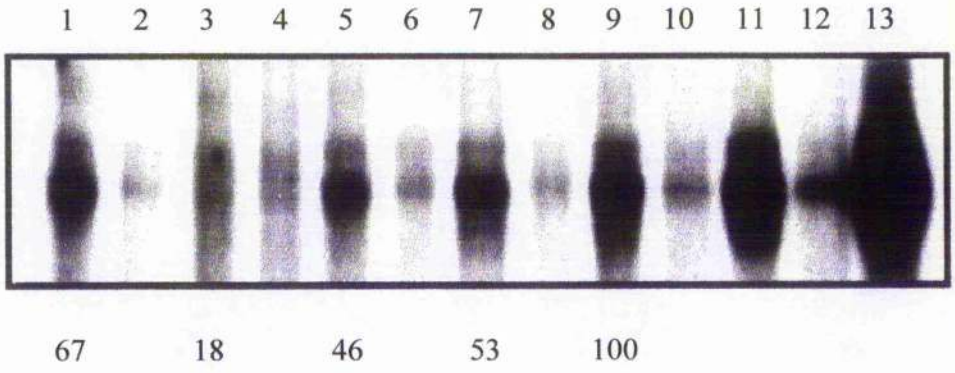
(C) Autoradiograph showing the PEPc kinase activity of *in vitro* translated mRNA from different disk samples, expressed as a percentage of the highest activity (lane 4) disregarding the control samples (lanes 6 - 8). The intensities of the bands have been corrected for efficiency of [^{35}S] methionine incorporation:
Lane 1 - control 8 am
Lane 2 - acetic acid 8 am
Lane 3 - acetic acid midday
Lane 4 - acetic acid 3 pm
Lane 5 - acetic acid 6 pm
Lane 6 - no RNA
Lane 7 - RNA from *K. fedtschenkoi* leaves in middle of night
Lane 8 - kinase control (partially purified maize PEPc kinase)

IA

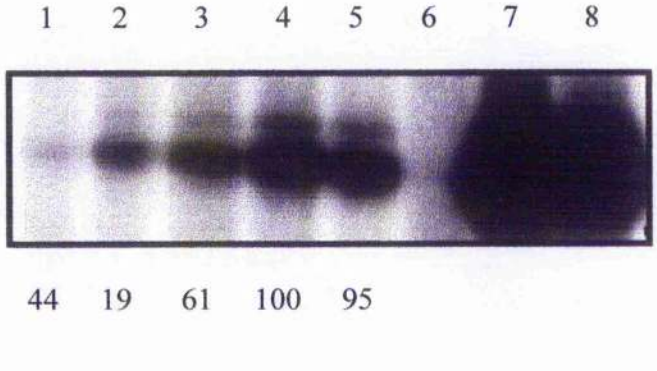
The effect of acetic acid treatment length on the malate sensitivity of PEPc from *K. fedtschenkoi* leaf disks



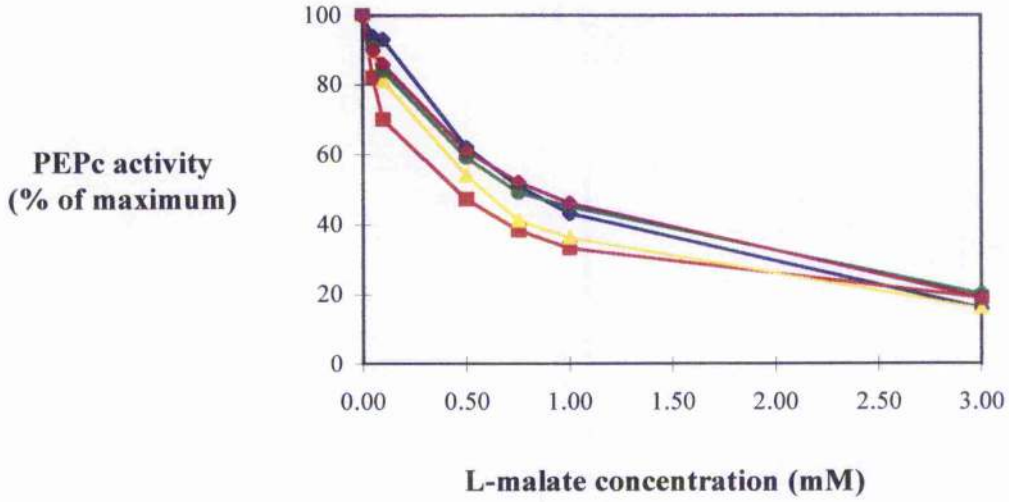
IB



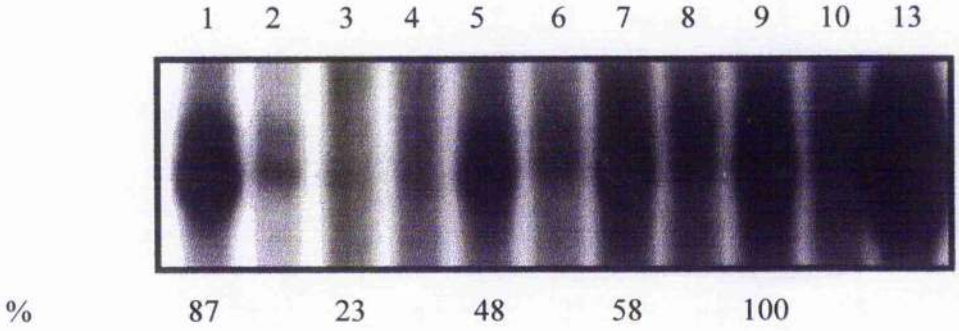
IC



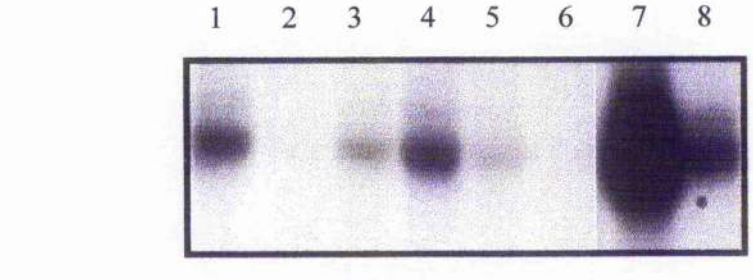
IIA **The effect of acetic acid treatment length on the malate sensitivity of PEPc from *K. fedtschenkoi* leaf disks**



IIB



IIC



kinase activity and the actual intensity of the band apparent from the phosphorimage (compare with Figure 3.5 IIC). The RNA isolation from the intact leaf at midnight (Figure 3.5 IC lane 7) was carried out by Dr. J. Hartwell using different plants which explains the apparently much greater level of mRNA. The level of PEPc kinase translatable mRNA was found to decrease with length of acetic acid treatment (Figure 3.5C). It can therefore be seen that 3 mM acetic acid affects the level of the PEPc kinase translatable RNA to decrease the kinase activity and the consequent sensitivity of PEPc to malate inhibition determined by the phosphorylation state of the PEPc protein. However, it would appear that disks have to be treated for at least 9-12 hours before the effect is noticeable i.e. disks transferred to acetic acid at 8 am are much more affected by the treatment than those transferred at 3 pm or later. In one experiment disks transferred at midday were similar to those transferred at 3 pm; in the other they resembled disks transferred at 8 am.

3.2.6 Cytosolic acidification prevents decarboxylation of intracellular malate

The total concentration of malate in the disks was also determined throughout the time course (see section 2.14). Figure 3.6 shows that just before the lights came on the malate concentration was high due to its accumulation in the cell vacuole throughout the night. By the end of the day malate levels in the control sample were very low as the malate had been decarboxylated by the activity of malic enzyme. Throughout the following night period the malate levels rose again. However, in the samples treated with acetic acid from 8 am the decline in malate by 4 pm was reduced and the subsequent rise in malate throughout the following dark period was blocked. With the later acetic acid treatments i.e. from midday, 3 pm or 6 pm, when malate had already fallen (nearly as far as the control), the treatment with acetic acid slightly reduced but did not prevent the subsequent rise in malate content.

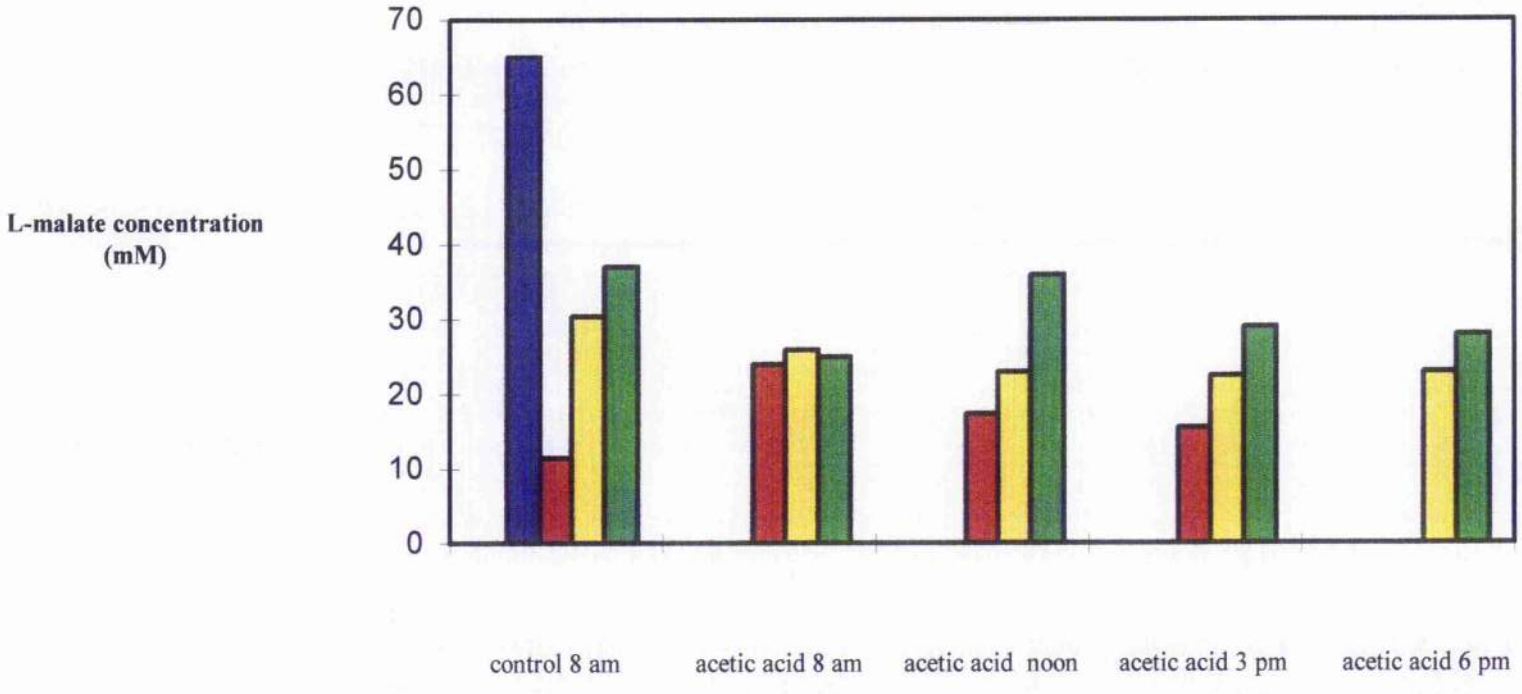
It appears that one effect of acetic acid is to reduce the decarboxylation during the day of the malate that accumulates in the cell vacuole throughout the night. This raised the question of whether treatment with acetic acid would affect the apparent K_i of PEPc for L-malate in *K. fedtschenkoi* disks if there was very little malate at the beginning of the time course. This was investigated by detaching leaves from plants at the beginning of the night and maintaining them in CO_2-free air throughout the night

Figure 3.6 The effect of acetic acid treatment length on the intracellular malate concentration of *K. fedtschenkoi* leaf disks

Leaf disks were cut from *K. fedtschenkoi* plants at the night/day transition. Disks were put into either water or 3 mM acetic acid. At 12 noon, 3 pm and 6 pm some disks were transferred from the control (water) incubation into 3 mM acetic acid. Samples were taken at 8 am (the beginning of the incubations), 4 pm, 12 midnight and 7 am the following morning and the tissue malate concentration determined.

The graph opposite shows the malate concentration at the different points of the time course thus: 8 am (■); 4 pm (■); 12 midnight (■); 7 am (■).

The effect of acetic acid treatment length on the malate concentration in *K. fedtschenkoi* leaf disks



(section 2.13). As only respiratory CO_2 is available for primary fixation by PEPc throughout the night there should be much less malate present in the leaves the following morning. Disks were then cut from these CO_2-deprived leaves and from control leaves the next morning and incubated throughout the day in water or 3 mM acetic acid. The malate content of the disks was determined at 8 am (i.e. at the end of the night incubation in CO_2-free air), 4 pm and 12 midnight. In the middle of the following night period, desalted extracts of the disks were also made and assayed for the malate sensitivity of PEPc. The CO_2-free air treatment reduced but did not completely eliminate malate accumulation during the night (Figure 3.7). By the end of the following day the malate levels in disks cut from these CO_2-deprived leaves and incubated in either water or 3 mM acetic acid had dropped further to a level similar to or just greater than the control disks and then rose, although by a lesser extent than the control disks, by the middle of the following night. Therefore, nocturnal treatment with the CO_2-free air seemed to eliminate the effect of acetic acid on intracellular malate levels. However, when the malate sensitivity of the PEPc in the disks was determined, it was seen that the CO_2-free air treatment did not alter the effect of the 3 mM acetic acid on the dark apparent K_i of PEPc for malate (Figure 3.8). Hence it appears that the disks can accumulate at least some malate even though PEPc remains dephosphorylated.

3.2.7 PEPc inhibitors fail to prevent dark CO_2 fixation

The PEPc inhibitors DCDP and DMDP were employed in an attempt to further reduce the levels of intracellular malate at the end of a night by using them in conjunction with CO_2-free air.

To test the effects of these compounds on PEPc itself, purified *K. fedtschenkoi* PEPc was diluted 30-fold in 50 mM Tris-HCl, pH 7.8 and 1 mM DTT and assayed in the presence of increasing concentrations of each inhibitor. From Figure 3.9 it can be seen that DCDP is over 10 times more potent than DMDP in the inhibition of PEPc, with apparent apparent K_i values of approximately 40 μM and 575 μM respectively at 2 mM PEP.

The inhibition of purified PEPc by DMDP in the presence of 0.2 mM PEP was also studied. The inhibition of PEPc by DMDP was approximately 10 times greater at the

Figure 3.7 The effect of CO_2-free air and 3 mM acetic acid on the intracellular malate concentration of *K. fedtschenkoi* leaf disks

Intact leaves were incubated in CO_2-free air throughout the night. Disks were cut from these leaves and from control leaves next morning and incubated in water or 3 mM acetic acid. Samples were taken at different time points and the tissue malate concentrations determined.

The graph opposite shows the malate concentration of disks sampled: 8 am (■); 4 pm (■) and 12 midnight (■).

The effect of CO_2-free air and 3 mM acetic acid on the malate content of *K. fedtschenkoi* disks

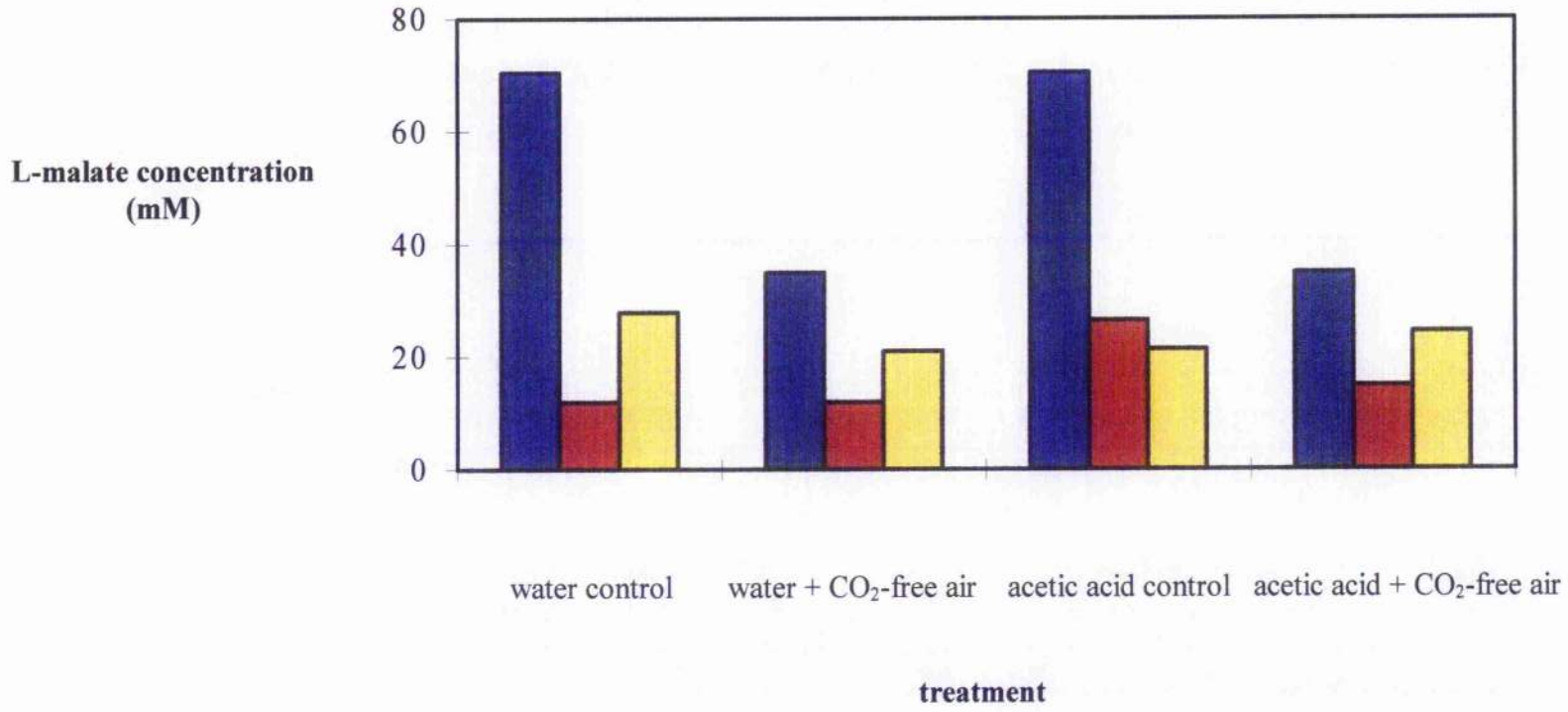


Figure 3.8 The effect of CO_2-free air and 3 mM acetic acid on the malate sensitivity of PEPc from *K. fedtschenkoi* leaf disks

Intact leaves were incubated in CO_2-free air throughout the night. Disks were cut from these leaves and control leaves next morning and incubated in water or 3 mM acetic acid until the middle of the following night period. Desalted extracts of the disks were made and assayed for the malate sensitivity of PEPc.

The graph below shows the sensitivity of PEPc to malate in the desalted extracts from the different disk treatments: water control (◆); water + CO_2-free (■); acetic acid control (▲); acetic acid + CO_2-free (●).

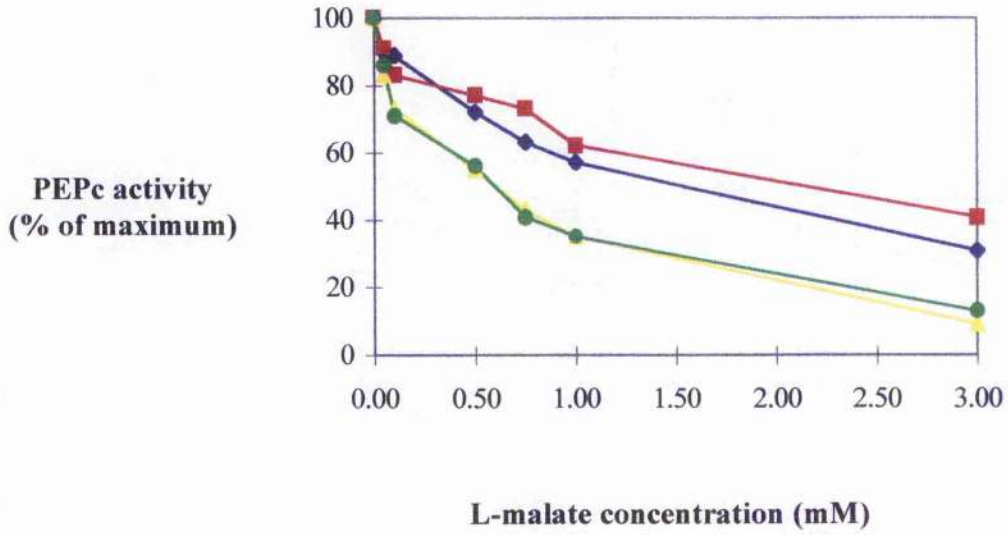
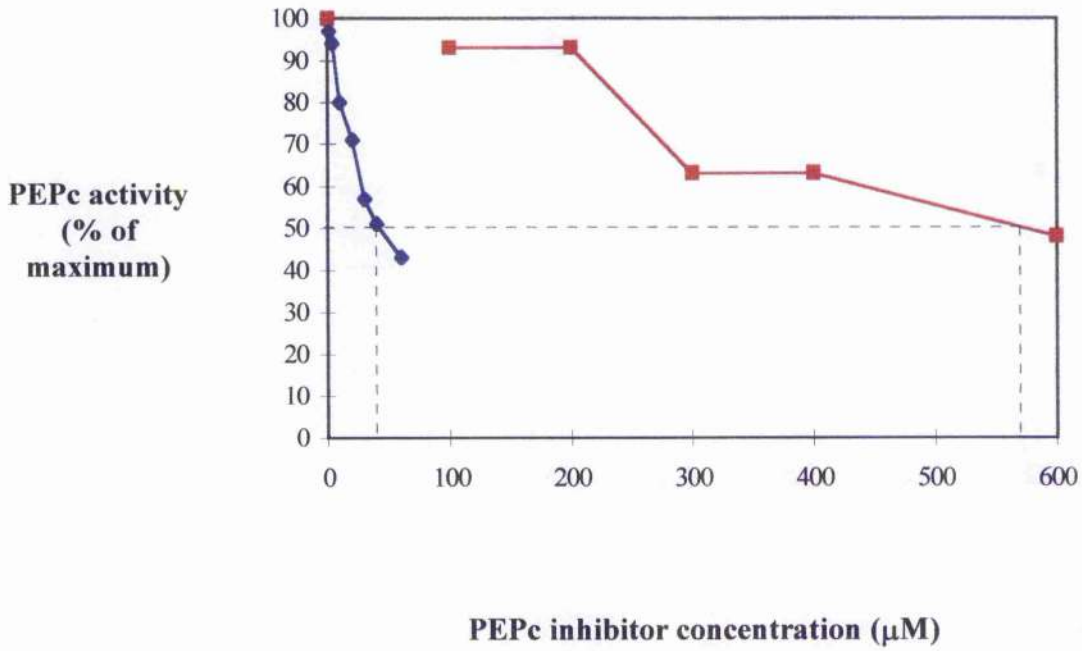


Figure 3.9 The effect of DCDP and DMDP on the activity of purified *K. fedtschenkoi* PEPc

Purified PEPc from *K. fedtschenkoi* was diluted 30-fold in 50 mM Tris-HCl, pH 7.8 and 1 mM DTT and assayed in the presence of different concentrations of the PEPc inhibitors DCDP and DMDP.

The graph below was used to determine the apparent K_i of PEPc for each of the inhibitors i.e. the inhibitor concentration at 50 % PEPc activity: DCDP (◆); DMDP (■).



lower substrate concentration of 0.2 mM giving an apparent K_i of 40 - 50 μM compared with 575 μM at 2 mM PEP (results not shown).

A desalted extract of *K. fedtschenkoi* leaves harvested in the middle of the day was also prepared and assayed for PEPc activity in the presence of increasing concentrations of each inhibitor. PEPc in the desalted extract had an apparent K_i for DCDP (40 - 50 μM) similar to that seen with purified PEPc and an apparent K_i of approximately 300 μM for DMDP, slightly lower than the value for purified PEPc (results not shown).

K. fedtschenkoi leaves were detached from plants at the end of a day period and allowed to take up either 2 mM DCDP or 10 mM DMDP in constant darkness, 15°C and a stream of CO_2-free air. CO_2 output by the leaves was monitored by infra red gas analysis (section 2.13). As shown by Wilkins (1959; 1962), control leaves show a persistent rhythm of CO_2 output that lasts for several days. This phenomenon has been attributed to oscillations in flux through PEPc. The troughs in output correspond to fixation of respired CO_2 by PEPc. The peaks in output correspond to escape of respired CO_2 from the leaf when PEPc is inactive.

The results (Figure 3.10) show that neither inhibitor reduced CO_2 fixation by the leaves, even though the leaves did take up the inhibitors. Hence it was not possible to prevent CO_2 assimmilation and malate accumulation by the use of these inhibitors.

3.2.8 The involvement of light in the effect of 3 mM acetic acid on the malate sensitivity of PEPc and malate content in *K. fedtschenkoi* disks

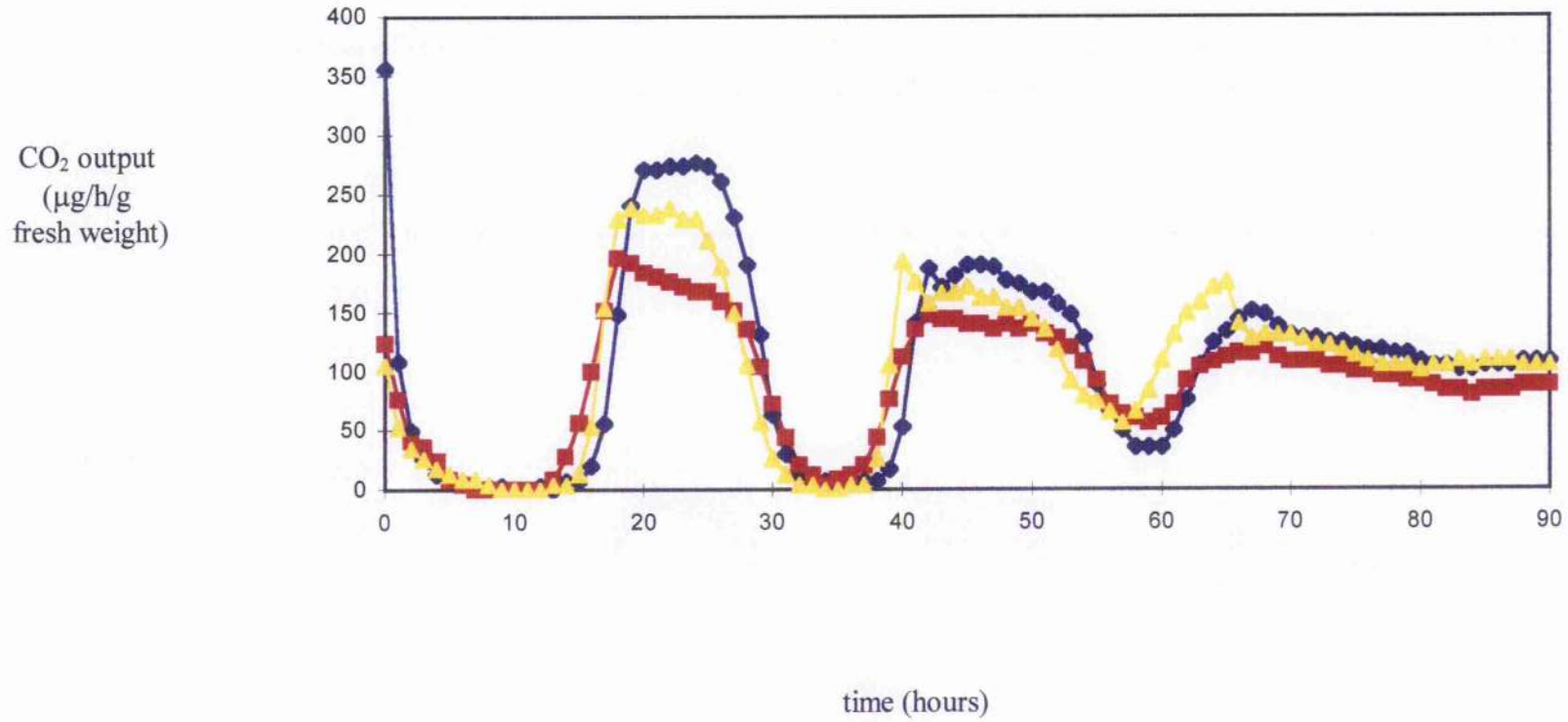
The present work has demonstrated that cytosolic acidification, in response to acetic acid treatment in *K. fedtschenkoi* disks, leads to a reduction in both malate content and the malate sensitivity of PEPc. This latter effect is a consequence of the reduction in the level of PEPc kinase translatable mRNA and PEPc kinase activity.

Light-induced cytosolic alkalinization has been shown to be involved in the light-induced change in PEPc activity observed in C_4 plants (Giglioli-Guivarc'h et al., 1996). Is it therefore possible that the effect of acetic acid observed thus far might be dampened by the counteraction of light-induced cytosolic alkalinization in the *K. fedtschenkoi* disks? To investigate this possibility, disks were cut from leaves just before the day began and shaken in water or 3 mM acetic acid in the normal

Figure 3.10 The effect of the PEPc inhibitors DCDP and DMDP on the circadian rhythm of CO_2 output in *K. fedtschenkoi* leaves maintained in CO_2-free air and constant darkness at 15°C

Detached *K. fedtschenkoi* leaves were placed in CO_2-free air and constant darkness at 15°C and the CO_2 output of the leaves was monitored using an infra-red gas analyser. The data from the experiment are shown in the graph opposite: 10 mM DMDP (◆); water (■); 2 mM DCDP (▲).

The effect of the PEPc inhibitors DCDP and DMDP on the circadian rhythm of CO_2 output in *K. fedtschenkoi* leaves maintained in CO_2-free air and constant darkness at 15°C



photoperiod either uncovered or in a light-tight box (continuous darkness and 28°C/15°C as normal growth regime, see section 3.2.1) until the middle of the following dark period. The malate content of the disks was determined at different points in the incubation and desalted extracts of the disks in the middle of the night were made and assayed for malate sensitivity of PEPc. From Figure 3.11 it can be seen that the disks incubated in water and continuous darkness gave a malate sensitivity greater than the light control and similar to the light/acetic acid-treated disks and the dark/acetic acid-treated disks.

The malate concentration of the disks at different points in the incubation is shown in Figure 3.12. The disks incubated under normal light conditions, and in water and acetic acid, behaved as previously. The malate concentration of disks incubated in continuous darkness in both water and acetic acid decreased between the start of the incubation at 8 am and 4 pm, although to a lesser extent than both light samples, and less in the dark acetic acid-treated disks than the dark control disks. In both dark samples the malate levels then continued to decrease until 12 midnight when they had reached a similar concentration. Incubation of disks in continuous darkness therefore seems to reduce the decarboxylation of malate even further than treatment with acetic acid alone.

3.3 DISCUSSION

Cytosolic pH affects the activity of both PEPc and PEPc kinase (Andreo et al., 1987; Rajagopalan et al., 1993; Wang and Chollet, 1993; Echevarria et al., 1994) and has been shown to be an important factor during the light activation of PEPc in mesophyll cells of C_4 plants (Devi and Raghavendra, 1992; Pierre et al., 1992; Raghavendra et al., 1993; Yin et al., 1993). *In situ* evidence for the involvement of cytosolic alkalinization in the light-activation of PEPc has also implicated increases in $[Ca^{2+}]$ which may modulate an upstream protein kinase (Pierre et al., 1992; Giglioli-Guivarc'h et al., 1996; Vidal et al., 1996). The pH optimum of PEPc is approximately pH 8 (O'Leary, 1982 and references therein) and so an increase in cytosolic pH could directly enhance PEPc activity or lead to an increase in PEPc kinase activity and the subsequent phosphorylation state of PEPc. As comparatively little was known about

Figure 3.11 The effect of light and 3 mM acetic acid on the malate sensitivity of PEPc in *K. fedtschenkoi* disks

Leaf disks were cut from *K. fedtschenkoi* plants at the beginning of the day period and put into either water or 3 mM acetic acid and then incubated in the normal photoperiod and left uncovered or put in a light-tight box (continuous darkness) until the middle of the following night. Desalted extracts of the disks were assayed for the malate sensitivity of PEPc.

The graph below shows the sensitivity of PEPc to malate in the desalted extracts from the different disk treatments: light/control (◆); light/acetic (■); dark/acetic (▲); dark/control (●).

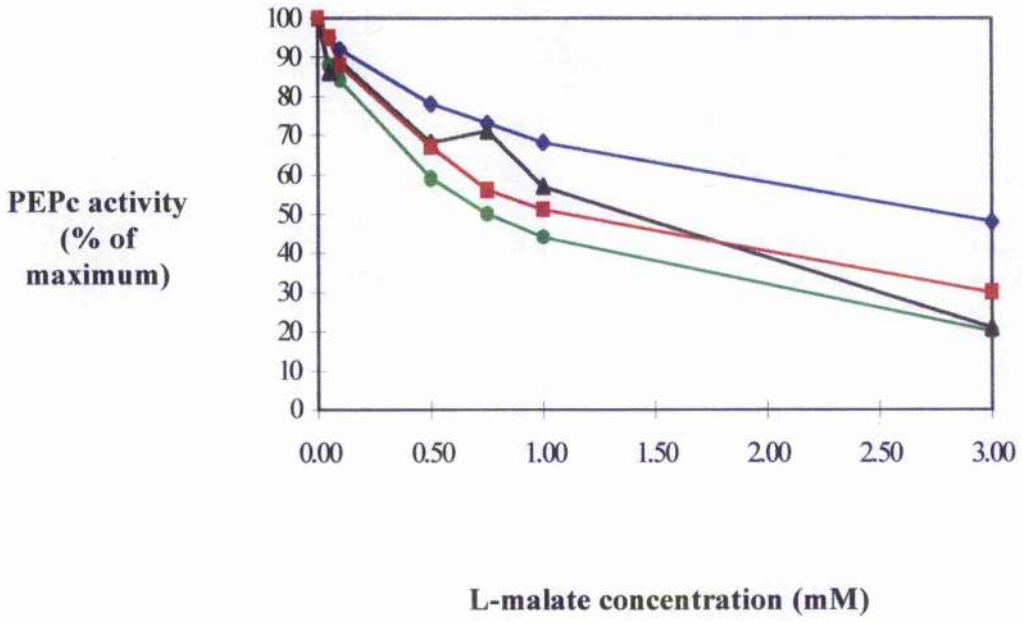
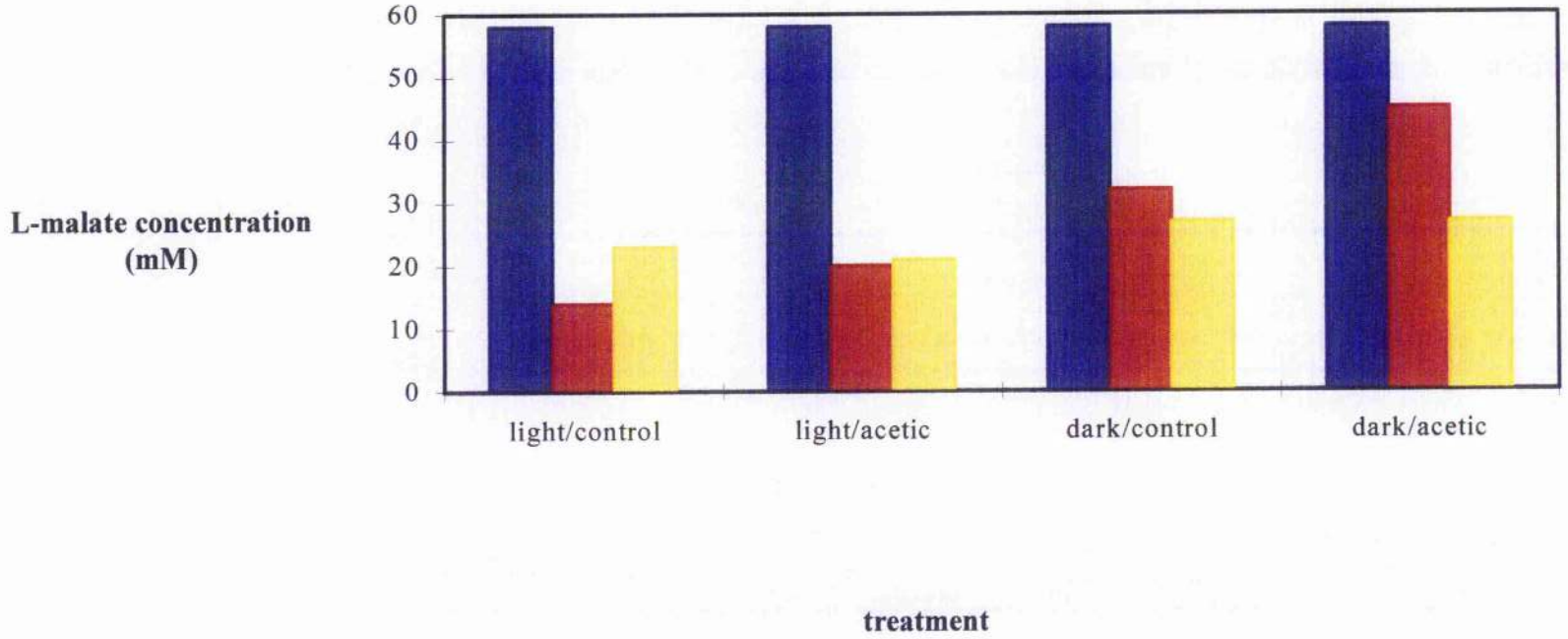


Figure 3.12 The effect of light and 3 mM acetic acid on the intracellular malate concentration of *K. fedtschenkoi* leaf disks

Leaf disks were cut from *K. fedtschenkoi* plants at the beginning of the day period and put into either water or 3 mM acetic acid. The disks were then incubated in the normal photoperiod and left uncovered or put in a light-tight box (continuous darkness) until the middle of the following night. Samples were taken at different time points and the malate concentration determined.

The graph opposite shows the malate concentration of disks sampled at 8 am (■); 4 pm (■) and 12 midnight ( ).

The effect of light and 3 mM acetic acid on the malate concentration in *K. fedtschenkoi* leaf disks



the involvement of pH in the regulation of PEPc in CAM species, attempts were made to manipulate the cytosolic pH of *K. fedtschenkoi* disks and the effects studied. Treatment of *K. fedtschenkoi* disks with NH_4Cl had no affect on the malate sensitivity of PEPc (section 3.2.1). Cytosolic alkalinization therefore does not appear to affect PEPc kinase in the CAM species as it has been reported to do in C_4 species (Pierre et al., 1992; Giglioli-Guivarc'h et al., 1996). In leaf protoplasts of the C_3 species barley, treatment with the weak base methylamine in darkness resulted in activation of PEPc (Lillo et al., 1995). Suprisingly, treatment with the acid DMO had a similar effect. When *K. fedtschenkoi* disks were incubated in 3 mM acetic acid, the nocturnal decrease in the malate sensitivity of PEPc was prevented (Figure 3.1A). A time course of the effect of acetic acid on the malate sensitivity of PEPc showed the effect to be slow, requiring at least 9-12 hours treatment (Figure 3.5A). This is in contrast to the activation of barley leaf protoplast PEPc by methylamine and DMO which was apparent after 40 minutes. Although the effect of acetic acid is too slow to be presumed significant in signal transduction affecting CAM PEPc, it caused a reduction in the level of translatable PEPc kinase mRNA (Figure 3.5C), a decrease in PEPc kinase activity (Figure 3.5B) and ultimately a reduced phosphorylation state of PEPc detectable as a low K_i of PEPc for L-malate (Figure 3.5A).

The comparison of treatment with acetic acid, propionic acid and HCl on the malate sensitivity of PEPc (Figure 3.4A) suggests that the effects of acetic and propionic acid are modulated by changes in intracellular pH as opposed to metabolic effects. Even though the H^+ concentration of the three acids is the same in the incubations no effect is seen with HCl. This can be explained by the pK_a values of the acids. Acetic acid and propionic acid have pK_a values of 4.75 and 4.87 respectively and so are both weak acids. This means that in their protonated forms the acid molecules are able to cross the plasma membrane lipid bilayer and dissociate in the cell cytosol, thus affecting intracellular pH. However, HCl is a strong acid and in its fully dissociated state is unable to cross the cell plasma membrane to affect intracellular pH. More direct evidence that the acetic acid effect is a result of pH changes could be provided by fluorescent dyes which could confirm the changes in cytosolic pH. However, this would require the use of protoplasts and the difficulty of obtaining protoplasts from *K. fedtschenkoi* has already been referred to in section 3.1.

Physiologically significant increases in PEPc activity (e.g. during the night in CAM plants) are thought to increase the cytosolic malate pool. The importance of phosphorylation of PEPc is to reduce the enzyme's sensitivity to inhibition by malate and allow CO_2 fixation in these conditions. However, it is known that malate inhibits PEPc kinase activity (Carter et al., 1991) and more recently (Borland et al., 1999), malate has been suggested to be a feedback inhibitor of PEPc kinase transcription. It was therefore of interest to measure the intracellular concentration of malate in *K. fedtschenkoi* disks treated with acetic acid.

The data suggested that acetic acid prevents the decarboxylation of malate (Figure 3.6). Disks put into 3 mM acetic acid at the end of a night period (i.e. 8 am) have a greater concentration of malate at the end of the day (4 pm) than the control disks incubated in water. The accumulation of malate in the subsequent dark period attributed to PEPc activity is prevented in the acid-treated disks. The malate sensitivity of PEPc in the middle of the dark period (12 midnight) increases with the length of acetic acid treatment i.e. the apparent K_i for malate decreases (Figure 3.5A). This seems to be due to a reduction in the expression of PEPc kinase as judged both by kinase activity (Figure 3.5B) and by kinase mRNA levels (Figure 3.5C). One possible explanation of these results is inhibition of PEPc kinase transcription by the high levels of malate present in the acid-treated disks at the end of the day. The PEPc would therefore not become phosphorylated during the dark period and would also be inhibited by the levels of malate present and unable to catalyze any further production of malate. The malate that accumulates at night during CAM is pumped into the cell vacuole. Presumably this doesn't occur in the acid-treated disks. It is conceivable that the cytosolic pH is so low that the leaves are unable to generate a big enough pH gradient across the tonoplast to pump malate; the low pH might also prevent the H^+ symport necessary for the activity of the vacuole H^+ATPase that pumps the malate into the vacuole. In addition, it seems that the activities of NAD-malic enzyme and/or NADP-malic enzyme are reduced when the cytosolic pH is low, thus inhibiting decarboxylation of malate. Further studies of these two enzymes would be valuable. This explanation implies that the failures to decarboxylate malate and induce PEPc kinase are related. However it is also possible that a decline in cytosolic pH reduces

expression of PEPc kinase irrespective of malate. It is not possible to distinguish between these at the present time.

Incubating leaves in CO_2-free air reduced the amount of malate accumulated throughout the night (Figure 3.7). The apparent mechanism by which acetic acid affects PEPc activity was thus made ineffective and the malate was able to accumulate during the following dark period to a similar extent as the control. This result nicely indicates that the effect of acetic acid is not a consequence of damage caused to the disks by the treatment. However, even with disks from leaves incubated in CO_2-free air, acetic acid prevented the nocturnal increase in the apparent K_i of PEPc for malate (Figure 3.8). One explanation of this result is that there is sufficient cytosolic malate left at the end of the day in these disks to inhibit PEPc kinase transcription (Borland et al., submitted) and thus the PEPc remains dephosphorylated and malate sensitive, but there is not enough malate to inhibit PEPc activity and therefore malate can be made in the following night (Figure 3.7). However, it seems suprising that PEPc could still function in the face of low pH and high cytosolic malate. In comparison acetic acid treatment alone results in higher levels of malate at the end of the day but the acid prevents re-uptake of the malate into the vacuole, and thereby blocks induction of PEPc kinase and phosphorylation of PEPc. Anderson and Wilkins (1989) reported the inhibition of the circadian rhythm of CO_2 fixation and a low malate content of *K. fedtschenkoi* leaves in response to high temperature (40°C) in continuous light. However, the leaves appeared to have a basal level of about 10 mM malate which apparently cannot be metabolized. It is possible that there is a threshold concentration of malate above which the transcription of PEPc kinase and PEPc activity will be inhibited. This could explain why in Figure 3.7 the malate level rises in the night following treatment of the *K.fedtschenkoi* disks with acetic acid and CO_2-free air but not after treatment with acetic acid alone. Likewise in Figure 3.6 malate accumulates when acetic acid treatment of disks begins at or after noon but not before. From close inspection of Figure 3.6 and 3.7 it would appear that the proposed threshold malate concentration is about 20 mM. A significant part of this is presumably not metabolizable perhaps due to compartmentation.

The reduction in malate accumulated overnight was not complete using CO_2-free air. However, even though the inhibition potential of DCDP and DMDP could be

demonstrated *in vitro* (Figure 3.9), neither PEPc inhibitor was able to reduce the fixation of respired CO_2 further than CO_2-free air alone (Figure 3.10). The incubation of leaves in N_2 might have been more effective in lowering the malate levels than CO_2-free air but the extent to which the leaves would be perturbed by this treatment eliminated this as an attractive experimental alternative.

Another possible factor in the effect of acetic acid treatment was light. The acid and base treatment of barley leaf protoplasts mentioned above (Lillo et al., 1995) had been given during darkness. From Figure 3.11 it would seem that the only condition that leaves PEPc phosphorylated in the middle of the night is the light control. With the other three treatments the malate level is a lot higher at 4 pm (Figure 3.12) so presumably the kinase is not induced thus accounting for the greater sensitivity to malate of PEPc from these disks. The failure of disks in continuous darkness to accumulate malate in the following "night" (Figure 3.12) is exactly as predicted from previous unpublished work (G.A. Nimmo and H.G. Nimmo, personal communication). When *K. fedtschenkoi* leaves were placed in continuous darkness as the end of a night, i.e. when malate is high, there is no rhythm in CO_2 fixation, malate does not decline and PEPc does not become phosphorylated in the following "night". A rhythm is only observed if the constant conditions (continuous darkness, 15°C, CO_2-free air) commence at the end of a day. This is presumably because malate is low under these conditions and the PEPc kinase can be induced. These findings all support the view that metabolites can over-ride circadian control. They are also consistent with the view that the circadian clock actually controls PEPc kinase via altering metabolites e.g. the primary target of circadian control could be vacuolar malate transport/permeability. However, the issue central to this discussion is the cytosolic malate concentration i.e. is there sufficient malate in the cytosol to inhibit PEPc kinase transcription and PEPc activity? It is difficult to measure cytosolic malate with any degree of accuracy because such a high proportion of total malate is in the vacuole. However, a rough estimate might be possible using ^{13}C nuclear magnetic resonance (vacuolar and cytosolic malate would give separate signals because of the pH difference) or non-aqueous fractionation to give physical separation of the various compartments.

In summary then, acetic acid reduced the dark apparent K_i of PEPc for malate in *K. fedtschenkoi* disks incubated in a normal 8 hour photoperiod and reduced the decarboxylation of malate during the day. However, the effect of acetic acid was sufficiently slow to suggest that cytosolic acidification is *not* involved in the CAM PEPc signal transduction pathway as such but instead has a less direct role to play in its regulation through preventing the decarboxylation of malate.

In consequence, while further experiments could have been done to ascertain that the acid treatments did indeed affect cytosolic pH, it was felt that these should not be attempted since the change in pH did not seem to be part of a physiologically important signalling process.

Chapter Four

CLONING AND SEQUENCING OF FIRST FULL-LENGTH PHOSPHOENOLPYRUVATE CARBOXYLASE cDNA FROM *ARABIDOPSIS THALIANA*

4.1 INTRODUCTION

For the purposes of investigating the regulation of PEPc in C_3 species it was decided that it would be useful to obtain a full-length cDNA of the enzyme from *Arabidopsis thaliana*. Cloning of the cDNAs for different PEPc isoforms that are known to exist is essential for investigation of the tissue specificity of their expression. It is also possible in principle to use a clone to overexpress a protein and facilitate studies of the enzyme. The purification of enough *A. thaliana* PEPc protein to investigate its regulation would be difficult using conventional sources of the enzyme. A cloning approach was therefore employed in preference to protein purification in the isolation of the C_3 isoform of PEPc. This chapter describes the results obtained. Data on the expression of PEPc using a probe resulting from the work described here will be presented in Chapter 5.

4.2 RESULTS

4.2.1 Cloning of a full-length putative PEPc from an *A. thaliana* cDNA library

The *A. thaliana* var. Columbia cDNA library to be screened for the PEPc gene was λPRL2, a library constructed in the vector λZipLox (see D'Alessio et al., 1992) and containing the plasmid pZL1. The cDNA was made by reverse transcription of an mRNA pool from four tissue types: i) roots ii) tissue culture iii) leaves and iv) germinating seedlings. The library was obtained from the Arabidopsis Biological Resource Center, Ohio State University. A search of the *A. thaliana* sequence database (http://genome-www.stanford-edu/Arabidopsis) using a *Z. mays* PEPc sequence found an EST (expressed sequence tag), 129G6T7, with good identity to the *Z. mays* PEPc sequence. The EST was a clone of the λPRL2 cDNA library and was therefore obtained for use as a probe to screen the library.

A pair of PEPc-specific primers were synthesized from a sequence given by Honda et al. (1996):

5' - GGCAAGCAGGAGGTCATGATCGG - 3'

5' - GAAGTACTCGACAAATCGAGGCTC - 3'

The region of the PEPc gene that these primers anneal to is shown diagrammatically in Figure 2.1 (section 2.9.7). Use of these primers had led to the amplification of a 0.5kb cDNA fragment from the RNA of *Aloe aborescens* leaves (a monocot CAM plant). The nucleotide sequence of this fragment showed a high degree of similarity to the conserved region of the ice plant, Sorghum and maize PEPc genes that the primer design was based upon (Cushman and Bohnert, 1989; Cretin et al., 1991; Izui et al., 1986). The *A. thaliana* PEPc EST 129G6T7 was approximately 1.6 kb and therefore because it was rather long to be used as a probe, the above primers were used in a PCR (section 2.9.5) with the cDNA (section 2.11.3) to amplify a 500 bp fragment of PEPc. The corresponding band was extracted from the gel using the QIAGEN gel extraction kit. The 500bp sequence from clone 129G6T7 was then radiolabelled using Amersham's Decaprime DNA labelling kit (section 2.8.2) and subsequently used to probe the λPRL2 library for PEPc (section 2.11.5).

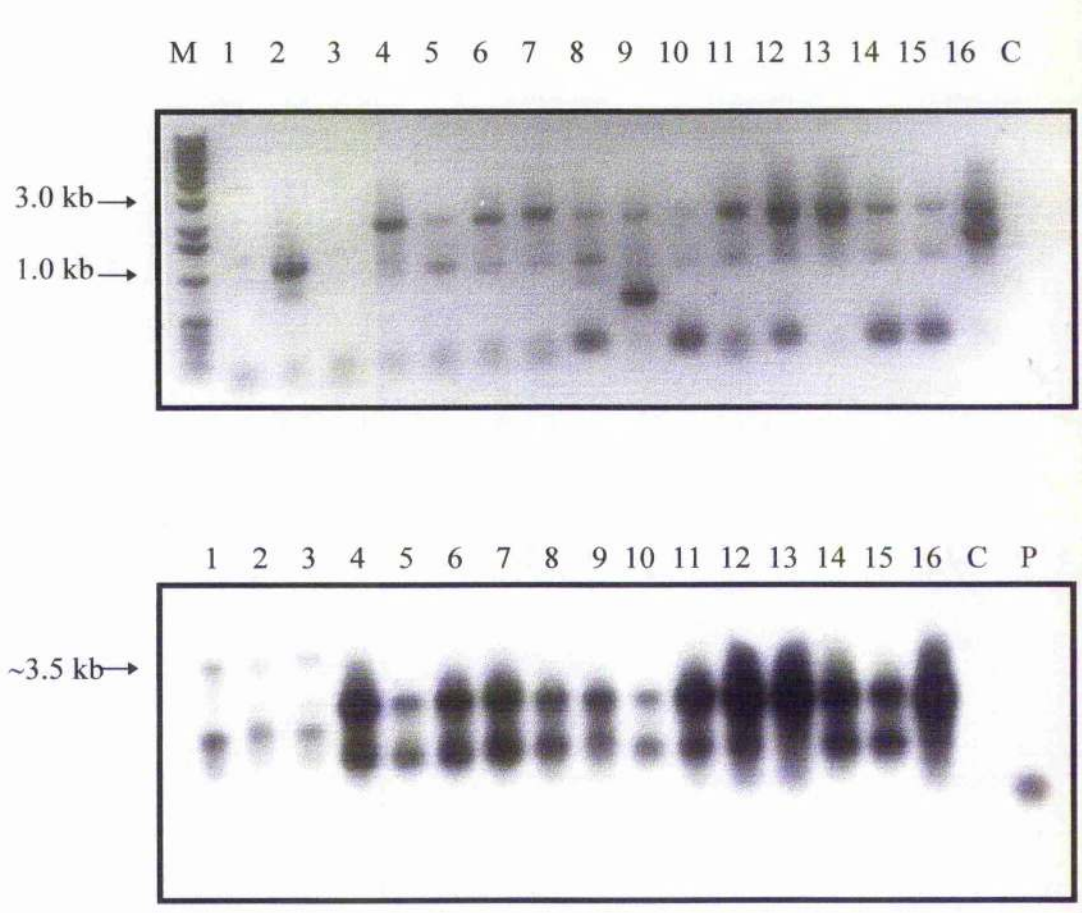
The λPRL2 library had a lower titre than specified (section 2.11.2). The full library (approximately 150,000 pfu) was therefore screened by nucleic acid hybridization analysis using the 500 bp ^{32}P-dCTP labelled PEPc probe (section 2.11.4 - 2.11.7). Plaques resulting from each round of screening were immobilized onto nitrocellulose membrane (section 2.8.1) and probed with the radiolabelled PEPc fragment. Autoradiographs (section 2.7.3) of the membranes allowed identification of phage plaques with putative PEPc cDNA inserts. These were cored out and the phage eluted into SM buffer. A PCR was then performed on these phage using the phage primers Sp6 and T7 and the PCR products run out on a 1% agarose gel in TAE buffer (section 2.7.4). The DNA in the agarose gel was then transferred by capillary action onto another nitrocellulose membrane and this was also probed with the radiolabelled PEPc fragment and autoradiographed. This combined PCR/Southern blotting approach facilitated the identification of phage containing the putative full-length PEPc cDNA inserts i.e. approximately 3 - 3.5 kb. These phage were then titred and plated out as described above and the screening procedure repeated until a cDNA of the desired size had been isolated from a single plaque. Figure 4.1 shows the PCR products of the third round screen run on an agarose gel and then blotted and probed with the radiolabelled 500 bp

Figure 4.1 Hybridization of radiolabelled PEPc cDNA to PCR products from λPRL2 cDNA library screen

A combined PCR/Southern hybridization analysis of putative positives from each round of the library screening facilitated the identification of phage containing putative full-length PEPc cDNA inserts i.e. approximately 3-3.5 kb.

(A) A photograph of the PCR products amplified from third round screen putative positives using the phage primers T7 and Sp6 and separated on a 1% agarose gel. The ethidium bromide-stained DNA fragments are visualised under UV light. The lanes are as follows:
M - 1 kb DNA ladder
Lanes 1-16 - third round screen putative positives 3.1-3.16 labelled 1-16
C - water control

(B) Autoradiograph of Southern hybridization of PCR products as shown in (A) with radiolabelled PEPc cDNA. The lanes are as follows:
Lanes 1-16 - third round screen putative positives 3.1-3.16 labelled 1-16
C - water control
P - 500 bp PEPc control



PEPc probe. In lanes 1 - 3 of Figure 4.1A (PEPc clones 3.1 - 3.3 respectively) a faint band of approximately 3 - 4 kb can be seen. The radiolabelled PEPc fragment then appeared to hybridize to these bands suggesting that they were PEPc fragments of the desired size (Figure 4.1B lanes 1 - 3). The radiolabelled PEPc probe hybridized to two bands in lanes 1 - 16 of Figure 4.1B. This indicates that the plaques from the third round of screening contain more than one colony of phage. After a further round of PCR/Southern blotting, a PEPc cDNA insert of approximately 3.5 kb was obtained in a plaque purified form (Figure 4.2 lane 2). An *in vivo* excision of the recombinant plasmid pKP42 (pZL1 containing PEPc clone 4.2) from the bacteriophage vector was then performed using *E.coli* strain DH10B and selection on NZYCM plates containing 100μg/ml ampicillin and 10mM $MgCl_2$. 5ml LB broth containing the antibiotic and magnesium were then inoculated with one of the colonies and grown to an approximate density of A_{600}= 0.5. A small scale plasmid preparation was performed on the culture using the QIAGEN mini-prep kit and some of the purified plasmid checked by agarose gel electrophoresis and a restriction digest (section 2.9.4) using *SalI* to linearize the recombinant plasmid (Figure 4.3). The restriction digest produced an approximately 8kb fragment on an agarose gel consistent with an approximately 3.5kb cDNA insert and a 4.08kb pZL1 plasmid.

4.2.2 Sequencing of the putative full length PEPC cDNA

Some of the recombinant plasmid pKP42 was denatured and then the single stranded DNA was sequenced (section 2.9.6) using the T7 Sequenase version 2.0 kit and the bacteriophage primers T7 and Sp6 (section 2.9.6). This allowed the sequencing of approximately 250 bases from both the 5'- and 3'- ends of the PEPc cDNA. The nucleotide sequence was read from the autoradiograph of the sequencing gel and translated in each of the three reading frames. A BLAST search was then performed on the DNA sequence databases available via the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). The search showed that the 5' end of the clone was very similar in sequence to PEPc, contained a possible start codon and the putative PEPc phosphorylation motif which is close to the N-terminal end of the protein. The 3' end contained a polyA tail but no similarity to other PEPc sequences. The size of the clone suggested it might contain a significant 3' untranslated region and

Figure 4.2 Purified phage containing putative full-length PEPc cDNA inserts

Four rounds of combined PCR/Southern hybridization analysis of putative positives from each round of the library screening resulted in the isolation of three purified phage containing putative full-length PEPc cDNA inserts. Below is an autoradiograph of the Southern hybridization of radiolabelled PEPc cDNA with PCR products amplified from fourth round screen putative positives using the phage primers T7 and Sp6. The lanes are as follows:

Lane 1-4 - fourth round screen putative positives 4.1 - 4.4 labelled 1- 4

C - water control

P - 500 bp PEPc control

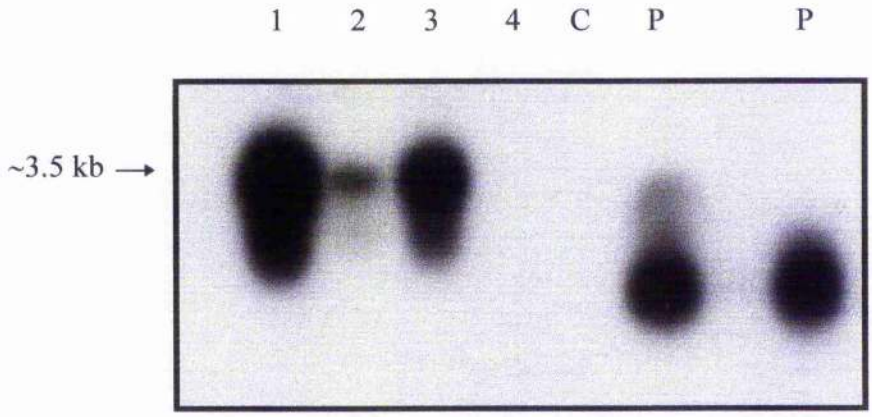
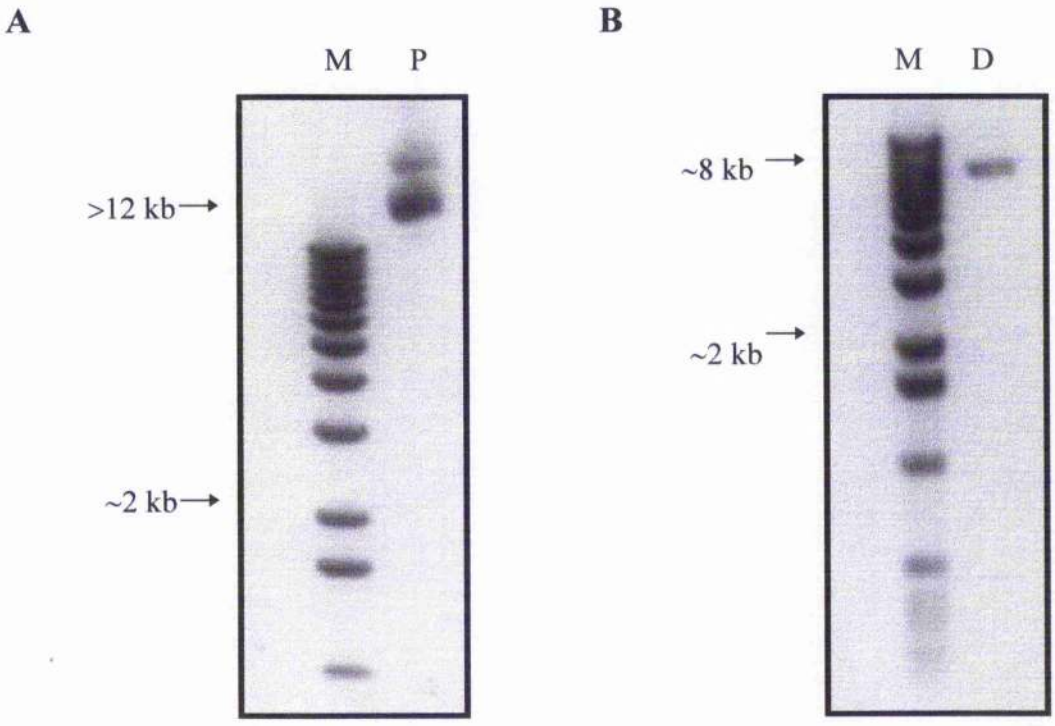


Figure 4.3 *Sal* I digest of purified recombinant plasmid pKP42

An *in vivo* excision of the recombinant plasmid pKP42 containing PEPc cDNA clone 4.2 was performed and the plasmid purified. The integrity of the plasmid was checked by agarose gel electrophoresis and the plasmid then linearized by *Sal* I to size the putative full-length PEPc cDNA insert.

(A) Photograph of undigested pKP42 separated on a 1% agarose gel. The ethidium bromide stained DNA fragments were visualised under UV light. The lanes are as follows:
M - 1 kb DNA ladder
P - undigested pKP42

(B) Photograph of *Sal* I digested pKP42 separated on a 1% agarose gel. The ethidium stained DNA fragments were visualised under UV light. The lanes are as follows:
M - 1 kb DNA ladder
D - *Sal* I digested pKP42



so it was possible that the 3' sequence read had not reached the PEPc coding region. The presence of a possible start codon and a polyA tail are indicative of a full length functional clone and so sequencing was continued.

As the clone to be sequenced was greater than 3 kb the remainder of the sequencing was carried out in collaboration with the Molecular Biology Sequencing Unit (MBSU) using an automated sequencer. Oligonucleotides primers internal to the 5' and 3' sequences were designed and synthesized and used to obtain further sequence. This procedure was repeated until both the sense and anti-sense strands of the cDNA had been completely sequenced twice.

Multiple alignments of all sequences were performed using GeneJockey II and finally used to produce a sequence contig of the full clone. When analysed the contig had an open reading frame (ORF) of 968 amino acid residues in frame +2. The multiple cloning site sequence was identified and this revealed the beginning and end of the 5' and 3' untranslated regions respectively. The interleaved format of the nucleotide and amino acid sequence for the PEPc clone from the λPRL2 *A. thaliana* cDNA library is shown in Figure 4.4.

4.2.3 Sequence analysis of full-length *A. thaliana* PEPc cDNA

The PEPc clone has an open reading frame of 968 amino acid residues (M_r 124, 890 and an isoelectric point of 7.31) beginning at base number 116 of the full length sequence including 5' untranslated sequence and finishing at base number 3022. There are two ATG triplets in the PEPc encoding ORF that are candidates for translation initiation (Figure 4.4 nucleotide nos. 116 and 140). Two possible start codons have been found in the genomic and cDNA sequences of other plant PEPcs (Relle and Wild, 1996), many of which initiated translation from the first ATG. However, until the protein encoded by the *A. thaliana* PEPc is sequenced it is not possible to say with any certainty which site initiates translation. A sequence corresponding to the typical polyadenylation signal, ATAA, described for higher plants (Joshi, 1987) and reported in other PEPc cDNA clones (e.g. Honda et al., 1996), is found at nucleotide number 3107, 142 bp upstream of the poly A sequence. The conserved PEPc phosphorylation motif SIDAQ is highlighted in Figure 4.4, indicating serine[11] as the phosphorylated residue. The *A. thaliana* PEPc cDNA sequence was aligned with other known PEPc sequences using the Genejockey II

Figure 4.4 Interleaved nucleotide and amino acid sequence of a full-length PEPc cDNA from *A. thaliana*

The numbers down the left hand side of the sequence correspond to nucleotide number. The open reading frame starts at nucleotide number 116 and ends at nucleotide number 3022. The shaded box is the PEPc phosphorylation motif conserved among all known PEPc sequences to date. ● is the stop codon.

```
   1                                  CCTGCAGGTACCGGTCCGGAATTCCCGGGTCGACCCACGCGTCCGAAT
  49 CCGCAGAGATTTCTTCTTTTCAGAAGAAGTAAGAGGGTGGCGAAGAAGATTTGATTGATCGGCGATA
     Met Ala Gly Arg Asn Ile Glu Lys Met Ala Ser Ile Asp Ala Gln Leu Arg
 116 ATG GCG GGT CGG AAC ATA GAG AAG ATG GCA TCT ATT GAT GCT CAG CTT CGG
     Gln Leu Val Pro Ala Lys Val Ser Glu Asp Asp Lys Leu Val Glu Tyr Asp
 167 CAA CTC GTT CCT GCT AAA GTC AGT GAA GAC GAT AAG CTT GTT GAG TAC GAT
      Ala Leu Leu Leu Asp Arg Phe Leu Asp Ile Leu Gln Asp Leu His Gly Glu
 218 GCT CTT CTC CTT GAT CGC TTT CTC GAC ATT CTC CAG GAT TTA CAC GGC GAG
     Asp Leu Arg Glu Thr Val Gln Glu Leu Tyr Glu Leu Ser Ala Glu Tyr Glu
 269 GAT CTC CGT GAA ACG GTT CAA GAG TTA TAC GAG CTT TCT GCT GAC TAT GAA
     Gly Lys Arg Glu Pro Ser Lys Leu Glu Glu Leu Gly Ser Val Leu Thr Ser
 320 GGG AAG CGT GAG CCT AGC AAG CTT GAG GAG CTA GGG AGT GTC CTA ACG ACT
     Leu Asp Pro Gly Asp Ser Ile Val Ile Ser Lys Ala Phe Ser His Met Leu
 371 TTG GAT CCT GGT GAC TCA ATT GTT ATC TCC AAG GCT TTC TCT CAC ATG CTT
     Asn Leu Ala Asn Leu Ala Glu Glu Val Gln Ile Ala His Arg Arg Arg Ile
 422 AAC TTA GCC AAT TTG GCT GAG GAG GTG CAG ATT GCT CAC CGT CGC AGG ATC
     Lys Lys Leu Lys Lys Gly Asp Phe Val Asp Glu Ser Ser Ala Thr Thr Glu
 473 AAG AAG CTG AAG AAA GGT GAT TTC GTT GAT GAG AGT TCT GCA ACT ACT GAA
     Ser Asp Ile Glu Glu Thr Phe Lys Arg Leu Val Ser Asp Leu Gly Lys Ser
 524 TCC GAT ATT GAA GAG ACT TTT AAG AGG CTC GTT TCG GAT CTT GGT AAG TCT
     Pro Glu Glu Ile Phe Asp Ala Leu Lys Asn Gln Thr Val Asp Leu Val Leu
 575 CCT GAA GAG ATC TTT GAT GCC TTG AAG AAT CAG ACT GTG GAT CTG GTT TTG
     Thr Ala His Pro Thr Gln Ser Val Arg Arg Ser Leu Leu Gln Lys His Gly
 626 ACT GCT CAT CCT ACT CAG TCT GTG CGT AGA TCA TTG CTT CAG AAG CAT GGG
     Arg Ile Arg Asp Cys Leu Ala Gln Leu Tyr Ala Lys Asp Ile Thr Pro Asp
 677 AGG ATA AGG GAC TGT CTT GCT CAA CTC TAT GCA AAG GAC ATT ACT CCT GAT
     Asp Lys Gln Glu Leu Asp Glu Ser Leu Gln Arg Glu Ile Gln Ala Ala Phe
 728 GAC AAG CAG GAG CTA GAT GAG TCT CTG CAA AGA GAG ATT CAA GCT GCA TTC
     Arg Thr Asp Glu Ile Arg Arg Thr Pro Pro Thr Pro Gln Asp Glu Met Arg
 779 CGA ACA GAT GAG ATT AGA AGA ACA CCT CCA ACC CCA CAA GAT GAA ATG AGA
     Ala Gly Met Ser Tyr Phe His Glu Thr Ile Trp Lys Gly Val Pro Lys Phe
 830 GCT GGA ATG AGT TAT TTC CAC GAG ACA ATC TGG AAA GGT GTC CCC AAG TTC
     Leu Arg Arg Val Asp Thr Ala Leu Lys Asn Ile Gly Ile Asp Glu Arg Val
 881 TTG CGC CGT GTG GAC ACA GCT CTG AAA AAC ATT GGG ATT GAT GAA CGT GTT
     Pro Tyr Asn Ala Pro Leu Ile Gln Phe Ser Ser Trp Met Gly Gly Asp Arg
 932 CCT TAC AAT GCC CCA TTG ATT CAA TTC TCT TCG TGG ATG GGC GGT GAT CGT
     Asp Gly Asn Pro Arg Val Thr Pro Glu Val Thr Arg Asp Val Cys Leu Leu
 983 GAT GGT AAT CCG AGG GTC ACA CCT GAG GTC ACT AGA GAT GTG TGC TTG TTG
     Ala Arg Met Met Ala Ala Asn Leu Tyr Tyr Asn Gln Ile Glu Asn Leu Met
1034 GCT AGA ATG ATG GCT GCC AAT CTC TAC TAT AAC CAA ATC GAG AAT CTG ATG
     Phe Glu Leu Ser Met Trp Arg Cys Thr Asp Glu Phe Arg Val Arg Ala Asp
1085 TTT GAG TTA TCT ATG TGG CGT TGC ACT GAT GAA TTC CGT GTG CGG GCG GAT
     Glu Leu His Arg Asn Ser Arg Lys Asp Ala Ala Lys His Tyr Ile Glu Phe
1136 GAA CTG CAC AGG AAC TCA AGG AAA GAT GCT GCA AAA CAT TAC ATA GAA TTC
     Trp Lys Thr Ile Pro Pro Thr Glu Pro Tyr Arg Val Ile Leu Gly Asp Val
1187 TGG AAG ACA ATT CCT CCA ACT GAG CCA TAC CGT GTG ATT CTT GGT GAT GTG
     Arg Asp Lys Leu Tyr His Thr Arg Glu Arg Ser Arg Gln Leu Leu Ser Asn
1238 AGG GAT AAG CTG TAT CAC ACA CGT GAG CGT TCC CGC CAA TTG CTG AGT AAT
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     Gly Ile Ser Asp Ile Pro Glu Glu Ala Thr Phe Thr Asn Val Glu Gln Phe
1289 GGA ATC TCG GAT ATT CCT GAA GAA GCT ACC TTC ACT AAT GTG GAA CAG TTC
     Leu Glu Pro Leu Glu Leu Cys Tyr Arg Ser Leu Cys Ser Cys Gly Asp Ser
1340 TTG GAG CCT CTT GAG CTC TGT TAC CGA TCA CTA TGT TCA TGT GGT GAC AGC
     Pro Ile Ala Asp Gly Ser Leu Leu Asp Phe Leu Arg Gln Val Ser Thr Phe
1391 CCG ATA GCT GAT GGA AGC CTT CTT GAT TTC TTG AGG CAA GTC TCT ACC TTT
     Gly Leu Ser Leu Val Arg Leu Asp Ile Arg Gln Glu Ser Glu Arg His Thr
1442 GGA CTC TCC CTT GTG AGA CTT GAC ATC AGG CAA GAG TCT GAA CGC CAC ACA
     Asp Val Leu Asp Ala Ile Thr Lys His Leu Asp Ile Gly Ser Ser Tyr Arg
1493 GAT GTC TTG GAT GCT ATC ACC AAG CAC TTG GAC ATC GGT TCC TCC TAT AGA
     Asp Trp Ser Glu Glu Gly Arg Gln Glu Trp Leu Leu Ala Glu Leu Ser Gly
1544 GAC TGG TCT GAA GAA GGC CGA CAG GAA TGG CTT CTT GCT GAA CTA AGC GGC
     Lys Arg Pro Leu Phe Gly Pro Asp Leu Pro Lys Thr Glu Glu Ile Ser Asp
1595 AAA CGT CCA CTT TTC GGA CCT GAT CTT CCC AAA ACC GAA GAA ATT TCT GAT
     Val Leu Asp Thr Phe Lys Val Ile Ser Glu Leu Pro Ser Asp Cys Phe Gly
1646 GTC CTG GAC ACA TTC AAA GTC ATA TCT GAG CTG CCT TCA GAT TGT TTT GGA
     Ala Tyr Ile Ile Ser Met Ala Thr Ser Pro Ser Asp Val Leu Ala Val Glu
1697 GCT TAT ATT ATC TCT ATG GCA ACT TCA CCT AGT GAT GTG CTT GCG CTT GAG
     Leu Leu Gln Arg Glu Cys His Val Lys Asn Pro Leu Arg Val Val Pro Leu
1748 CTT TTA CAG CGC GAA TGC CAT GTG AAA AAT CCA CTT AGA GTT GTT CCA CTC
     Phe Glu Lys Leu Ala Asp Leu Glu Ala Ala Pro Ala Ala Val Ala Arg Leu
1799 TTT GAG AAG CTA GCT GAT CTT GAA GCA GCT CCT GCC GCT GTT GCA AGA CTC
     Phe Ser Ile Asp Trp Tyr Lys Asn Arg Ile Asn Gly Lys Gln Glu Val Met
1850 TTT TCT ATA GAC TGG TAC AAA AAC CGT ATT AAC GGT AAA CAA GAG GTT ATG
     Ile Gly Tyr Ser Asp Ser Gly Lys Asp Ala Gly Arg Leu Ser Ala Ala Trp
1901 ATT GGT TAC TCA GAT TCA GGC AAA GAT GCA GGG CGT CTC TCA GCT GCT TGG
     Glu Leu Tyr Lys Ala Gln Glu Glu Leu Val Lys Val Ala Lys Lys Tyr Gly
1952 GAG CTA TAC AAA GCT CAA GAA GAG CTT GTG AAG GTT GCT AAG AAA TAT GGA
     Val Lys Leu Thr Met Phe His Gly Arg Gly Gly Thr Val Gly Arg Gly Gly
2003 GTG AAG CTA ACT ATG TTC CAT GGC CGT CGT GGC ACA GTC GGA AGA GGA GGT
     Gly Pro Thr His Leu Ala Ile Leu Ser Gln Pro Pro Asp Thr Val Asn Gly
2054 GGT CCT ACT CAT CTT GCT ATA TTG TCT CAG CCA CCA GAT ACA GTT AAT GGC
     Ser Leu Arg Val Thr Val Gln Gly Glu Val Ile Glu Gln Ser Phe Gly Glu
2105 TCT CTT CGA GTC ACG GTT CAG GGT GAA GTC ATT GAG CAA TCA TTT GGG GAG
     Ala His Leu Cys Phe Arg Thr Leu Gln Arg Phe Thr Ala Ala Thr Leu Glu
2156 GCA CAC TTA TGC TTT AGA ACA CTT CAA CGT TTC ACA GCA GCT ACT CTA GAG
     His Gly Met Asn Pro Pro Ile Ser Pro Lys Pro Glu Trp Arg Ala Leu Leu
2207 CAC GGA ATG AAC CCT CCG ATT TCA CCA AAA CCC GAG TGG CGT GCT TTG CTT
     Asp Glu Met Ala Val Val Ala Thr Glu Glu Tyr Arg Ser Val Val Phe Gln
2258 GAT GAA ATG GCG GTT GTT GCA ACT GAG GAA TAC CGA TCT GTC GTT TTC CAA
     Glu Pro Arg Phe Val Glu Tyr Phe Arg Leu Ala Thr Pro Glu Leu Glu Tyr
2309 GAA CCT CGA TTC GTC GAG TAT TTC CGC CTC GCT ACT CCG GAG CTG GAG TAT
     Gly Arg Met Asn Ile Gly Ser Arg Pro Ser Lys Arg Lys Pro Ser Gly Gly
2360 GGA CGT ATG AAT ATT GGA AGT AGA CCT TCA AAG CGA AAA CCA AGC GGT GGG
     Ile Glu Ser Leu Arg Ala Ile Pro Trp Ile Phe Ala Trp Thr Gln Thr Arg
2411 ATC GAA TCT CTC CGT GCA ATC CCA TGG ATC TTT GCT TGG ACG CAA ACA AGA
     Phe His Leu Pro Val Trp Leu Gly Phe Gly Ala Ala Phe Arg Tyr Ala Ile
2462 TTC CAT CTT CCT GTA TGG TTA GGT TTC GGA GCA GCA TTT AGG TAT GCG ATC
     Lys Lys Asp Val Arg Asn Leu His Met Leu Gln Asp Met Tyr Lys Gln Trp
2513 AAG AAG GAT GTG AGA AAC CTT CAC ATG CTG CAA GAT ATG TAT AAA CAA TGG
     Pro Phe Phe Arg Val Thr Ile Asp Leu Ile Glu Met Val Phe Ala Lys Gly
2564 CCC TTT TTC CGA GTC ACC ATC GAT CTA ATT GAA ATG GTG TTC GCC AAG GGA
     Asp Pro Gly Ile Ala Ala Leu Tyr Asp Lys Leu Leu Val Ser Glu Asp Leu
2615 GAC CCC GGG ATC GCT GCT TTG TAC GAC AAA CTT CTT GTC TCA GAA GAT TTA
     Trp Ala Phe Gly Glu Lys Leu Arg Ala Asn Phe Asp Glu Thr Lys Asn Leu
2666 TGG GCT TTT GGA GAG AAA CTC AGA GCC AAC TTT GAT GAA ACC AAG AAC CTC
     Val Leu Gln Thr Ala Gly His Lys Asp Leu Leu Glu Gly Asp Pro Tyr Leu
2717 GTC CTC CAG ACT GCT GGA CAT AAA GAC CTT CTT GAA GGA GAT CCT TAC TTG
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     Lys Gln Arg Leu Arg Leu Arg Asp Ser Tyr Ile Thr Thr Leu Asn Val Cys
2768 AAA CAG AGA CTA AGG CTA CGT GAC TCT TAC ATT ACG ACC CTC AAC GTT TGC
     Gln Ala Tyr Thr Leu Lys Arg Ile Arg Asp Ala Asn Tyr Asn Val Thr Leu
2819 CAA GCC TAC ACA TTG AAG AGG ATC CGT GAT GCA AAC TAC AAT GTG ACT CTG
     Arg Pro His Ile Ser Lys Glu Ile Met Gln Ser Ser Lys Ser Ala Gln Glu
2870 CGA CCA CAC ATT TCT AAA GAG ATC ATG CAA TCA AGC AAA TCA GCA CAA GAG
     Leu Val Lys Leu Asn Pro Thr Ser Glu Tyr Ala Pro Gly Leu Glu Asp Thr
2921 CTC GTC AAG CTT AAC CCC ACG AGT GAA TAC GCG CCT GGA CTT GAG GAC ACA
     Leu Ile Leu Thr Met Lys Gly Ile Ala Ala Gly Leu Gln Asn Thr Gly  ●
2972 CTT ATC TTA ACC ATG AAG GGT ATT GCT GCA GGA TTG CAA AAC ACC GGT TAA
3023 GTGAGTCAGTGAAAGAAAACAAAACTTCGAATCTCTCTTTTTTATCTACCCTTTTTAATAATCTCTT
3090 TITTTCTAGAATCCAAAATAATTACGGTTGGATTACAGTTTACTTTATGTATCCACCGTTGAAATCT
3157 TAATCTTCCATTGTATCAAACGTCACTGACTCTGTTTTCTGGAAGTGTAAACAAGAACAGAGACAGTG
3224 AATCTTAATGTTATCTTCTTTGTCTAAAAAAAAAAAAAAAAAAGGCGGCCGCTCTAGAGGATCCAAG
3291 CTTAC
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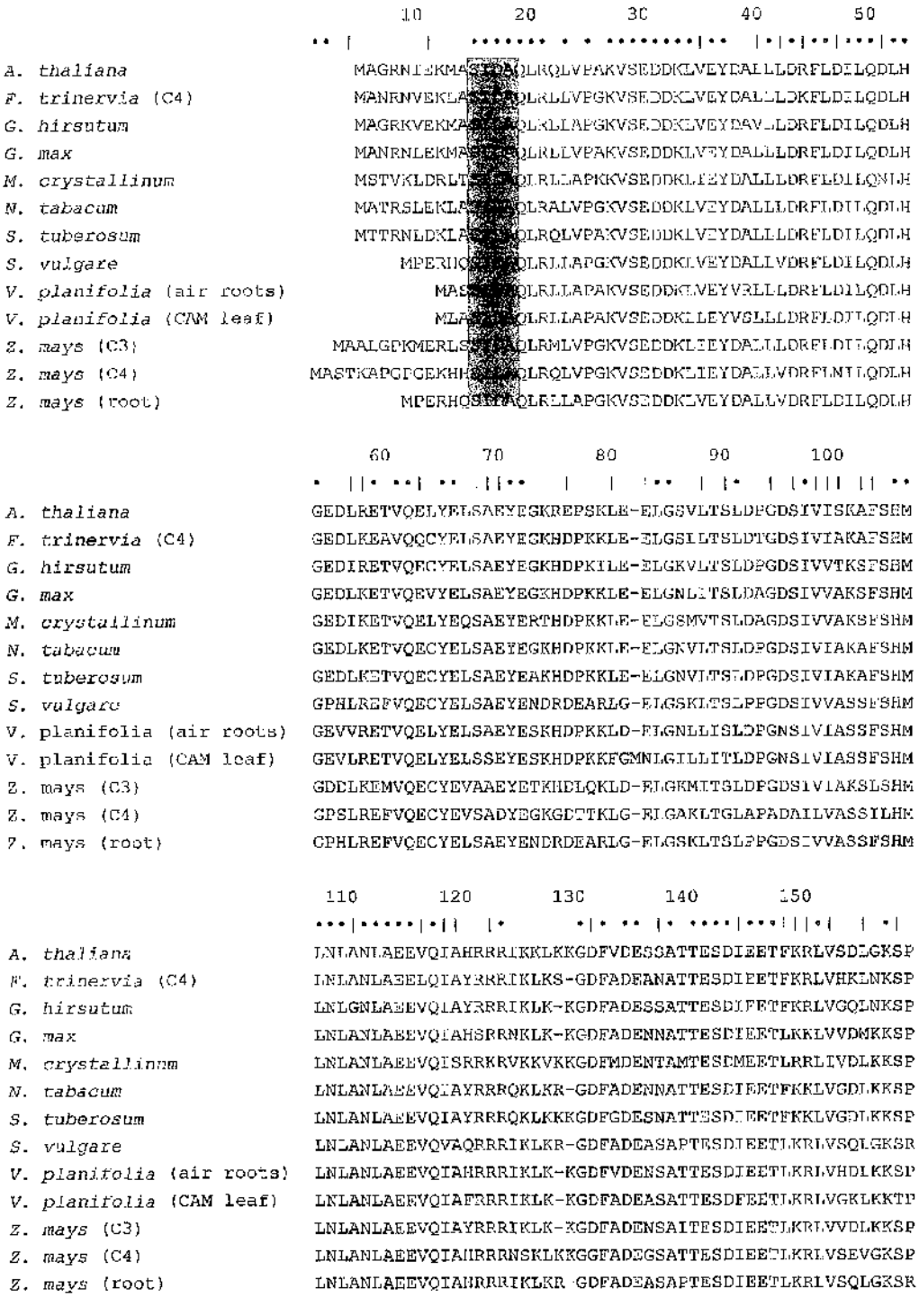
Figure 4.5 Sequence alignments of higher plant PEPcs

The numbers correspond to amino acid residue number. The shaded box is the PEPc phosphorylation motif conserved among all PEPc sequences known to date. Small black circles (•) in the sequence alignment denote conserved residues. Small black vertical lines (|) denote similar residues.



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                                160       170       180       190       200       210
                                 .|.|||*.***|**|******* *******||***| * ** |**** ****
A. thaliana                     EEIFDALKNQTVDLVLTAHPTQSVRRSLLQKHGRIRDCLAQLYAKDITPDDKQ
F. trinervia (C4)               EEVFDALKNQTVELVLTAHPTQSVRRSLLQKHGRIRNCLAQLYAKDITPDDKQ
G. hirsutum                     EEVFDALKNQTVDLVLTAHPTQSVRRSLLQKHGRIRNCLTQLYAKDITPDDKQ
G. max                          QEVFDALKNQTVDLVLTAHPTQSVRRSLLQKHGRIRKNLTQLYAKDITPDDKQ
M. crystallinum                 QEIFETLKNQTVDLVFTAHPTQSVRRSLLQKHGRIRDCLAQLYAKDITPDDKQ
N. tabacum                      QEVFDALKNQTVDLVLTAHPTQSVRRSLLQKHGRIRDCLAQLYAKDITPDDKQ
S. tuberosum                    QEVFDALKNQTVDLVLTAHPTQSVRRSLLQKHGRIRDCLAQLYAKDITPDDKQ
S. vulgare                      EEVFDALKNQTVDLVFTAHPTQSVRRSLLQKHGRIRNCLRQLYAKDITADDKQ
V. planifolia (air roots)       EEVFDALKNQTVDLVLTAHPTQSVRRSLLQKHARIRNCLAQLYAKDITPDDKQ
V. planifolia (CAM leaf)        QEIFDALKSQTVDLVLTAHPTQSVRRSLLQKHGRIRNCLTQLYAKDITPDDKQ
Z. mays (C3)                    AEVFDALKSQTVDLVLTAHPTQSVRRSLLQKHSRIRNCLVQLYSKDITPDDKQ
Z. mays (C4)                    EEVFEALKNQTVDLVFTAHPTQSARRSLLQKNARIRNCLTQLNAKDITDDDKQ
Z. mays (root)                  EEVFDALKNQTVDLVFTAHPTQSVRRSLLQKHGRIRNCLRQLYAKDITADDKQ

                                     220       230       240       250       260
                                ****|* |  **********  **** *** ****|***|*|*** ****|*
A. thaliana                     ELDESLQREIQAAFRTDEIRRTPPTPQDEMRAGMSYFHETIWKGVPKFLRRVD
F. trinervia (C4)               ELDEALQREIQAAFRTDEIRRTPPTPQDEMRAGMSYFHETIWKGVPKFLRRVD
G. hirsutum                     ELDEALQREIQAAFRTDEIRRNPPTPQDEMRAGMSYFHETIWKGVPKFLRRVD
G. max                          ELDEALQREIQAAFRTDEIRRTPPTPQDEMRAGMSYFHETIWKGVPTFLRRVD
M. crystallinum                 ELDEALQREIQAAFRTDEIRRTQPTPQDEMRAGMSYFHETIWNGVPKFLRRLD
N. tabacum                      ELDEALQREIQAAFRTDEIRRTAPTPQDEMRAGMSYFHETIWKGVPKFLRRVD
S. tuberosum                    ELDEALQREIQAAFRTDEIRRTPPTPQDEMRAGMSYFHETIWKGVPKFLRRVD
S. vulgare                      ELDEALQREIQAAFRTDEIRRTPPTPQDEMRAGMSYFHETIWKGVPKFLRRID
V. planifolia (air roots)       ELDEAL-KGGSAAFRTDEIRRTPPTPQDEMRAGMSYFHETIWKGVPKFLRRVD
V. planifolia (CAM leaf)        ELDEALHREIQAAFRTDEIRRIPPTPQDEMRAGMSYFHETIWKGVPQFLRRVD
Z. mays (C3)                    ELDEALQREIQAAFRTDEIRRTQPTPQDEMRAGMSYFHETIWKGVPKFLRRVD
Z. mays (C4)                    ELDEALQREIQAAFRTDEIRRAQPTPQAEMRYGMSYIHETVWKGVPKFLRRVD
Z. mays (root)                  ELDEALQREIQAAFRTDEIRRTPPTPQDEMRAGMSYFHETIWKGVPKFLRRID

                                  270       280       290       300       310
                                *****|** ** *** |**|**|************ ************||*
A. thaliana                     TALKNIGIDERVPYNAPLIQFSSWMGGDRDGNPRVTPEVTRDVCLLARMMAAN
F. trinervia (C4)               TALKNIGINERVPYNAPLIQFSSWMGGDRDGNPRVTPEVTRDVCLLARMMTSN
G. hirsutum                     TALKNIGINERVPYNAPLIQFSSWMGGDRDGNPRVTPEVTRDVCLLARMMAAN
G. max                          TALKNIGINERVPYNAPLIQFSSWMGGDRDGNPRVTPEVTRDVCLLARMMAAN
M. crystallinum                 TALKNIGITERVPYNAPLIQFSSWMGGDRDGNPRVTPEVTRDVCLLARMMAAN
N. tabacum                      TALKNIGINERLPYNAPLIQFSSWMGGDRDGNPRVTLEVTRDVCLLARMMAAN
S. tuberosum                    TALKNIGINERVPYNAPLIQFSSWMGGDRDGNPRVTPEVTRDVCLLARMMAAN
S. vulgare                      TALKNIGINERLPYNAPLIQFSSWMGGDRDGNPRVTPEVTRDVCLLARMMAAN
V. planifolia (air roots)       TALKNIGINERVPYNAPLIQFSSWMGGDRDGNPRVTPEVTRDVCLLARMMAAN
V. planifolia (CAM leaf)        TALKNLGINERLPYNAPLIQFSPWMGGDRDGNPRVTPEVTRDVCLLARMMAAN
Z. mays (C3)                    TALKNIGINERVPYNAPLIQFSSWMGGDRDGNPRVTPEVTRDVCLLARMMASN
Z. mays (C4)                    TALKNIGINERLPYNVSLIRFSSWMGGDRDGNPRVTPEVTRDVCLLARMMAAN
Z. mays (root)                  TALKNIGINERLPYNAPLIQFSSWMGGDRDGNPRVTPEVTRDVCLLARMMAAN
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                                320       330       340       350       360       370
                                |*  **||**|* *** *| ||* **  *   |       * |***** |*| |
A. thaliana                     LYYNQIENLMFELSMWRCTDEFRVRADELHRNSRKDA-AKHYIEFWKTIPPTE
F. trinervia (C4)               MYFSQIEDLMIEMSMWRCNSELRVRAEELYRTARKD--VKHYIEFWKRIPPNQ
G. hirsutum                     LYFSQIEDLMFELSMWRCSDELRIRADELHRSSKKDA--KHYIEFWKQIPPNE
G. max                          LYYSQIEDLMFELSMWRCNDELRVRADELNRSSKKNSVAKHYIEFWKAIPPNE
M. crystallinum                 MYFSQIDELMFELSMWRCTDELRERAEELHKYSKRDS--KHYIEFWKQIPSSE
N. tabacum                      LYYSQIEDLMFELSMWRCNDDLRIRAAELYRSSRRD--TKHYIEFWKTIPPSE
S. tuberosum                    LYYSQIEDLMFELSMWRCNEELRVRADDLQRSSRRD--EKHYIEFWKQVPPNE
S. vulgare                      LYFSQIEDLMFELSMWRCSDELRIRADELHRSSKRA--AKHYIEFWKQVPPNE
V. planifolia (air roots)       LYFSQIEDLMFELSMWRCSDELRIRADELHRSSKRDA--KHYIEFWKQVPPNE
V. planifolia (CAM leaf)        LYFSQIEDLMFELSMWPCSDELRLRADELNVSSKKDA--KHYIEFWKKVPPNE
Z. mays (C3)                    LYCSQIEDLMFELSMWRCSDELRMRADVLHLSTKKDA--KHYIEFWKKVPPNE
Z. mays (C4)                    LYIDQIEELMFELSMWRCNDELRVRAEELHSSSG-SKVTKYYIEFWKQIPPNE
Z. mays (root)                  LYFSQIEDLMFELSMWRCSDELRIRADELHRSSRKA--AKEYIEFWKQVPPNE

                                      380       390       400       410       420
                                ***|*||**||** ****|* |*    *||       ||||********|*
A. thaliana                     PYRVILGDVRDKLYHTRERSRQLLSNGISDIPEEATFTNVEQFLEPLELCYRS
F. trinervia (C4)               PYRVILGDVRDKLYNTRERSRHLLVDGKSDIPDEAVYTNVEQLLEPLELCYRS
G. hirsutum                     PYRIILGDVRDKLYNTRERARSLLANGFSDIPEEAAFTNVEQFLEPLELCYRS
G. max                          PYRVLLGEVRNRLYQTRERSRHLLAHGYSDIPEEETFTNVEEFLEPLELCYRS
M. crystallinum                 PYRVILADVRDKLYYTRERSRQLLASEVSEIPVEATFTEIDQFLEPLELCYRS
N. tabacum                      PYRVILGDVRDKLYQTRERTRQMLAHGISDIPEDATYNNVEQFLEPLELCYRS
S. tuberosum                    PYRVILGDVRDKLYQTRERARQLLCHGYSEIPEEATYTNIEQFLEPLELCYRS
S. vulgare                      PYRVILGDVRDKLYYTRERSRHLLSSGISEIPEEATFTNVEQFLEPLELCYRS
V. planifolia (air roots)       PYRVVLADVRDKLYNTRERSRHLLSSGISDVPEESSMTNVEQLLEPLELCYRS
V. planifolia (CAM leaf)        PYRVILGDVRDKLYHTRERARQILSHGVSEIPE-ATFTNVEQFLEPLELCYRS
Z. mays (C3)                    PYRVILSDVRDKLYNTRERSRELLSSGHSDIPEEATLTNVEQLLEPLELCYRS
Z. mays (C4)                    PYRVILGHVRDKLYNTRERARHLLASGVSEISAESSFTSIEEFLEPLELCYKS
Z. mays (root)                  PYRVILGDVRDKLYYTRERSRHLLTSGISEILEEATFTNVEQFLEPLELCYRS

                                   430       440       450       460       470
                                ** *|| |***|***||*** *** |*|*******|***.*|*|**  | *
A. thaliana                     LCSCGDSPIADGSLLDFLRQVSTFGLSLVRLDIRQESERHTDVLDAITKELDI
F. trinervia (C4)               LCDCGDHVIADGSLLDFLRQVSTFGLSLVKLDIRQESDRHTEVLDAITQELGI
G. hirsutum                     LCACGDRPIADGSLLDFLRQVSTFGLSLVRLDIRQESDRHTDVLDAITKELDI
G. max                          LCACGDRAIADGSLLDFLRQVSTFGLSLVRLDIRQESDRHTDVLDAITKHLEI
M. crystallinum                 LCACGDRPVADGSLLDFMRQVATFGLCLVKLDIRQESERHTDVMDAITTHLGI
N. tabacum                      LCECGDRPIADGSLLDFLRQVSTFGLSFVRLDIRQESDRHTDVLDAITQHLEI
S. tuberosum                    LCACGDLSIADGSLLDFLRQVSTFGLSLVRLDIRQESDRHTDVLDAITQHLEI
S. vulgare                      LCACGDKPIADGSLLDFLRQVFNFGLALVKLDIRQESDRHTDVLDSITTHLGI
V. planifolia (air roots)       LCSCGDRPIADGSLLDFMRQVSTFGLALVKLDIRQESDRHTDVIDAITTHLGI
V. planifolia (CAM leaf)        LCACAHRPIADGSLLDFLRQVSTFGLSLVKLDIRQESERHTDVMDAITRHIEI
Z. mays (C3)                    LCACGDSVIADGTLLDFLRQVSTFGLSLVRLDIRQESDRHTDVLDAITTYLGI
Z. mays (C4)                    LCDCGDKAIADGSLLDLLRQVFTFGLSLVKLDIRQESERHTDVIDAITTHLGI
Z. mays (root)                  LCACGDKPIADGSLLDFLRQVSTFGLALVKLDIRQESDRHTDVLDSITTHLCI
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                                480       490       500       510       520       530
                                ** * |*|*| **|***,**   *  * | |*** *    * |||| *||***
A. thaliana                     GSSYRDWSEEGRQEWLLAEL-SGKRPL-FGPDLPKTEEISDVLDTFKVISELP
F. trinervia (C4)               GS-YREWSEEKRQEWLLAEL-SGKRPL-IGPDLPKTEEVKDCLDTFKVLAELP
G. hirsutum                     GS-YREWPEERRQEWLLSEL-RGKRPL-FGPDLPKTEEVADVLDTFHVISELP
G. max                          GS-YQEWSEEKRQQWLLSEL-SGKRPL-FGPDLPQTEEIRDVLETFHVIAELP
M. crystallinum                 GS-YRDWTEEKRQDWLLSEL-RGKRPL-FGPDLPRTDEIADVLDTINVIAELP
N. tabacum                      GS-YREWSEERRQEWLLSEL-SGKRPL-FGPDLPRTEEIADVLDTLHVIAELP
S. tuberosum                    GS-YRDWSEERRQEWLLSEL-SGKRPL-FGPDLPKTEEIADVLDTFHVIAELP
S. vulgare                      GS-YAEWSEEKRQDWLLSEL-RGKRPL-FGSDLPQTEETADVLGTFHVLAELP
V. planifolia (air roots)       GS-YREWSED-RQEWLLSELASGKGDLQIGSDLPATCSV-DVIDTFLVIAELP
V. planifolia (CAM leaf)        GS-YREWSEEQRQQWLLSELAE-KRPL-FGPDLPRTEEIADVLDTFQVIAELP
Z. mays (C3)                    GS-YREWTEERRQEWLLSEL-NGKRPL-FGSDLPKTEEISDVLDTFSVIAELP
Z. mays (C4)                    GS-YREWPEDKRQEWLLSEL-RGKRPL-LPPDLPQTDEIADVIGAFHVLAELP
Z. mays (root)                  GS-YAEWSEEKRQDWLLSEL-RGKRPL-FGSDLPQTEETADVLGTFHVLAELP

                                      540       550       560       570       580
                                * **|*|*****| ********** |  ** ******|* **||*|*||
A. thaliana                     SDCFGAYIISMATSPSDVLAVELLQRECHVKNPLRVVPLFEKLADLEAAPAAV
F. trinervia (C4)               SDCFGAYIISMATSTSDVLAVELLQREYHIKHPLRVVPLFEKLADLEAAPAAM
G. hirsutum                     SDSFGAYIISMATAPSDVLAVELLQRECHVKQPLRVVPLFEKLADLEAAPAAV
G. max                          LDNFGAYIISMATAPSDVLAVELLQRECHVKHPLRVVPLFEKLADLEAAPAAL
M. crystallinum                 SDSFGAYVISMATAPSDVLAVELLQRECKVKKPLRVVPLFEKLADLEAAPASM
N. tabacum                      SDCFGAYIISMATAPSDVLAVELLQRECHVKQPLRVVPLFEKLDDLESASAAV
S. tuberosum                    ADCFGAYIISMATAPSDVLAVELLQRECRVRQPLRVVPLFEKLADLDAAPAAV
S. vulgare                      ADCFGAYIISMATAPSDVLAVELLQRECEVKQPLRVVPLFEKLADLEAAPAAV
V. planifolia (air roots)       PDNFGAYIISMATAPSDVLAVELLQRECHVRQPLRVVPLFEKLADLEAAPAAL
V. planifolia (CAM leaf)        SDGFGAYIISMATSSSDVLAVELLQREFRVPKPLRVVPLFEKLADLEAAPAAV
Z. mays (C3)                    SDNFGAYIISMATAPSDVLAVELLQRECHVKTPLRVVPLFEKLADLEAAPAAL
Z. mays (C4)                    PDSFGPYIISMATAPSDVLAVELLQRECGVRQPLPVVPLFERLADLQSAPASV
Z. mays (root)                  ADCFGAYIISMATAPSDVLAVELLQRECEVKHPLRVVPLFEKLADLEAAPAAV

                                   590       600       610       620       630
                                ****||** |**|***|**|**********      |*| **||  ||*
A. thaliana                     ARLFSIDWYKNRINGKQEVMIGYSDSGKDAGRLSAAWELYKAQEELVKVAKKY
F. trinervia (C4)               TRLFSMDWYRNRIDGKQEVMIGYSDSGKDAGRFSAAWQLYKTQEQIVKIAKEF
G. hirsutum                     ARLFSIDWYRDRINGKQEVMIGYSDSGKDAGRLSAAWQLYKAQEELVKVAKQY
G. max                          ARLFSVDWYRNRINGKQEVMIGYSDSGKDAGRFSAAWQLYKAQEELIMVAKQY
M. crystalliinum                TRLFSVDWYKNRIDGKQEVMIGYSDSGKDAGRLSAAWQMYKVQEELLKVSKKF
N. tabacum                      ARLFSIEWYRNRINGKQEVMVGYSDSGKDAGRFSAAWQLYKAQEELIKVAKEH
S. tuberosum                    ARLFSIEWYRNRINGKQEVMIGYSDSGKDAGRLSAAWQLYKAQEELIQVAKEF
S. vulgare                      ARLFSIDWYMNRINGKQEVMIGYSDSGKDAGRLSAAWQMYKAQEELIKVAKHY
V. planifolia (air roots)       ARLFSIEWYRNRIDGKQEVMIGYSDSGKDAGRLSAAWQLYKGQEDLIKVAKKY
V. planifolia (CAM leaf)        SRLFSIDWYRDRINGKQEVMIGYSDSGKDAGRTFCCLAVYKTQEELVQVAKQF
Z. mays (C3)                    ARLFSIDWYRQRINGKQEVMIGYSDSGKDAGRLSAAWQLYKAQEELIKVAKDF
Z. mays (C4)                    ERLFSVDWYMDRIKGKQQVMVGYSDSGKDAGRLSAAWQLYRAQEEMAQVAKRY
Z. mays (root)                  ARLFSIDWYMDRINGKQEVMIGYSDSGKDAGRLSAAWQMYKAQEELIKVAKHY
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                                640       650       660       670       680
                                |***  |**************|***|*|**||*| **|***·******  ***
A. thaliana                     GVKLTMFHGRGGTVGRGGGPTHLAILSQPPDTVNGSLRVTVQGEVIEQSFGEA
F. trinervia (C4)               GVKLVIFHGRGGTVGRGGGPTHLALLSQPPDTINGSLRVTVQGEVIEQSFGEE
G. hirsutum                     GVKLTMFHGRGGTVGRGGGPTHLAILSQPPDTIHGSLRVTVQGEVIEQSFGEE
G. max                          GVKLTMFHGRGGTVGRGGGPTHLAILSQPPETIHGSLRVTVQGEVIEQSFGEQ
M. crystallinum                 GVKLTMFHGRGGTVGRGGGPTHLAILAQPAETIGGSLRVTIQGEVIEQSFGEQ
N. tabacum                      GVKLTMFHGRGGTVGRGGGPTHLAILSQPPDTIQGSLRVTVQGEVIEQSFGEE
S. tuberosum                    DVKLTMFHGRGGTVGRGGGPAHLAILSQPPETIHGSLRVTVQGEVIEQSFGEE
S. vulgare                      GVKLTMFHGRGGTVGRGGGPTHLAILSQPPDTIHGSLRVTVQGEVIEHSFGEE
V. planifolia (air roots)       GVKLTMFHGRGGTVGRGGGPTHLAILSQPPDTIHGSLRVTVQGEVIEQSFGEE
V. planifolia (CAM leaf)        GVKLTMFHGRGGTVGRGGGPTHLAILSQPPDTINGSLRVTVQGEVIEQCFGEE
Z. mays (C3)                    GVKLTMFHGRGGTVGRGGGPTHLAILSQPPDTIHGSLRVTVQGEVIEQSFGEE
Z. mays (C4)                    GVKLTLFHGRGGTVGRGGGPTHLAILSQPPDTINGSIRVTVQGEVIEFCFGEE
Z. mays (root)                  GVKLTMFHGRGGTVGRGGGPTHLAILSQPPDTIHGSLRVTVQGEVIEHSFGEE

                          690       700       710       720       730       740
                            ***|****| ********|*   ||**** *|*||**:**  ** |* |*|*
A. thaliana                 HLCFRTLQRFTAATLEHGMNPPISPKPEWRALLDEMAVVATEEYRSVVFQEPR
F. trinervia (C4)           HLCFRTLQRFCAATLEHGMNPPISPRPEWRELMDQMAVVATEEYRSVVFKEPR
G. hirsutum                 HLCFRTLQRFTAATLEHGMHPPVSPNPEWRALMDEMAVVATKEYRSVVFQEPR
G. max                      HLCFRTLQRFTAATLEHGMHPPISPKPEWRALMDEMAVIATEEYRSIVFKEPR
M. crystallinum             HLCFRTLQRYTAATLEHGMNPPKSPKPEWRALLDQMAVVATEEYRSIVFKEPR
N. tabacum                  HLCFRTLQRFTAATLEHGMHPPVSPKPEWRALMDEIAVIATEKYRSIVFKEPR
S. tuberosum                HLCFRTLQRFTAATLEHGMHPPVSPKPEWRALMDEIAVVATEKYRSIVFKEPR
S. vulgare                  LLCFRTLQRYTAATLEHGMHPPISPKPEWRALMDEMAVVATKEYRSIVFQEPR
V. planifolia (air roots)   HLCFRTLQRFTAATLEHGMHPPISPKPEWRTLLDEMAVVATEEYRSIVFQEPR
V. planifolia (CAM leaf)    RLCFRTLQRYTAATLEHGMNP-YFSKPEWRSLLDEMAVIATNRYRWIVFQEPR
Z. mays (C3)                HLCFRTLQRFTAATLEHGMHPPNAPKPEWRALLDEMAVVATEEYRSIVFKEPR
Z. mays (C4)                HLCFQTLQRFTAATLEHGMHPPVSPKPEWRKLMDEMAVVATEEYRSVVVKEAR
Z. mays (root)              LLCFRTLQRYTAATLEHGMHPPISPKPEWRALMDEMAVVATKEYRSIVFQEPR

                                750       760       770       780       790
                                ****  **** *|*********|**|*|***  ********|**********
A. thaliana                     FVEYFRLATPELEYGRMNIGSRPSKRKPSGGIESLRAIPWIFAWTQTRFHLPV
F. trinervia (C4)               FVEYFRLATPELEFGRMNIGSRPSKRKPSGGIESLRAIPWIFSWTQTRFHLPV
G. hirsutum                     FVEYFRLATPELEYGRMNIGSRPSKRKPSGGIESLRAIPWIFAWTQTRFHLPV
G. max                          FVEYFRLATPELEYGRMNIGSRPAKRRPSGGIETLRAIPWIFAWTQTRFHLPV
M. crystallinum                 FVEYFRLATPETEYGRMNIGSRPSKRKPSGGIESLRAIPWIFAWTQTRFHLPV
N. tabacum                      FVEYSALATPELEYGRMNIGSRPSKRKPSGGIESLRAIPWIFAWTQTRFHLPV
S. tuberosum                    FVEYFRLATPELEYGRMNIGSRPSKRKPSGGIESLRAIPWIFAWTQTRFHLPV
S. vulgare                      FVEYFRSATPETEYGRMNIGSRPSKRKPSGGIESLRAIPWIFAWTQTRFHLPV
V. planifolia (air roots)       FVEYGRLATPELEYGRMNIGSRPSKRKPSGGIESLRAIPWIFAWTQTRFHLPV
V. planifolia (CAM leaf)        FVEYFRLATPELEYGRMNIGSRPSKRKPSGGIESLRAIPWIFAWTQTRFHLPV
Z. mays (C3)                    FVEYFRLATPETEYGRMNIGSRPSKRKPSGGIESLRAIPWIFAWTQTRFHLPV
Z. mays (C4)                    FVEYFRSATPETEYGRMNIGSRPAKRRPGGGITTLRAIPWIFSWTQTRFHLPV
Z. mays (root)                  FVEYFRSATPETEYGRMNIGSRPSKRKPSGGIESLRAIPWIFAWTQTRFHLPV
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                                  800       810       820       830       840
                                  ••• •|•|| |    ••  •• | ||||• |  •••••|••|••••|••|• ••||
A. thaliana                       WLGFGAAFRYAIKKDVRNLHMLQDMYKQWPFFRVTIDLIEMVFAKGDPGIAAL
F. trinervia (C4)                 WLGFGAAFKHAIQKDSKNLQMLQEMYKCWPFFRVTIDLVEMVFAKGNPGIAAL
G. hirsutum                       WLGFGAAFKHVIQKDIKNLHMLQEMHNQWPFFRVTMDLIEMVFAKGDPGIAAL
G. max                            WLGFGAAFKHVIEKDVRNIHVLQEMYNQWPFFRVTIDLVEMVFAKGDPGIAAL
M. crystallinum                   WLGVGGALKHVLEKDIRNHNMLRDMYNKFPFFRVTIDLLEMVFAKGDPEIAAL
N. tabacum                        WLGFGAAFKYAIDKDIKNLRMFHEMYNEWPFFRVTIDLVEMVFAKGNPGIAAL
S. tuberosum                      WLGFGAAFKYAIEKDIKNLRMLQEMYNAWPFFRVTIDLVEMVFAKGDPGIAAL
S. vulgare                        WLGFGAAIKHIMQKDIRNIHVLKEMYNEWPFFRVTLDLLEMVFAKGDPGIAAV
V. planifolia (air roots)         WLGFGTAFKHVSPKDIKNLRILQEMYNEWPFFRVTIDLVEMVFAKGNPGIASL
V. planifolia (CAM leaf)          WLGFGAAFKHVISKDNKNLHTLREMYNGWPFFRVTIDLIEMVFTKGNPGIASL
Z. mays (C3)                      WLGFGAAFKNVLQKDIRNLHMLQEMYNEWPFFRVTIDLVEMVFAKGNPGIAAV
Z. mays (C4)                      WLGVGAAFKFAIDKDVRNFQVLKEMYNEWPFFRVTLDLLEMVFAKGDPGIAGL
Z. mays (root)                    WLGFGAAIKHIMQKDIRNIHILREMYNEWPFFRVTLDLLEMVFAKGDPGIAAV

                                850       860       870       880       890       900
                                  | •••| |• ||•  ••  | |•|  |•  •••| |•  |       | ••|
A. thaliana                       YDKLLVSEDLWAFGEKLRANFDETKNLVLQTAGHKDLLEGDPYLK-QRLRLRD
F. trinervia (C4)                 NDKLLVSEDLRPFGESLRANYEETKNYLLKIAGHKDLLEGDPYLK-QGIRLRD
G. hirsutum                       YDKLLVSKELWPFGENLRANYEDTKRLVLQVAGHRDLLEGDPYLK-QRLRLRD
G. max                            YDRLLVSEDLWSFGEQLRTMYEETKELLLQVAGHRDLLEGDPYLK-QRLRLRD
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N. tabacum                        YDKLLVSEDLLPFGELLRSNYEETRSLLLQTAGHKDLLEGDPYLK-QRLRLRD
S. tuberosum                      FDKLLVSEDLWSFGELLRSKYEETKSLLLQIAGEKDLLEGDPYLK-QRLRLRD
S. vulgare                        YDKLLVAEDLQSFGEQLRKNYEETKELLLQVAGHKDVLEGDPYLK-QRLRLRE
V. planifolia (air roots)         YGKLLVSSELWSFGERLRANVQRTKDLLLQVAGHKVFLK-ESLSE-AEVGLRD
V. planifolia (CAM leaf)          YDKLLVSSELLPFGAQLRENYGETKRLLLLVAGHKDLLEGDPYLKDQRLRLRD
Z. mays (C3)                      YDKLLVSEELHPLGEKLRANYEETQKLLLQVAGHRDLLEGDLYLK-QRLRLRD
Z. mays (C4)                      YDELLVAEELKPFGKQLRDKYVETQQLLLQIAGHKDILEGDPFLKQG-LVLRN
Z. mays (root)                    YDKLLVADDLQSFGEQLRKNYEETKELLLQVAGHKDVLEGDPYLK-QRLRLRE

                                      910       920       930       940       950
                                  |••••••| •••••••••|| | •  |• | ||   |       || ••  ••
A. thaliana                       SYITTLNVCQAYTLKRIRDANYNVTLRPHISKEIM-QSSKSAQELVKLNPTSE
F. trinervia (C4)                 PYITTLNVCQAYTLKRIRDPNYHVTLRPHISKEYAAEPSKPADELIHLNPTSE
G. hirsutum                       AYITTLNVCQAYTLKRIRDPDYHVKVRPHLSREYM-ESSKAAAELVKLNPTSE
G. max                            SYITTLNVCQAYTLKRIRDPNYNVKLRPHISKESI-EISKPADELITLNPTSE
M. crystallinum                   PYITTLNVCQAYTLKRIRDPDFKVTERPHLSKEIM-DAHKAAAELVKLNPTSE
N. tabacum                        SYITTLNLLQAYTLKRIRDPNYHVTLRPHISKDYM-E-SKSAAELVQLNPTSE
S. tuberosum                      SYITTLNVCQAYTLKRIRDPDYSVTPRPHISKEYM-E-AKPATELVNLNPTSE
S. vulgare                        SYITTLNVCQAYTLKRIRDPSFQVSPQPPLSKEFT-DESQPV-ELVQLNQQSE
V. planifolia (air roots)         SYITTLNVCQAYTLKRIRDPNFHVKVRPHISKE-ISDASKPAAELVKLNPTSE
V. planifolia (CAM leaf)          PYITTLNVCQAYTLKRIREPGYHVTARPHLLKE-TDESIKSAAELVKLNPTSE
Z. mays (C3)                      AYITTLNVCQAYTLKRIRDPDYHVALRPHISKEIM-DSTKAAADVVKLNPGSE
Z. mays (C4)                      PYITTLNVFQAYTLKRIRDPNFKVTPQPPLSKEFADENK--PAGLVKLNPASE
Z. mays (root)                    SYITTLNVCQAYTLKRIRDPSFQVSPQPPLSKEFT-DESQPA-ELVQLNQQSE
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                                960       970
                                .|........|....|...|....
A. thaliana                     YAPGLEDTLILTMKGIAAGLQNTG
F. trinervia (C4)               YAPGLEDTLILTMKGIAAGMQNTG
G. hirsutum                     YAPGLEDTLILTMKGIAAGMQNTG
G. max                          YAPGLEDTLILTMKGIAAGLQNTG
M. crystallinum                 YAPGLEDTLILTMKGVAAGLQNTG
N. tabacum                      YAPGLEDTLILTMKGIAAGLQNTG
S. tuberosum                    YAPGLEDTLILTMKGIAAGMQNTG
S. vulgare                      YAPGLEDTLILTMKGIAAGMQNTG
V. planifolia (air roots)       YAPGLEDTLILTMKGIAAGMQNTG
V. planifolia (CAM leaf)        YGPGLEDTLIITMKGIAAGLQNTG
Z. mays (C3)                    YAPGLEDTLILTMKGIAAGLQNTG
Z. mays (C4)                    YPPGLEDTLILTMKGIAAGMQNTG
Z. mays (root)                  YAPGLEDTLILTMKGIAAGMQNTG
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software (Figure 4.5) and this allows the identification of several conserved sites. These are discussed in section 4.3.

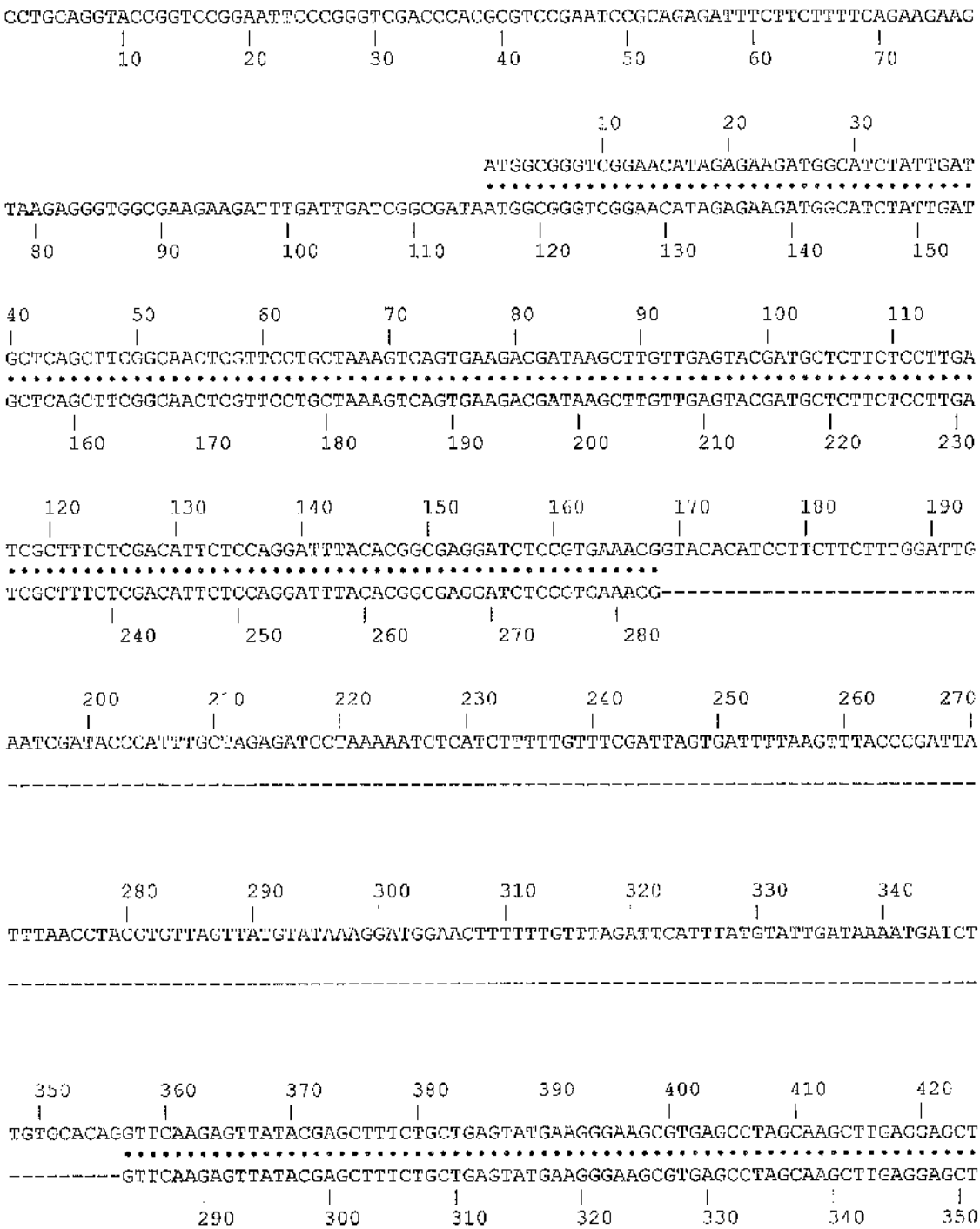
The peptide sequence of the *A. thaliana* PEPc cDNA was used to search the Genbank database of *A. thaliana* sequences using BLAST. The search revealed that the sequence reported in this work is the first full-length PEPc cDNA to be cloned from *A. thaliana*. Six months after submission of this sequence to the Genbank database (accession no. AF071788), the genomic sequence of a PEPc gene cloned from young seedlings of *A. thaliana* var. Columbia (accession no. ATH13170) was submitted to the database (Hartung, F., 1998). A gapped alignment of this genomic sequence with the *A. thaliana* PEPc cDNA nucleotide sequence is shown in Figure 4.6. The genomic sequence contains nine introns as do most PEPc genes (Yanai et al., 1994). All the introns have the conserved intron/exon splice sites (GT....AG) observed in the six plant PEPc genes discussed by Lepiniec et al. (1993). Figure 4.7 is a diagrammatic representation of the genomic sequence of the *A. thaliana* PEPc with some other plant PEPcs, highlighting the conserved location of the introns in the genes with respect to the coding region. However, as shown in Table 4.1, the intron length of the genes varies quite considerably (Lepiniec et al., 1993).

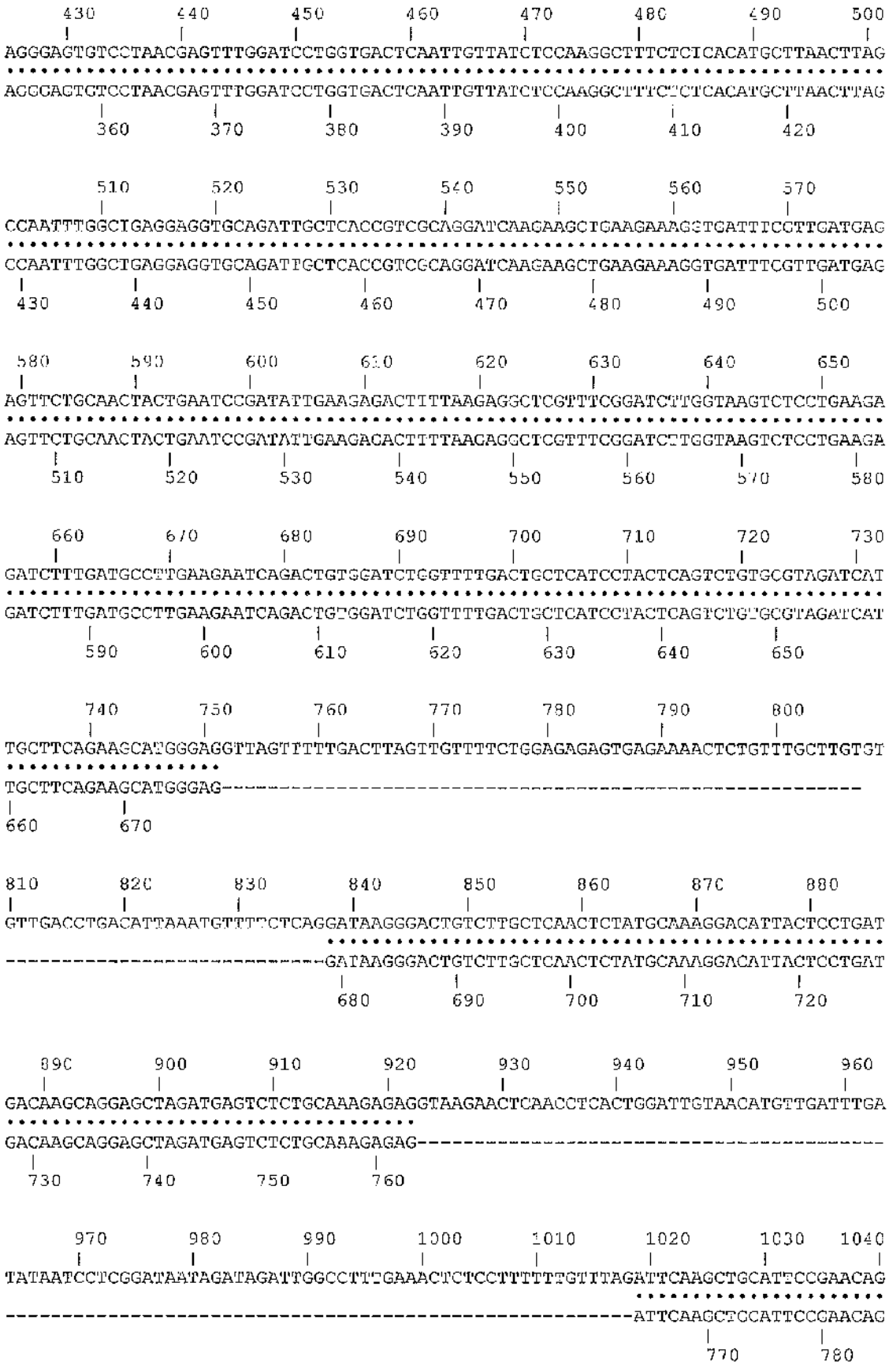
Table 4.1 A comparison of intron length from different PEPc genes

	A. thaliana **(ATH131710)**	***M. crystallinum*** **(x14588)**	***F. trinervia*** **(x64143)**	***Z. mays*** **(x15642)**
intron I (bp)	187	568	833	111
intron II (bp)	85	103	78	111
intron III (bp)	105	1453	92	898
intron IV (bp)	86	530	89	450
intron V (bp)	82	106	79	126
intron VI (bp)	105	324	83	138
intron VII (bp)	172	260	740	97
intron VIII (bp)	82	377	617	98
intron IX (bp)	82	805	605	89

Figure 4.6 Alignment of *A. thaliana* PEPc cDNA and genomic sequences

The numbers in the figure below correspond to nucleotide number. The top line is the PEPc genomic sequence and the bottom line is the PEPc cDNA sequence. Small black circles (•) between the two sequences show identical nucleotides at that point in the alignment. A series of dashes (---) in the genomic sequence represents an intron.

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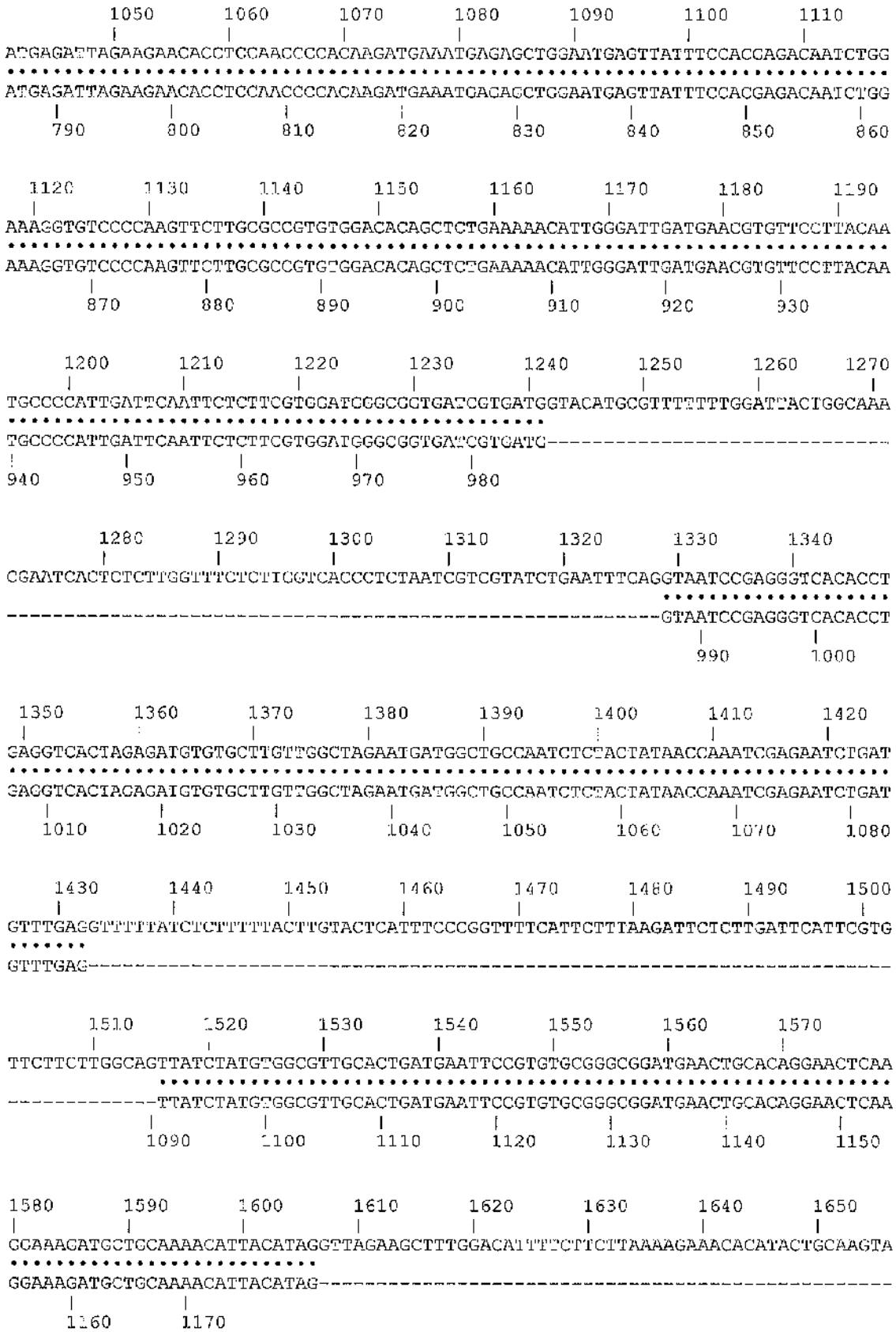
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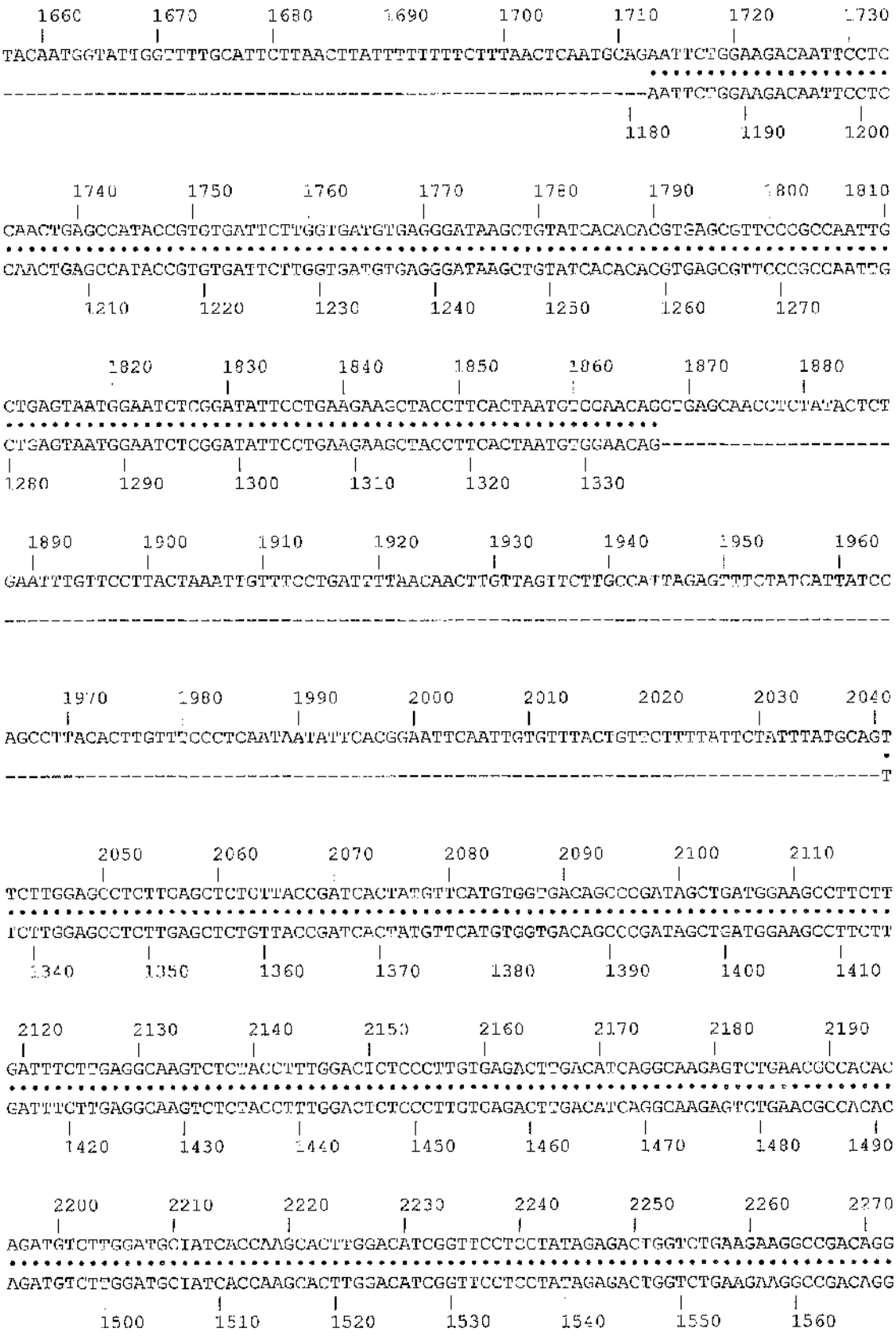
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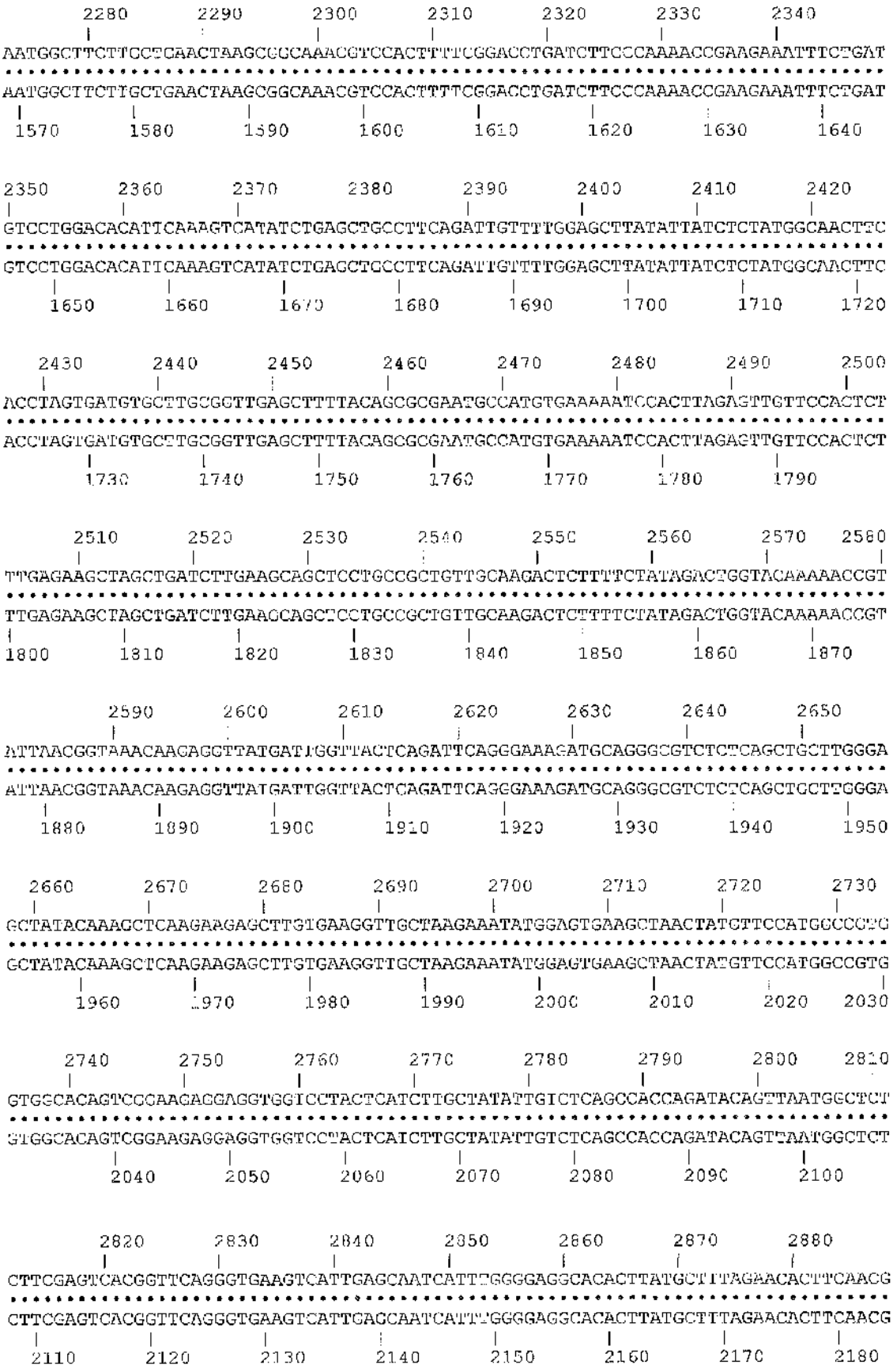
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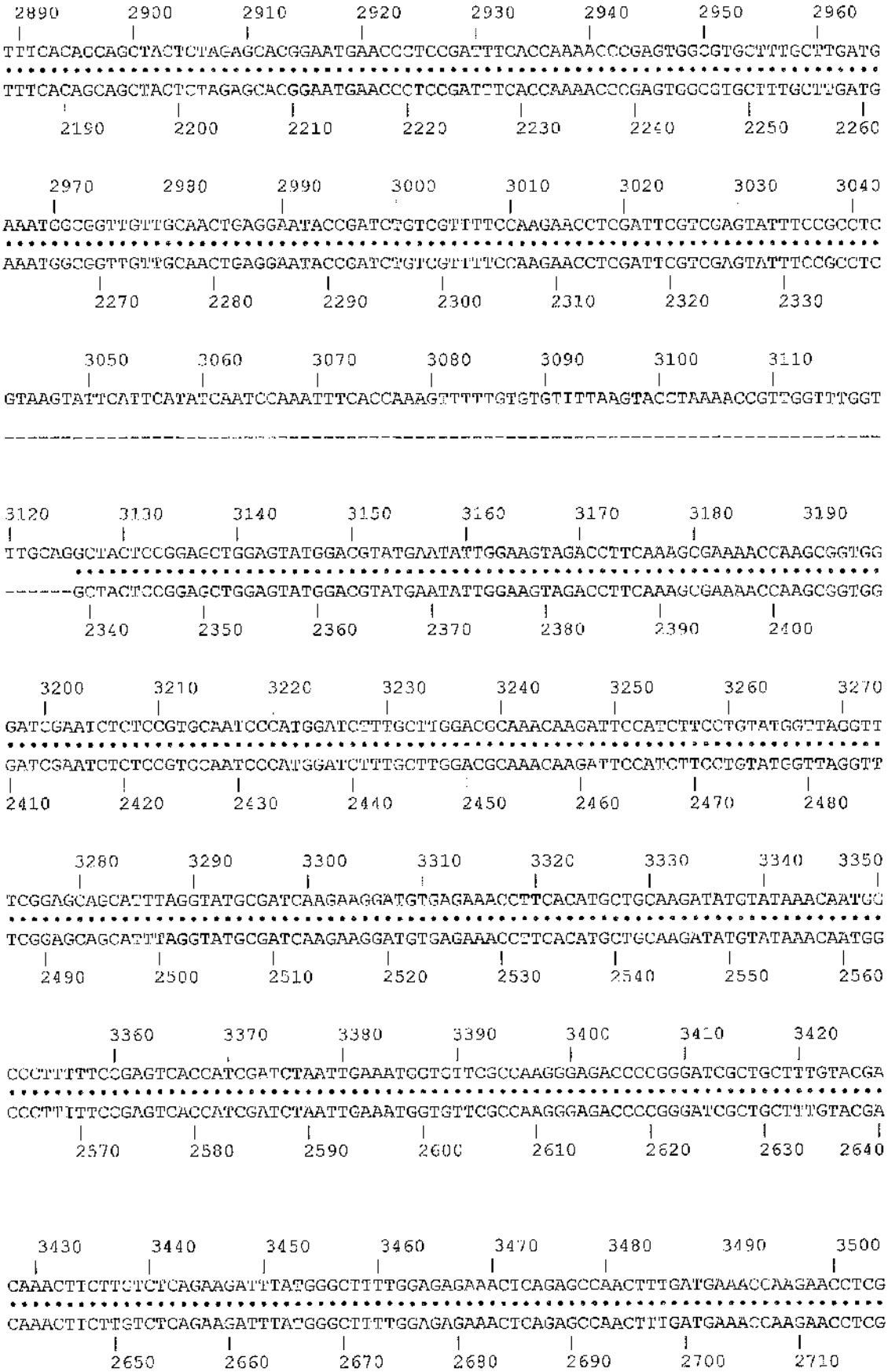
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3360 3370 3380 3390 3400 3410 3420
CCCTTTTTCCGAGTCACCATCGATCTAATTGAAATGGTGTTCGCCAAGGGAGACCCCGGGATCGCTGCTTTGTACGA
CCCTTTTTCCGAGTCACCATCGATCTAATTGAAATGGTGTTCGCCAAGGGAGACCCCGGGATCGCTGCTTTGTACGA
2570 2580 2590 2600 2610 2620 2630 2640
3430 3440 3450 3460 3470 3480 3490 3500
CAAACTTCTTGTCTCAGAAGATTTATGGGCTTTTGGAGAGAAACTCAGAGCCAACTTTGATGAAACCAAGAACCTCG
CAAACTTCTTGTCTCAGAAGATTTATGGGCTTTTGGAGAGAAACTCAGAGCCAACTTTGATGAAACCAAGAACCTCG
2650 2660 2670 2680 2690 2700 2710

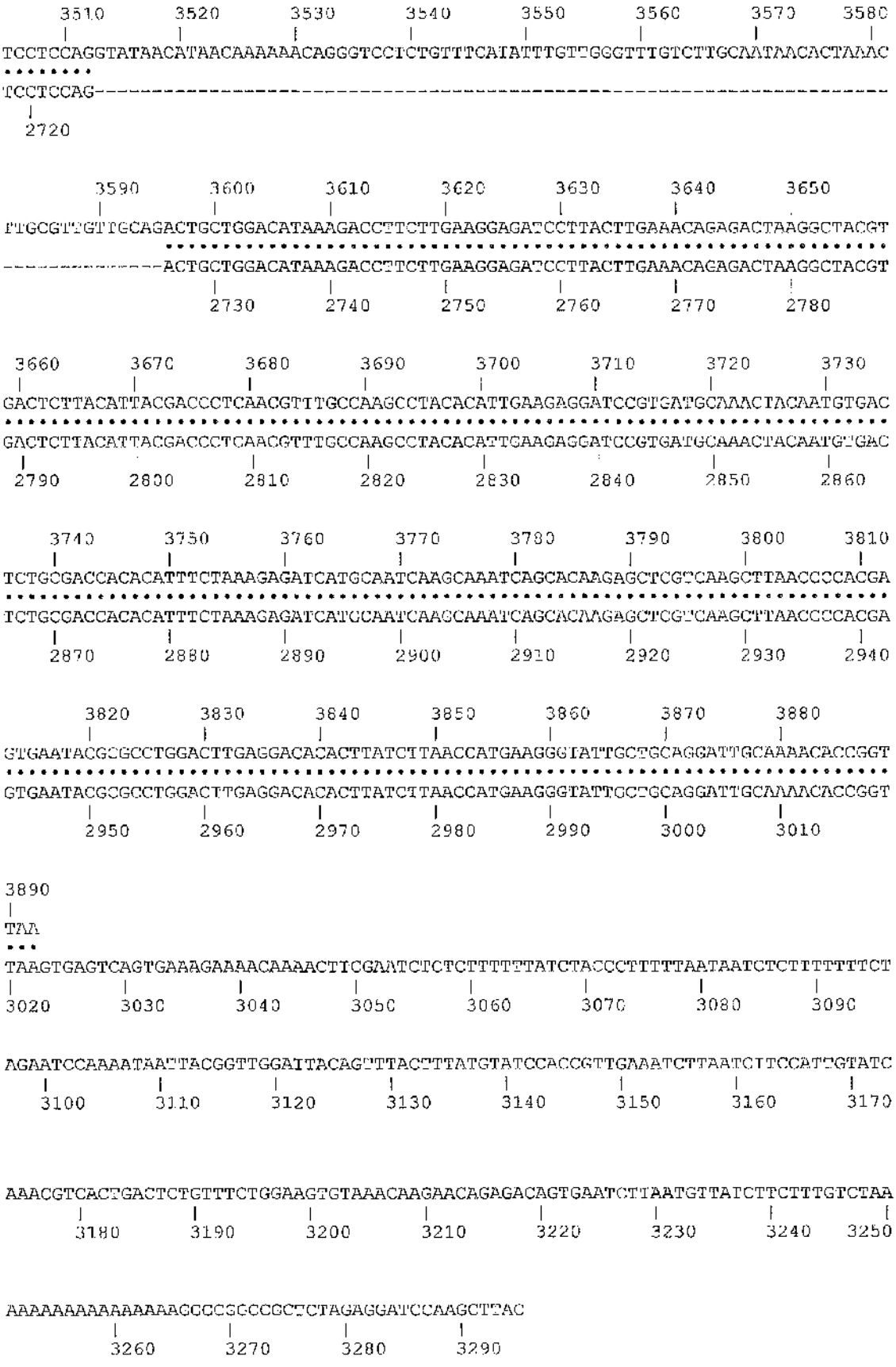
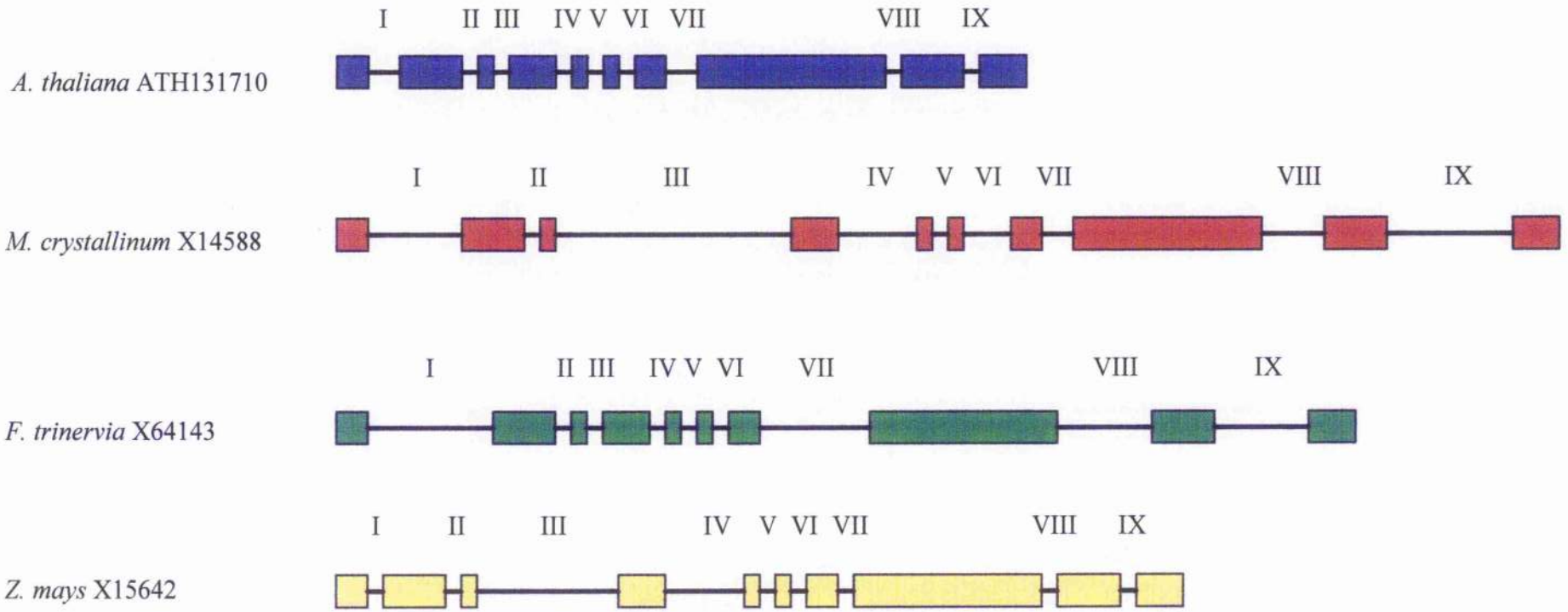

3510 3520 3530 3540 3550 3560 3570 3580
TCCTCCAGGTATAACATAACAAAAAACAGGGTCCTCTGTTTCATATTTGTTGGGTTTGTCTTGCAATAACACTAAAC
TCCTCCAG
2720
3590 3600 3610 3620 3630 3640 3650
TTGCGTTGTTGCAGACTGCTGGACATAAAGACCTTCTTGAAGGAGATCCTTACTTGAAACAGAGACTAAGGCTACGT
ACTGCTGGACATAAAGACCTTCTTGAAGGAGATCCTTACTTGAAACAGAGACTAAGGCTACGT
2730 2740 2750 2760 2770 2780
3660 3670 3680 3690 3700 3710 3720 3730
GACTCTTACATTACGACCCTCAACGTTTGCCAAGCCTACACATTGAAGAGGATCCGTGATGCAAACTACAATGTGAC
GACTCTTACATTACGACCCTCAACGTTTGCCAAGCCTACACATTGAAGAGGATCCGTGATGCAAACTACAATGTGAC
2790 2800 2810 2820 2830 2840 2850 2860
3740 3750 3760 3770 3780 3790 3800 3810
TCTGCGACCACACATTTCTAAAGAGATCATGCAATCAAGCAAATCAGCACAAGAGCTCGTCAAGCTTAACCCCACGA
TCTGCGACCACACATTTCTAAAGAGATCATGCAATCAAGCAAATCAGCACAAGAGCTCGTCAAGCTTAACCCCACGA
2870 2880 2890 2900 2910 2920 2930 2940
3820 3830 3840 3850 3860 3870 3880
GTGAATACGCGCCTGGACTTGAGGACACACTTATCTTAACCATGAAGGGTATTGCTGCAGGATTGCAAAACACCGGT
GTGAATACGCGCCTGGACTTGAGGACACACTTATCTTAACCATGAAGGGTATTGCTGCAGGATTGCAAAACACCGGT
2950 2960 2970 2980 2990 3000 3010
3890
TAA
TAAGTGAGTCAGTGAAAGAAAACAAAACTTCGAATCTCTCTTTTTTATCTACCCTTTTTAATAATCTCTTTTTTTCT
3020 3030 3040 3050 3060 3070 3080 3090
AGAATCCAAAATAATTACGGTTGGATTACAGTTTACTTTATGTATCCACCGTTGAAATCTTAATCTTCCATTGTATC
3100 3110 3120 3130 3140 3150 3160 3170
AAACGTCACTGACTCTGTTTCTGGAAGTGTAAACAAGAACAGAGACAGTGAATCTTAATGTTATCTTCTTTGTCTAA
3180 3190 3200 3210 3220 3230 3240 3250
AAAAAAAAAAAAAAAGCGCGCCGCTCTAGAGGATCCAAGCTTAC
3260 3270 3280 3290

Figure 4.7 A comparison of intron length and location in PEPc genes from different species

In the diagram below introns are numbered 1 to 9 in Roman numerals and exons are represented by coloured boxes.



A chromosome location has not yet been assigned to this gene and unfortunately no sequence on the 5' or 3' side of the coding sequence has been submitted to allow identification of the promoter sequence for the gene or other regulatory elements. Recently (Lin et al., 1999), the sequence of an *A. thaliana* BAC clone (accession no. AC007087) from chromosome 2 was submitted to GenBank. This clone contains a PEPc gene different from the one for the PEPc cDNA clone 4.2 (81% identity) thus assigning an *A. thaliana* PEPc gene to chromosome 2 and providing 5'-UTR and 3'-UTR sequence for future analysis.

The PEPc cDNA (accession no. AF071788) also showed homology to several other *A. thaliana* sequences. Those sequences with greater than 90% identity to the PEPc cDNA were presumed to be PEPc sequences, and of these sequences, those with greater than 90% identity to one another were presumed to be part of the same gene. Using these criteria, the sequences were analysed and 7 distinct PEPc sequences were found from this search of the *A. thaliana* Genbank database (Figure 4.8). However, only the genomic (accession no. ATH131710) and cDNA (accession no. AF071788) sequences for the PEPc gene discussed in this chapter, and the other PEPc genomic clone from *A. thaliana* (accession no. AC007087) contained the characteristic start codon nucleotides ATG indicative of a full-length PEPc sequence.

4.2.4 Tissue expression study of *A. thaliana* PEPc clone

Rosette leaves, bracts, bolts, flowers and buds were taken from 5-week old *A. thaliana* var. Landsberg erecta plants. Root tissue was grown as described in section 2.2.2(i). After 14 days the roots were removed from the seedlings, washed with distilled water and carefully blotted dry with paper towel. All plant tissue was snap-frozen upon harvesting and stored at -80°C until required. Figure 4.9 is a labelled diagram of an *A. thaliana* var. Landsberg erecta plant showing the different tissues used for the Northern analysis of PEPc and PEPc kinase transcripts. Total RNA was isolated from 1-2 g of tissue by the method described in section 2.10.2(i) and from this poly A^+ RNA was isolated using Promega's Poly ATract kit (section 2.10.3). These poly A^+ RNA samples were run on a denaturing formaldehyde gel (section 2.7.5) and the RNA then immobilized onto Hybond-N membrane by overnight capillary transfer.

Primers were designed to the 3'-untranslated region (3'-UTR) of the *A. thaliana* PEPc clone (section 2.9.7 and Figure 2.1):

Figure 4.8 Alignment of *A. thaliana* PEPc cDNA with other *A. thaliana* PEPc sequences

All sequences represented have ≥ 90% identity to the *A. thaliana* PEPc cDNA (accession no. AF071788) and are therefore assumed to be PEPc. Those sequences with ≥ 90 % identity to AF071788 *and* ≥ 90 % identity to one another are assumed to be the same PEPc gene and are coloured blue ( ). All other sequences are assumed to represent different *A. thaliana* PEPc genes and as such are coloured differently. The sequences are given by their accession numbers. A scale is provided to show the length of the sequences in amino acid residues.

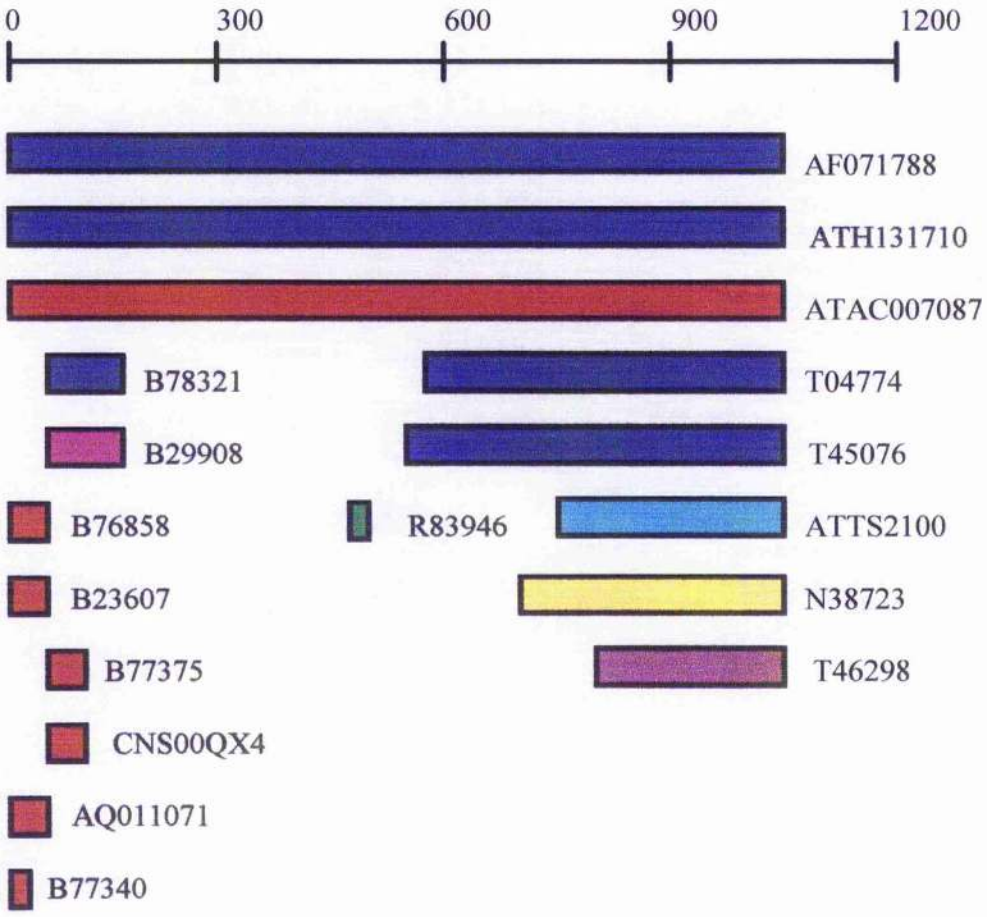


Figure 4.9 **Labelled photograph of a flowering *A. thaliana* var. Landsberg erecta plant**

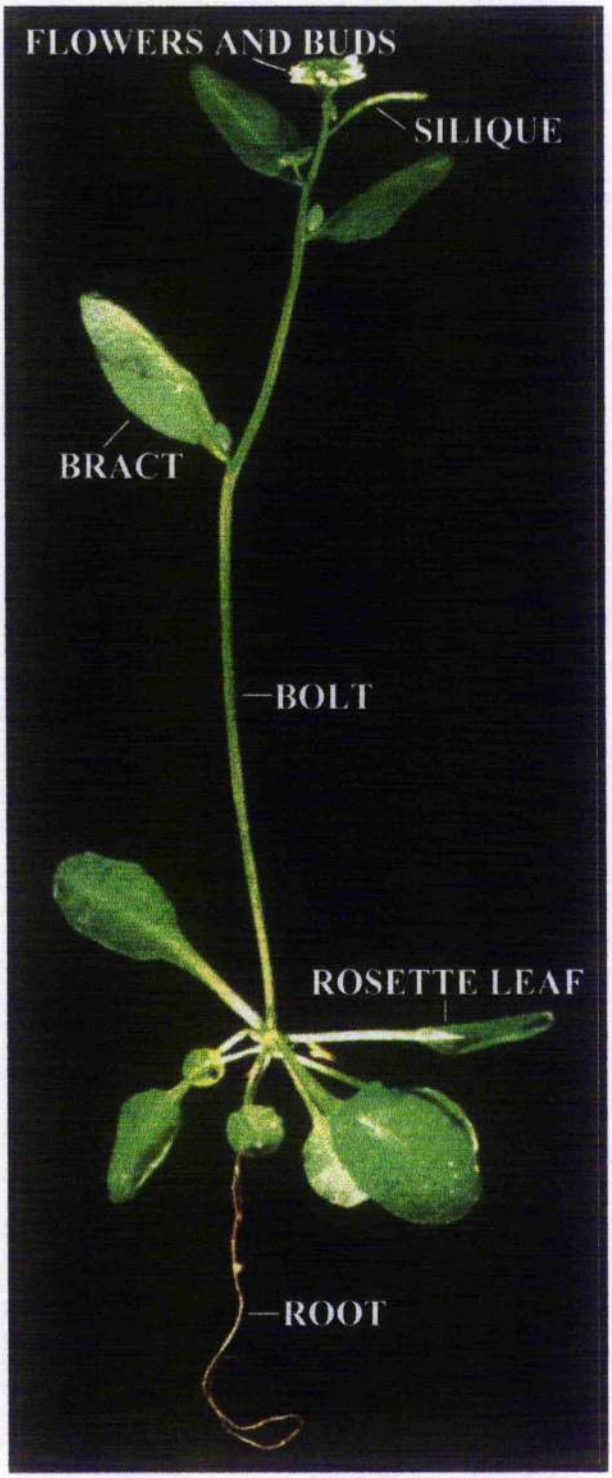
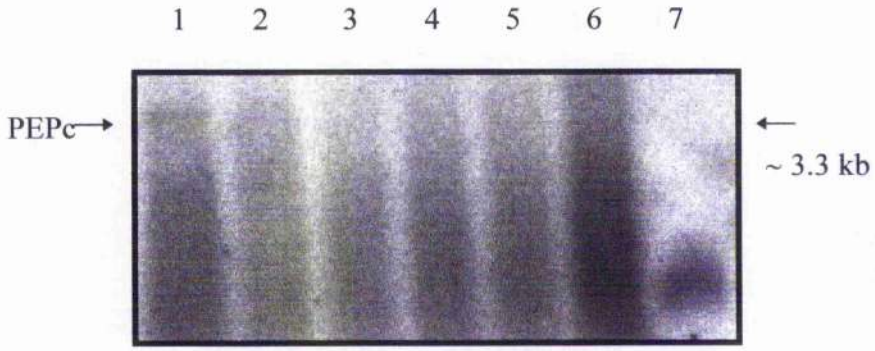


Figure 4.10 Expression of *A. thaliana* PEPc transcript in different tissues of *A. thaliana* var. Landsberg erecta

Poly A^+ RNA was isolated from different tissues harvested from *A. thaliana* var. Landsberg erecta plants. RNA samples were separated by denaturing agarose electrophoresis, immobilized on Hybond-N membrane and probed with a radiolabelled fragment of the 3'- untranslated region of *A. thaliana* PEPc cDNA. A phosphorimage of the membrane after hybridization is shown below. The samples in the lanes of the phosphorimage are as follows:

Lane 1 - roots
Lane 2 - siliques
Lane 3 - leaves
Lane 4 - bolts
Lane 5 - bracts
Lane 6 - flowers and buds
Lane 7 - *A. thaliana* PEPc kinase transcript



5' - ACCGGTTAAGTGAGTCAGTG - 3'

5' - ATTCACTGTCTCTGTTCTTG - 3'

These primers were used in a PCR with some *A. thaliana* PEPc template DNA to amplify an approximately 200 bp fragment of the PEPc. This fragment was then radiolabelled as described previously and used to probe the nylon membrane containing the *A. thaliana* tissue poly A^+ RNA.

Figure 4.10 shows the autoradiograph of the nylon membrane after probing with the radiolabelled 3'UTR PEPc fragment. There is a clear hybridization signal with the root poly A^+ RNA. There is no apparent hybridization signal with any other tissue.

root poly A^- RNA. There is no apparent hybridization signal with any other tissue.

4.2.5 Phylogenetic analysis of *A. thaliana* PEPc

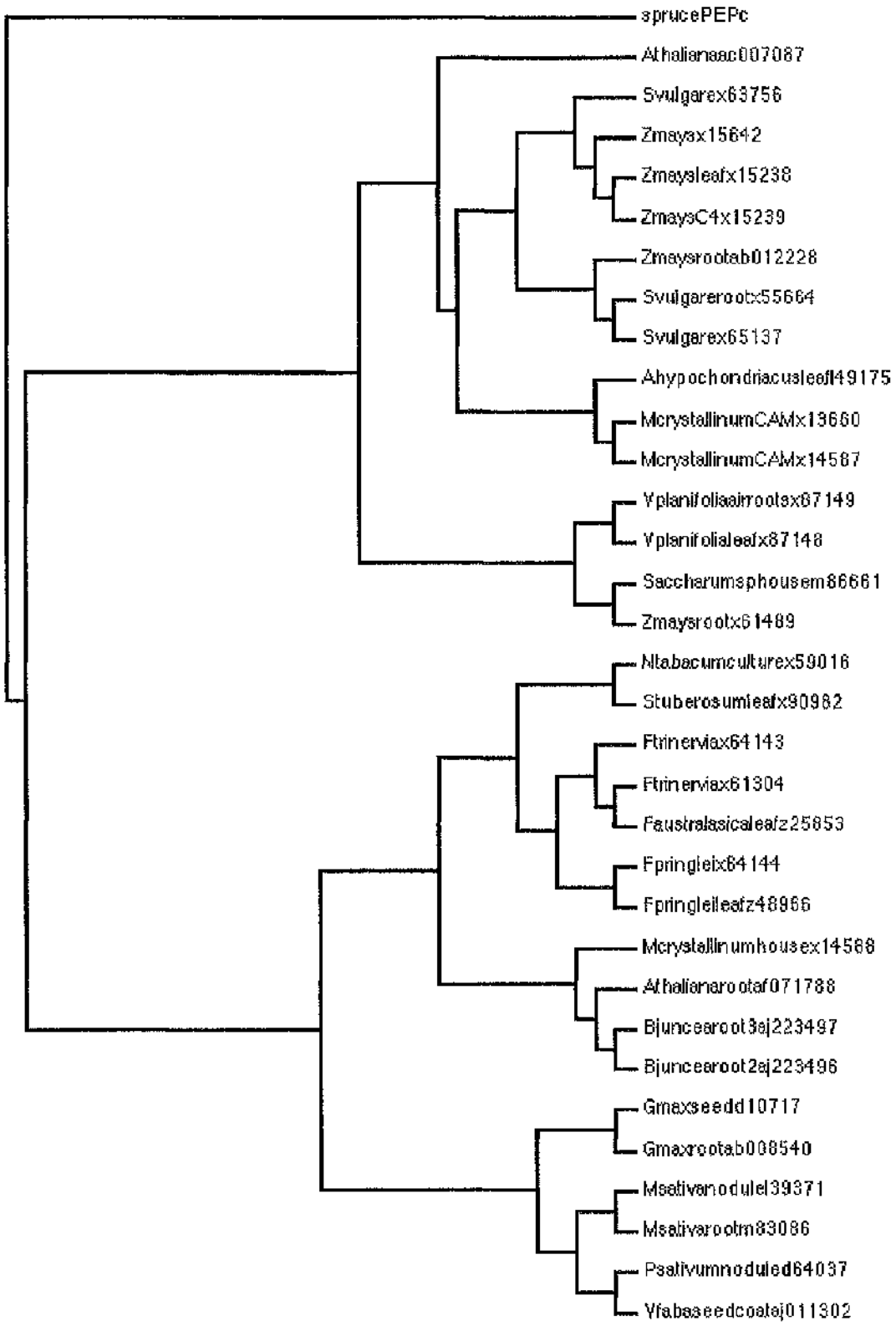
A phylogenetic analysis of the *A. thaliana* PEPc cDNA described in this chapter was carried out. PEPc nucleotide sequences from different species and tissues were aligned using Clustalx software and a phylogenetic tree produced using the Neighbour-joining method. The tree was viewed using Treeview software. A PEPc sequence from the gymnosperm *P. abies* (spruce) was specified as the root of the tree based on the assumption that angiosperms diverged from gymnosperms at some point in evolution. The phylogenetic tree is shown in Figure 4.11. It is difficult to draw any conclusions from this analysis. However, the information is consistent with previous data suggesting that the *A. thaliana* PEPc discussed in this chapter is different from the PEPc contained within the *A. thaliana* chromosome 2 BAC (section 4.2.3) and with the northern blot data presented in Figure 4.10 which suggests that the *A. thaliana* PEPc cloned is a root isoform.

4.3 DISCUSSION

It is a generally accepted fact that the diverse functions of PEPc are attributable to the existence of different PEPc isoforms which have specific functional and regulatory properties. However, one property common to plant PEPcs is their control by phosphorylation. For example, similar to their C_4 and CAM photosynthetic counterparts (e.g. Nimmo et al., 1984; Brulfert et al., 1986; Budde and Chollet, 1986; Nimmo et al., 1987a; Weigend, 1994), non-photosynthetic PEPcs from C_3 and C_4 plants undergo

Figure 4.11 Phylogenetic tree of *A. thaliana* PEPc and various other PEPc isoforms

The data was obtained as described in section 4.2.5. The different isoforms are given by the name of the species and tissue that they were obtained from and their accession number. The accession number of spruce (*P. abies*) is x79090.


sprucePEPc
Athalianaac007087
Svulgarex63756
Zmayax15642
Zmaysleafx15238
ZmaysC4x15239
Zmaysrootab012228
Svulgarerootx55664
Svulgarex65137
Ahypochondriacusleafl49175
McrystallinumCAMx13660
McrystallinumCAMx14587
Vplanifoliaairrootax87149
Vplanifolialeafx87148
Saccharumsphousem86661
Zmaysrootx61489
Ntabacumculturex59016
Stuberosumleafx90982
Ftrinerviax64143
Ftrinerviax61304
Faustralasicaleafz25853
Fpringleix64144
Fpringleileafz48966
Mcrystallinumhousex14588
Athalianarootaf071788
Bjuncearoot3aj223497
Bjuncearoot2aj223496
Gmaxseedd10717
Gmaxrootab008540
Msativanodulel39371
Msativarootm83086
Psativumnoduled64037
Vfabaseedcoataj011302

regulatory phosphorylation e.g. wheat (Champigny and Foyer, 1992), tobacco leaves (Wang and Chollet, 1993), soybean root nodules (Schuller and Verner, 1993), *Vicia* guard cells (Schnabl et al., 1992) and *Sorghum* roots (Pacquit et al., 1993). PEPc kinase has been partially purified from tobacco leaves (Wang and Chollet, 1993) and detected in several different C_3 tissues (e.g. Schuller and Verner, 1993; Pacquit et al., 1993) including *A. thaliana* leaf, root, bracts, bolts, flower and bud (see Chapter 5). These observations, together with the strict conservation of an N-terminal phosphorylation site in all known plant PEPc sequnces, support the hypothesis that this type of reversible covalent control is a general regulatory feature of the different higher plant PEPcs.

The first full-length PEPc cDNA from the C_3 species *A. thaliana*, cloned and sequenced as described in this chapter, contains the phosphorylation motif SIDAQ, with serine[11] as the phosphorylated residue (Figure 4.4). Previous alignments of deduced PEPc amino acid sequences have revealed several highly conserved residues and motifs other than the phosphorylation site. These likely contribute to the domains involved in the active site and/or regulation of the enzyme (Lepiniec et al., 1994; Toh et al., 1994). The importance of some of these conserved sites has been confirmed by site-directed mutagenesis (Duff et al., 1995; Wang et al., 1992) and chemical modification studies which have also helped to relate the primary structure of the enzyme to its function. Recently, the structure of PEPc from *E.coli* was determined (Kai et al., 1999) and this information will greatly advance understanding of the structure/function relationship of the enzyme. In general, the C-terminal half of the ~110kD PEPc polypeptide contains most of these presumed active-site determinants, whereas the N-terminal half appears to include the motifs that are regulatory in nature (Jiao and Chollet, 1990; Jiao et al., 1990; Terada et al., 1990; Toh et al., 1994). The histidine residue of the amino acid motif VLTAHPT (Figure 4.5, amino acid residue no. 176 - 182) is essential for the carboxylation activity of PEPc (Terada et al., 1992). The highly conserved sequence QqVMvGYSDSgKDaG (Figure 4.5, amino acid residue no. 602 - 616; the residues noted with capital letters being absolutely conserved) contains the species invariant lysine residue implicated in the active site by Jiao et al. (1990) and the recent determination of the three-dimensional structure of *E. coli* PEPc confirms the involvement of this lysine in the putative active site (Kai et al., 1999).

The glycine-rich motif FHGRGGtvGRGGgP (Figure 4.5 no. 645 - 658) has been proposed to be part of the substrate binding site (Terada et al., 1992). The second arginine residue (R) interacts with aspartate which is known to inhibit *E. coli* PEPc (Kai et al., 1999). In the active enzyme this region probably folds over the active site i.e. the inhibitor site is quite close to the active site. Toh et al. (1994) postulated that this region is a phosphate binding site after comparing it with the conserved region in the PEPcs of *E.coli* and *A. nidulans* which were shown to share some common features with known nucleotide- or phosphate-binding motifs. Such motifs construct flexible loops containing conserved Gly residues. In addition, the motifs contain conservative positively charged residues to interact with phosphate. The conserved region of PEPc referred to contains two-fold short repeats, Gly-Arg-Gly-Gly (Figure 4.5 no. 647 - 650 and 653 - 656). The pattern of the conservative sequence required to construct a phosphate-binding flexible loop in PEPc is therefore present and as the reaction catalysed by PEPc includes liberation of phosphate from phosphoenolpyruvate, it is quite plausible that PEPc may contain a phosphate-binding motif in its primary structure.

Of the preserved areas of unknown function, the conservation of the carboxy- terminus with regard to length as well as sequence should be noticed. The C-terminal sequence motif LTMKGIAAGMQNTG (Figure 4.5 no. 967 - 980) exists slightly modified in the carboxy-terminus of all plant PEPcs and the PEPcs of several bacteria (Relle and Wild, 1996 and references therein). It seems to be an essential component serving the stability of the enzyme (Izui et al., 1986). The binding sites of allosteric effectors have not been identified yet (Toh et al., 1994).

Further insight into the structure/function relationships of PEPc must await continued mutagenesis of these and other highly conserved domains and, most importantly, high resolution crystallographic analysis of the plant and microbial proteins.

Consistent with the enzyme's functional diversity, small multigene families of PEPc have been found (see Lepiniec et al., 1993 and references therein). *A. thaliana* has previously been suggested to have a PEPc gene family (Newman et al., 1994). A search and analysis of an *A. thaliana* sequence database with the *A. thaliana* PEPc cDNA clone described in this chapter, suggested that there are at least 7 *A. thaliana* PEPc sequences (section 4.2.3 and Figure 4.8). The Southern analysis of *A. thaliana* genomic DNA digested with

a variety of restriction enzymes would also give some indication of the number of PEPc genes.

As has been reported previously for some C_4- and C_3-type genes, the typical TATA or CAAT box sequences, a common feature of eukaryotic genes, are not found in the 5'-flanking sequence of the *A. thaliana* PEPc sequence presented here. This is in contrast to the data of Lepiniec et al., (1993) who reported a typical CAAT box and TATA box in *Sorghum* PEPc. Neither does the *A. thaliana* PEPc sequence contain evidence of the multiple polyadenylation sites reported in the 3'-UTR of some other PEPc sequences (Crétin et al., 1991; Matsuoka and Minami, 1989; Yanagisawa et al., 1988).

Having obtained the first full-length *A. thaliana* PEPc cDNA, it was of immediate interest to determine which isoform of PEPc had been cloned. Northern analysis of poly A^+ RNA from different *A. thaliana* plant tissues led to the hybridization of the sequence specific probe to root tissue only (Figure 4.10). This expression pattern is similar to the root-specific isoform reported in maize by Kawamura et al. (1990) and is distinct from the maize anaplerotic isoform which was constitutively expressed in green and etiolated leaves and roots (Kawamura et al., 1992). The result of phylogenetic analysis of the *A. thaliana* PEPc (Figure 4.11) is consistent with the Northern data suggesting that the PEPc cloned is a root isoform. However, it is not possible to draw any definitive conclusions regarding the origin of the clone from this analysis. Comparison of the hydropathy profile of the *A. thaliana* PEPc with those of known PEPc isoforms e.g. the root and anaplerotic maize isoforms, would highlight local structural differences or similarites in regions which may be associated with differences in catalytic and regulatory properties of the isozymes, and thus help confirm the identity of the *A. thaliana* PEPc clone.

The *A. thaliana* PEPc clone pKP42 was one of three clones of >3 kb isolated in the screening of the λPRL2 cDNA library (Figure 4.2). It would be worthwhile to check if the three clones are different PEPc isoforms as it is valuable to have more than one member of a gene family from a single plant species (e.g. Lepiniec et al., 1993) for comparisons of isoform function and regulation. *In situ* hybridization would clarify the tissue expression of each gene.

Now that clones for both a PEPc gene and PEPc kinase from *A. thaliana* are available, the over-expression of the genes is a desirable target. The purified proteins would not

only facilitate the investigation of PEPc regulation at the protein level, for example by providing a good control for kinase assays, but for the first time would enable studies of the interaction of the kinase with PEPc to be carried out. Mr J. Jardine has been trying different vectors to express *Kalanchoë fedtschenkoi* PEPc with the intention of then using any successful system for the expression of the *A. thaliana* PEPc and PEPc kinase. At present a suitable vector has still not been found.

The recent Genbank submission of an *A. thaliana* BAC containing a PEPc gene (accession no. AC007087) has revealed the presence of a PEPc gene on chromosome 2 of *A. thaliana*. However, the chromosomal location of the gene for the PEPc cDNA cloned during this research is not yet known but could be determined by restriction fragment length polymorphism (RFLP) analysis using the specific probe for the *A. thaliana* PEPc cDNA and other PEPc probes whose chromosomal location is known. In all C_3- and C_4-monocots and dicots studied so far, the various PEPc genes are not located together (Lepiniec et al., 1994). Kawamura et al. (1992) found that the genes for the C_4 and C_3 isoenzymes of PEPc in maize are located apart on different chromosomes. Chao et al. (1989), located PEPc to chromosome number 7 in the C_3 species wheat, although the RFLP analysis also gave hybridization of the *Sorghum* probe used to a number of fragments from the group 3 chromosomes, suggesting that there are also several other related sequences in wheat.

To summarise therefore, sequence analysis and comparison of the first full-length PEPc cDNA from *A. thaliana* with other PEPc sequences is consistent with the importance assigned to certain conserved sites in the regulation and activity of PEPc. The tissue expression study (Figure 4.10) and phylogenetic analysis (Figure 4.11) suggest that the isoform cloned is root-specific and a search of an *A. thaliana* sequence database indicates that the clone is only one of at least seven PEPc genes. The isolation of clones for these other genes will provide great insight into the different expression patterns of the isoforms of PEPc in the C_3 species *A. thaliana* and their regulation.

Chapter Five

CHARACTERIZATION OF PHOSPHOENOLPYRUVATE CARBOXYLASE AND ITS KINASE FROM THE C_3 SPECIES *ARABIDOPSIS THALIANA*

5.1 INTRODUCTION

The work described in Chapter 1 highlights the fact that the regulation of PEPc and its kinase in C_3 species appears to be significantly different from C_4 and CAM species. It was therefore of interest to investigate the nature and extent of PEPc regulation in C_3 species and more specifically the involvement of PEPc kinase in the regulation.

The purification and characterization of PEPc from photomixotrophically cultured green tobacco cells (Sato et al., 1988) showed the purified enzyme to display typical C_3-, as opposed to C_4-, plant type behaviour with regards to substrate affinity and substrate saturation kinetics. Furthermore, immunochemical studies suggested that the cultured cells possibly have a very similar enzyme to intact tobacco leaves. Therefore, for the purpose of this present research, a photomixotrophic culture of green *A. thaliana* cells was chosen as a source of the C_3 PEPc isoform and its kinase(s). *A. thaliana* is a system in which biochemical parameters can be defined alongside gene cloning and genetic analyses and the culture provides a simple model to study because of the undifferentiated state of the cells and the ease with which metabolic conditions can be manipulated Experiments were also carried out using *A. thaliana* plant tissue.

During the course of this work, a full length cDNA for PEPc kinase from *A. thaliana* became available. This was identified following the cloning of PEPc kinase from a *K. fedtschenkoi* cDNA library. The first full length cDNA for PEPc from *A. thaliana* was also cloned and sequenced as described in Chapter 4. These discoveries facilitated the study of the expression of both the PEPc and PEPc kinase genes, and the steady-state transcript levels of both PEPc and its kinase were examined in the culture and different tissues of *A. thaliana*. In consideration of the supposed involvement of C_3 PEPc activity with nitrogen metabolism, the effects of nitrate and sucrose concentrations on the transcript level of PEPc and PEPc kinase in the culture were also investigated.

5.2 RESULTS

5.2.1 Development of an extraction protocol for PEPc activity from *A. thaliana* cell culture

The *A. thaliana* cell culture was established and maintained as described (section 2.2.1). As the culture had not previously been assayed for PEPc activity, a method had to be developed for the extraction of the enzyme activity.

2 ml samples of 3 day old cells were spun in a microfuge and the cell pellet resuspended in 1 ml of sonication buffer (section 2.4). The cell suspension was then sonicated on ice for 30 s at an amplitude of 5 microns using a Soniprep sonicator. When a little of the sonicated sample was viewed under a light microscope it was seen that the majority of the *A. thaliana* cells had been broken open by the sonication. The sonicated cells were then microfuged and the supernatant removed by micropipetting to a clean 1.5 ml microfuge tube. The supernatant was assayed for PEPc, but very little activity, approximately 0.02 units/ml, was detected. By comparison, the same samples had approximately 0.5 units/ml of MDH activity (section 2.6.3). These values suggested that the culture was metabolically active but exhibited low levels of PEPc activity under normal growth conditions. It was therefore necessary to develop the extraction protocol to obtain samples in which the PEPc was more concentrated.

After sonication, 750 µl of sample was ultracentrifuged for 20 mins at 30,000 rpm and 4°C to remove as much cell debris as possible. The resulting supernatant was brought to 75% saturation with ground ammonium sulphate and left to precipitate on ice for 30 mins. The sample was then spun in a microfuge for 2 mins at 13,000 rpm and 4°C and the pellet resuspended in 50 µl sonication buffer.

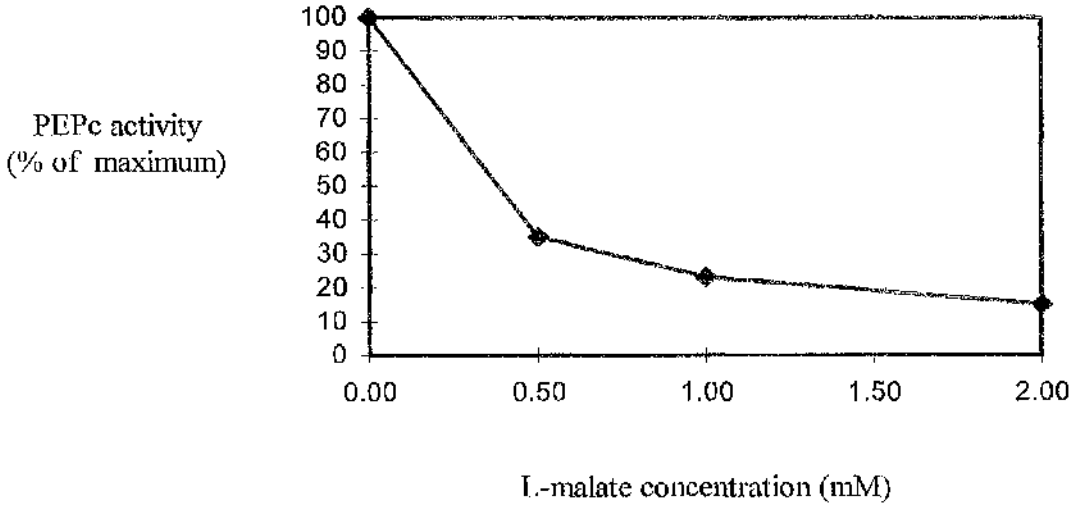
When the sample was assayed for PEPc activity, an activity of 0.3 units/ml was obtained, that is an approximately 15-fold concentration. It was now also possible to measure the malate sensitivity of the culture sample. The sensitivity of PEPc to L-malate is measured as the apparent K_i of the enzyme i.e. the inhibitor concentration which reduces the velocity of the enzyme by 50%. Figure 5.1 shows that an apparent K_i of 0.4 mM L-malate was obtained for the culture PEPc.

In subsequent experiments the sonicate was microfuged and the ammonium sulphate added without ultracentrifugation of the supernatant. The extraction protocol for PEPc activity from *A. thaliana* cell culture is summarised in section 2.5.1.

Figure 5.1 Determination of the malate sensitivity (apparent K_i) of PEPc in *A. thaliana* cell culture

Cell culture samples were prepared and assayed for PEPc activity in the presence of different concentrations of L-malate (see section 2.6.1 for assay conditions).

The graph below was used to determine the malate sensitivity or apparent K_i of the PEPc i.e. the L-malate concentration at 50% maximum PEPc activity.



The assay mixture used to measure PEPc activity in the cell culture was adapted from Van Quy et al. (1991) (section 2.4). The assay mixture was at pH 7.6 and contained 0.3 mM PEP. An extract of cell culture was made as described above and then assayed for PEPc activity at pH 7.0, 7.2, 7.4 and 7.6. The activity of PEPc detected was highest at pH 7.6 (data not shown). The malate sensitivity of PEPc from cell culture extract was then measured at pH 7.6 and 0.3 mM PEP and and at pH 8.0 and 3.0 mM PEP. The results in Table 5.1 show that higher PEPc activities were detected under "optimal" assay conditions (higher pH and PEP concentration) but the enzyme was not sensitive to malate. By comparison, under the standard assay conditions of pH 7.6 and 0.3 mM PEP, PEPc activity was slightly lower but the enzyme displayed malate sensitivity, having an apparent K_i between 0.5 and 1.0 mM L-malate.

Table 5.1 The activity and malate sensitivity of PEPc from *A. thaliana* cell culture assayed under different conditions

	PEPc activity (units/ml) pH 8.0 and 3.0 mM PEP	**PEPc activity (units/ml)** pH 7.6 and 0.3 mM PEP
0 mM L-malate	0.23	0.17
0.1 mM L-malate	0.29	0.16
0.3 mM L-malate	0.32	0.18
0.5 mM L-malate	0.28	0.10
1.0 mM L-malate	0.30	0.07

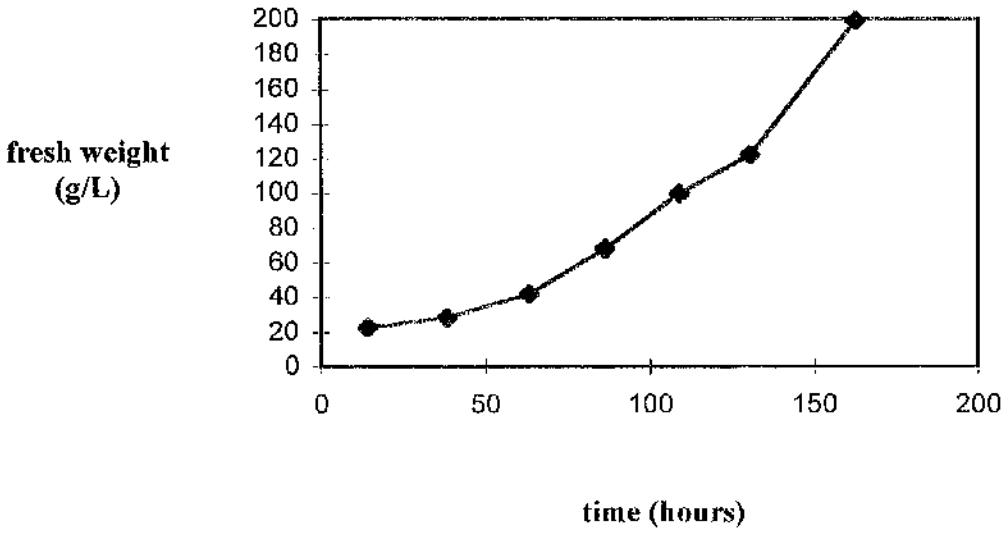
5.2.2 Characterization of *A. thaliana* cell culture growth, PEPc activity and the malate sensitivity of PEPc

The *A. thaliana* cell culture was maintained by subbing 20 ml of culture into 180 ml of fresh medium every 7 days. The growth of the culture throughout this 7 day period was recorded as the fresh weight (g/L). 2 ml of freshly subbed cells were aliquoted into twenty-one sterile 50 ml glass conical flasks each containing 18 ml of culture medium

Figure 5.2 Time course of cell growth in an *A. thaliana* cell culture

Cell culture samples were collected every day throughout the growth phase of the culture, spun down and the fresh weight measured.

The graph below shows the results obtained.



and shaken as for the 200 ml volumes of culture. Each day of the time course, duplicate flasks were taken and the contents filtered using Buchner apparatus, then weighed to measure fresh weight. Figure 5.2 shows that the growth of the cell culture was exponential during this period. Culture maintained longer than 7 days entered a stationary phase by day 10 (data not shown).

The specific activity of PEPc and its sensitivity to malate were also followed throughout the 7-day growth phase of the culture. One 50 ml flask containing 20 ml culture was taken each day and an extract prepared (section 2.5.1). 5 µl samples of the extract were used to measure the activity and malate sensitivity of PEPc (section 2.6.1). The protein concentration of samples was determined by the dye-binding assay of Bradford (section 2.12) to enable PEPc specific activity to be calculated. No clear trends were apparent for either kinetic characteristic throughout the time course (Figure 5.3). The average values obtained were a specific activity of 0.13 units/mg protein and an apparent K_i of 0.54 mM L-malate when measured under sub-optimal conditions of pH 7.6 and 0.3 mM PEP. This value for specific activity is consistent with those obtained for PEPc from other C_3 tissues e.g. 0.16 units/mg protein from tobacco cell culture (Sato et al., 1988) and 0.04 - 0.13 units/mg protein from a range of C_3 species (Gupta et al., 1994).

A desalted, ammonium sulphate precipitated extract of rosette leaves from flowering *A. thaliana* plants var. Columbia (prepared as described in section 2.5.2) was also assayed for PEPc activity and malate sensitivity. The apparent K_i for malate (0.5 mM) obtained from the leaf extract was comparable to that obtained from the *A. thaliana* cell culture.

5.2.3 Manipulation of the malate sensitivity of PEPc in *A. thaliana*: effects of the protein phosphatase inhibitor cantharidin and light

The malate sensitivity of PEPc is a qualitative measure of the *in vivo* phosphorylation state of PEPc. It is therefore possible to assess whether particular treatments affect the phosphorylation state of PEPc by monitoring the apparent K_i for malate.

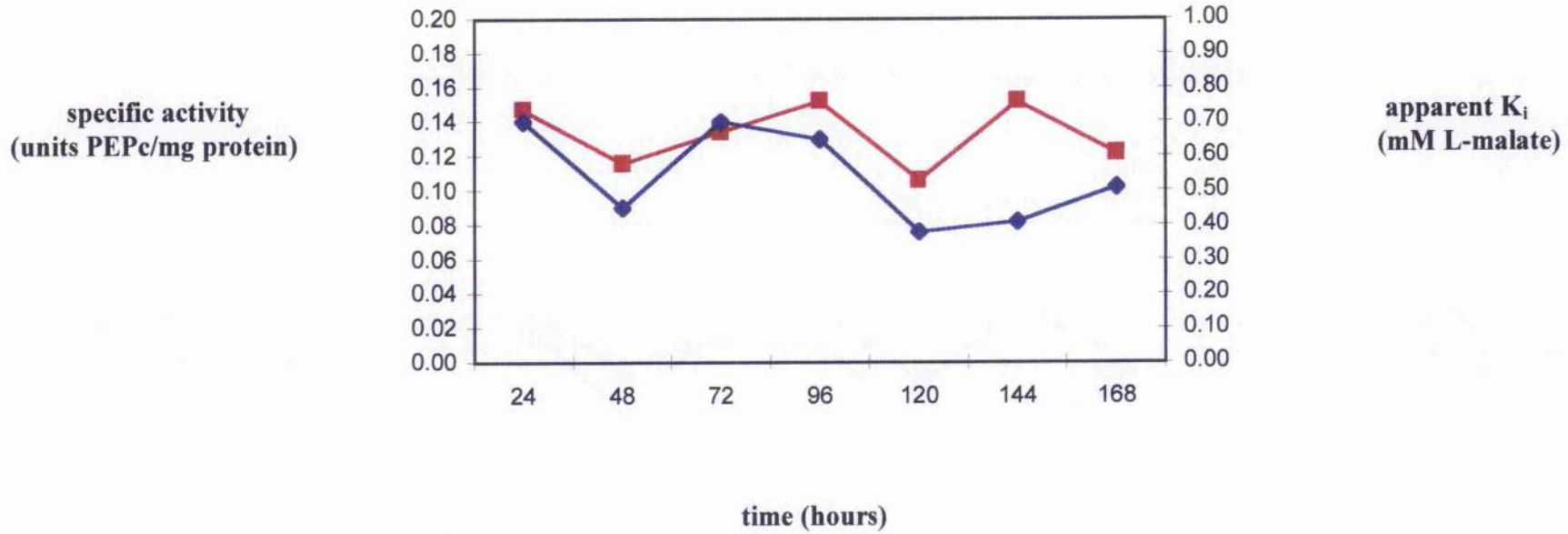
To investigate whether the cell culture contained the machinery to phosphorylate PEPc, the apparent K_i of PEPc for L-malate was assayed after incubation of the cells with cantharidin which is an inhibitor of protein phosphatase type 1 and 2A activity (MacKintosh and MacKintosh, 1994; Smith and Walker, 1996). It should therefore

Figure 5.3 The specific activity and malate sensitivity of PEPc in *A. thaliana* cell culture

Cell culture samples were collected every day throughout the growth phase of the culture and assayed for the specific activity and malate sensitivity of PEPc.

The graph opposite shows the results obtained: specific activity (◆); apparent K_i (■).

The specific activity and malate sensitivity of PEPc in *A. thaliana* cell culture



increase the phosphorylation state of PEPc and decrease its sensitivity to feedback inhibition by L-malate.

10 ml volumes of 3 day old culture were incubated for 6 hours in 25 ml tissue culture bottles with or without 100 μM cantharidin and shaken gently at 75 rpm. The PEPc activity from the different incubation conditions was assayed in the presence and absence of 0.5 mM L-malate. The means of the results agreed to within 15% and, as shown in Figure 5.4, PEPc from cells incubated with cantharidin showed significantly less sensitivity to malate than control cells. The cantharidin was dissolved in ethanol, so as a further control some cells had been incubated in ethanol but this did not appear to affect the malate sensitivity of PEPc.

Since a pharmacological agent had been effective in manipulating the malate sensitivity of the culture PEPc, the potential of light, an environmental factor, to alter the malate sensitivity of the culture PEPc was also investigated. 10 ml volumes of 3 day old cell culture were aliquoted into 25 ml tissue culture bottles and shaken for 24 hours at 75 rpm under normal temperature and light intensity growth conditions for culture. Some of the tissue culture bottles were wrapped in aluminium foil so that the cells were shaken in the dark. After 24 hours, extracts of the light- and dark- incubated culture samples were prepared and assayed for the malate sensitivity of PEPc. The light and dark samples had apparent K_i values of approximately 0.5 mM and 0.7 mM L-malate respectively, that is, there appeared to be no significant difference in the malate sensitivity of the PEPc from the two treatments (Figure 5.5).

Detached leaves of *A. thaliana* var. Columbia were also used to study the effect of light on the malate sensitivity of C_3 PEPc. Flowering plants grown under a 16 hour photoperiod (50 μmoles/m^2/s white light) at 20°C were transferred to a higher intensity white light (500 μmoles/m^2/s) and 27°C growth regime at the beginning of a 12 hour photoperiod. Leaves were detached 8 hours into the dark/18°C period (dark samples) and some were put into water and incubated for a further 4 hours darkness followed by 3 hours in the light (light samples). Desalted, ammonium sulphate precipitated extracts of the leaves were then prepared and assayed for the malate sensitivity of PEPc. No appreciable difference in malate sensitivity was observed between the light and dark samples (Figure 5.5). Therefore, consistent with the results using the cell culture, no evidence was found to suggest light/dark changes in the malate sensitivity of *A. thaliana*

Figure 5.4 The effect of the phosphatase inhibitor cantharidin on the malate sensitivity of PEPc

Cell culture samples were incubated in either ethanol or 100 μM cantharidin. The samples were then assayed for sensitivity to 0.5 mM L-malate expressed as a percentage of PEPc activity from control samples from the same incubation conditions assayed with and without L-malate.

The graph below shows the results obtained.

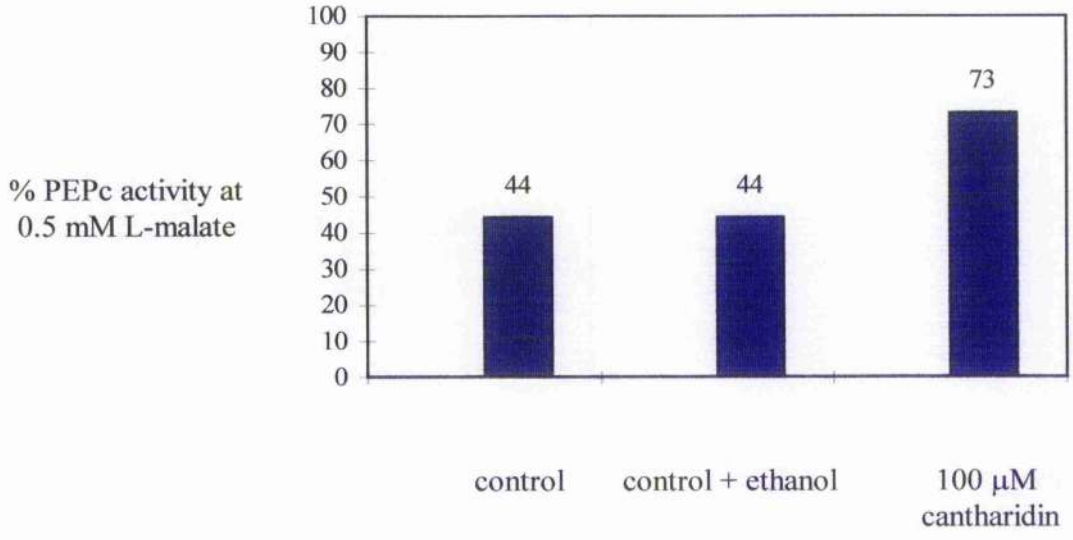
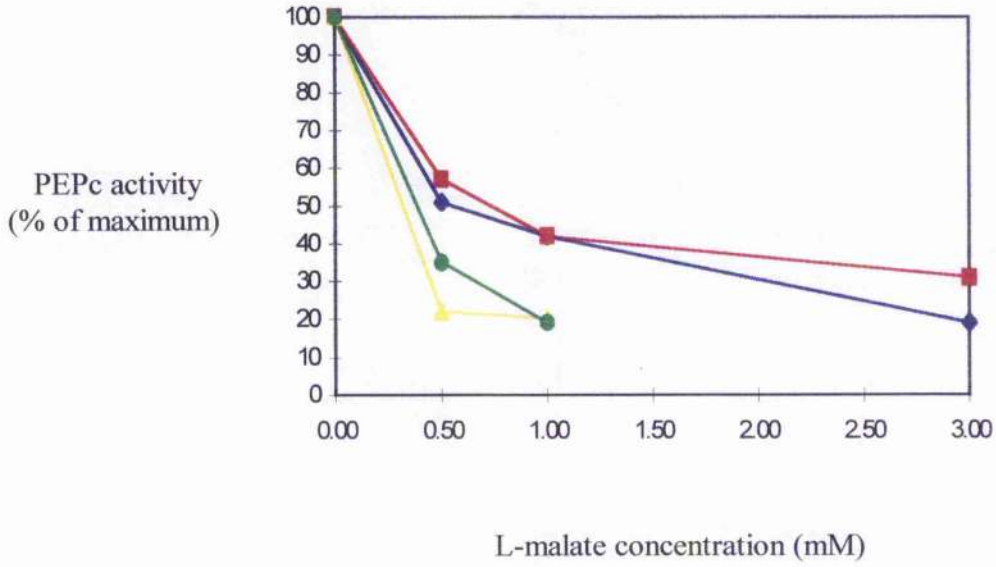


Figure 5.5 The effect of light and dark on the PEPc malate sensitivity in *A. thaliana* cell culture and leaves

3-day cell culture samples were incubated in the light (20 μmoles/m^2/s, 20°C) or dark for 24 hours and then the malate sensitivity of PEPc was assayed. Leaves were detached from flowering *A. thaliana* plants 8 hours into a 12 hour dark period (18°C). Some detached leaves were then assayed for the malate sensitivity of dark-treated samples. Other leaves were incubated in water for the remainder of the dark period and were then collected and assayed for malate sensitivity 3 hours into a subsequent 12 hour light period (500 μmoles/m^2/s, 27°C).

The graph below shows the results obtained: light-treated culture (◆); dark-treated culture (■); light-treated leaves (▲); dark-treated leaves (●).



PEPc from culture or leaves. The increases in light intensity and temperature were used because other plant species demonstrating a light enhancement of PEPc activity had been grown under similar conditions (e.g. Jiao and Chollet, 1988). However, it is not impossible that the dramatic change in light intensity and temperature stressed the leaves of the *A. thaliana* plants thus preventing the effect of the light/dark treatment alone to be studied.

5.2.4 PEPc and PEPc kinase transcript levels in *A. thaliana* cell culture and plant tissue

The failure to detect clear changes in the malate sensitivity of PEPc raised questions about the occurrence of phosphorylation of the protein in *A. thaliana*, at least under the experimental conditions employed here. The isolation of the *A. thaliana* PEPc kinase cDNA (see 5.1) meant that it was possible to test whether the PEPc kinase gene was expressed. Northern analysis of total RNA from the culture and leaf tissue failed to detect the kinase transcript (data not shown).

The more sensitive approach of RT-PCR (see section 2.10.5) allowed the detection of the PEPc kinase transcript in the culture and 3 week-old *A. thaliana* var. Landsberg erecta plants that had been grown under continuous high white light (500 μmoles/m^2/s). Figure 5.6 is a photograph of some RT-PCR products separated on a 1% agarose gel (section 2.7.4) and visualised by ethidium bromide staining. Total RNA was isolated from culture and plant tissue (section 2.10.2(i)) and oligodT (polythymine oligonucleotide) was used to prime the reverse transcription reaction catalysed by Promega's AMV Reverse Transcriptase (RT reactions 2-5). Some transcript was also made from the *A. thaliana* PEPc kinase cDNA after excision from the plasmid pZL1 by digestion with *Hind* III and *Pst* I (Figure 5.7). This transcript was then reverse transcribed using a primer to the antisense strand of the PEPc kinase cDNA (RT1). PCR (section 2.9.5) was performed on the different reverse transcription reaction products to amplify PEPc kinase, PEPc and tubulin from the different samples using primers designed to *A. thaliana* PEPc kinase cDNA, a conserved region of PEPc (Honda et al., 1996) and α-tubulin from *Chlamydomonas reinhardtii* (Silflow et al., 1985), respectively (section 2.9.7). Plasmid and water controls were included where appropriate. Conserved PEPc kinase primers were used in reverse transcription reaction 1 and control PCR (Figure

Figure 5.6 RT-PCR of PEPc kinase, PEPc and α-tubulin steady-state transcript from *A. thaliana* culture

Total RNA was isolated from *A. thaliana* cell culture and 3-week old *A. thaliana* plants var. Landsberg erecta which had been grown under a high white light (~500 μmoles/m^2/s, 27°C) 12 hour photoperiod. The following reverse transcription (RT) reactions were performed as described in section 2.10.5:

RT1. 2 μl *A. thaliana* kinase transcript + 3.6 μl antisense conserved PEPc kinase primer

RT2. 1 μl RNA from 3% sucrose stationary culture cells + 3.6 μl oligo dT primer

RT3. 0.9 μl RNA from 3% sucrose exponential culture cells + 3.6 μl oligo dT primer

RT4. 0.75 μl RNA from 1% sucrose exponential culture cells + 3.6 μl oligo dT primer

RT5. 0.7 μl RNA from *A. thaliana* plants + 3.6 μl oligo dT primer

5 μl of each reverse transcription reaction product was then used as the template in a PCR. The products of each PCR were run out on a 1% agarose gel with 1 kb DNA ladder (outside lanes) and are shown in the photographs opposite. The reactions in the lanes of each photograph are as follows:

(A)
Lane 1 - 1 kb DNA ladder
Lane 2 - RT 1
Lane 3 - RT 2
Lane 4 - RT 3
Lane 5 - RT 4
Lane 6 - RT 5
Lane 7 - linearized *A. thaliana* PEPc kinase cDNA

A. thaliana kinase-specific primers

Lane 8 - RT 1
Lane 9 - RT 2
Lane 10 - RT 3
Lane 11 - RT 4
Lane 12 - RT 5
Lane 13 - linearized *A. thaliana* PEPc cDNA

PEPc primers

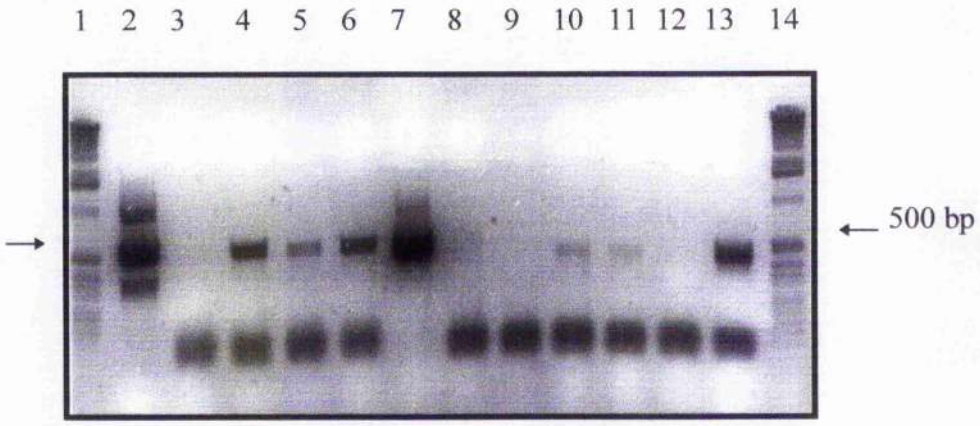
Lane 14 - 1 kb DNA ladder

(B)
Lane 1 - 1 kb DNA ladder
Lane 2 - RT 1 + conserved PEPc kinase primers
Lane 3 - water + conserved PEPc kinase primers
Lane 4 - water + *A. thaliana* specific PEPc kinase primers
Lane 5 - water + PEPc primers
Lane 6 - RT 1
Lane 7 - RT 2
Lane 8 - RT 3
Lane 9 - RT 4
Lane 10 - RT 5
Lane 11 - water

α-tubulin primers

Lane 12 - 1 kb DNA ladder

A



B

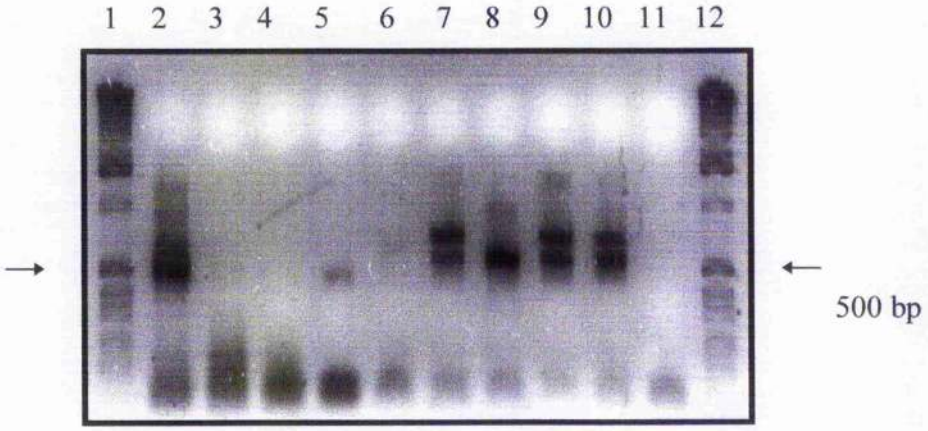
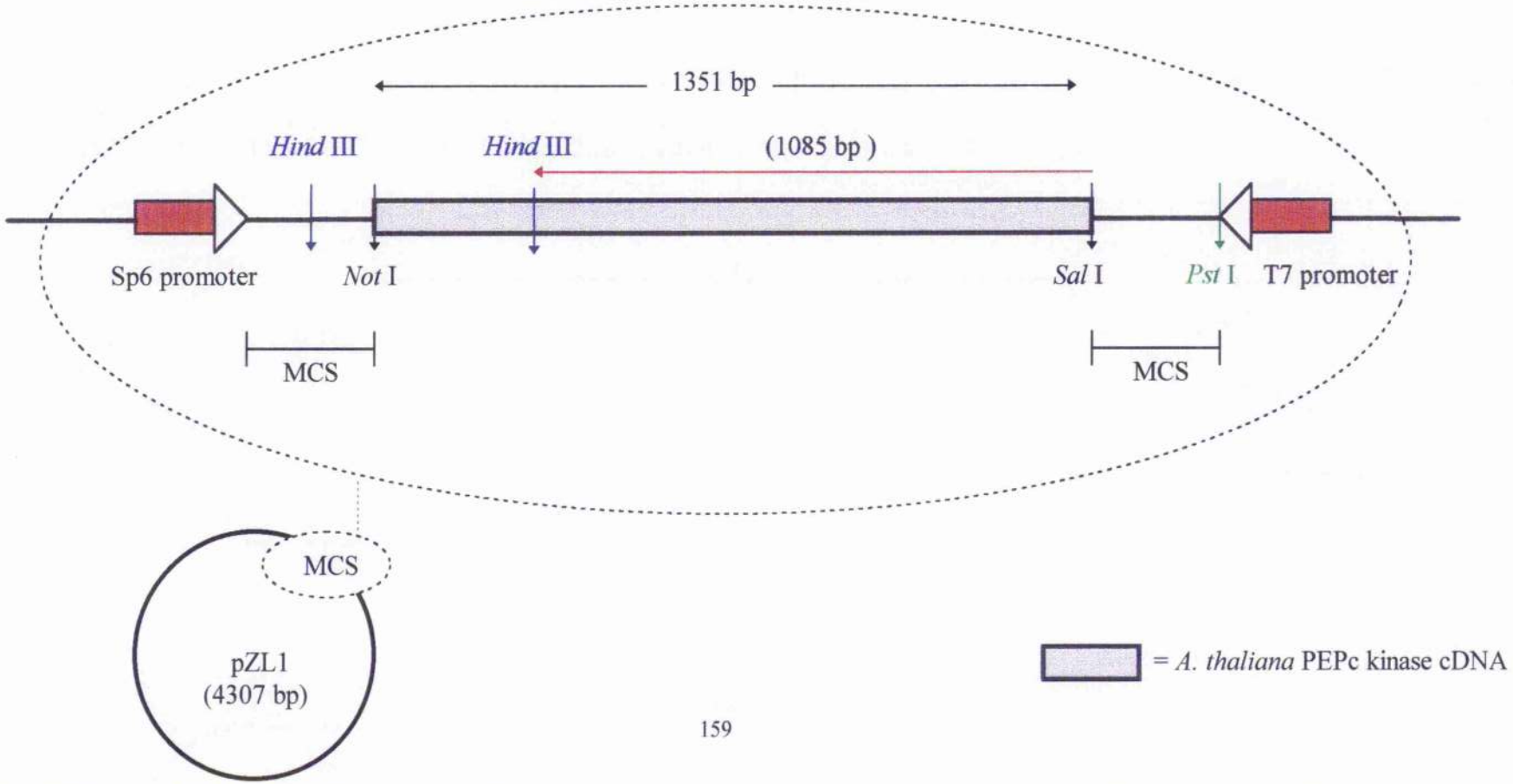


Figure 5.7 Diagram of the *A. thaliana* PEPc kinase EST in plasmid pZL1

Plasmid pZL1 is represented in a simple diagram at the bottom left hand corner of the figure. The main figure is a more detailed view of the multiple cloning site (MCS) of pZL1 showing the *A. thaliana* PEPc kinase cDNA insert, the Sp6 and T7 promoters and important restriction sites. *In vitro* transcription of the cDNA was from the T7 promoter (section 2.10.6) and is represented by a thin red arrow (←).



5.6) because they had previously successfully primed the reverse transcription of PEPc kinase from *A. thaliana* RNA (data not shown) and the *A. thaliana* PEPc kinase-specific primers had not yet been used in any reaction. A putative PEPc kinase was amplified from the reverse transcription products of RNA from exponential cells grown in 1%- and 3%-sucrose grown *A. thaliana* culture and the *A. thaliana* leaf tissue (Figure 5.6A lanes 4-6). PCR with an annealing temperature of 55°C using the tubulin specific primers unexpectedly resulted in more than one amplification product from culture and leaf RNA RT products (Figure 5.6B lanes 7-10). Surprisingly, when the annealing temperature of the reaction was lowered to 46°C (Jackson et al., 1995), the higher molecular weight amplification product was no longer apparent (data not shown). The PEPc kinase and PEPc plasmid controls gave the expected bands (Figure 5.6A lanes 7 and 13) but the water control for the PEPc primers (Figure 5.6B lane 5) showed contamination making it impossible to interpret anything from the bands present in the products of PCR using the PEPc primers from RT reactions (Figure 5.6A lanes 8-12). Such problems were not consistently encountered but overall difficulties experienced obtaining reproducible data meant that it was not possible to quantify transcript levels of PEPc or PEPc kinase using RT-PCR.

Northern analysis of poly A^+ RNA (sections 2.8 and 2.10) was therefore employed, initially to detect the transcripts of both PEPc and PEPc kinase in the culture, and then to investigate the level of the transcripts in *A. thaliana* plant tissue. The plant tissue was harvested as described in Chapter 4 and section 2.2.2(i).

Total RNA was isolated from 1-2 g of tissue by the method described in section 2.10.2(i) and from this poly A^+ RNA was isolated using Promega's Poly ATract kit (section 2.10.3). Poly A^+ RNA was also isolated by the same methods from approximately 950 μg of total RNA from 4-day old *A. thaliana* cell culture grown in 3% sucrose (a gift from O. Oswald).

Poly A^+ RNA samples were separated by denaturing agarose gel electrophoresis and the samples then transferred to Hybond-N membranes (sections 2.7.5 and 2.8.1).

The *A. thaliana* PEPc kinase cDNA (Hartwell et al., submitted) was cut out of the plasmid pZL1 with *Hind* III and *Pst* I to give a fragment of PEPc kinase cDNA to be radiolabelled for use as a probe. Figure 5.7 is a diagram of the plasmid pZL1 with the PEPc kinase cDNA insert. Primers to the 3'-untranslated region (3'-UTR) of the *A.*

thaliana PEPc clone (Chapter 4 and section 2.9.7)) were used in a PCR to amplify an approximately 200 bp fragment from *A. thaliana* PEPc cDNA (section 4.2.4.). An approximately 400 bp fragment of coding sequence conserved between different PEPc genes was also isolated from *A. thaliana* PEPc cDNA (section 2.9.7).

These three cDNA fragments and a fragment of an α-tubulin gene from *Chlamydomonas reinhardtii* (Silflow et al., 1985) (a gift from H. Wade) were radiolabelled (section 2.8.2 and 2.8.3) and used to probe the nylon membranes (section 2.8.4) containing poly A^+ RNA from *A. thaliana* tissue and culture. The specific activity of each probe was $1.14x10^9$ dpm/μg or greater (section 2.8.3).

Phosphorimages (section 2.7.3) of the membranes containing the tissue and culture poly A^+ RNA after hybridization with the four different radiolabelled probes are shown in Figures 5.8 and 5.9 respectively. A hybridization signal was seen in the culture poly A^+ RNA with all four probes (Figure 5.9). A clear hybridization signal was apparent in all the plant tissues with the PEPc kinase probe (Figure 5.8). As mentioned already, the PEPc kinase cDNA was excised from the plasmid pZL1 by digestion with *Hind* III and *Pst* I, restriction enzymes with sites in the multiple cloning site of plasmid pZL1 (section 2.10.6). Since there is also a *Hind* III site in the 3'UTR of the kinase cDNA, the kinase transcript run off from this is smaller (<300 bp smaller) than the *in vivo* transcript. The hybridization signals of kinase with plant tissue in Figure 5.8C would confirm this.

Hybridization with the PEPc 3'UTR probe was seen in root tissue (Figure 5.9A lane 1). The conserved PEPc sequence probe only hybridized to poly A^+ RNA from the root and flower/bud tissue and culture (Figure 5.8B lanes 1 and 6; Figure 5.9B lane 2). The tubulin probe (Figure 5.8D) was used as a constitutive control to determine the relative loading of poly A^+ RNA for each tissue. The intensity of the tubulin hybridization signal with each tissue was then used to standardize the level of the other three transcripts in each tissue.

5.2.5 The effect of sucrose and nitrate on the transcript levels of PEPc and PEPc kinase in *A. thaliana* cell culture.

A. thaliana cell culture was grown in 3% or 1% sucrose. After 3 days, 555 ml of each culture was transferred to sterile centrifuge bottles and spun at 5000 rpm in a Beckman centrifuge using a JA-14 rotor for 10 mins at 20°C. The pellets of both cultures were

Figure 5.8 Northern blot analysis of PEPc and PEPc kinase steady-state transcript levels in *A. thaliana* tissue

Poly A^+ RNA was isolated from different tissues harvested from *A. thaliana* var. Landsberg erecta plants. RNA samples were separated by denaturing agarose electrophoresis, immobilized on Hybond-N membrane and probed with radiolabelled fragments as indicated below. Phosphorimages of the membrane after each hybridization are shown opposite. The intensity of the α-tubulin signal for each lane was used to standardize all other signals for RNA loading. Where relevant, the corrected intensities of bands relative to the most intense band for each probe is shown below the phosphorimages. The samples in the lanes of each phosphorimage are as follows:
Lane 1 - roots
Lane 2 - siliques
Lane 3 - leaves
Lane 4 - bolts
Lane 5 - bracts
Lane 6 - flowers and buds
Lane 7 - *A. thaliana* PEPc kinase transcript

(A) Phosphorimage of membrane after hybridization to a radiolabelled fragment of the 3'-untranslated region of *A. thaliana* PEPc.

(B) Phosphorimage of membrane after hybridization to a radiolabelled fragment of an *A. thaliana* PEPc conserved between different PEPc genes.

(C) Phosphorimage of membrane after hybridization to a radiolabelled fragment of *A. thaliana* PEPc kinase.

(D) Phosphorimage of membrane after hybridization to a radiolabelled fragment of α-tubulin.

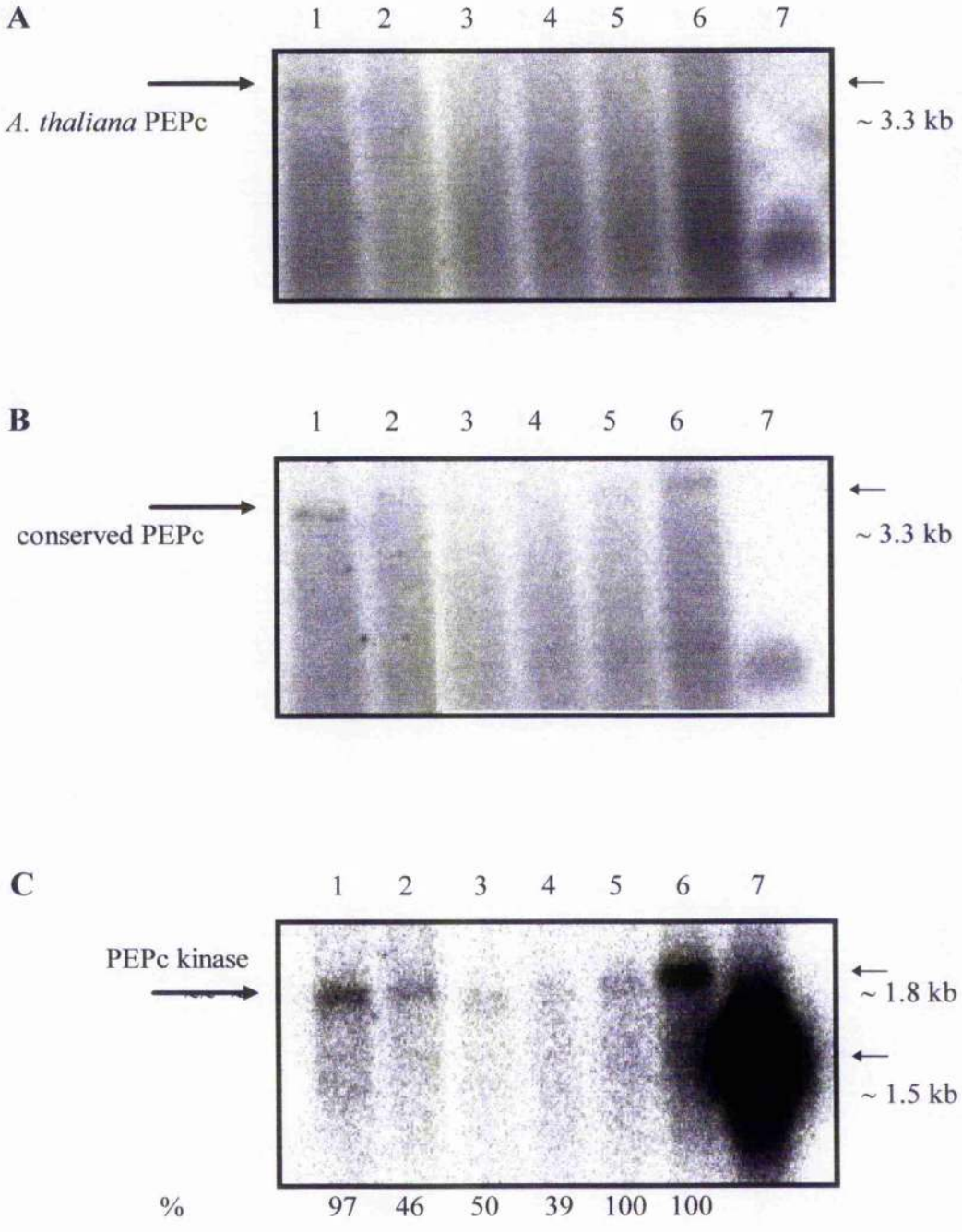

A
1 2 3 4 5 6 7
A. thaliana PEPc
~ 3.3 kb
B
1 2 3 4 5 6 7
conserved PEPc
~ 3.3 kb
C
1 2 3 4 5 6 7
PEPc kinase
~ 1.8 kb
~ 1.5 kb
%
97 46 50 39 100 100
D
1 2 3 4 5 6 7
α-tubulin
~ 1.9 kb
%
46 66 11 50 25 100

Figure 5.9 Northern blot analysis of PEPc and PEPc kinase steady-state transcript levels in *A. thaliana* culture

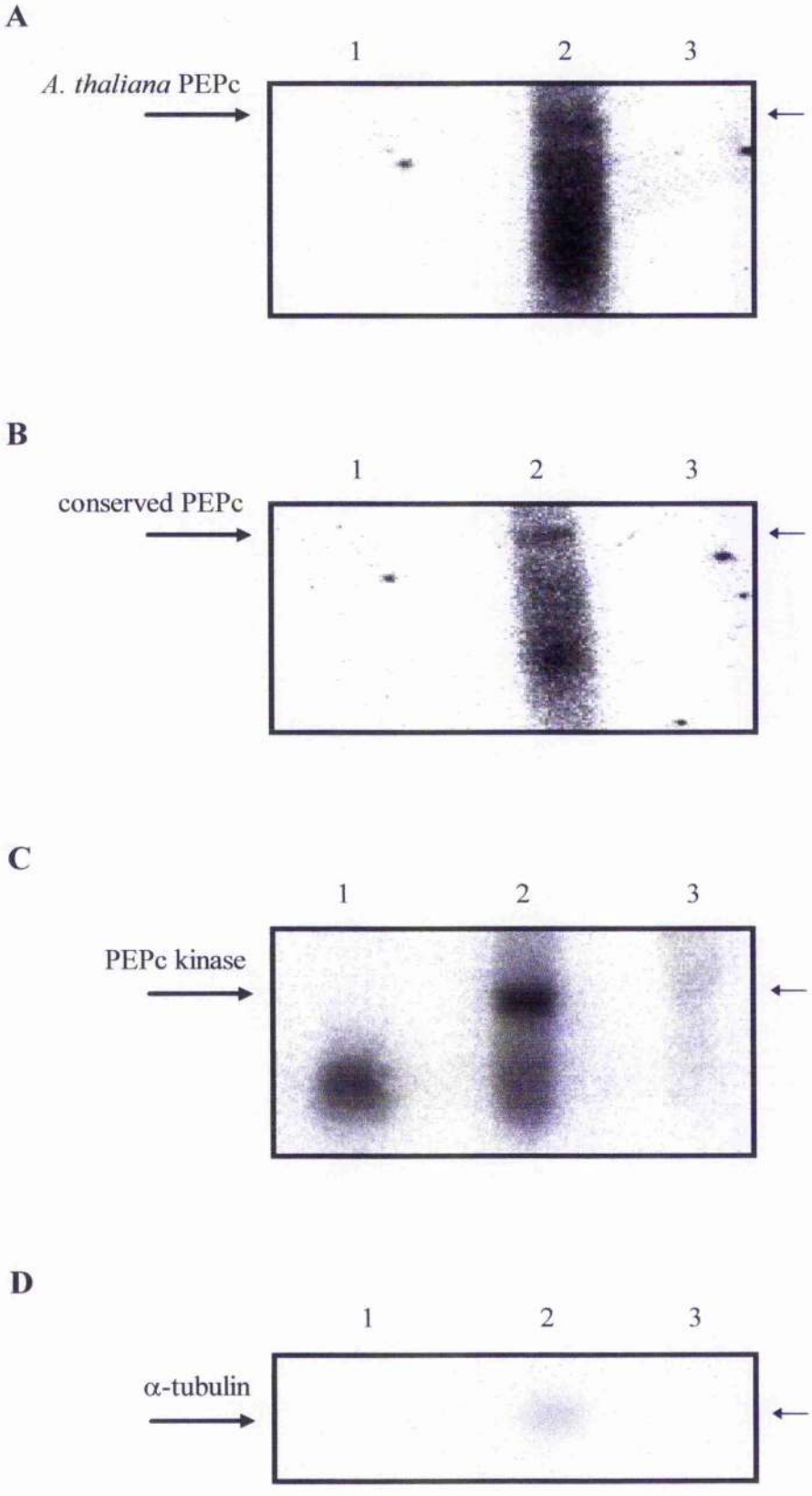
Poly A^+ RNA was isolated from *A. thaliana* cell culture. The poly A^+ RNA and supernatant (containing transfer RNA and ribosomal RNA) from the Poly ATract kit column were separated by denaturing agarose electrophoresis, immobilized on Hybond-N membrane and probed with radiolabelled fragments as indicated below. Phosphorimages of the membrane after each hybridization are shown opposite. Transcript sizes are as shown in Figure 5.8. The samples in the lanes of each phosphorimage are as follows:
Lane 1 - *A. thaliana* PEPc kinase transcript (1:1000 dilution)
Lane 2 - poly A^+ RNA
Lane 3 - Poly ATract column supernatant

(A) Phosphorimage of membrane after hybridization to a radiolabelled fragment of the 3'-untranslated region of *A. thaliana* PEPc.

(B) Phosphorimage of membrane after hybridization to a radiolabelled fragment of an *A. thaliana* PEPc conserved between different PEPc genes.

(C) Phosphorimage of membrane after hybridization to a radiolabelled fragment of *A. thaliana* PEPc kinase.

(D) Phosphorimage of membrane after hybridization to a radiolabelled fragment of α-tubulin.


A
1
2
3
A. thaliana PEPc
B
1
2
3
conserved PEPc
C
1
2
3
PEPc kinase
D
1
2
3
α-tubulin

then washed three times with MS salts made up without any nitrate, centrifuging to remove the supernatant after each wash. After the final centrifugation step, the pellets were resuspended in MS salts (section 2.4) with either 3% or 1% sucrose. 60 ml volumes of the 3% sucrose culture were then transferred to sterile 200 ml glass conical flasks containing filter sterilised NH_4NO_3/KNO_3 solution to make the medium to a final nitrate concentration of 0 mM, 0.6 mM, 6 mM or 60 mM. 100 ml volumes of the 1% sucrose culture were transferred to sterile 250 ml glass conical flasks containing sterile nitrate solution to the specified concentration. One flask from both the 3% and 1% sucrose cultures containing 60 mM nitrate was wrapped in tin foil to control for the effect of light, and then all the flasks were shaken as normal for a further 48 hours. Prior to washing the 3% sucrose culture, 60 ml had been removed as a control for washing, and, immediately following washing, 60 ml of 3% sucrose culture containing 60 mM nitrate was removed as a control for growing the culture in the smaller flasks. Both controls were immediately filtered using Buchner apparatus and Whatman 3MM filter paper and the cells snap-frozen in liquid nitrogen, then stored at -80°C until required. After incubation, all the other culture samples were harvested and stored in the same way. Total RNA was isolated from each sample (by Mr. J. Jardine) and Promega's polyAtract system was then used to obtain poly A^+ RNA from each of the samples. The poly A^+ RNA was separated using denaturing agarose gel electrophoresis and the RNA immobilised on nylon membrane as described previously. The nylon membrane was probed with the radiolabelled fragments used in section 5.2.4. The phosphorimages of the washed membrane after hybridization to each probe are shown in Figure 5.10. Hybridization of each probe to the membrane was evident and the hybridization with tubulin (Figure 5.10D) was used to standardize the level of the other three transcripts. The hybridization signals in Figure 5.10A are rather faint but this is most likely due to the nylon membrane being stripped (section 2.8.5) several times before hybridization with the 3'UTR probe. For ease of interpretation, the standardised intensity of hybridization for each sample is presented in graphical form for both the 3% sucrose and 1% sucrose cultures in Figure 5.11.

The only consistent and easily interpretable observation from these results is that the level of transcripts detected by the PEPc kinase probe and both PEPc probes was higher when the cells were grown in 1% sucrose compared with 3% sucrose. Light-treated cells

Figure 5.10 Northern blot analysis of PEPc and PEPc kinase steady-state transcript levels in *A. thaliana* culture in response to altered sucrose and nitrate levels

3-day old *A. thaliana* cell culture was washed and samples then resuspended in different media and shaken for a further 24 hours as described in section 5.2.5. Poly A^+ RNA was isolated from the *A. thaliana* cell culture after treatment. The poly A^+ RNA from the Poly ATract kit column was separated by denaturing agarose electrophoresis, immobilized on Hybond-N membrane and probed with radiolabelled fragments as indicated below. Phosphorimages of the membrane after each hybridization are shown opposite. Transcript sizes are as shown in Figure 5.8. The percentage intensities of bands relative to the most intense band for each probe is shown below the phosphorimages. The intensity of the α-tubulin signal for each lane was used to standardize all other signals for RNA loading. The samples in the lanes of each phosphorimage are as follows:
Lane 1 - unwashed
Lane 2 - washed
Lane 3 - 3% sucrose + 60mM NO_3^-
Lane 4 - 3% sucrose + 60mM NO_3^- + dark
Lane 5 - 3% sucrose - NO_3^-
Lane 6 - 3% sucrose + 6mM NO_3^-
Lane 7 - 3% sucrose + 0.6mM NO_3^-
Lane 8 - 1% sucrose + 60mM NO_3^-
Lane 9 - 1% sucrose + 60mM NO_3^- + dark
Lane 10 - 1% sucrose - NO_3^-
Lane 11 - 1% sucrose + 6mM NO_3^-
Lane 12 - 1% sucrose + 0.6mM NO_3^-
Lane 13 - *A. thaliana* PEPc kinase transcript

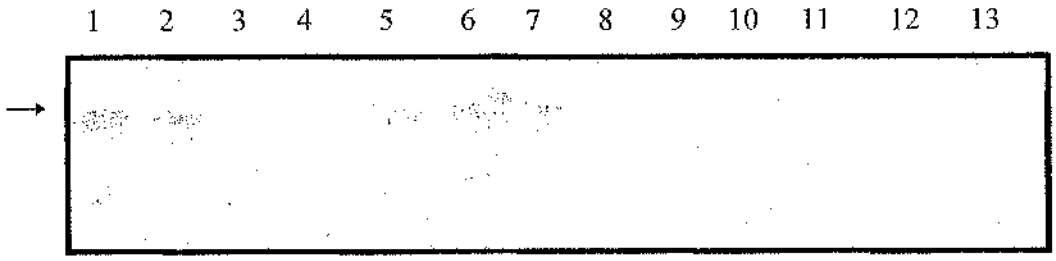
(A) Phosphorimage of membrane after hybridization to a radiolabelled fragment of the 3'-untranslated region of *A. thaliana* PEPc.

(B) Phosphorimage of membrane after hybridization to a radiolabelled fragment of an *A. thaliana* PEPc conserved between different PEPc genes.

(C) Phosphorimage of membrane after hybridization to a radiolabelled fragment of *A. thaliana* PEPc kinase.

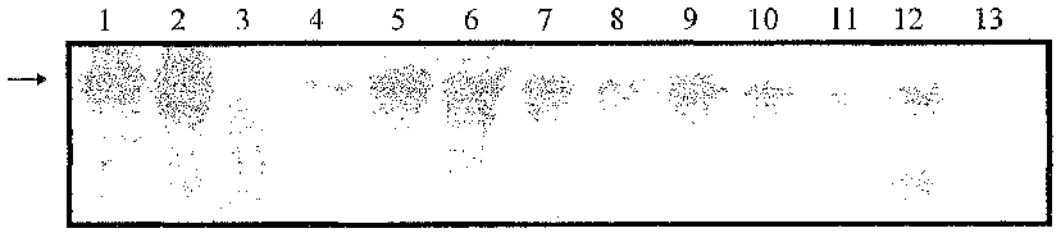
(D) Phosphorimage of membrane after hybridization to a radiolabelled fragment of α-tubulin.

A



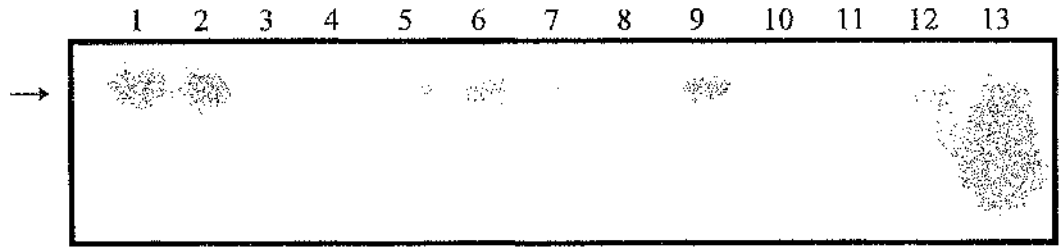
%	100	159	333	337	249	315	400	530	423	578	425	856

B



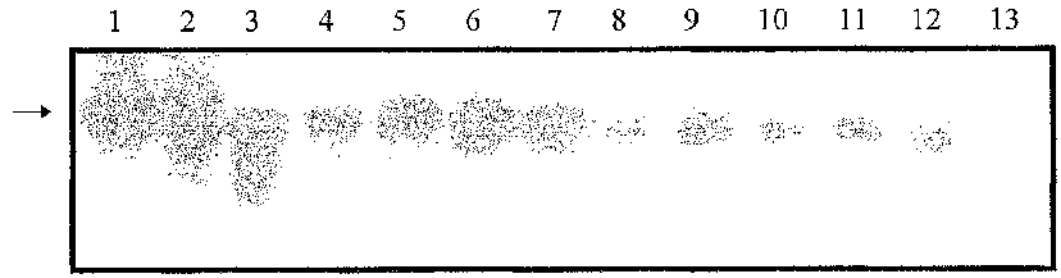
%	100	161	140	169	243	252	320	360	331	322	200	400

C



%	100	163	147	163	109	159	140	330	477	267	217	478

D



%	100	51	15	16	35	27	20	10	13	9	12	9

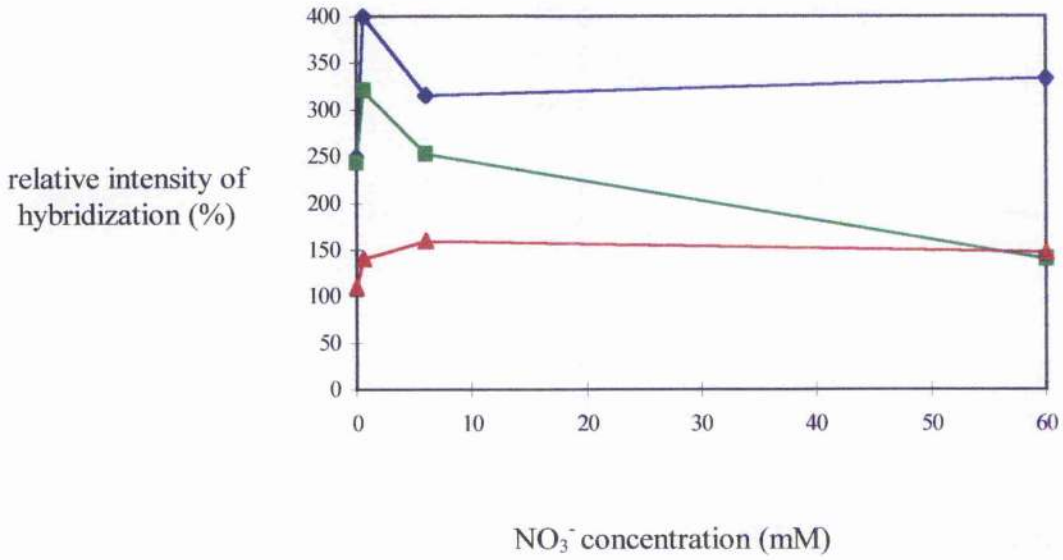
Figure 5.11 PEPc and PEPc kinase steady-state transcript levels in *A. thaliana* culture in response to altered sucrose and nitrate levels

The data shown in Figure 5.10 as northern blot analysis is displayed opposite in graphical form. Unwashed cells showed the highest level of each transcript, including the control α-tubulin, and so were used as a control for relative intensity of hybridization (100%). The different transcripts are represented thus: *A. thaliana* PEPc (◆); conserved PEPc (■); *A. thaliana* PEPc kinase (▲).

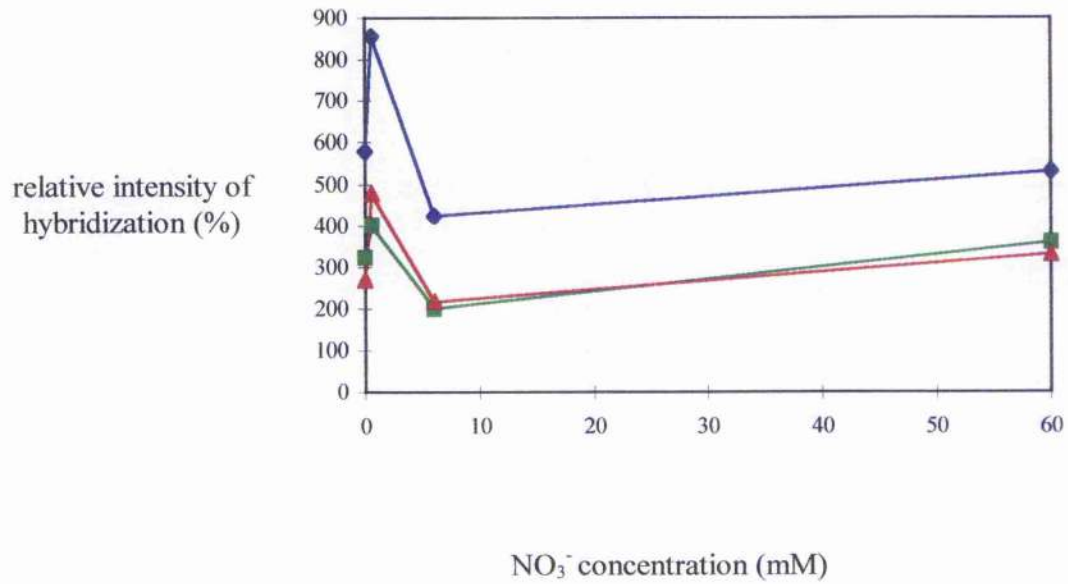
(A) The effect of nitrate on the transcript level of PEPc and PEPc kinase in 3% sucrose-grown *A. thaliana* culture.

(B) The effect of nitrate on the transcript level of PEPc and PEPc kinase in 1% sucrose-grown *A. thaliana* culture.

A **The effect of nitrate on transcript level of PEPc and PEPc kinase in 3% sucrose-grown *A. thaliana* culture**



B **The effect of nitrate on the transcript level of PEPc and PEPc kinase in 1% sucrose-grown *A. thaliana* culture**



grown in 1% sucrose had a higher level of the *A. thaliana* PEPc transcript than dark-treated cells. In contrast, the PEPc kinase transcript level in 1% sucrose-grown cells was higher with the dark treatment.

The introduction of nitrate at 0.6 mM concentration to the culture medium, supplemented with either 1% or 3% sucrose, increased the transcript level of PEPc and PEPc kinase (Figure 5.10A, B and C lanes 5, 7, 10 and 12). In 1% sucrose-grown cells, all three transcript levels then fell below the level in the absence of nitrate as the concentration was increased to 6 mM nitrate. The levels then increased again as the nitrate concentration was increased to 60 mM. Likewise, in 3% sucrose-grown cells, the *A. thaliana*-specific PEPc transcript level initially rose, then declined only to rise again as the nitrate concentration increased. The conserved PEPc transcript also initially rose with the introduction of 0.6 mM nitrate to the culture medium but then steadily declined as the nitrate concentration decreased. The PEPc kinase transcript level increased between 0 and 6 mM nitrate but no further as the nitrate concentration was increased to 60 mM.

5.3 DISCUSSION

The first objective of the work described in this chapter was to investigate the use of the *A. thaliana* cell culture as as experimental system in which the effects of metabolite availability on PEPc and PEPc kinase could be tested. To some extent this was successful but progress was hindered by fungal contamination - a problem in other laboratories throughout the project - hence not as much progress was made in this research as had been hoped. Nevertheless some positive results did emerge. In general, the work showed that the cell culture is indeed a useful system for the study of the regulation of PEPc and PEPc kinase from a C_3 species.

The response of PEPc from various sources to malate and glucose 6-phosphate is known to be affected by assay pH (Israel and Jackson, 1982; Podesta and Andreo, 1989; Schuller et al., 1990; Wedding et al., 1990). Malate is a stronger inhibitor (Schuller et al., 1990; Wedding et al., 1990) and glucose 6-phosphate a stronger activator (Schuller et al., 1990) at pH 7.0 than at pH 8.0. The sensitivity of PEPc from the *A. thaliana* culture to the effector malate was also found to be greater at an assay pH of 7.6 than 8.0 (Table 5.1). These data indicate that pH fluctuations may be significant for the regulation of

PEPc by malate, consistent with the proposed pH stat function of soybean nodule PEPc (Israel and Jackson, 1982).

No apparent trend was evident in either the malate sensitivity or the specific activity of the culture PEPc over the 7 days of the culture growth phase (Figure 5.3). This contrasts with the results of Sato et al. (1988) who showed that the PEPc activity in a photomixotrophic culture of tobacco cells was highest during or at the onset of exponential growth. However, the comparability of the malate sensitivity of PEPc in *A. thaliana* culture with that in *A. thaliana* leaf tissue (section 5.2.2) suggests that the culture is a convenient experimental system to use for the purposes of this research.

After the characterization of the specific activity and malate sensitivity of PEPc in the culture, the potential to manipulate the malate sensitivity of PEPc from both culture and leaf tissue was investigated as a tool by which to elucidate some of the factors regulating C_3 PEPc. The result of the cantharidin experiment (section 5.2.3) is consistent with the view that reversible phosphorylation is the means by which the C_3 isoform of PEPc, like the C_4 and CAM isoforms, modulates its sensitivity to allosteric effectors. In the presence of the phosphatase inhibitor cantharidin, the PEPc will remain in its malate insensitive (high apparent K_i), phosphorylated state and so will demonstrate less inhibition by malate. However, there is no evidence that light can cause a change in the K_i of *A. thaliana* PEPc for malate (section 5.2.3). Fluorescence studies on the culture have shown that the cells' photosystems I and II have photosynthetic capacity (Dr. PJ Dominy, personal communication). It is therefore possible that the high photosynthate (3% sucrose) in the culture medium inhibits the photosynthetic activity of the cells. Further information might be obtained by repeating the study of the light effect on culture PEPc using cells washed with medium to remove sucrose and then resuspended in medium containing 3% sucrose or 1.6% mannitol. Mannitol is not metabolized and so at this concentration would act only to maintain the osmotic pressure of the cells, thus controlling for any involvement of photosynthate in a light effect on the malate sensitivity of PEPc.

No effect of light on the malate sensitivity of PEPc from the cell culture was seen (section 5.2.3) suggesting that the *A. thaliana* culture is not a good model for studying leaf PEPc. In contrast, barley leaf protoplasts were found to be a suitable model for the demonstration of light activation of C_3 PEPc (Lillo et al., 1995), resulting in a reduction

in the enzyme's sensitivity to the inhibitor L-malate (Smith et al., 1996). However, PEPc kinase activity increased with time after isolation of the barley leaf protoplasts in both light and darkness, although more so in the light than in the dark. The increase in kinase activity in dark-incubated protoplasts contrasts with the increase in malate sensitivity of PEPc over the same period, and indicates that, in contrast with the situation in C_4 and CAM plants, in C_3 barley leaf protoplasts PEPc kinase activity and the malate sensitivity of PEPc are not necessarily linked (Smith et al., 1996).

The availability of full-length cDNAs for both PEPc (see Chapter 4; Cloning and Sequencing of First Full-Length cDNA for Phosphoenolpyruvate Carboxylase from *A. thaliana*) and PEPc kinase from *A. thaliana* (see section 5.1) made it possible to study expression of the enzymes using a molecular approach.

Northern analysis of poly A^+ RNA from different *A. thaliana* tissues provided information on the level of transcript for the *A. thaliana*-specific PEPc cDNA (discussed in Chapter 4), any other PEPc isoforms and *A. thaliana*-specific PEPc kinase cDNA in each tissue. A radiolabelled fragment from the 3'-untranslated region of the *A. thaliana*-specific PEPc cDNA resulted in hybridization to root tissue only (Figure 5.8A lane 1). As the probe used was specific to the sequence of that PEPc cDNA, it would appear that the cDNA cloned is a root isoform of PEPc. Phylogenetic analysis of this PEPc sequence (section 4.2.5) is consistent with this expression data. A PEPc sequence conserved between different PEPc genes was amplified from the *A. thaliana* PEPc cDNA and also used to probe the tissue poly A^+ RNA. This resulted in hybridization to root and flower and bud tissue only (Figure 5.8B lanes 1 and 6). As the sequence used is highly similar to other PEPcs, it would appear that PEPc isoforms are simply not detectable at the transcript level in silique, leaf, bract and bolt tissue. In contrast, the *A. thaliana* PEPc kinase probe gave a hybridization signal in all plant tissues (Figure 5.8C). These observations of the two enzymes at the transcript level are consistent with the knowledge that PEPc kinase protein turns over very rapidly (Nimmo, 1993; Hartwell et al., 1996).

Tubulin was employed as a constitutive control for the quantification of PEPc and PEPc kinase transcript levels using poly A^+ RNA northerns. It has been shown using RNA gel blot hybridizations and gene-specific probes, that the transcript levels of all fifteen tubulin genes in *A. thaliana* are decreased by 24 hours of continuous white light, although to different degrees (Leu et al., 1995). Tubulin is therefore, strictly speaking, not

constitutively expressed but, in the absence of any known constitutively expressed gene, tubulin was used for the purpose of standardizing the levels of the other transcripts examined.

A hybridization signal was also detected using both PEPc probes and the PEPc kinase probe on poly A^+ RNA from *A. thaliana* cell culture. As discussed earlier, one of the attractions of using cell culture is the ease with which metabolic conditions can be altered. The ability to measure the transcript level of both PEPc and PEPc kinase in the *A. thaliana* culture meant that the system could be used to investigate the effect of different metabolites on the transcript level of the two enzymes. PEPc is one of many genes known to be regulated by both sugars and nitrate (see Koch, 1996 for review), not suprisingly considering the central role it is assumed to have in the control of photosynthetic carbon flow between sucrose synthesis and amino acid biosynthesis (Champigny and Foyer, 1992; Huber et al., 1994). Both photosynthetic carbon metabolism and nitrogen metabolism have also been implicated in the regulation of PEPc kinase activity in C_3 species (Duff and Chollet, 1995; Li et al., 1996; Scheible et al., 1997; Wadham et al., 1996). The regulatory effect of sucrose and nitrate on PEPc and PEPc kinase transcript level was therefore studied in the *A. thaliana* cell culture.

When the sucrose concentration of the culture medium was altered, the level of transcripts detected by the PEPc kinase probe and both PEPc probes was higher when the cells were grown in 1% sucrose compared with 3% sucrose. This would suggest that high photosynthate levels cause a reduction in the level of transcript for PEPc and PEPc kinase in the culture. Considering the presumed anaplerotic role of PEPc in the C_3 cell culture, it is possible that PEPc kinase acts as a "pull" for carbon and so when less carbon is available there would be higher levels of PEPc and PEPc kinase transcript to utilise what available carbon there is for amino acid biosynthesis. There was no significant effect of light on the transcript levels in 3% sucrose grown cells, consistent with the results obtained when investigating the effect of light on the malate sensitivity of culture PEPc (section 5.2.3). However, there was a definite light enhancement of the 3'-UTR transcript in 1% sucrose grown cells (Figure 5.10A lanes 8 and 9). This is rather surprising considering that this transcript encodes a putative root isoform of PEPc. Under the same metabolic conditions, the PEPc kinase transcript level was higher in the dark than the light (Fig 5.10C lanes 8 and 9).

In addition to the effects of sucrose mentioned above, it would be interesting to study the effect of nitrogen supply on PEPc and PEPc kinase. Duff and Chollet (1995) studied the short-term effect of nitrate feeding on the activity and malate sensitivity of C_3 PEPc, and observed little difference between illuminated or darkened wheat leaves detached from low-N-grown plants. In comparison, a rather dramatic increase in PEPc kinase activity was observed with illuminated excised leaves fed exogenous nitrate. However, no trend was apparent in the response of either of the PEPc transcripts or PEPc kinase transcript to increasing concentrations of nitrate in the cell culture medium.

It would appear that washing the cells to remove traces of growth medium affects the level of all three transcripts and the tubulin transcript grown under 3% sucrose (Figure 5.10A-D lanes 1 and 2). An effect of resuspending the cells, even in similar medium, can also be seen on transcript level (Figure 5.10A-D lanes 1 and 3). Therefore, it is quite plausible that the cells are stressed as a result of the experimental treatments and this might explain the absence of any consistent response of PEPc and PEPc kinase transcript to the alteration of nitrate levels. Equally these data might just reflect a lack of precision in this type of experiment, especially when the tubulin control is not ideal. In either case, it is questionable whether manipulation of the metabolic conditons of the cell culture is a suitable experimental system for studying the control of PEPc

Close inspection of Figure 5.10B reveals the presence of a band much lower in its molecular weight than the PEPc hybridization signal (lanes 8-10 and 12). Its size would suggest that it is not an isoform of PEPc and the hybridization pattern is not similar to that of any other transcript analyzed to be explained by filters not being stripped completely of previously hybridized probes. The band is only evident in 1% sucrose-grown cells but again there is no obvious trend in response to nitrate concentration. Its identity is still unclear.

The aim of these experiments was to discover something of the regulation of PEPc in the C_3 species *A. thaliana*. The work described in this chapter has established that the activity of PEPc and its malate sensitivity in the *A. thaliana* suspension culture is comparable to that of other C_3 species and is affected by the phosphorylation state of the PEPc protein. However, the light activation of PEPc demonstrated previously in other C_3 species was not seen with either *A. thaliana* culture or leaves (Rajagopalan et al., 1993; Lillo et al., 1995; Smith et al., 1996). Northern analysis of poly A^+ RNA from *A.*

thaliana culture and different plant tissues showed the presence of PEPc kinase transcript, a putative root isoform PEPc transcript and the transcripts of other PEPc isoforms. The level of all three transcripts was higher in culture grown in 1% sucrose as opposed to 3% sucrose (Figure 5.10) but there was no obvious trend in the effect of nitrate level on any of the transcripts.

Therefore, whilst these experiments have not facilitated any further understanding of the regulation of PEPc in C_3 species, they have confirmed what was already known from studies using other C_3 species and have highlighted the realistic potential and pitfalls of using the *A. thaliana* culture as an experimental system for future research.

Chapter Six

DISCUSSION

The challenge of scientific research lies not only in understanding the working of the world in which we live but how our improved understanding can be applied to better the way we live. In consideration of its important role in photosynthesis, its involvement in nitrogen metabolism and its contribution to good "house-keeping" in the plant cell, the regulation of PEPc has been the focus of intensive research for about 15 years. As knowledge and understanding of PEPc regulation increases so does appreciation of the complex mechanisms involved and the ability to manipulate these mechanisms to modify plant metabolism.

The specific objectives of this present work as outlined in Chapter 1 were to investigate the involvement of cytosolic pH in the regulation of the CAM isoform of PEPc and to study the regulation of PEPc in C_3 species using an *A. thaliana* cell culture. The findings of these investigations and the conclusions drawn from them are presented in the preceding chapters.

The regulatory phosphorylation of PEPc is probably the most studied example in plants of this form of reversible covalent modification. However, very little is known about the signal transduction processes that lead to phosphorylation of PEPc, particularly in CAM and C_3 plants. The work described in Chapter 3 tested the idea that intracellular pH changes may be involved in this signalling in a CAM plant, as they are in C_4 species. While an effect of cytosolic acidification was observed, it seemed to be too slow to play a role in signal transduction.

The cloning of the first *A. thaliana* PEPc cDNA is described in Chapter 4. The sequence confirms that it can be phosphorylated. Unfortunately the clone was not expressed *in vitro* to provide a substrate for further studies during the course of this project. Use of an *A.* thaliana cell culture confirmed that the machinery required to phosphorylate PEPc is present in this C_3 species. Furthermore, it seems that there is more PEPc kinase present in culture grown in 1% than 3% sucrose.

The biggest development during the course of this project has been the cloning of the first PEPc kinase. This allows numerous developments. Results from previous investigations of PEPc kinase activity in different plant species suggest transcriptional regulation of the enzyme. However, the kinase activity assay of *in vitro* translation products and RNA synthesis inhibitors used in these experiments only in fact gives information about the steady-

involves extending previously initiated transcripts for a time in the presence of ^{32}P-UTP and then isolating the total RNA and using as a probe for slot blots containing S-S DNA (Dean et al, 1969)

state level of the transcript i.e. the balance between synthesis and degradation. The RT-PCR and Northern analyses of PEPc kinase described in Chapter 5 do not overcome this limitation. The existence of a PEPc kinase clone will now enable nuclear run-on assays to be performed which should clarify the exact nature of the regulation of PEPc kinase at the transcriptinal level. It is also possible to make constructs of the PEPc kinase promoter and a reporter gene like β-glucuronidase which will facilitate studies of PEPc kinase expression in different tissues, under different conditions and at different stages of development. Isolation of the promoter would also allow investigation of transcription factors which regulate PEPc kinase, providing access to steps of the PEPc signalling pathway upstream of those already examined. It is difficult to extrapolate *in vitro* study of enzyme regulation to the *in vivo* situation of an intact plant. The genetic transformation of plants, although having its own limitations, provides an excellent means of studying the regulation and importance of an enzyme in its usual environment. Given the importance of protein phosphorylation in the regulation of PEPc in CAM plants and other species, it is likely that large-scale changes in the activity of PEPc kinase by overexpression or suppression of the PEPc kinase gene would result in easily observed changes in the plant phenotype which might enhance understanding of the role of the kinase in the plant. Transgenic plants in which the control of PEPc, a key enzyme of central metabolism, is altered could have advantageous phenotypic changes. The cloning of a PEPc and PEPc kinase from *A. thaliana* is particularly exciting due to the advantages of this species for genetic studies; it grows from seed to seed in about 6 weeks, it has a relatively small genome which has been extensively mapped, it has been successfully transformed and collections of *A. thaliana* tagged lines exist which could be screened for PEPc or PEPc kinase mutants. Knockout mutations of PEPc or PEPc kinase would be more revealing than the plants in which a gene has been antisensed.

A second recent development in PEPc research is the determination of the first crystallographic structure of PEPc from *E. coli* (Kai et al., 1999). Nucleotide sequence comparisons have been indispensable in understanding the biochemistry and regulation of PEPc. However, it is hoped that plant PEPc crystallographic structures will soon be available which will facilitate further site-directed mutagenesis studies and analysis of structure/function relationships.

Therefore two major hurdles in understanding the regulation of PEPc appear to have been cleared - the cloning of PEPc kinase and the elucidation of the first three-dimensional structure of PEPc. These achievements will make possible many research strategies which

could greatly advance the understanding of PEPc regulation in higher plants in the next few years.

Chapter Seven

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