The Regulation of Phosphoenolpyruvate Carboxylase in Higher Plants

by
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Abbreviations

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

ADP, adenosine diphosphate; AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; ATP, adenosine triphosphate; [γ–^{32}P]ATP, radioactively labelled adenosine triphosphate; ATPase, adenosine triphosphatase; CAM, Crassulacean acid metabolism; cAMP, cyclic adenosine monophosphate; cDNA, complementary DNA; CDPK, calcium-dependent protein kinase (or calmodulin-like domain protein kinase); DMSO, dimethyl sulphoxide; DNA, deoxyribonucleic acid; EtOH, ethanol; GA, gibberellic acid; Gln, glutamine; Glu, glutamate; GOGAT, glutamine 2-oxoglutarate aminotransferase (glutamate synthase); GS, glutamine synthetase; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; KAPP, kinase associated protein phosphatase; MAPK, MAPKK or MAPKKK, mitogen activated protein kinase, kinase kinase or kinase kinase kinase; ME, malic enzyme; MDH, malate dehydrogenase; MeOH, methanol; [^{35}S]Met, radioactively labelled methionine; NAD^+/NADH, nicotinamide adenine dinucleotide oxidised/ reduced form; $NADP^+$/NADPH, nicotinamide adenine dinucleotide phosphate oxidised/reduced form; NR, nitrate reductase; OAA, oxaloacetate; PDC, pyruvate dehydrogenase complex; PEP, phosphoenolpyruvate; PEPc, phosphoenolpyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; Pi, inorganic phosphate; PKA, PKG and PKC, protein kinase A, G and C; PPDK, pyruvate, phosphate dikinase; PPi inorganic pyrophosphate; PP1, protein phosphatase type-1; PP2A, 2B, 2C, protein phosphatase type-2A, -2B or -2C; RLK, receptor-like protein kinase; RNA, ribonucleic acid (mRNA, messenger ribonucleic acid); RNase, ribonuclease; Rubisco, ribulose bisphosphate carboxylase/oxygenase; SA, salycylic acid; 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; SS or SuSy, sucrose synthase; TP, triose phosphate; UDP, uridine diphosphate.

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Summary

Phosphoenolpyruvate carboxylase (PEPc) is a ubiquitous enzyme in higher plants, performing primary CO_2 fixation in C_4 and Crassulacean acid metabolism (CAM) plants and a range of housekeeping functions in C_3, C_4 and CAM plants. In C_3 and C_4 plants, PEPc activity is regulated in response to light whilst in CAM plants it is regulated by an endogenous circadian oscillator. The regulation of PEPc is brought about through reversible protein phosphorylation which modulates the allosteric properties of the enzyme. The phosphorylated form of the enzyme is less sensitive to feedback inhibition by malate. The phosphorylation state of PEPc is itself regulated by the activity of the Ca^{2+}-independent PEPc kinase which is induced by light in C_3 and C_4 plants, and by a circadian oscillator in CAM plants. Both the light- and circadian-induction of PEPc kinase activity require *de novo* protein synthesis. The aim of the work in this thesis was to investigate the requirement for protein synthesis in the induction of PEPc kinase in higher plants.

A novel assay was developed in which isolated plant RNA was translated *in vitro* using a rabbit reticulocyte lysate system and the translation products were assayed directly for PEPc kinase activity. A series of experiments were performed which demonstrate that the assay provides a valid estimate of the amount of PEPc kinase translatable mRNA in any sample of RNA. The extent of labelling of exogenous PEPc in the assay is proportional to the volume of translation products used, the amount of RNA translated and (after a short lag) the duration of the *in vitro* translation. Inclusion of EGTA in the kinase assays of translation products demonstrated that the kinase activity produced was Ca^{2+}-independent, like the activity detected in plant extracts. Size fractionation of a sample of mRNA revealed that PEPc kinase mRNA has a length of between 0.9 and 1.3 Kb which corresponds well with the predicted size calculated from the molecular weight of partially purified PEPc kinase polypeptides.

In the leaves of the CAM plant *Bryophyllum* (*Kalanchoë*) *fedtschenkoi*, in normal diurnal conditions, kinase mRNA was high at night and below the limits of detection during the day. In constant environmental conditions (continuous darkness, CO_2-free air, 15°C) kinase mRNA exhibited circadian oscillations. The circadian disappearance of

kinase mRNA and kinase activity was delayed by lowering the temperature to 4°C and accelerated by raising the temperature to 30°C. Pharmacological agents were used to dissect the signal transduction cascade that mediates circadian regulation of PEPc kinase mRNA levels and PEPc kinase activity. The nocturnal appearance of kinase mRNA and activity was blocked by a number of protein and RNA synthesis inhibitors which act on different components of the transcription and translation machinery. In addition to transcription and translation, the circadian appearance of PEPc kinase mRNA and activity requires protein dephosphorylation and a calcium/calmodulin interaction.

The physiological relevance of changes in PEPc kinase mRNA and activity to CAM with respect to CO_2 assimilation and total leaf malate content was investigated using intact plants of *Kalanchoë daigremontiana*. The leaf malate content was manipulated by placing leaves in an atmosphere of pure nitrogen during the night for various lengths of time to prevent CO_2 fixation and respiration. The effects of blocking nocturnal CO_2-fixation and malate accumulation on PEPc kinase mRNA and activity levels were examined with a view to determining whether the metabolite status of the leaf could influence the circadian control of the allosteric properties of PEPc. Changes in CO_2 fixation and PEPc kinase activity reflected those in kinase mRNA except in leaves transferred from nitrogen to ambient air at the start of the light period. In these leaves, kinase activity fell even though kinase mRNA rose, probably due to a rapid increase in cytosolic malate. The highest rates of CO_2 fixation and levels of kinase mRNA were observed in leaves subjected to anaerobic treatment for the first half of the night and then transferred to ambient air. A temperature increase from 19°C to 27°C led to a rapid reduction in kinase mRNA and activity. However, this was not observed in leaves in which malate accumulation had been prevented by anaerobic treatment. These results demonstrate that the metabolite status of the leaf and in particular the leaf malate status can override the circadian clock control of PEPc kinase mRNA and activity. The discrepancies between kinase mRNA and activity in some conditions suggest that (a) kinase turnover may be regulated, independent of kinase synthesis, and (b) light may either block translation of

kinase mRNA, increase its rate of destruction or cause the appearance of a tight-binding inhibitor of PEPc kinase.

In the leaves of C_3 and C_4 plants PEPc kinase activity and the apparent K_i of PEPc for malate increase in response to illumination. Maize and barley were used as representative C_4 and C_3 plants respectively. In both maize and barley, PEPc kinase mRNA was found to increase in response to light. This light induction was found to require transcription but not translation. In fact, protein synthesis inhibitors caused an accumulation of PEPc kinase mRNA above control levels in response to light. The light induction of kinase mRNA in maize was found to involve protein dephosphorylation and a calcium/calmodulin interaction. Collectively these results indicate that the light induction of PEPc kinase mRNA in C_4 and C_3 plants involves transcription of the PEPc kinase gene itself and that the activation of the PEPc kinase promoter involves both protein dephosphorylation and a possible calcium derived signal propagated via calmodulin.

Chapter 1

Introduction

1.1. The C_3 pathway of photosynthesis

1.1.1. The physiology and biochemistry of the C_3 pathway

In the majority of higher plant species, primary carbon dioxide fixation occurs in the chloroplasts and is catalysed by the enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) in the Calvin-Benson cycle (reductive pentose phosphate cycle) (Macdonald and Buchanan, 1990). For every three molecules of ribulose 1,5-bisphosphate (Rbu-1,5-P_2) carboxylated by Rubisco six molecules of 3-phosphoglycerate (3-PGA) are formed. Energy from the light reactions of photosynthesis is used to reduce 3-PGA to glyceraldehyde 3-phosphate (G3P). One molecule of G3P is exported from the Calvin-Benson cycle and is converted to dihydroxyacetone phosphate (DHAP). The DHAP represents the net gain of fixed carbon from the Calvin-Benson cycle. It can either be used for starch synthesis inside the chloroplast or exchanged into the cytosol, via the phosphate translocator, for sucrose synthesis. The remaining steps of the Calvin-Benson cycle regenerate the Rbu-1,5-P_2 via a series of isomerizations, condensations and rearrangements. These steps convert five molecules of triose phosphate into three molecules of pentose phosphate (Rbu-5-P), which is then phosphorylated using ATP to regenerate three molecules of Rbu-1,5-P_2 (see figure 1.1). This pathway of photosynthetic CO_2 fixation is termed C_3 photosynthesis because the product of CO_2 assimilation (3-PGA) possesses three carbon atoms.

1.1.2. Photorespiration

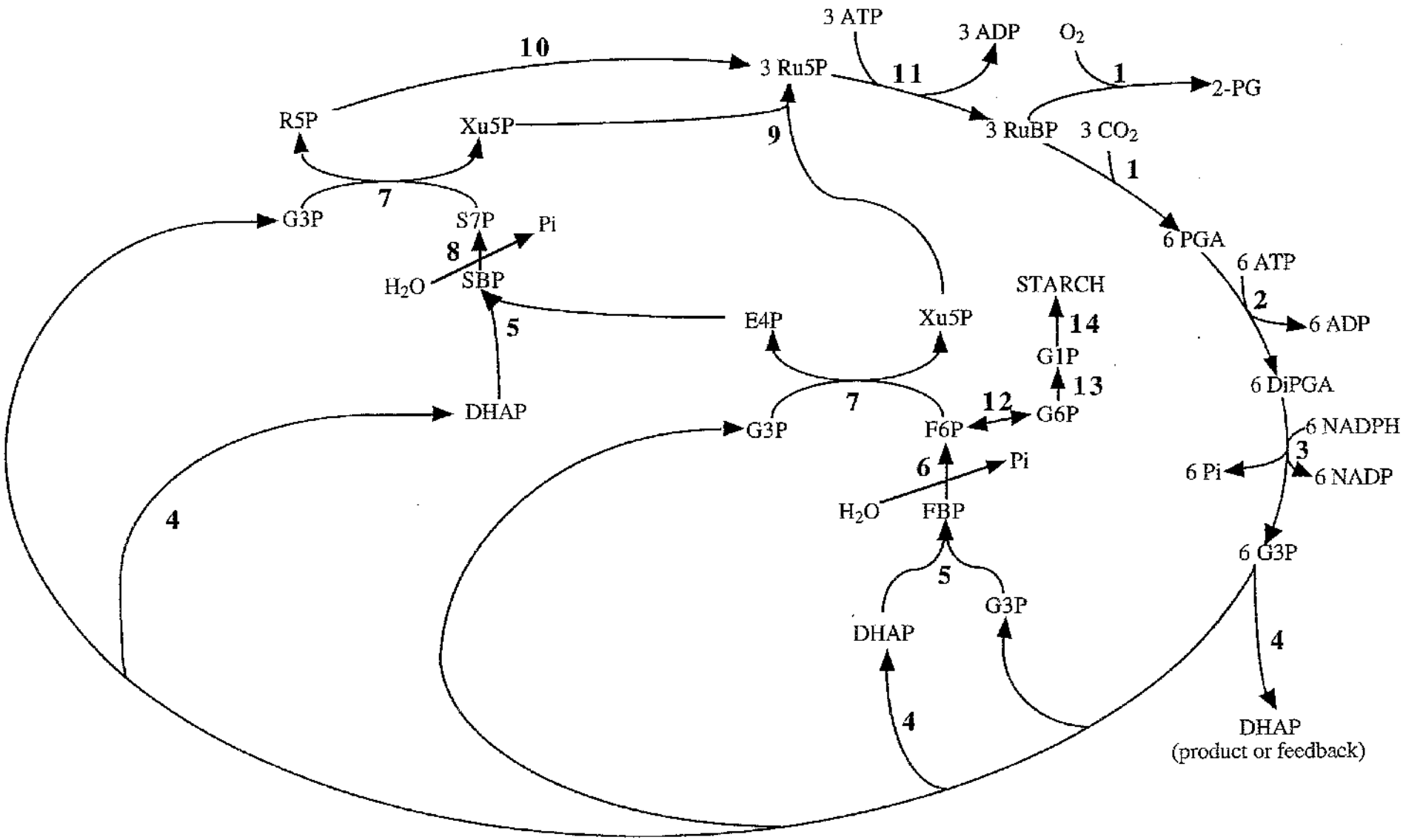
Despite the majority of higher plants performing primary CO_2 assimilation via Rubisco, the process is inherently inefficient because Rubisco also catalyses the oxygenation of Rbu-1,5-P_2 forming one molecule of 3-PGA and one molecule of 2-phosphoglycolate (Canvin, 1990). Subsequent metabolism of the phosphoglycolate leads to the light-dependent evolution of CO_2, which is called photorespiration. The phosphoglycolate passes through the photosynthetic carbon oxidation (PCO) cycle. This pathway is localized in three separate organelles; namely the chloroplast, peroxisome and

Figure 1.1. The C_3 pathway of photosynthesis (the reductive pentose phosphate pathway)

Abbreviations: RuBP, ribulose 1,5-bisphosphate; PGA, 3-phosphoglycerate; DiPGA, 1,3-diphosphoglycerate; Ru5P, ribulose 5-phosphate; G3P, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; FBP, fructose 1,6-bisphosphate; F6P, fructose 6-phosphate; Xu5P, xylulose 5-phosphate; E4P, erythrose 4-phosphate; SBP, sedoheptulose 1,7-bisphosphate; S7P, sedoheptulose 7-phosphate; R5P, ribose 5-phosphate; G6P, glucose 6-phosphate; G1P, glucose 1-phosphate; 2-PG, 2-phosphoglycolate.

The enzymes which catalyse each step are as follows:

1. ribulose 1,5-bisphosphate carboxylase oxygenase
2. phosphoglycerate kinase
3. glyceraldehyde 3-phosphate dehydrogenase
4. triose-phosphate isomerase
5. aldolase
6. fructose 1,6-bisphosphatase
7. transketolase
8. sedoheptulose 1,7-bisphosphatase
9. phosphopentoepimerase
10. phosphoriboisomerase
11. phosphoribulokinase
12. hexose phosphate isomerase
13. phosphoglucomutase
14. ADP-glucose pyrophosphorylase and starch synthase


3 ATP
3 ADP
O_2
10
11
1
3 Ru5P
2-PG
R5P
Xu5P
9
3 RuBP
3 CO_2
1
7
G3P
S7P
Pi
6 PGA
8
6 ATP
H_2O
SBP
STARCH
2
E4P
Xu5P
14
6 ADP
5
G1P
6 DiPGA
13
12
DHAP
7
G3P
F6P
G6P
6 NADPH
3
6 Pi
6 NADP
6
Pi
H_2O
FBP
6 G3P
5
G3P
DHAP
4
4
DHAP
(product or feedback)
4

mitochondrion (see figure 1.2) . Photorespiration leads to the loss of NH_3 and CO_2 in the mitochondrion, whilst 3-PGA is eventually returned to the chloroplast to replenish the Calvin cycle. The NH_3 must be reassimilated in the chloroplast by glutamine synthetase to provide the glutamine necessary for the continued operation of the PCO cycle. The CO_2 is released and represents a loss of carbon due to the oxygenase activity of Rubisco. Photorespiration is an energy consuming process that can reduce the net rate of photosynthesis in C_3 plants by 25-50 %. Increasing leaf temperature increases the affinity of Rubisco for oxygen and decreases its affinity for CO_2. This means that, in C_3 plants, increasing temperature takes the leaf beyond the CO_2 compensation point (the concentration of CO_2 at which the release and assimilation of CO_2 are equal) and hence the loss of CO_2 through photorespiration becomes greater than the assimilation of CO_2 in the Calvin-Benson cycle. C_3 plants are therefore poorly adapted to surviving in environments where high temperatures are commonplace such as the tropics and sub-tropics. However, a small proportion of higher plants have evolved a specialized biochemistry of photosynthesis for limiting or abolishing photorespiration which entails concentrating CO_2 at the active site of Rubisco. These plants are highly efficient at surviving in high temperature, high irradiance environments and fall into two distinct photosynthetic classes according to the temporal and spatial separation of their primary and secondary CO_2 fixation steps; they exhibit Crassulacean acid metabolism (CAM) or C_4 photosynthesis.

1.2. The C_4 pathway of photosynthesis

1.2.1. CO_2 assimilation in C_4 plants

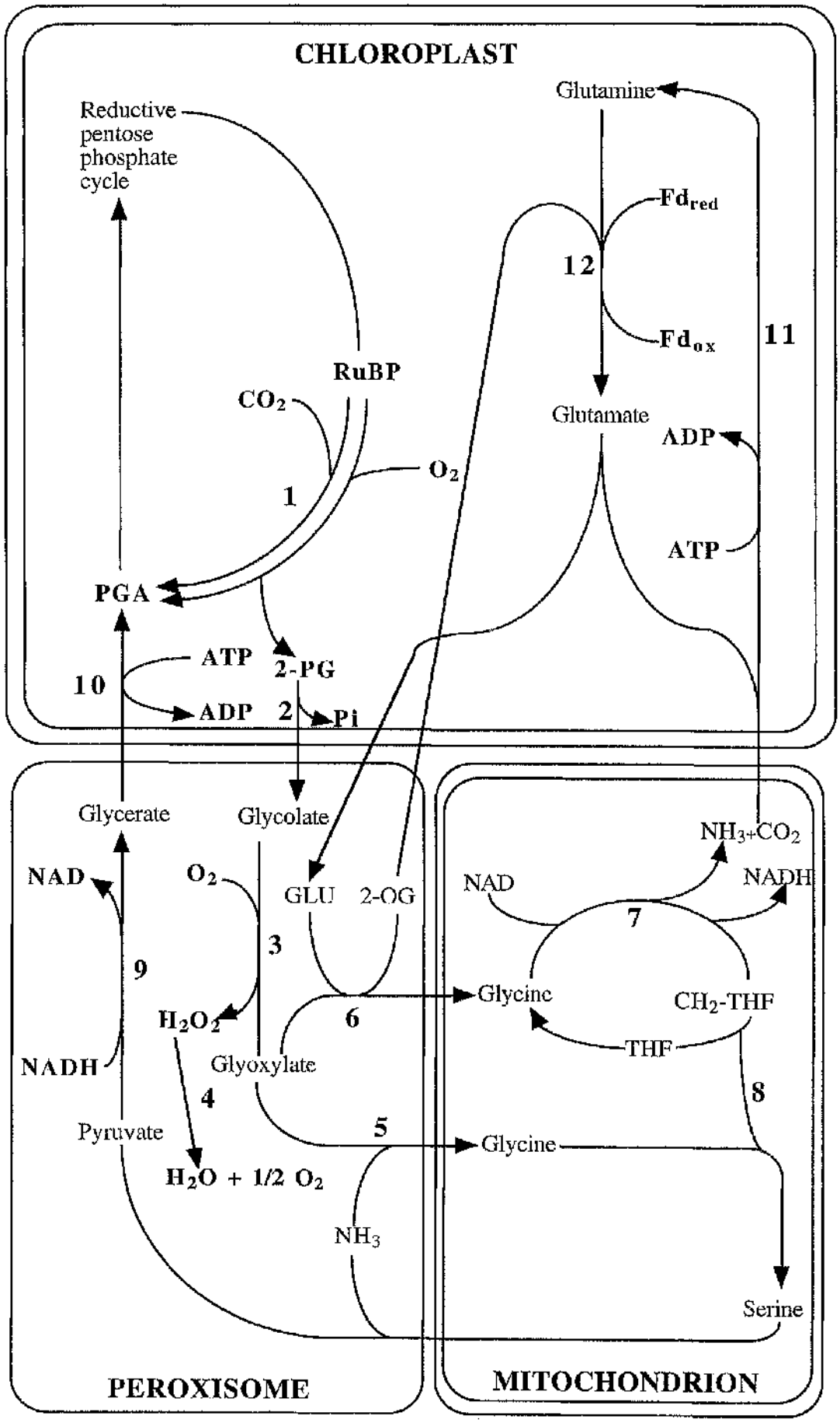
Plants which perform C_4 photosynthesis have a specialized leaf anatomy. The bundle-sheath cells are arranged in a ring or sheath ('Kranz' anatomy) around the vascular strands and have prominent starch-filled chloroplasts and thickened walls (Leegood, 1997). The mesophyll cells occur throughout the lamina and possess small chloroplasts and thin walls. Primary CO_2 fixation occurs in the mesophyll cells and is catalysed by the cytosolic enzyme phosphoenolpyruvate carboxylase (PEPc). PEPc is localized specifically in the mesophyll cells and actually uses HCO_3^- as its substrate rather than CO_2. The product of the initial CO_2 fixation step is oxaloacetate which is rapidly converted to malate by malate

Figure 1.2. The photosynthetic carbon oxidation (PCO) cycle

Abbreviations: RuBP, ribulose 1,5-bisphosphate; 2-PG, 2-phosphoglycolate; PGA, 3-phosphoglycerate; THF, tetrahydrofolic acid; Fd(red or ox), ferredoxin (reduced or oxidised). The figure was adapted from Canvin (1990).

The enzymes which catalyse each step are as follows:

1. 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


CHLOROPLAST
Reductive pentose phosphate cycle
Glutamine
Fd_{red}
12
Fd_{ox}
11
RuBP
CO_2
O_2
1
Glutamate
ADP
ATP
PGA
ATP
2-PG
10
ADP
2
Pi
Glycerate
Glycolate
NAD
O_2
GLU
2-OG
3
9
H_2O_2
6
NADH
Glyoxylate
4
5
Pyruvate
$H_2O + 1/2\ O_2$
NH_3
NH_3+CO_2
NAD
NADH
7
Glycine
CH_2-THF
THF
8
Glycine
Serine
PEROXISOME
MITOCHONDRION

dehydrogenase (in some types of C_4 plant aspartate is formed from the oxaloacetate by transamination) (Hatch, 1987). The malate (or aspartate) moves down a concentration gradient through the plasmodesmata which link the mesophyll cells to the bundle sheath cells. In the bundle sheath cells, malate is decarboxylated by either NAD-malic enzyme (NAD-ME) or NADP-malic enzyme (NADP-ME), or oxaloacetate is decarboxylated by phosphoenolpyruvate carboxykinase (PEPCK), depending on the type of C_4 plant. The CO_2 which is liberated is then fixed by Rubisco in the Calvin cycle (see figure 1.3).

C_4 plants therefore display a spatial separation of primary and secondary carbon dioxide fixation. This effectively acts as a 'pump' which concentrates CO_2 in the bundle sheath cells where the Rubisco is localized in the chloroplasts. Mesophyll cell chloroplasts lack granal stacks and Rubisco which prevents them from fixing CO_2 via the Calvin cycle. Hence, the oxygenase activity of Rubisco is inhibited by the presence of high concentrations of CO_2 in the bundle sheath cells. Furthermore, a combination of the internal position of the bundle-sheath cells, around the vascular strand, and their thick cell walls (and in some plants the presence of suberin in the cell wall) helps to prevent the diffusion of oxygen (and gaseous CO_2) into the bundle sheath.

1.2.2. Photorespiration in C_4 plants?

For many years, C_4 plants were believed to perform little, if any, photorespiration due to their efficient mechanism for concentrating CO_2 at the site of Rubisco. Increasing the CO_2 concentration increases the carboxylase reaction of Rubisco and effectively out competes the oxygenase reaction. However, evidence has recently come to light which indicates that photorespiration occurs in the NAD-ME type C_4 plant *Amaranthus edulis*. In this plant, approximately 6 % of the total net photosynthetic CO_2 assimilation was lost through the photorespiratory cycle (Lacuesta *et al.*, 1997). It is therefore evident that photorespiration does occur in at least some C_4 plants. It has been suggested that photorespiration may be a mechanism for protecting C_3 plants from photooxidation caused by the failure to dissipate excess light energy (Kozaki and Takeba, 1996). Transgenic tobacco plants possessing elevated levels of plastidic glutamine synthetase (GS2) were found to have an improved capacity for photorespiration and an increased tolerance to

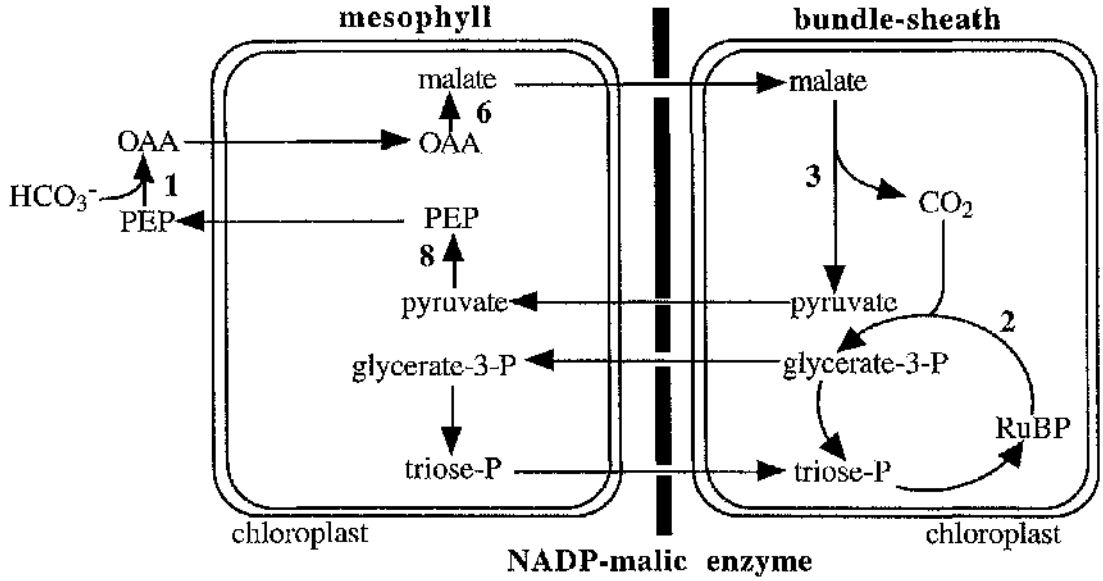
Figure 1.3. The C_4 pathway of photosynthesis

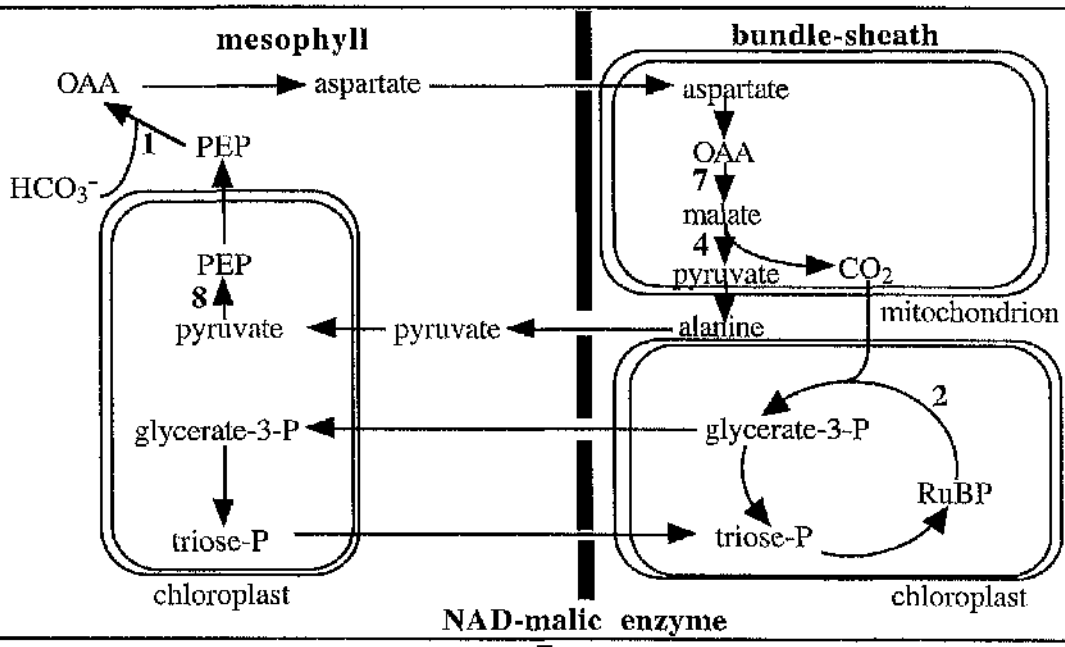
The diagram gives an overview of the pathway and its intracellular compartmentation for each of the three subgroups of C_4 plants. For most reactions, cofactors, transaminations etc. have been omitted for clarity. In PEP carboxykinase type C_4 plants NAD-malic enzyme also performs malate decarboxylation. The diagram was adapted from Leegood (1997).

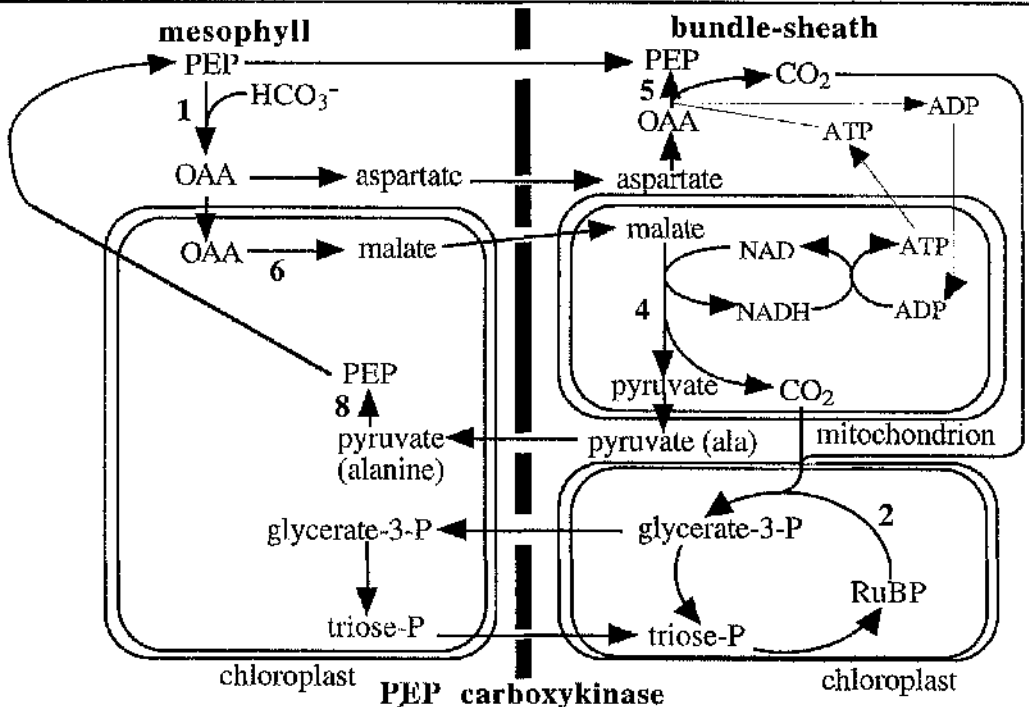
Abbreviations: RuBP, ribulose 1,5-bisphosphate; OAA, oxaloacetate; ala, alanine; PEP, phosphoenolpyruvate.

The enzymes which catalyse key steps in each subgroup are as follows:

1. phosphoenolpyruvate carboxylase
2. ribulose 1,5-bisphosphate carboxylase/oxygenase
3. NADP-malic enzyme
4. NAD-malic enzyme
5. phosphoenolpyruvate carboxykinase
6. NADP-malate dehydrogenase
7. NAD-malate dehydrogenase
8. pyruvate, phosphate dikinase


mesophyll
bundle-sheath
malate
malate
6
OAA
OAA
HCO_3^-
1
PEP
PEP
3
CO_2
8
pyruvate
pyruvate
2
glycerate-3-P
glycerate-3-P
RuBP
triose-P
triose-P
chloroplast
chloroplast
NADP-malic enzyme


mesophyll
bundle-sheath
OAA
aspartate
aspartate
1
PEP
OAA
HCO_3^-
7
malate
4
pyruvate
CO_2
PEP
8
mitochondrion
pyruvate
pyruvate
alanine
2
glycerate-3-P
glycerate-3-P
RuBP
triose-P
triose-P
chloroplast
chloroplast
NAD-malic enzyme


mesophyll
bundle-sheath
PEP
PEP
CO_2
1
HCO_3^-
5
ADP
OAA
ATP
OAA
aspartate
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malate
OAA
6
malate
NAD
ATP
4
NADH
ADP
PEP
8
pyruvate
CO_2
pyruvate
(alanine)
pyruvate (ala)
mitochondrion
glycerate-3-P
glycerate-3-P
2
RuBP
triose-P
triose-P
chloroplast
chloroplast
PEP carboxykinase

high-intensity light. Kozaki and Takeba (1996) demonstrated that photorespiration can dissipate excess energy from photosynthetic electron transport in the absence of photosynthesis. It is therefore possible that photorespiration plays a similar role in C_4 plants, particularly considering that they are usually found in high irradiance environments.

1.2.3. Development and regulation of the C_4 pathway

In dark-grown, developing leaves of a C_4 plant such as maize, the chloroplasts of both the mesophyll and the bundle-sheath cells possess low levels of Rubisco, both at the transcript and protein level (Langdale *et al.*, 1988; Sheen and Bogorad, 1985). Upon illumination Rubisco accumulates in the bundle-sheath cells and is turned over in the mesophyll cells. However, nuclei isolated from illuminated bundle-sheath and mesophyll cells were both found to synthesize the *rbcS* transcript suggesting that the repression of Rubisco in mesophyll cells occurs post-transcriptionally (Nelson and Langdale, 1992; Schaffner and Sheen, 1991). Illumination of dark-grown leaves causes an increase in the steady-state levels of a number of C_4 enzymes and mRNAs (Nelson *et al.*, 1984; Sheen and Bogorad, 1987a; Sheen and Bogorad, 1987b). For example, the C_4 isoform of PEPc and its gene (*Ppc*) are induced and expressed specifically in the mesophyll cells during greening (Sheen and Bogorad, 1987a). Similarly pyruvate orthophosphate dikinase (PPdK) and NAD-ME, NADP-ME or PEPCK are induced at the transcriptional level by light during greening of etiolated leaves (Nelson and Langdale, 1992). PPdK is induced in mesophyll chloroplasts (Sheen, 1991), NAD-ME is induced in bundle-sheath mitochondria (Long and Berry, 1996), NADP-ME is induced in bundle-sheath chloroplasts (Sheen and Bogorad, 1987a) and PEPCK is induced in bundle-sheath cytoplasm (Finnegan and Burnell, 1995). The promoters of a number of the genes for C_4 specific enzymes have been isolated and characterized. Stockhaus *et al.* (1997) demonstrated that 2 Kb of the 5' flanking region of the *Flaveria trinervia* C_4 *PpcA1* gene is sufficient to direct mesophyll-specific expression of the ß-glucuronidase reporter gene in transgenic *F. bidentis* (C_4) plants. However, despite extensive comparisons of the promoters of C_4 genes, no conserved elements responsible for C_4 bundle-sheath or mesophyll cell specific gene expression have been identified (Nelson and Langdale, 1992).

1.2.4. Post-translational regulation of C_4 enzymes

In addition to the transcriptional and post-transcriptional regulation of specific isoforms of C_4 genes described above, a number of C_4 enzymes have also been demonstrated to be post-translationally regulated (Budde and Randall, 1990; Chollet *et al.*, 1996; Hatch, 1987). Whilst transcriptional regulation ensures developmental and light-mediated cell and organelle specific expression, post-translational regulation permits fine tuning of an enzyme's regulatory properties in response to subtle changes in light-intensity, temperature and the metabolic status of the cell. Three key enzymes of the C_4 pathway have been found to undergo post-translational regulation in response to light. This regulation occurs in leaves which are fully green and actively utilising the C_4 pathway.

The C_4 isoform of pyruvate orthophosphate dikinase (PPdK, E.C. 2.7.9.1) is a mesophyll cell-localized enzyme found in the chloroplast stroma which is rapidly and reversibly light activated. It catalyses the conversion of pyruvate plus ATP and Pi to PEP plus AMP and PPi. The reversible light activation was found to occur via phosphorylation/dephosphorylation. Unusually, this phosphorylation involves ADP as the phosphate donor. The catalytic enzyme-phosphoryl intermediate of PPdK (E-His-P) is the target for ADP-dependent phosphorylation. Only the E-His-P form of the enzyme can undergo inactivation by the phosphorylation of a neighbouring threonine (Thr) residue. The His-P is thought to be extremely labile and is probably lost non-enzymatically. This results in the inactive configuration E-His/Thr-P. Activation occurs via the phosphorylytic removal of Pi forming PPi and active E-His/Thr. Light activation is thought to be mediated by the light-dependent transport of pyruvate into the chloroplast (Ohnishi and Kanai, 1987). The presence of pyruvate means that the E-His-P/Thr phosphoryl catalytic intermediate can continue with catalysis generating PEP, rather than being inactivated by the ADP-dependent phosphorylation of the neighbouring Thr. The inactivation and activation processes are thought to be catalysed by a single bifunctional regulatory protein (RP) which possesses two physically distinct active sites.

The mesophyll-chloroplast located NADP-malate dehydrogenase of C_4 plants is regulated by light (Hatch, 1987). This process involves interconversion between an active

dithiol form of the enzyme and an inactive disuphide form. Activation of NADP-malate dehydrogenase from maize leaves *in vitro* involves the reduction of two disulphide groups resulting in four thiol groups (Jenkins *et al.*, 1986). Light intensity is sensed through the redox state of ferredoxin, a component of the photosynthetic electron transport chain (Nakamoto and Edwards, 1986). A specific thioredoxin regulates the interconversion between the two forms of the enzyme (Droux *et al.*, 1987). A number of other plant enzymes have been found to be regulated in response to light by this ferredoxin/thioredoxin system, including a number of enzymes involved in carbohydrate biosynthesis and degradation (Cseke and Buchanan, 1986).

The C_4 isoform of phosphoenolpyruvate carboxylase (PEPc, E.C. 4.1.1.31) is a cytosolic mesophyll-cell enzyme (Perrot-Rechenmann *et al.*, 1982). This enzyme undergoes post-translational regulation via the phosphorylation of a single N-terminal serine residue catalysed by a highly specific serine/threonine kinase (Chollet *et al.*, 1996). This will be discussed in detail in section 1.4.5.

1.3. Crassulacean acid metabolism (CAM)

1.3.1. The physiology and biochemistry of CAM

CAM is a unique and specialised form of photosynthesis which is found in a wide range of plant families. It was first discovered in the family Crassulaceae, which lent its name to CAM (Ranson and Thomas, 1960). It has since been discovered in a diverse group of plant families including Cactaceae, Euphorbiaceae, Asclepiadaceae, Agavaceae, Aloeaceae, Liliaceae, Aizoaceae, Asteraceae, Vitaceae, Orchidaceae and Bromeliaceae (Dittrich *et al.*, 1973; Winter and Smith, 1996). These include families of monocotyledonous and dicotyledonous angiosperms. This indicates that CAM probably evolved prior to the separation of monocotyledons and dicotyledons into distinct lineages and that CAM has evolved on a number of separate occasions (Toh *et al.*, 1994). The occurrence of CAM in the Isoetaceae (Lycopodiopsida), Polypodiaceae (Filicopsida), Vitarriaceae (Filicopsida) and Welwitschiaceae (Gnetopsida), all of which are representatives of classes which evolved before the angiosperms, indicates that CAM most probably did exist in the ancestors of the angiosperms (Smith and Winter, 1996).

The key physiological features of CAM include the following (see figure 1.4):

a) the stomatal pores open at night, allowing gas exchange, and close in the day (Osmond and Holtum, 1981; Winter and Smith, 1996).

b) CO_2 is initially fixed at night via PEPc and the malate produced is stored in the vacuole as malic acid. This malic acid greatly increases the titratable acidity of the leaves of CAM plants at night (Osmond and Holtum, 1981; Winter and Smith, 1996). A few species accumulate significant amounts of citric acid as well as malic acid (e.g. *Clusia minor*) (Borland and Griffiths, 1997).

c) during the day the malic acid stored in the vacuole is released and decarboxylated by either malic enzyme or PEP carboxykinase (PEPCK, which actually decarboxylates OAA produced from the malate) depending on the type of CAM plant (Dittrich, 1976; Dittrich *et al.*, 1973; Walker and Leegood, 1996). Only at this point is the CO_2 refixed by Rubisco using photosynthetically captured energy in the Calvin cycle. CAM is thus a CO_2 concentrating mechanism which overcomes the O_2 inhibition of Rubisco (Osmond *et al.*, 1982).

CO_2 fixation via Rubisco is associated with a larger discrimination against $^{13}CO_2$ compared to the much more abundant $^{12}CO_2$ than is CO_2 fixation via PEPc (Winter and Smith, 1996). Thus, when the tissues of plants are examined by mass-spectrometry, the relative abundance of ^{13}C compared to ^{12}C can be used as a strong indication that primary CO_2 fixation is occurring via either Rubisco or PEPc. A value called the carbon isotope discrimination ratio ($\delta^{13}C$ value) can be calculated from the levels of ^{13}C and ^{12}C in a sample (Winter and Smith, 1996). For example, an analysis of a wide range of tropical and subtropical species of epiphytic Orchidaceae from Australia revealed a continuous spectrum of $\delta^{13}C$ values from those with full CAM (-15 to -12 ‰) to those with values characteristic of the C_3 pathway (-33 to -24 ‰) (Winter and Smith, 1996). Application of this method to fossils of Miocene and Pliocene grasses revealed that fossils with C_3 anatomy had C_3 $\delta^{13}C$ values whilst fossils with Kranz anatomy had C_4-like $\delta^{13}C$ values

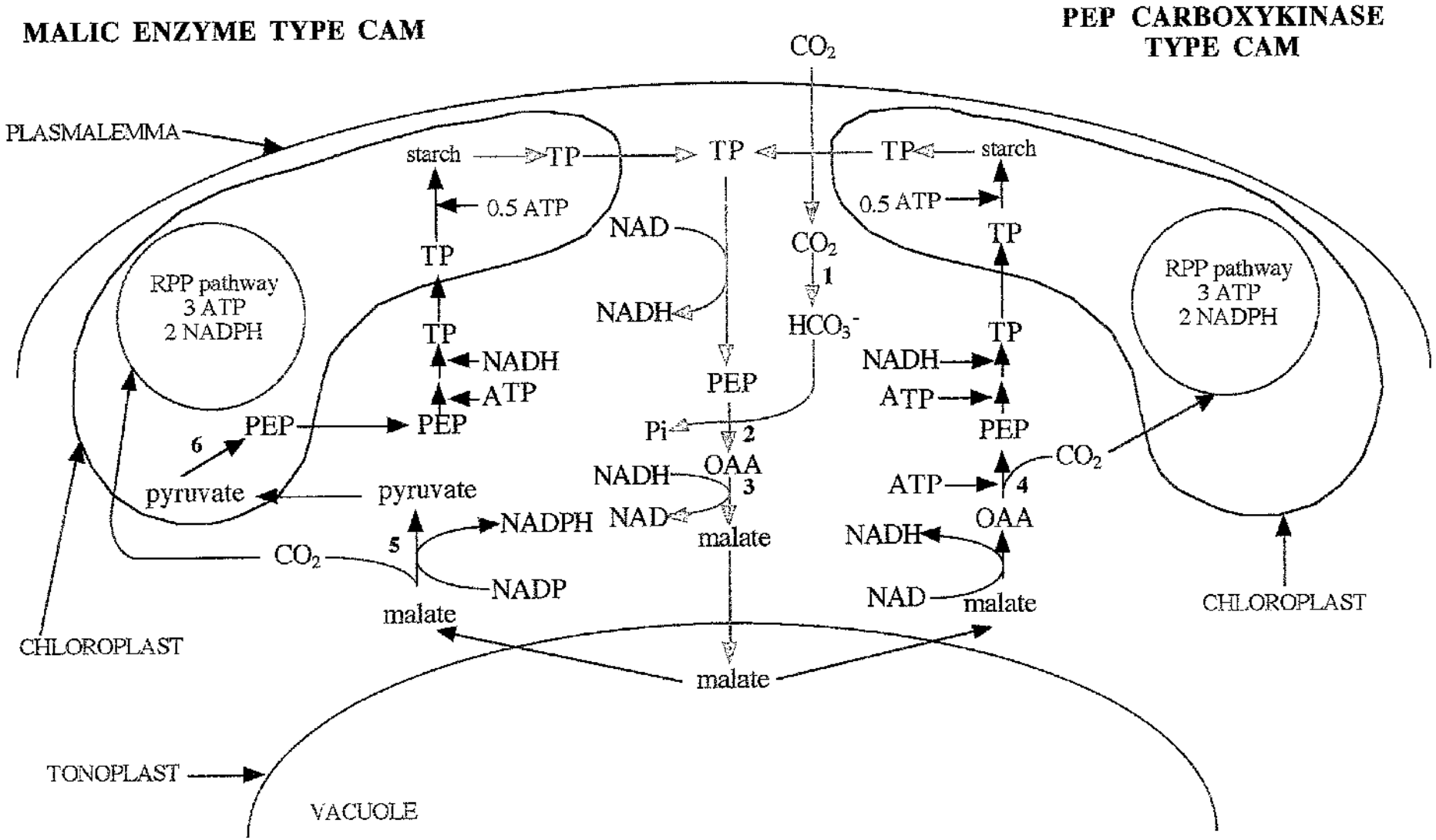
Figure 1.4. Crassulacean acid metabolism (CAM)

The arrows in red represent steps which occur during the dark period, whilst the arrows in black represent steps which occur during periods of illumination.

Abbreviations: RPP, reductive pentose phosphate pathway; PEP, phosphoenolpyruvate; OAA, oxaloacetate; TP, triose phosphate.

The key enzymes of the CAM pathway are as follows:

1. carbonic anhydrase
2. phosphoenolpyruvate carboxylase
3. malate dehydrogenase
4. phosphoenolpyruvate carboxykinase
5. NADP-malic enzyme
6. pyruvate, phosphate dikinase


MALIC ENZYME TYPE CAM
PEP CARBOXYKINASE TYPE CAM
CO_2
PLASMALEMMA
starch
TP
TP
TP
starch
0.5 ATP
0.5 ATP
NAD
CO_2
TP
RPP pathway
3 ATP
2 NADPH
1
NADH
HCO_3^-
RPP pathway
3 ATP
2 NADPH
TP
NADH
TP
NADH
ATP
PEP
ATP
PEP
PEP
PEP
Pi
2
6
CO_2
NADH
OAA
ATP
4
pyruvate
pyruvate
3
OAA
NADPH
NAD
malate
NADH
5
CO_2
NADP
NAD
malate
malate
CHLOROPLAST
CHLOROPLAST
malate
TONOPLAST
VACUOLE

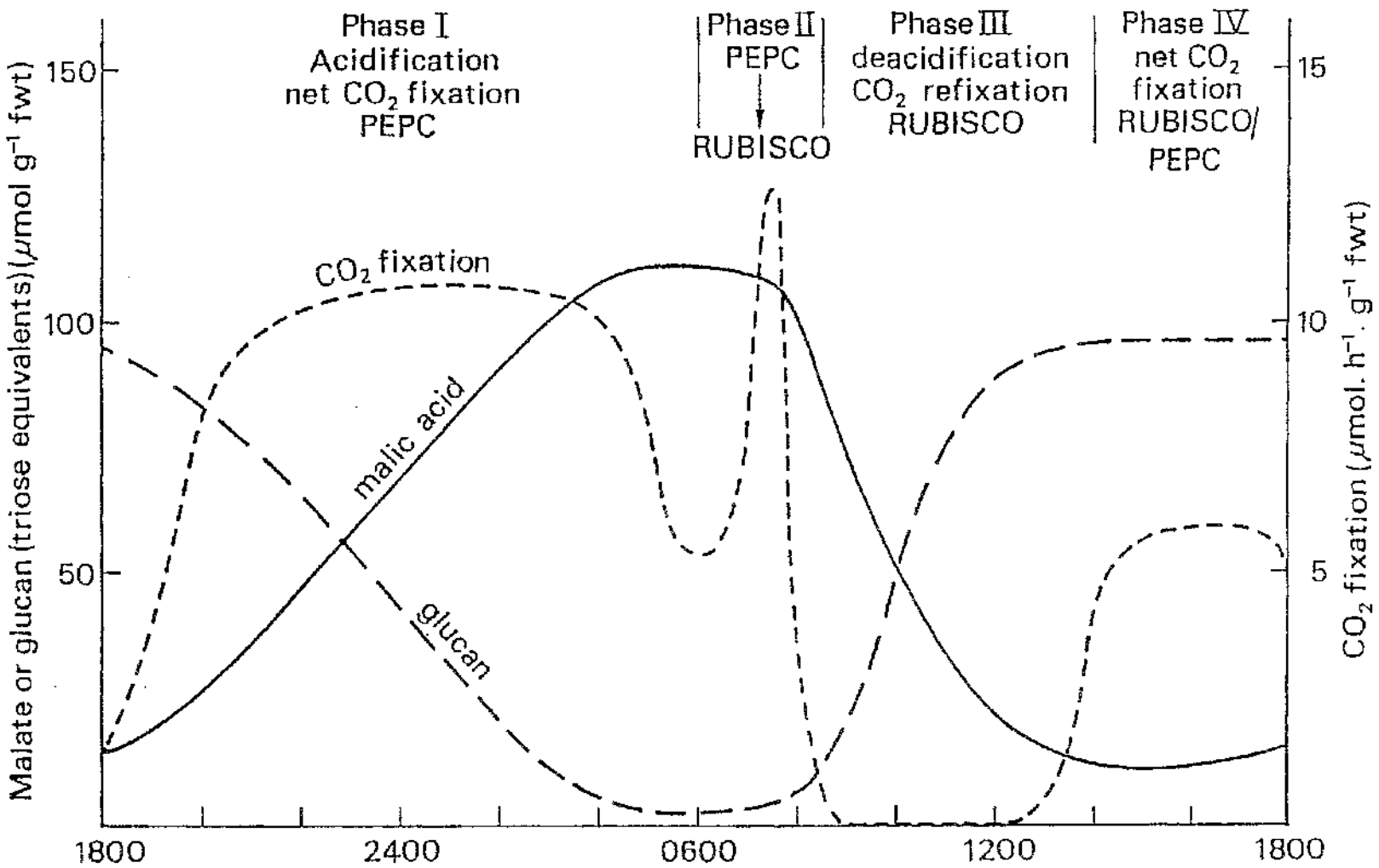
(Raven and Spicer, 1996). However, examination of various Palaeozoic and Mesozoic fossils revealed only $\delta^{13}C$ values indicative of C_3 plants and no values indicative of CAM plants have been obtained (Raven and Spicer, 1996).

The temporal sequence of CAM in a well-watered obligate CAM plant has been categorized into four distinct phases (figure 1.5) (Osmond, 1978). Phase I occurs during the dark period and is characterized by a marked increase in the assimilation of CO_2 via PEPc, the accumulation of malic acid in the vacuole and a reciprocal decrease in the storage carbohydrate (soluble sugars or glucans). Towards the end of phase I CO_2 fixation begins to decline due to the inhibition of PEPc by the high concentration of accumulated malate and an increase in the malate sensitivity of PEPc (Nimmo *et al.*, 1984; Winter, 1980; Winter, 1982). The properties of PEPc are discussed fully in section 1.4. Rubisco becomes active in response to illumination and the combined action of Rubisco and PEPc during phase II results in a brief burst of CO_2 fixation into both C_4 acids and C_3 compounds. As the stomata close, fixation of atmospheric CO_2 via PEPc ceases. Also during phase II, malic acid levels peak and begin to decline due to the commencement of decarboxylation. During the middle of the day the stomata are closed due to the high intercellular partial pressure of CO_2 generated by malate decarboxylation (phase III). During phase III, vacuolar malic acid levels drop to their basal value and net CO_2 uptake reaches a minimum. Once all the vacuolar malic acid has been decarboxylated, the internal CO_2 concentration is very low. This causes the stomata to open in favourable environmental conditions (e.g. well-watered plants). Atmospheric CO_2 uptake then begins to increase due to the activity of Rubisco and Rubisco-mediated CO_2 uptake peaks during phase IV. PEPc may also be active towards the end of phase IV but malic acid does not accumulate. The cycle is then repeated upon the return to darkness.

This pattern of carbon dioxide fixation and stomatal opening makes CAM plants extremely efficient at surviving in deserts, semi-arid regions, salt marshes and epiphytic sites (as for the epiphytic and lipophytic orchids) (Osmond, 1984). Some CAM plants utilise the CAM mode of metabolism at all stages of their life cycle and these are termed "obligate" CAM plants. A second class of CAM plants only utilise CAM when they require

Figure 1.5. The phases of CAM

Generalized schematic representation of malic acid and glucan levels, and rates of net CO_2 fixation in air, used to identify four phases of metabolism in ME-type CAM plants (taken from Leegood and Osmond, 1990)


Phase I
Acidification
net CO_2 fixation
PEPC
Phase II
PEPC
RUBISCO
Phase III
deacidification
CO_2 refixation
RUBISCO
Phase IV
net CO_2
fixation
RUBISCO/
PEPC
CO_2 fixation
malic acid
glucan
Malate or glucan (triose equivalents) (μmol g^{-1} fwt)
CO_2 fixation (μmol. h^{-1}. g^{-1} fwt)
150
100
50
15
10
5
1800
2400
0600
1200
1800

the greatly increased water use efficiency it affords. At other times they use C_3 photosynthesis. These are termed "facultative" CAM plants and they only invoke CAM when induced to do so by drought stress, day-length, salt level in the soil water or severe day-night temperature fluctuations (Osmond, 1984).

A variation of the method of examining the $\delta^{13}C$ value of plant material, called instantaneous on-line discrimination of carbon isotopes, allows the relative contribution of PEPc and Rubisco to CO_2 fixation by a living leaf to be examined. Changes in the $\delta^{13}C$ ratio of CO_2 in air as it passes over a leaf will reflect changes in both diffusional resistance to CO_2 and carboxylation pathway (Evans *et al.*, 1986). Discrimination (Δ) is a positive term with the extent of depletion in ^{13}C directly related to the magnitude of discrimination processes. Typical values of Δ would be 3.0 ‰ for CAM and 17.4 ‰ to 22.7 ‰ for C_3 photosynthesis (Borland and Griffiths, 1996). Application of this approach to facultative CAM plants has allowed an accurate determination of the relative contribution of PEPc to CO_2 fixation throughout the 24 h cycle in response to drought stress both in the natural environment and in the laboratory (Borland and Griffiths, 1996). In *Sedum telephium*, well-watered plants display Δ values around 23 ‰ throughout the diurnal cycle. However, when the leaf relative water content has been reduced to 60 % by water-stressing the plant, the Δ values drop to about 12 ‰ at night indicating significant nocturnal fixation of CO_2 by PEPc. When leaf relative water content is 52 %, Δ values are less than 15 ‰ even during the photoperiod indicating significant CO_2 fixation via PEPc even during the photoperiod (Borland and Griffiths, 1996).

Clusia minor was examined in the field in Trinidad during the wet and dry season using on-line discrimination (Borland *et al.*, 1993). During the wet season plants displayed on-line discrimination values of 15 to 25 ‰ throughout the diel cycle in shaded and exposed leaves, indicating that little, if any, CAM was occurring in these leaves. However, during the dry season both exposed and shaded leaves performed significant nocturnal uptake of CO_2 and had Δ values below 10 ‰ at night indicating significant CO_2 fixation by PEPc. Furthermore, these leaves showed significant fixation of CO_2 via PEPc during the early part of phase II (first 3 h after dawn) and the latter part of phase IV (Borland and

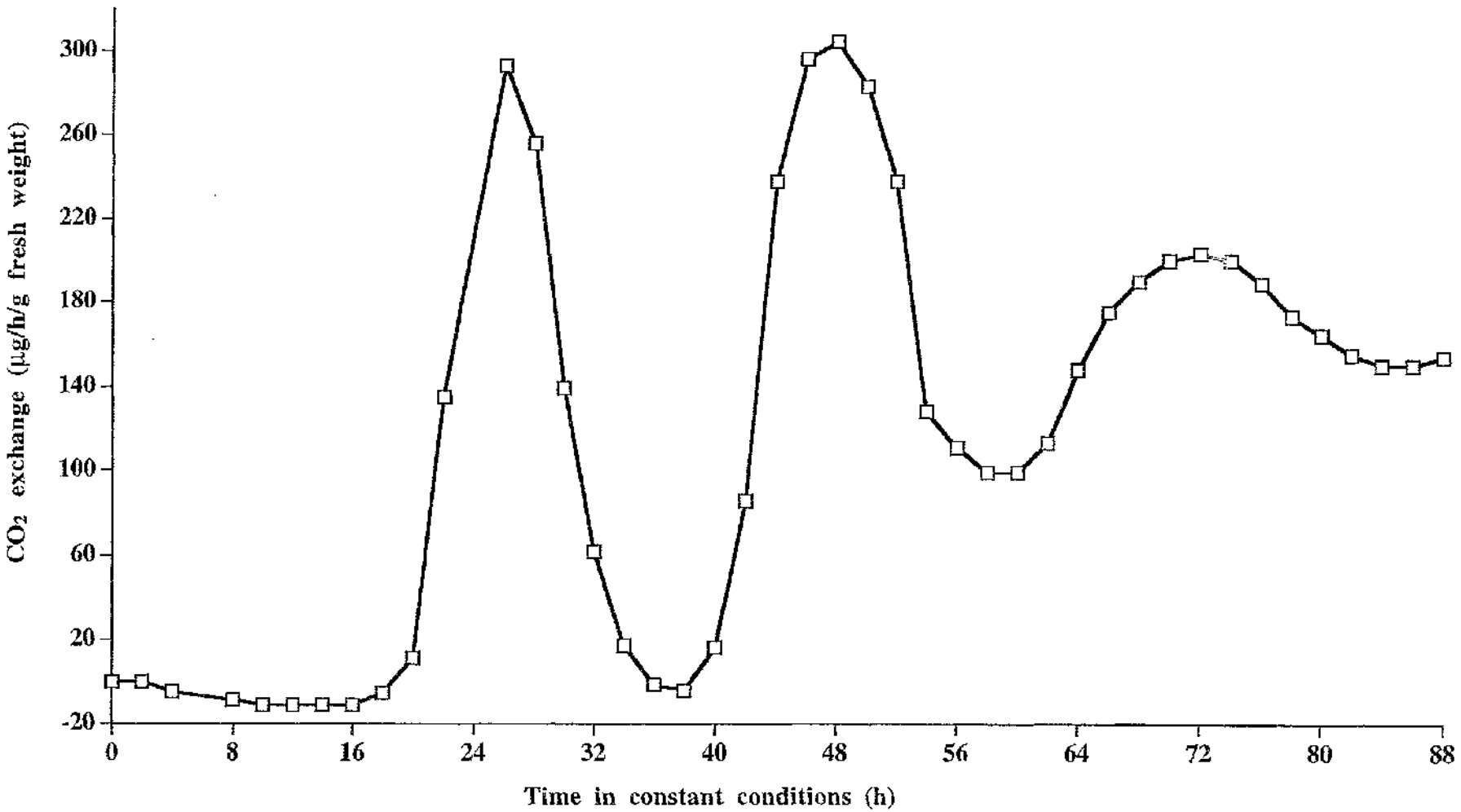
Griffiths, 1996). Exposed, dry season, leaves also kept their stomata open during the transition from dusk to darkness, with the decrease in Δ indicating an increase in PEPc activity (Borland and Griffiths, 1996). Such physiological examinations of the relative contributions of C_4 and C_3 carboxylation to the carbon budget of facultative CAM plants provide fascinating insights into the phenotypic plasticity of these plants and indicate the substantial natural variations in the processes occurring during the four phases of CAM (e.g. continued fixation of CO_2 by PEPc well into phase II coupled with delayed stomatal closure and decarboxylation) (Borland and Griffiths, 1996). Combination of such physiological approaches with detailed biochemical and molecular analyses of the underlying processes will provide invaluable insights into the operation of the CAM pathway.

1.3.2. CAM in *Bryophyllum (Kalanchoë) fedtschenkoi*

B. fedtschenkoi is an obligate CAM plant which is endemic to Madagascar. There it is exposed to low night temperatures and high day temperatures, which means that CAM is a physiological advantage to the plant. Since the late 1950's a great deal of research has been carried out on the persistent circadian rhythm of carbon dioxide output or assimilation which is observed in detached leaves of *B. fedtschenkoi* (Wilkins, 1992). When leaves are detached from the plant at 16.00 h (the end of the light period), and placed at 15°C in continuous darkness and a CO_2-free air stream, a circadian rhythm of peaks in CO_2 output is observed (see figure 1.6) (Wilkins, 1959; Wilkins, 1962). This rhythm persists for 3-4 days, and is observed between temperatures of 10-28°C (Wilkins, 1959; Wilkins, 1962). The observed rhythm of CO_2 output seems to be generated by the oscillation of the flux through PEPc. This can be inferred from the observation that incorporation of trace $^{14}CO_2$, in an otherwise CO_2-free air stream, results in the periodic labelling of malate (Warren and Wilkins, 1961). When the enzyme is active then respired CO_2, the only CO_2 available, is refixed and this generates corresponding troughs in the measured CO_2 output (Wilkins, 1959). By contrast, when PEPc is inactive respired CO_2 escapes from the leaf generating corresponding peaks in CO_2 output. When malate synthesized during periods of PEPc activity accumulates in the cytosol it will inhibit PEPc, curtailing the troughs in CO_2

Figure 1.6. The circadian rhythm of CO_2-output from *B. fedtschenkoi* leaves maintained in continuous darkness and CO_2 -free air at 15°C

Detached *B. fedtschenkoi* leaves were placed in continuous darkness and CO_2-free air at 15°C and the CO_2-output from the leaves was measured using an infra-red gas analyser.

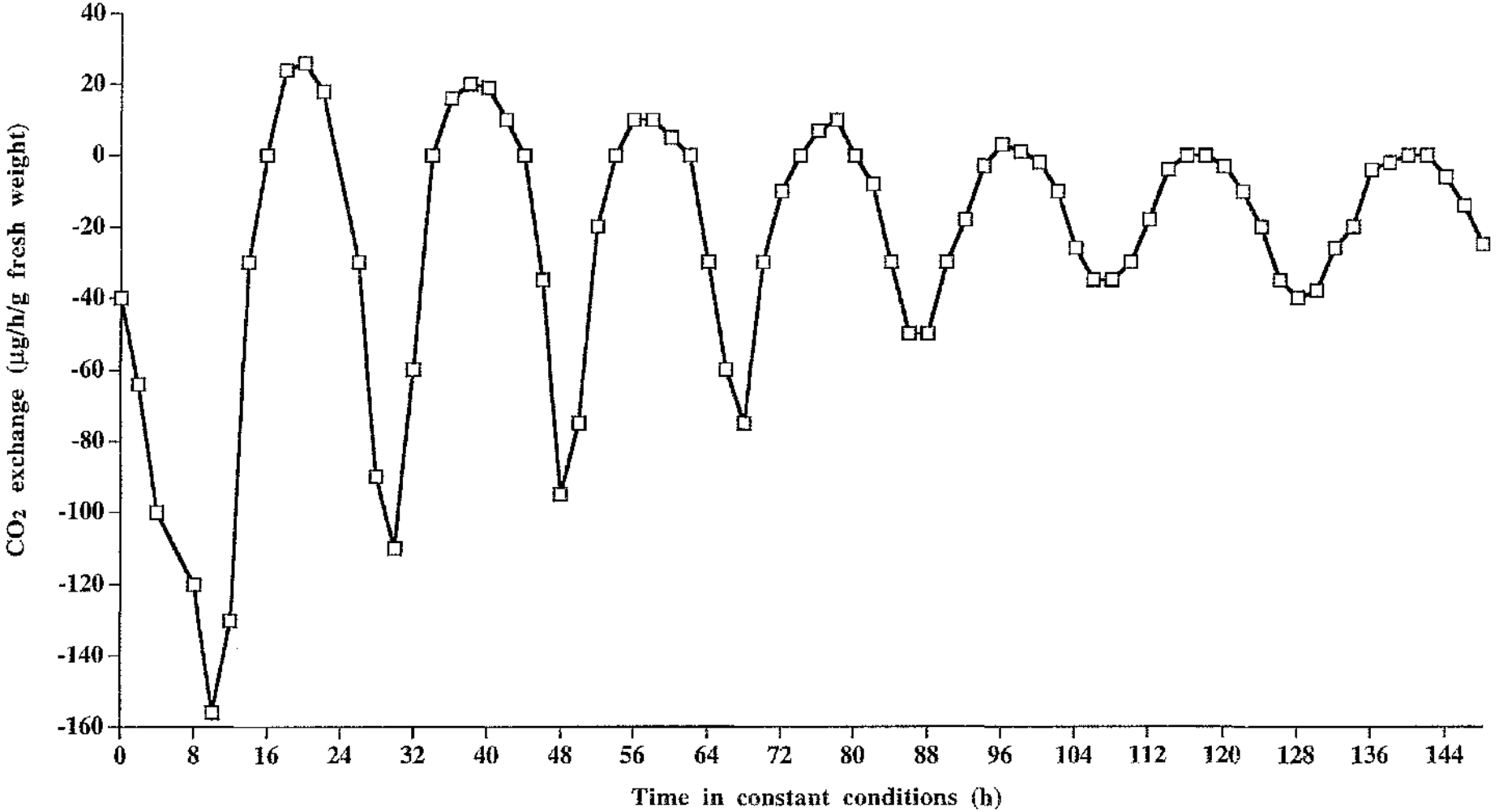

300
260
220
180
140
100
60
20
-20
CO_2 exchange (µg/h/g fresh weight)
0
8
16
24
32
40
48
56
64
72
80
88
Time in constant conditions (h)

output. However, the malate is pumped into the vacuole and this then allows PEPc to be active again leading to a second trough in the CO_2 output of the leaf. In conditions of constant darkness and CO_2-free air the rhythm of CO_2 output is thought to disappear after 3 to 4 days due to the vacuole reaching its capacity for malate. Under these circumstances, the malate synthesized during periods of PEPc activity will remain in the cytoplasm and therefore prevent the refixation of respired CO_2 by PEPc. The CO_2 output from the leaves therefore plateaus after 3 to 4 days as indicated by the constant escape of respired CO_2 from the leaves. Exposure of such leaves to a 4 h period of illumination allows all the malate to come out of the vacuole into the cytoplasm, where it is decarboxylated, causing a large peak in CO_2 output. The rhythm will then reinitiate until the vacuole has once more become saturated with malate (Wilkins, 1992). It must be noted that although this theory has a number of attractive features, such as the fact that reinitiation of the rhythm by light can be explained by decarboxylation of the accumulated malate, other factors must also be important. For example, the total malate content of leaves which have been in CO_2-free air at 15°C in the dark for 3-4 days does not reach anything like the malate content of leaves after a single normal night. Stored glucan provides the PEP which is the substrate for PEPc activity and the possibility that the rhythm damps out after 3-4 days due to depletion of the glucan stores has not been tested. The molecular mechanism that underlies the changes in flux through PEPc is discussed in section 1.4.3.

In continuous light and a stream of normal air, detached *Bryophyllum* leaves show a rhythm of CO_2 assimilation, rather than output, which lasts for at least 10 days (figure 1.7) (Anderson and Wilkins, 1989; Wilkins, 1984). However, at any particular temperature, with the exception of 31°C, the period of the rhythm is shorter than in darkness and a CO_2-free air stream (Wilkins, 1992). When PEPc is least active then CO_2 assimilation becomes negligible or CO_2 output is detected and CO_2 assimilation is occurring only via the Calvin-Benson cycle (Wilkins, 1992). There is no evidence that the rate of photosynthesis is regulated by a circadian rhythm in *B. fedtschenkoi* leaves (Wilkins, 1992). The rhythm of CO_2 assimilation by leaves in continuous light and a stream of normal air is believed to continue for up to 10 days because malate does not accumulate in the vacuole. The

Figure 1.7. The circadian rhythm of CO_2-exchange in *B. fedtschenkoi* leaves maintained in normal air and continuous light at 15°C

Detached *B. fedtschenkoi* leaves were placed in continuous light and normal air at 15°C and the CO_2-exchange of the leaves was monitored using an infra-red gas analyser.


40
20
0
-20
-40
-60
-80
-100
-120
-140
-160
CO_2 exchange (µg/h/g fresh weight)
0
8
16
24
32
40
48
56
64
72
80
88
96
104
112
120
128
136
144
Time in constant conditions (h)

conditions of continuous illumination mean that malate moves easily from the vacuole to the cytoplasm so the vacuole is not available as a malate storage compartment. Wilkins (1984) suggested that malate which accumulates during the first period of PEPc activity inhibits the enzyme and then the malate is removed via decarboxylation. This would require the circadian oscillator to drive a circadian alteration of the properties of the malate removal system (i.e. malic enzyme) and there is some evidence for this (Cook *et al.*, 1995). The CO_2 released may be refixed by Rubisco or escape from the leaf. Hence, the malate synthesized during each period of PEPc activity will subsequently be decarboxylated and so each cycle of the rhythm will begin with the leaf having a low malate content.

1.4. Phosphoenolpyruvate carboxylase in higher plants

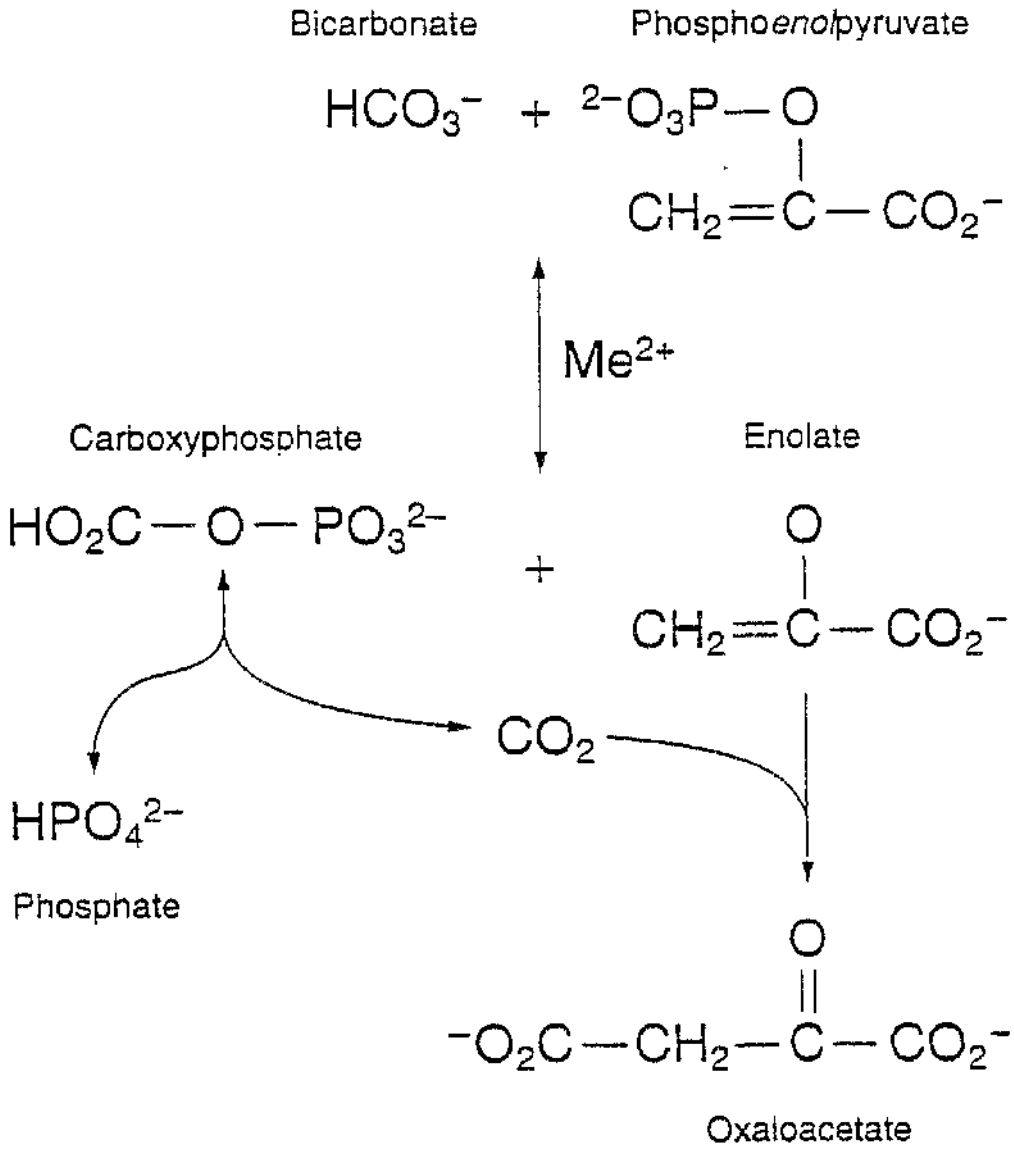
1.4.1. The reaction mechanism, effectors and roles of phosphoenolpyruvate carboxylase in plants

Phosphoenolpyruvate carboxylase [orthophosphate oxaloacetate carboxylase (phosphorylating)] (EC 4.1.1.31., PEPc) catalyses the ß-carboxylation of phosphoenolpyruvate (PEP) using HCO_3^- to yield oxaloacetate (OAA) and inorganic phosphate (P_i) (Andreo *et al.*, 1987). A number of divalent cations can act as cofactors for PEPc *in vitro*. The most effective cofactor is Mg^{2+}, but Mn^{2+} and Co^{2+} are also active (Andreo *et al.*, 1987). Ca^{2+} is inhibitory (Andreo *et al.*, 1987). The reaction proceeds in two steps and is strongly exergonic (ΔG° = -25 to -33 kJ mol^{-1}) to the extent that it is effectively unidirectional (Vennesland *et al.*, 1954) . The first, reversible step involves the transfer of phosphate to form carboxyphosphate and the enolate of pyruvate (figure 1.8). The enolate is then irreversibly carboxylated to give OAA and P_i (Andreo *et al.*, 1987; Chollet *et al.*, 1996). The OAA is rapidly reduced into malate by NADP-malate dehydrogenase (MDH, E.C. 1.1.1.37) or transaminated into aspartate by aspartate aminotransferase (EC 2.6.1.1).

Several metabolic effectors of PEPc are known. Malate is a potent feedback inhibitor and is thought to act, at least partly, allosterically at a number of sites mediating competitive, non-competitive and mixed inhibition (Doncaster and Leegood, 1987; Duff *et*

Figure 1.8. The proposed reaction mechanism of phosphoenolpyruvate carboxylase

Mechanism of the biotin-independent carboxylation of PEP (phosphoenolpyruvate) by PEP carboxylase. Abbreviation: Me^{2+}, divalent cation. The figure was taken from Vidal and Chollet (1997).


Bicarbonate
Phosphoenolpyruvate
HCO3−
2−O3P—O
CH2=C—CO2−
Me2+
Carboxyphosphate
Enolate
HO2C—O—PO32−
O
CH2=C—CO2−
CO2
HPO42−
Phosphate
O
−O2C—CH2—C—CO2−
Oxaloacetate

al., 1995; Wedding *et al.*, 1990). The pattern and extent of malate inhibition is dependent on the assay pH, phosphorylation status of the enzyme and whether the N-terminal region of the enzyme has been removed by proteolysis (Ausenhus and Oleary, 1992; Duff *et al.*, 1995; Jiao and Chollet, 1991; McNaughton *et al.*, 1991; Wang *et al.*, 1992). The apparent K_i (L-malate) of recombinant *Sorghum* PEPc at pH 7.3 was 0.17 mM and 1.2 mM for the dephosphorylated and phosphorylated forms respectively (Duff *et al.*, 1995). Glucose 6-phosphate is an allosteric activator of PEPc and is thought to act at a different site to malate (Doncaster and Leegood, 1987; O'Leary, 1982). The sensitivity of PEPc to activation by G6P varies according to its phosphorylation state. For example the K_a (G6P) of recombinant *Sorghum* PEPc at pH 7.3, 1 mM pyruvate was 1.3 mM and 0.28 mM for the dephospho- and phospho-forms of PEPc respectively (Duff *et al.*, 1995). PEPc is also inhibited by organic acids and PEP/pyruvate analogues, although the significance of this *in vivo* is not known. Interestingly, flavanoids and shikimic acid have been demonstrated to be potent inhibitors of PEPc (Colombo *et al.*, 1996; Pairoba *et al.*, 1996). It has been suggested that this may allow plants to divert PEP into the shikimic acid pathway for subsequent secondary metabolite (flavanoid) biosynthesis under conditions of excess products of glycolysis (including PEP), or photosynthetic metabolites (including PEP in C_4 plants) (Colombo *et al.*, 1996). A thorough coverage of the inhibitors and activators of PEPc is given in Rajagopalan *et al.* (1994).

Higher plant PEPc carries out a number of metabolic processes. In the leaves and non-photosynthetic tissues of C_3 plants PEPc's major role is anaplerotic, providing four carbon skeletons (OAA, malate or aspartate) for nitrogen assimilation and amino acid biosynthesis (Melzer and O'Leary, 1987; Van Quy *et al.*, 1991a). PEPc also maintains pH and ionic balance, recaptures respiratory CO_2 and plays a role in the transfer of reducing power (NADH) (Lepiniec *et al.*, 1993). More specialised functions of PEPc include the regulation of stomatal movement (Schulz *et al.*, 1992; Tarczynski and Outlaw, 1993; Zhang *et al.*, 1994) and nitrogen assimilation in roots and root nodules (Deroche and Carrayol, 1988; Schuller *et al.*, 1990; Schuller and Werner, 1993). In the leaves of C_4 and CAM plants, distinct isoenzymes of PEPc carry out the initial, photosynthetic fixation of

atmospheric CO_2 (as HCO_3^-) (Chollet *et al.*, 1996; Cushman and Bohnert, 1997; Lepiniec *et al.*, 1994; Toh *et al.*, 1994; Vidal and Chollet, 1997).

1.4.2. The structure and evolution of plant PEPc

The PEPc protein and corresponding cDNA have now been characterized from a wide range of species encompassing C_3, C_4 and CAM plants. C_4 and CAM plants possess a specific isoform of PEPc involved in the C_4 and CAM pathways respectively. In addition they possess C_3, stomatal and housekeeping isoforms of PEPc which are not directly involved in the C_4 or CAM pathway. For example, 2 genes encoding PEPc have been cloned from the facultative CAM plant *M. crystallinum* whilst four bands can be detected on Western blots; and 3 genes which code for PEPc have been cloned from the C_4 plant *Sorghum vulgare*, corresponding to the C_3, C_4 and root isoforms of the enzyme (Bohnert *et al.*, 1992; Cretin *et al.*, 1990). In *M. crystallinum* the 3.2 Kb *ppc2* gene encodes the 109 kDa C_3-form of PEPc whilst the 3.4 Kb *ppc1* gene encodes the 110 kDa, salt-inducible, CAM-form of PEPc (Bohnert *et al.*, 1992; Cushman and Bohnert, 1989a; Cushman and Bohnert, 1989b; Rickers *et al.*, 1989). In maize there are at least five members of the PEPc gene family (Grula and Hudspeth, 1987). The monocot C_4 species, maize and *Sorghum*, possess a single gene for the C_4 isoform of PEPc, whilst in the C_4 dicot *Flaveria trinervia*, a small subgroup of the genes in the PEPc multigene family encode the C_4 isoform of PEPc (Grula and Hudspeth, 1987; Hermans and Westhoff, 1990; Hermans and Westhoff, 1992; Lepiniec *et al.*, 1993; Poetsch *et al.*, 1991).

When all of the published, deduced amino acid sequences for PEPc are aligned, a number of well conserved residues and motifs become evident. In particular, the phosphorylation motif near the N-terminus has been found in all higher plant PEPcs including a gymnosperm, but is absent from the bacterial and cyanobacterial PEPc sequences (Relle and Wild, 1996; Vidal and Chollet, 1997). This motif has the consensus sequence - E/DR/KxxSIDAQL/MR (the phosphorylated serine is highlighted in bold) (Chollet *et al.*, 1996). The presence of this phosphorylation motif in all the higher plant PEPc deduced amino acid sequences indicates that they are all capable of undergoing phosphorylation on this N-terminal serine residue. The plant PEPcs also possess five

conserved cysteine residues which may be involved in subunit interactions to maintain the tetrameric structure or redox regulation (Andreo *et al.*, 1987; Chardot and Wedding, 1992). Other species invariant motifs in the PEPc sequences include TA**H**PT, VMxGYSDSx**K**DxG, and F**H**GRGxxxx**R**GxxP which contain the Arg, His and Lys residues (indicated in bold) that have been implicated in the active site domain (Chollet *et al.*, 1996). The region surrounding the lysine in the VMxGYSDSx**K**DxG motif is thought to be involved in the binding of PEP/catalytic activity (Jiao *et al.*, 1990). For the *E. coli* enzyme, the histidine in the TA**H**PT motif has been proposed to be involved in the formation of OAA by using a site-directed mutagenesis approach (Terada and Izui, 1991). Further characterization of the structure/function relationships of PEPc may result from work involving the site-directed mutagenesis of additional conserved sites in the enzyme and the production of a model of the three dimensional structure of the enzyme as a consequence of X-ray crystallography studies (Chollet *et al.*, 1996). The X-ray structure of *Eschericia coli* PEPc has recently been resolved and hopefully this will permit modelling of the structure of the plant PEPcs using the *E. coli* structure as a basis (Kai *et al.*, 1997).

Site-directed mutagenesis of the phosphorylatable N-terminal serine residue found in the plant PEPc sequences has demonstrated the importance of this residue for the regulation of the enzyme. Examination of the C_4 isoform of PEPc from *Sorghum vulgare* has demonstrated that Ser^8 undergoes phosphorylation (Cretin *et al.*, 1991a; Jiao *et al.*, 1991b). Site-directed mutagenesis of this serine residue to an aspartate (S8D) mimicked phosphorylation by introducing a negative charge at this position (Wang *et al.*, 1992). The S8D form of the enzyme could not be phosphorylated by the C_4-leaf PEPc kinase but was malate-insensitive like the phosphorylated (Ser^8-P) form of the enzyme found *in planta* (Duff *et al.*, 1995; Wang *et al.*, 1992). However, when Ser^8 was mutagenised to a cysteine (S8C) the resulting enzyme could not be phosphorylated by PEPc kinase and was malate sensitive like the dephosphorylated enzyme found *in planta* (Wang *et al.*, 1992).

When the S8C form of the enzyme was subjected to S-carboxymethylation by iodoacetic acid, the resulting negative charge a position 8 caused the $I_{0.5}$ (L-malate) of the enzyme to increase from 0.12 mM to 0.35 mM which mimics the change mediated by

phosphorylation of the wild type enzyme at Ser^8 (0.15 to 0.40 mM) (Duff *et al.*, 1993). Further, evidence of the cardinal role of Ser^8 in *Sorghum* PEPc with regard to the changes in the kinetic properties of the enzyme brought about by phosphorylation at this residue has been obtained by using specific anti-phosphorylation site antibodies. Anti-phosphorylation site antibodies reacted specifically with the N-terminal phosphorylation domain of PEPc. Binding of the antibodies to dephospho-PEPc blocked the subsequent phosphorylation of the enzyme by mammalian cyclic AMP-dependent protein kinase (PKA) and caused a decrease in the L-malate sensitivity of the enzyme which mirrored the change brought about by the phosphorylation of Ser^8 (Pacquit *et al.*, 1995). Hence, binding of an antibody to the N-terminus of PEPc appears to cause the same changes to the properties of PEPc as phosphorylation. It would seem likely from this data that in the dephospho-form of the enzyme the N-terminal region interacts with the rest of the protein and this can be prevented by either phosphorylation or antibody binding.

The deduced amino acid sequences of PEPc from over 20 species of plant and bacteria have been published. Analysis of the phylogenetic relationships between these sequences provides an insight into the evolutionary transition from C_3 to C_4 and CAM plants. The C_4 isoforms of PEPc from monocots fall on their own branch of the phylogenetic tree and are distinct from a dicot C_4 sequence, various monocot, dicot and gymnosperm C_3 and CAM sequences and also their indigenous C_3 isoforms (Lepiniec *et al.*, 1994; Relle and Wild, 1996). It has been suggested that this may indicate that the monocot C_4 PEPc genes appeared due to a duplication event which occurred prior to the divergence of the monocots and dicots, and the appearance of C_4 plants. The gene for C_4 PEPc may have evolved from this original duplication in some species (such as maize and *Sorghum*) whilst it was lost from other species that did not evolve the C_4 pathway of photosynthesis (Chollet *et al.*, 1996). This hypothesis would explain the separate position of monocot C_4 PEPc sequences on the phylogenetic tree. The sequence of the C_4 isoform of PEPc from the dicot *Flaveria trinervia* is more closely related to that of the C_3 and CAM isoforms than it is to the sequences of the C_4 isoforms from the monocots maize and *Sorghum*. This indicates that the C_4 pathway has most probably evolved on numerous

separate occasions and that the mechanism of evolution probably differs in each case. For example, the C_4 *Ppc* gene from *F. trinervia* has a promoter sequence very similar to that of an orthologous gene in the closely related C_3-species *F. pringlei*. (Hermans and Westhoff, 1992). Thus, it has been suggested that, in the case of the genus *Flaveria*, the C_4 isoform of PEPc has evolved via the 'fine tuning' of the promoter of the orthologous C_3 gene (Hermans and Westhoff, 1992). This 'fine tuning' allowed an alteration to the expression pattern of PEPc such that it is expressed in a mesophyll-cell cytosol specific manner in C_4 *F. trinervia* (Hermans and Westhoff, 1992).

Interestingly, the PEPc gene for the CAM isoform of PEPc from the monocot CAM plant *Aloe arborescens* does not group with the C_4-monocot PEPcs, but instead groups with the root isoform of PEPc from *Sorghum* and the CAM isoform from the dicot *Mesembryanthemum crystallinum* (Honda *et al.*, 1996). This indicates that the monocot CAM-form of PEPc is unlikely to have originated via a gene duplication event which occurred prior to the separation of monocots and dicots, as has been proposed for the gene from C_4 monocots (Chollet *et al.*, 1996). Instead, the monocot CAM isoform of PEPc seems to have evolved from the monocot C_3 isoform, and this possibly occurred at or after the separation of the monocots and dicots. The CAM isoform of PEPc from *M. crystallinum* also falls within the C_3-type grouping, on a separate branch from the C_4-monocots. Hence, both the CAM PEPc sequences published to date seem to have evolved from their C_3 counterparts either around the time of the divergence of monocots and dicots or after this divergence. Whether this will remain the case as further CAM-PEPc sequences are produced, or whether there proves to be some CAM-PEPcs which occurred prior to the separation of monocots and dicots must await the publication of other CAM-PEPc sequences. Particularly interesting will be the analysis of the phylogenetic relationships of the CAM isoform of PEPc from pteridophytes and *Isoetes*, because this may reveal whether the CAM isoforms of PEPc in the angiosperms are closely related to the CAM isoforms in some of the ancestors of the angiosperms (Griffiths, 1988; Raven and Spicer, 1996).

1.4.3. PEPc regulation in *B. fedtschenkoi*

When the diurnal changes in CO_2 metabolism in *B. fedtschenkoi* leaves were studied at the biochemical level, it was discovered that the sensitivity of PEPc to the feedback inhibitor L-malate also changes between day and night (Nimmo *et al.*, 1984). Rapidly desalted extracts prepared during the day contained PEPc with a low apparent K_i for malate of 0.3 mM. The PEPc in desalted extracts made from *B. fedtschenkoi* leaves at night showed an apparent K_i ten-fold greater at 3.0 mM (Nimmo *et al.*, 1984). The specific activity of PEPc within the extracts did not vary over a 24 h period indicating that the amount of PEPc protein in the leaves remains constant throughout. This was confirmed immunologically (Nimmo *et al.*, 1986). This suggested that PEPc was regulated post-translationally. In order to determine the nature of the post-translational regulation of PEPc, detached leaves were allowed to take up $^{32}P_i$ for 72 hours followed by rapid extraction and immunoprecipitation of PEPc (Nimmo *et al.*, 1984). The immunoprecipitated PEPc was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Autoradiography of the resulting gel demonstrated that the night form of PEPc contained $^{32}P_i$ but the day form did not (Nimmo *et al.*, 1984). The phosphate group was shown to be bound to serine using two dimensional thin-layer electrophoresis of hydrolysed ^{32}P-labelled PEPc (Nimmo *et al.*, 1986). This suggested that protein phosphorylation was the post-translational modification responsible for controlling the circadian rhythm of PEPc activity.

Further investigations led to the purification of the phosphorylated night form and dephosphorylated day form of PEPc from *Bryophyllum* leaves and the subsequent characterisation of the enzyme in the two phosphorylation states (Nimmo *et al.*, 1986). Both forms of the enzyme contained two proteins, a major one (subunit Mr 112 000) and a minor one (subunit Mr 123 000) as determined by SDS PAGE. These subunits are related as judged by proteolysis patterns and this is not an artefact of proteolysis *in vitro* (i.e. both subunits exist *in planta*). Both subunits became phosphorylated at night (Nimmo *et al.*, 1986). Alkaline phosphatase was found to remove the phosphate group from the night

form PEPc *in vitro*. This gave a concomitant increase in the apparent K_i of the PEPc for malate to that of the day form (Nimmo *et al.*, 1984).

Analysis of PEPc from detached *Bryophyllum* leaves maintained in continuous darkness and a stream of CO_2-free air, showed a good correlation between periods when the leaves were fixing respiratory CO_2 and periods when the PEPc was in the phosphorylated night form (Nimmo *et al.*, 1987b). With all the evidence pointing to phosphorylation as the cause of the circadian rhythm of PEPc activity in *Bryophyllum* the search for the enzyme(s) responsible for regulating the phosphorylation state of PEPc began. There were obviously two possible candidates for the role, namely the kinase and the phosphatase.

The first evidence as to the nature of the phosphatase which dephosphorylates PEPc at the end of the dark period came from experiments in which the purified catalytic subunits of rabbit skeletal muscle type 1 and 2A protein phosphatases were tested for *in vitro* activity against phosphorylated PEPc. This lead to the discovery that phosphorylated PEPc from *Bryophyllum* could only be dephosphorylated by protein phosphatase type 2A and not type 1 (Carter *et al.*, 1990). Furthermore, by using specific inhibitors, Carter *et al.* (1990) were able to demonstrate that *Bryophyllum* leaves contain a type 2A protein phosphatase that can dephosphorylate PEPc. Examination of the specific activity of this protein phosphatase type 2A over the 24 h period showed that there was no significant diurnal variation in its activity (Carter *et al.*, 1991).

Subsequent work in the same laboratory lead to the discovery of a kinase in *Bryophyllum* leaves capable of phosphorylating PEPc (Carter *et al.*, 1991). In plants under short day conditions (photoperiod 08.00-16.00), the kinase activity appeared between 20.00h and 22.00 h and persisted at a high level throughout the middle of the dark period, but was virtually undetectable by 06.00 h (Carter *et al.*, 1991). Presence of kinase activity correlated with the K_i of PEPc for malate being high. This pattern of activity indicated that it is the kinase and not the phosphatase that is responsible for the observed circadian rhythm of PEPc phosphorylation.

This raised the question as to what regulates the activity of the kinase such that it is active at night but not in the day. Detached *Bryophyllum* leaves were allowed to take up puromycin, cycloheximide (protein synthesis inhibitors), cordycepin and actinomycin D (RNA synthesis inhibitors) during the day in an attempt to ascertain whether the nocturnal appearance of PEPc kinase activity is controlled at the level of protein synthesis and/or RNA synthesis (Carter *et al.*, 1996; Carter *et al.*, 1991; Nimmo, 1993). Rapidly desalted extracts prepared from these leaves in the middle of the following night were found to contain no PEPc kinase activity. This was true whether the inhibitor was applied during the previous day or just 1-2 h prior to kinase assays in the middle of the night (Carter *et al.*, 1996; Nimmo, 1993). The PEPc in these extracts was dephosphorylated, as judged by its malate sensitivity. This work suggested that both *de novo* protein synthesis and an increase in the steady state level of an mRNA, possibly PEPc kinase mRNA, are necessary for the appearance of PEPc kinase activity. Furthermore, the fact that application of the inhibitors only 1-2 h prior to assay lead to loss of activity suggested that kinase activity was being rapidly turned over. It would appear from these results that the activity of PEPc kinase plays a key role in the control of the circadian rhythm of PEPc activity and CO_2 metabolism in *B. fedtschenkoi*.

Recent work on the effect of temperature on PEPc kinase activity and the phosphorylation state of PEPc in *Bryophyllum* has brought to light some interesting effects that are of great significance to the physiology of the plant in the wild (Carter *et al.*, 1995a). At 30°C a distinct circadian rhythm of CO_2 output was observed in the absence of a detectable rhythm in PEPc kinase activity. Therefore, it would appear that kinase activity is not completely essential for the generation of a circadian rhythm in PEPc activity and CO_2 output (Carter *et al.*, 1995b). Rather, circadian changes in kinase activity may increase the amplitude of the rhythm. Decreasing temperature increases the apparent K_i of PEPc for malate irrespective of whether or not it is phosphorylated. At 3°C the apparent K_i of dephosphorylated PEPc for malate is 9 mM whereas the K_i of the phosphorylated form is 20 mM. These values are markedly higher than the values of 0.3 mM and 3.0 mM respectively obtained at 25°C (Carter *et al.*, 1995a). The catalytic activity of PEPc is

greatly reduced at 3°C. However, this seems to be overcome *in vivo* by the greatly increased K_i of the enzyme for malate which renders PEPc physiologically active. This finding is supported by measurements of the malate concentration in leaves of *Bryophyllum* at a range of temperatures (Carter *et al.*, 1995b. Between 10 and 25°C leaf malate concentrations were around 50 mM after 72 h in the dark with a supply of normal air (Carter *et al.*, 1995b). At 3°C and 0°C the malate concentration was much higher, reaching up to 90 mM (Carter *et al.*, 1995b). This supports the finding that the PEPc in leaves held at this temperature is stabilised in the phosphorylated form and achieves a K_i of around 20 mM (Carter *et al.*, 1995a; Carter *et al.*, 1995b). Decreasing temperature, as is experienced by *Bryophyllum* as night approaches in Madagascar, thus promotes maximal fixation of CO_2 into malate throughout the night. This allows the plant to make optimum use of the high concentration of CO_2 caused by the nocturnal opening of the stomata. Increasing temperature has the reverse effect and thus helps prevent any futile recycling of CO_2 by PEPc in the day (Carter *et al.*, 1995b).

In C_4 plants a number of the possible links in the signal transduction cascade which regulates PEPc kinase activity have already been established (Vidal and Chollet, 1997) (see section 1.4.5). In *Bryophyllum*, however, the signal transduction process is less well understood. It has been speculated that, in the light of the effects of RNA and protein synthesis inhibitors, it could be the kinase itself that is synthesised / degraded in response to a circadian oscillator (Nimmo, 1993). However, the inhibitors used are not specific to PEPc kinase regulation (i.e. they inhibit all protein and RNA synthesis). Therefore, it is equally feasible that some other protein or proteins is / are required to activate and / or inactivate the kinase. An example of such a protein would be a kinase-kinase, which phosphorylates the PEPc kinase causing it to be physiologically active or inactive. In fact there could be a whole cascade of kinases involved, but ultimately there must be at least one gene, and a protein that it encodes, which form the molecular basis of the circadian clock in *Bryophyllum*. In *Neurospora crassa* and *Drosophila melanogaster* two such loci have already been found and characterised at the molecular level (see section 1.5.2) (Page, 1994). The *per* locus in *Drosophila* and the *frq* locus in *Neurospora* have both been shown

to display rhythmic RNA and protein levels (Aronson *et al.*, 1994; Page, 1994). The product of *frq* negatively regulates its own transcription and this results in the daily oscillation of the amount of *frq* transcript (Aronson *et al.*, 1994). A gene with similar properties to *per* and *frq* probably controls the circadian rhythm of PEPc kinase activity in *Bryophyllum* and it is the identification of this gene which must be the ultimate goal of research into the molecular basis of the circadian rhythm. The first step towards this will be discovering exactly how PEPc kinase is regulated.

1.4.4. PEPc regulation in other CAM species

The regulation of PEPc has also been studied in a number of other CAM plants, including both constitutive CAM plants like *Kalanchoë daigremontiana* and facultative CAM plants such as *Mesembryanthemum crystallinum* and *Clusia minor*. For example, Brulfert *et al.* (1986) demonstrated that the PEPc from *K. daigremontiana*, *K. tubiflora* and *K. blossfeldiana* was phosphorylated *in vivo* at night. When the night-form of PEPc from *K. blossfeldiana* was incubated with acid phosphatase the L-malate sensitivity of the enzyme increased to that of the day-form of the enzyme (Brulfert *et al.*, 1986).

K. uniflora is an epiphytic species that develops long trailing shoots which possess a pair of CAM performing leaves and minute roots at each leaf node. Cutting these shoots at internodes allows multiple self-sufficient CAM performing units to be obtained. This system has been used to examine the diel course of CO_2 metabolism, malic acid accumulation and the malate sensitivity and phosphorylation status of PEPc simultaneously (Kluge and Brulfert, 1996). Using this system, PEPc was found to become phosphorylated and malate-insensitive at night. This was accompanied by an increase in CO_2 assimilation and malate accumulation (Kluge and Brulfert, 1996). These results demonstrate that the conclusions of Nimmo *et al.* (1984) are valid for another species of *Kalanchoë* which has a markedly different growth habit to *B. fedtschenkoi*.

The common ice plant (*M. crystallinum*) is a facultative CAM plant. During the early stages of its development it performs C_3 photosynthesis, but from about four weeks after germination it can be induced to perform CAM by salt or water stress (Edwards *et al.*, 1996). Salt stress causes increases in the activity of a number of enzymes associated with

CAM, including PEPc and both NAD- and NADP-malic enzyme (ME) (Holtum and Winter, 1982). It has also been found that the induction of CAM leads to an increase in the transcript levels for a wide range of genes including *Ppc1* (PEPc, cytosol), *Mdh1* (NADP-MDH, chloroplast), *Mdh2* (NAD-MDH, mitochondria/cytosol), *Mod1* (NADP-ME, cytosol), *Mod 2* (NAD-ME, mitochondria) and *Ppdk1* (PPdK, chloroplast) (Cushman and Bohnert, 1996; Cushman and Bohnert, 1997). Furthermore, it has been shown that CAM-induced *M. crystallinum* leaves perform the *in vivo* phosphorylation of PEPc and contain PEPc kinase activity during the dark period (Baur *et al.*, 1992; Weigend, 1994). This indicates that the induction of CAM is accompanied not only by the induction of both the transcripts, proteins and enzyme activities necessary for CAM, but also by the regulatory machinery responsible for modulating the diurnal pattern of CO_2 metabolism in CAM.

This was supported by the work of Li and Chollet (1994) who demonstrated that a Ca^{2+}-independent PEPc kinase is induced concomitantly with PEPc by salt stress in *M. crystallinum*. PEPc kinase activity was detected only from stressed plants at night. Maximal induction of both PEPc protein and PEPc kinase activity occurred 10 days after salt-stressing the plants with 0.5 M NaCl (Li and Chollet, 1994). The PEPc kinase activity from darkened CAM-performing leaves of *M. crystallinum* was partially purified about 3500-fold and found to have a native molecular weight of ~33, 000 (Li and Chollet, 1994). However, "in gel" PEPc kinase assays on night extracts of CAM-induced *M. crystallinum* leaves indicated two Ca^{2+}-independent PEPc kinase polypeptides with molecular weights of 39 and 32 kDa (Li and Chollet, 1994). Determination of the exact molecular weight of PEPc kinase from *M. crystallinum* will require its complete purification and/or the cloning of its gene.

1.4.5. The regulation of PEPc in C_4 plants

Following the discovery that PEPc from CAM plants undergoes post-translational regulation via protein phosphorylation (Nimmo *et al.*, 1984), it was subsequently found that PEPc in C_4 plants undergoes a similar process of post-translational regulation (Budde and Chollet, 1986; Nimmo *et al.*, 1987a). However, whilst the phosphorylation of PEPc and the appearance of PEPc kinase occur at night in CAM plants, the equivalent processes

in C_4 plants were found to occur during periods of illumination (Budde and Chollet, 1986; Carter *et al.*, 1991; Jiao and Chollet, 1989; McNaughton *et al.*, 1991; Nimmo *et al.*, 1987a; Nimmo *et al.*, 1984). Initial work by Budde and Chollet (1986) demonstrated that PEPc can be phosphorylated *in vitro* when ATP is supplied as substrate, but failed to link this to changes in the allosteric properties of the enzyme. However, Nimmo *et al.* (1987) found that the K_i of PEPc for L-malate in rapidly desalted extracts of illuminated leaves was 2-3 times higher than that of PEPc in extracts from leaves kept in darkness. This change in the malate sensitivity of PEPc was accompanied by an increase in the extent of phosphorylation of PEPc *in vivo*. This was determined by supplying detached maize leaves with $^{32}P_i$ and then subjecting them to periods of illumination or darkness. Subsequent immunoprecipitation of the PEPc from extracts made from these leaves and separation of the immunoprecipitates by SDS-PAGE allowed the determination of the degree of phosphorylation of the PEPc. The PEPc from illuminated leaves was found to be phosphorylated and have a high K_i for malate (1.2 mM) whilst the PEPc from darkened leaves was found to be dephosphorylated and have a low K_i for malate (0.5 mM) (Nimmo *et al.*, 1987a). Phosphopeptide analysis of the *in vivo* phosphorylated PEPc indicated that maize PEPc was phosphorylated on a serine residue, confirming the findings of Budde and Chollet (1986) for the *in vitro* phosphorylated maize enzyme.

Subsequently, it was discovered that illuminated maize leaves contain a soluble protein kinase capable of phosphorylating PEPc on a serine residue (Jiao and Chollet, 1989; McNaughton *et al.*, 1991). This protein kinase was partially purified by ammonium sulphate precipitation and affinity chromatography on blue-dextran agarose such that PEPc contamination was removed. A reconstituted, *in vitro* PEPc kinase assay was developed. This involved incubating purified dark-form maize leaf PEPc with the partially purified protein kinase from illuminated leaves and [γ-^{32}P]ATP. In this assay system, the protein kinase preparation activated the purified dark-form (dephospho-form) of maize leaf PEPc by decreasing its L-malate sensitivity (Jiao and Chollet, 1989). This activation was accompanied by a concomitant increase in the phosphorylation status of the PEPc. The maximal molar ^{32}P-incorporation value was 0.25 per 100 kDa subunit, which is equivalent

to 1 per holoenzyme. However, later work by McNaughton *et al.* (1991) showed that the maximum extent of phosphorylation of PEPc by a partially purified, illuminated maize leaf protein kinase was 0.7-0.8 molecules per PEPc subunit. They proposed that the lower value (0.25 molecules/subunit) reported earlier by Jiao and Chollet (1989) may have been due to partial proteolysis of the PEPc, which they had overcome by the inclusion of a cocktail of protease inhibitors in their extraction buffers (McNaughton *et al.*, 1991).

Earlier work by Jiao and Chollet (1988) had demonstrated that incubating the light form of maize leaf PEPc with alkaline phosphatase caused an increase in the L-malate sensitivity of the enzyme to that of the dark-form of the enzyme, whereas the dark-form was unaffected. This observation was complemented and extended by the work of McNaughton *et al.* (1991). They found that the purified catalytic subunit of rabbit skeletal muscle protein phosphatase type 2A (PP-2A) was capable of dephosphorylating maize PEPc which had been labelled *in vitro* by a partially purified kinase. This dephosphorylation was accompanied by an increase in the L-malate sensitivity of the PEPc. However, the PP-1 from rabbit skeletal muscle did not dephosphorylate PEPc nor affect its L-malate sensitivity (McNaughton *et al.*, 1991). Furthermore, the specific activities of PP-1 and PP-2A (using phosphorylase a as substrate) in maize leaf extracts were unaffected by illumination whilst the activity of the PEPc protein kinase was shown to increase in response to illumination (Echevarria *et al.*, 1990; McNaughton *et al.*, 1991).

The amino acid sequence around the phosphorylated, N-terminal serine of maize PEPc was determined by performing automated Edman degradation analysis on the ^{32}P-labelled regulatory site phosphopeptide purified from a tryptic digest of the *in vitro* phosphorylated, purified dark-form maize leaf PEPc (Jiao and Chollet, 1990; Terada *et al.*, 1990). The sequence of the phosphopeptide was His-His-Ser(P)-Ile-Asp-Ala-Gln-Leu-Arg, regardless of whether the PEPc had been phosphorylated with mammalian cyclic AMP-dependent protein kinase or an illuminated maize-leaf protein kinase (Jiao and Chollet, 1990; Terada *et al.*, 1990). This corresponds exactly to residues 13-21 of the deduced amino acid sequence of maize leaf PEPc (Jiao and Chollet, 1990; Terada *et al.*, 1990). This means that it is serine 15 in the maize PEPc sequence that is the target for phosphorylation.

This *in vitro* phosphorylation site was confirmed as the *in vivo* phosphorylation site by performing automated Edman sequencing on *in vivo* phosphorylated PEPc from both maize and *Sorghum* (Jiao *et al.*, 1991b). Only a single N-terminal serine residue (Ser-15 for maize and Ser-8 for *Sorghum*) was phosphorylated *in vivo* (Jiao *et al.*, 1991b). Examination of the deduced N-terminal sequences of other PEPcs, encompassing C_3, C_4 and CAM species indicated a conserved N-terminal motif, Lys/Arg-X-X-Ser, which corresponds to this phosphorylation site (Jiao and Chollet, 1990).

At the beginning of the 1990's, research on the regulation of C_4 PEPc began to focus more specifically on the protein kinase responsible for phosphorylating PEPc *in vivo* because it became clear that the phosphorylation status of PEPc changed largely due to changes in the activity of this kinase, rather than that of the PP-2A responsible for dephosphorylating PEPc (Echevarria *et al.*, 1990; McNaughton *et al.*, 1991). Maize PEPc kinase activity increased and decreased according to the duration of periods of illumination or darkness respectively (Echevarria *et al.*, 1990; McNaughton *et al.*, 1991). The light induction of PEPc kinase was found to require a threshold photosynthetic photon flux density (PPFD) of about 300 μmol m^{-2} s^{-1} as inferred from estimates of the malate sensitivity of PEPc at a range of PPFDs (Bakrim *et al.*, 1992; Nimmo *et al.*, 1987a). Furthermore, light quality had no effect on the light-induced decrease in the L-malate sensitivity of PEPc, with white, red and blue light proving equally effective (McNaughton *et al.*, 1991). This suggested that the induction of PEPc kinase was not controlled by phytochrome or the blue-light photoreceptor. The requirement for at least 300 μmol m^{-2} s^{-1} of light for a change in the malate sensitivity of PEPc suggested that flux through photosynthesis was involved in the light-induction of PEPc kinase. This possibility was tested by supplying detached maize leaves with photosynthetic electron transport and Calvin cycle inhibitors prior to illuminating them. Photosystem II (DCMU, isocil), photosystem I (methyl viologen) and Calvin cycle (DL-glyceraldehyde) inhibitors all blocked the subsequent light activation of PEPc kinase and the resulting change in the L-malate sensitivity of PEPc (Jiao and Chollet, 1992; McNaughton *et al.*, 1991). This finding was confirmed in leaves of *Sorghum* suggesting that the signal transduction

pathway for the regulation of PEPc kinase and PEPc in C_4 plants is conserved (Bakrim *et al.*, 1992). Additionally, Bakrim *et al.* (1992) supplied an ATP synthesis inhibitor (gramicidin) to detached *Sorghum* leaves and demonstrated that ATP synthesis is also required for the light-induction of C_4-PEPc kinase (Bakrim *et al.*, 1992).

One of the most important findings concerning the regulation of C_4-PEPc kinase was the discovery that the light-induction of the kinase requires *de novo* protein synthesis (Bakrim *et al.*, 1992; Bakrim *et al.*, 1993; Jiao *et al.*, 1991a). This was determined by feeding cytosolic protein synthesis inhibitors to detached leaves and attempting to induce PEPc kinase activity by illuminating the leaves. Subsequent analysis of the PEPc kinase activity in these leaves showed that the protein synthesis inhibitors prevented the light-induction of PEPc kinase without affecting the light activation of other photosynthesis-related enzymes in maize (cytosolic sucrose-phosphate synthase, chloroplast stromal NADPH-malate dehydrogenase and pyruvate P_i dikinase), stomatal conductance or the Calvin-Benson cycle (Bakrim *et al.*, 1992; Bakrim *et al.*, 1993; Jiao *et al.*, 1991a). However, treatment of leaves with chloramphenicol (a chloroplast protein synthesis inhibitor) had no effect on the light activation of maize leaf PEPc kinase (Jiao and Chollet, 1991). This requirement for cytosolic protein synthesis in the light-induction of PEPc kinase represents evidence that the kinase itself or a secondary component undergoes *de novo* protein synthesis during its induction. This is very unlike the typical animal paradigm, in which protein kinase activity is regulated by a second messenger, a metabolite or a protein phosphorylation cascade without net synthesis/degradation of the kinase.

Unlike the situation in CAM-plants, where the circadian-clock mediated, dark-induction of PEPc kinase has been shown to require both protein and RNA synthesis using an inhibitor based approach, the light-induction of PEPc kinase in C_4 plants apparently does not require RNA synthesis. This conclusion is drawn from the findings of Giglioli-Guivarc'h *et al.* (1996) that the RNA synthesis inhibitors α–amanitin and actinomycin D did not block the light- and weak-base induced decrease in the L-malate sensitivity of PEPc in isolated mesophyll protoplasts from leaves of the C_4 grass *Digitaria sanguinalis*.

It was concluded from this result that the increase in PEPc kinase activity most likely results from the increased translation of pre-existing mRNA (Giglioli-Guivarc'h *et al.*, 1996). However, these authors failed to demonstrate the effectiveness of their RNA synthesis inhibitors at blocking RNA synthesis in *D. sanguinalis* protoplasts and it therefore remains possible that C_4-PEPc kinase is transcriptionally regulated.

The use of mesophyll protoplasts and cells from both *Sorghum* and *D. sanguinalis* has permitted a more detailed analysis of the light-transduction cascade responsible for inducing PEPc kinase activity in C_4 plants. Initial findings with *Sorghum* mesophyll protoplasts demonstrated that PEPc kinase can be light activated in isolated protoplasts (Pierre *et al.*, 1992). The process of light-activation was found to require protein synthesis, alkalinization of the cytosol and an increase in the cytosolic calcium concentration (Duff *et al.*, 1996; Pierre *et al.*, 1992). A more detailed analysis of the signal cascade in *D. sanguinalis* mesophyll protoplasts involved the use of a wide variety of highly specific inhibitors of possible signal transduction events (Giglioli-Guivarc'h *et al.*, 1996). Light induction of PEPc kinase and an increase in the apparent phosphorylation state of PEPc were both found to require light plus a weak-base such as NH_4Cl or methylamine, which caused the alkalinization of the cytosol. The role of the weak-base was mimicked by supplying 3-PGA, which may indicate that, in the intact leaf, 3-PGA is transported from the bundle-sheath cells (where the chloroplasts possess a complete Calvin cycle) to the mesophyll cells as one of the early steps in the light induction of PEPc kinase. A variety of calcium channel blockers were tested for their ability to perturb the light- and weak base dependent induction of PEPc kinase. Only verapamil and TMB-8 inhibited the decrease in the L-malate sensitivity of PEPc, whilst the plasmalemma calcium channel blockers diltiazem and nifedipine had no effect. TMB-8 was also shown to inhibit the light-induction of PEPc kinase activity. Both verapamil and TMB-8 block the activity of tonoplast membrane calcium channels and it has therefore been suggested that the light-induction of PEPc kinase requires the flux of vacuolar Ca^{2+} into the mesophyll cytosol. Giglioli-Guivarc'h *et al.* (1996), also demonstrated that the light- and weak-base mediated activation of PEPc kinase requires a calcium/calmodulin-dependent protein kinase activity,

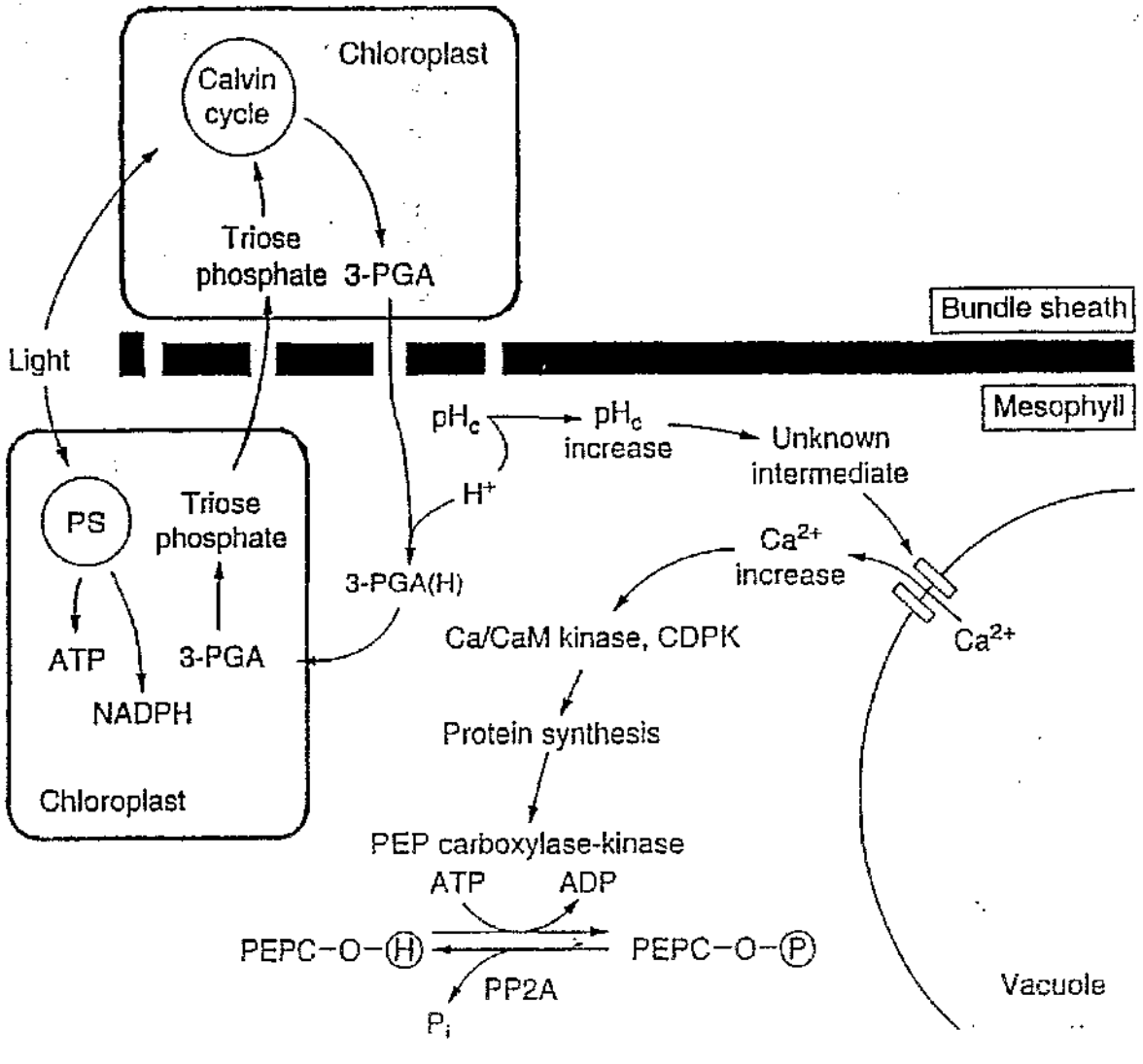
as implied from the inhibition of the decrease in the L-malate sensitivity of PEPc by W7, W5 and compound 48/80. W7 also inhibited the increase in PEPc kinase activity. Protein synthesis, ATP synthesis and PSII electron transport were also found to be necessary for the light and weak-base dependent induction of PEPc kinase, whilst, as already mentioned, RNA synthesis was not required (Giglioli-Guivarc'h *et al.*, 1996). This data led to the development of a model of the signal-transduction pathway involved in the light induction of PEPc kinase in C_4 mesophyll cells (see figure 1.9) (Vidal and Chollet, 1997).

1.4.6. Establishing the properties of the 'real' C_4-PEPc kinase

The identity of the 'real' C_4-PEPc kinase has been the subject of some confusion since C_4-PEPc kinase was first described in 1989 (Jiao and Chollet, 1989). Whilst, Echevarria *et al.* (1990) and McNaughton *et al.* (1991) defined maize PEPc kinase as a calcium-independent kinase, Ogawa *et al.* (1992) described a Ca^{2+}-dependent kinase capable of phosphorylating PEPc and activating it, although they did not display any data to demonstrate how their kinase activated PEPc. Other work has demonstrated the existence of both Ca^{2+}-dependent and Ca^{2+}-independent PEPc kinases in both *Sorghum* and maize (Bakrim *et al.*, 1992; Echevarria *et al.*, 1988; Li and Chollet, 1993; Wang and Chollet, 1993b). Bakrim *et al.* (1992) partially purified both a Ca^{2+}-dependent and a Ca^{2+}-independent kinase from illuminated *Sorghum* leaves. Only the Ca^{2+}-independent kinase mediated a decrease in the L-malate sensitivity of PEPc concomitant with phosphorylation of PEPc (Bakrim *et al.*, 1992). Similarly, both Ca^{2+}-dependent and -independent PEPc kinases have been identified in maize (Li and Chollet, 1993; Ogawa *et al.*, 1992; Wang and Chollet, 1993b). Ogawa *et al.* (1992) partially purified a Ca^{2+}-dependent maize PEPc kinase which they characterized using specific protein kinase inhibitors. Their kinase was inhibited by specific inhibitors of myosin light chain kinase which belongs to the Ca^{2+}-calmodulin-dependent protein kinase family. It was also inhibited by a calmodulin antagonist (W7) and in the presence of EGTA. The effect of EGTA was overcome by adding excess calcium at twice the EGTA concentration confirming that their kinase was indeed Ca^{2+}-dependent (Ogawa *et al.*, 1992). However, Wang and Chollet (1993) partially

Figure 1.9. The proposed signalling pathway which mediates the light-induction of PEPc kinase in C_4 plants

Abbreviations: 3-PGA, 3-phosphoglyceric acid; PS, photosystem; pHc, cytosolic pH; 3-PGA(H), partially protonated (2-) form of 3-PGA; CaM, calmodulin; CDPK, calmodulin-like domain protein kinase; PP2A, type 2A protein phosphatase; Pi, inorganic phosphate. The figure was taken from Vidal and Chollet (1997).


Chloroplast
Calvin cycle
Triose phosphate
3-PGA
Bundle sheath
Light
Mesophyll
pH_c
pH_c increase
Unknown intermediate
H^+
PS
Triose phosphate
3-PGA(H)
Ca^{2+} increase
ATP
3-PGA
NADPH
Ca/CaM kinase, CDPK
Ca^{2+}
Chloroplast
Protein synthesis
PEP carboxylase-kinase
ATP
ADP
PEPC–O–(H)
PEPC–O–(P)
PP2A
P_i
Vacuole

purified a Ca^{2+}-independent PEPc kinase from illuminated maize leaves. This kinase was purified 4000-fold and shown to be a ~30 kDa monomer. Despite applying rapid purification protocols in the presence of a complete arsenal of protease inhibitors (as suggested by Ogawa *et al.*, 1992) they failed to demonstrate the presence of a Ca^{2+}-dependent PEPc kinase in their purified PEPc kinase preparation. Their Ca^{2+}-independent kinase was shown to cause an increase in the $I_{0.5}$ (L-malate) of recombinant dephospho-*Sorghum* PEPc which was concomitant with the exclusive phosphorylation of Ser-8.

As had been reported previously for less purified preparations of maize PEPc kinase, the Ca^{2+}-independent kinase studied by Wang and Chollet (1993) was inhibited by L-malate (Jiao and Chollet, 1991; McNaughton *et al.*, 1991; Wang and Chollet, 1993b). This was most probably due an effect of L-malate on the structure of the kinase substrate (PEPc) because the phosphorylation of PEPc by mammalian cAMP-dependent protein kinase (PKA) was also inhibited by L-malate, whilst L-malate had no effect on the phosphorylation of casein by PKA (Wang and Chollet, 1993b).

Concurrent work in the same laboratory used a method of "in gel" kinase assay which involves separation of the kinase sample by denaturing electrophoresis. This is followed by the *in situ* renaturation of the proteins in the gel which allows them to be assayed *in vitro* for PEPc kinase activity by incubating gel segments in the presence of [γ-^{32}P]ATP and dephospho-PEPc. This approach reveals proteins in the gel capable of phosphorylating PEPc and allows the determination of their molecular weight by virtue of their separation through the gel (Li and Chollet, 1993). Using this approach Li and Chollet (1993) resolved one Ca^{2+}-dependent (~57 kDa) and two Ca^{2+}-independent (~37 and ~30 kDa) polypeptides capable of phosphorylating PEPc *in vitro* exclusively at the target serine residue. The ~57 kDa and ~37 kDa polypeptides were capable of autophosphorylation whilst the ~30 kDa polypeptide was not, and was therefore assumed to correspond to the ~30 kDa PEPc kinase partially purified by Wang and Chollet (1993). Furthermore, the activity of the ~57 kDa polypeptide was abolished by EGTA indicating it was Ca^{2+}-dependent, whilst the ~37 and ~30 kDa polypeptides were unaffected and therefore Ca^{2+}-independent. However, all three kinases were capable of phosphorylating the single N-

terminal serine of PEPc which is involved in the regulation of the enzyme. In order to resolve which of these polypeptides was the physiologically relevant PEPc kinase, Li and Chollet (1993) performed experiments in which maize leaves were supplied with inhibitors known to prevent the light-induction of PEPc kinase activity (cycloheximide and methyl viologen). Subsequent analysis of extracts from these leaves using the *in situ* renaturation approach revealed that the ~57 kDa polypeptide was present in darkness and unaffected by the inhibitor treatments, whilst the ~37 and ~30 kDa polypeptides were not present in darkness and their light-induction was blocked by inhibitors of protein synthesis and photosynthetic electron transport. The authors argued that the physiologically relevant PEPc kinase, which undergoes light activation that is blocked by cycloheximide and methyl viologen, is Ca^{2+}-independent and is either one or both of the ~37 kDa and ~30 kDa polypeptides (Li and Chollet, 1993). Since 1993, no further work has been published concerning the purification and characterization of the Ca^{2+}-independent C_4-PEPc kinase. This is most probably due to the extreme difficulties encountered when trying to purify a protein which represents «0.02 % of the total soluble leaf protein in a fully light-induced maize leaf (Vidal and Chollet, 1997; Wang and Chollet, 1993b). This means that, to date, there are no antibodies available to PEPc kinase and its corresponding cDNA has not been cloned. Thus, detailed analysis of the expression of the kinase in C_4 plants has been restricted to analysis of factors which affect the level of kinase activity rather than the level of kinase protein or mRNA.

1.4.7. The regulation of PEPc in C_3 plants

Phosphoenolpyruvate carboxylase performs a range of housekeeping functions in the tissues of C_3, C_4 and CAM plants in addition to its central role in primary CO_2 fixation in C_4 and CAM plants. 'C_3' isoforms of PEPc (so called purely because they are not involved in the C_4 pathway or CAM) occur and have specific roles in roots, root nodules, guard cells, fruits, seeds and leaves of C_3 plants (Deroche and Carrayol, 1988; Law and Plaxton, 1995; Melzer and O'Leary, 1987; Osuna *et al.*, 1996; Pacquit *et al.*, 1993; Sangwan *et al.*, 1992; Zhang *et al.*, 1994). The major 'C_3' role of PEPc is the provision of C_4 acids (oxaloacetate, malate or aspartate) to replenish the intermediates removed from

the TCA cycle for use as the skeletons for amino acid biosynthesis. In this role, PEPc is considered an anaplerotic enzyme due to its replenishing function (Melzer and O'Leary, 1987). PEPc also performs specialized roles in the carbon metabolism of nitrogen fixing nodules of Legume roots and stomatal guard cells during opening (Du *et al.*, 1997; Nimmo *et al.*, 1995; Schnabl *et al.*, 1992; Schuller *et al.*, 1990; Schuller and Werner, 1993; Zhang *et al.*, 1994; Zhang *et al.*, 1995). Early work suggested that the C_3 isoforms of PEPc did not undergo changes in activity and sensitivity to effectors (Chastain and Chollet, 1989; Tarczynski and Outlaw, 1990). However, it would be advantageous for a plant to be able to regulate the activity of a number of these C_3 PEPc isoforms. In particular, an ability to regulate the root nodule enzyme in response to photosynthate supply, the guard cell enzyme during stomatal opening and the anaplerotic enzyme during periods of favourable conditions for amino acid biosynthesis (high light and NO_3^-) would allow optimization of each of these processes. These possibilities have now been investigated by a number of research groups.

Sequence analysis of a variety of PEPcs from C_3 plants and the C_3 isoforms from C_4 and CAM plants reveals that they all possess the highly conserved N-terminal serine residue and phosphorylation motif previously characterized in C_4 plants (Cretin *et al.*, 1991b; Jiao *et al.*, 1991b; Lepiniec *et al.*, 1993). Thus, it was feasible that C_3 isoforms of PEPc also undergo *in vivo* phosphorylation as part of their regulation. *In vivo* phosphorylation of wheat leaf PEPc was first demonstrated by Van Quy *et al.* (1991). When detached wheat leaves from NO_3^- depleted plants were supplied with $^{32}P_i$ for 16 h in darkness the PEPc was only minimally labelled with ^{32}P. However, when these leaves were then subjected to 2 h of illumination in low-NO_3^- and high-NO_3^- the ^{32}P labelling of the PEPc was 416 and 665 % of the dark control respectively. These treatments also altered the L-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-NO_3^- and illuminated high NO_3^- leaves respectively (Van Quy *et al.*, 1991a). These results were later confirmed by Duff and Chollet (1995), who extended these observations by performing *in vitro* PEPc kinase assays and demonstrating that wheat-leaf PEPc kinase activity was highest in illuminated,

high NO_3^- leaves. The wheat-leaf PEPc kinase activity was Ca^{2+}-independent and did not phosphorylate the phosphorylation site mutant (S8D) form of *Sorghum* PEPc. This latter point indicates that this PEPc kinase exclusively phosphorylates PEPc at the N-terminal serine residue which is phosphorylated by other PEPc kinases (Duff and Chollet, 1995). Wheat-leaf PEPc kinase activity was completely abolished by feeding leaves with methyl viologen prior to inducing them with light and high NO_3^- and diminished by cycloheximide. This implies that the induction of PEPc kinase activity in N-depleted wheat-leaves requires photosynthetic electron transport from H_2O to NADP and at least part of the increase requires *de novo* protein synthesis. Hence, it was clear that the increase in the $I_{0.5}$(malate) of wheat leaf PEPc was accompanied by an increase in the phosphorylation state of the enzyme and PEPc kinase activity.

Interestingly, both illumination and high NO_3^- were required to achieve the greatest phosphorylation of wheat-leaf PEPc (Van Quy *et al.*, 1991a). Other concurrent work on N-depleted wheat-leaves demonstrated that the resupply of inorganic nitrogen to the 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). Feeding inorganic nitrogen to N-depleted leaves led to the diversion of ^{14}C-labelled photosynthetic carbon away from sucrose synthesis into organic acid and amino acid biosynthesis (Champigny and Foyer, 1992). Whilst PEPc was activated by the resupply of NO_3^-, sucrose phosphate synthase (SPS) was transiently inactivated (Champigny *et al.*, 1992; Van Quy and Champigny, 1992; Van Quy *et al.*, 1991a). When mannose was supplied to leaves to block protein kinase reactions, by sequestering P_i as mannose 6-phosphate and thereby inhibiting the synthesis of ATP, NO_3^--dependent PEPc activation and SPS inactivation was inhibited (Van Quy and Champigny, 1992). Furthermore, feeding leaves with okadaic acid, to block PEPc and SPS phosphatase activity, did not block the light- and high NO_3^--dependent induction of PEPc activity and inactivation of SPS (Van Quy and Champigny, 1992; Van Quy *et al.*, 1991a). This indicated that the resupply of high NO_3^- to N-depleted leaves did not activate PEPc and inactivate SPS by inactivating the relevant phosphatases, but rather by activating the relevant kinases (Van Quy and Champigny, 1992).

The exact nature of the NO_3^--mediated signal which stimulates the light-induction of PEPc phosphorylation and the increase in the $I_{0.5}$(malate) of PEPc in wheat leaves was dissected by feeding detached, N-depleted wheat leaves with NO_3^-, NH_4^+, glutamine, glutamate, alanine and aspartate. N-depleted wheat leaves were also supplied with NO_3^- plus either sodium tungstate, methionine sulphoximine, azaserine or amino oxyacetate in order to inhibit nitrate reductase (NR), glutamine synthetase (GS), glutamate synthase (GOGAT) or amino transferases respectively (Manh *et al.*, 1993). Glutamine enhanced the light-activation of PEPc and the concomitant decrease in sucrose synthesis. However, glutamate and aspartate prevented the light-activation of PEPc. Treatment of N-depleted leaves with sodium tungstate (NR inhibitor) and methionine sulphoximine (GS inhibitor) blocked the light and NO_3^--dependent induction of PEPc activity. By contrast, azaserine (a GOGAT inhibitor which causes the accumulation of glutamine) resulted in lower net sucrose synthesis and PEPc was activated above light plus NO_3^- control levels. Similar results for the effect of inhibiting GS activity, and thus blocking glutamine synthesis *in vivo*, on PEPc activity were obtained when N-limited barley leaves were supplied with phosphoinothricine (another type of GS inhibitor) (Diaz *et al.*, 1996; Diaz *et al.*, 1995). Collectively, these results demonstrate that the effect of NO_3^- on the light activation of PEPc activity and the inhibition of sucrose synthesis in N-depleted C_3 leaves is due to an increase in the *in vivo* glutamine concentration and not a direct effect of NO_3^- itself (Manh *et al.*, 1993).

In vitro assays on a partially purified PEPc kinase from wheat leaves demonstrated that the activity was enhanced by glutamine and inhibited by glutamate (Manh *et al.*, 1993). However, this observation was later refuted by Duff and Chollet (1995) who found no direct effect of glutamine on the *in vitro* activity of the Ca^{2+}-independent wheat-leaf PEPc kinase using a [γ-^{32}P]ATP-based *in vitro* kinase assay. It should be noted that Manh *et al.* (1993) did not include EGTA in their *in vitro* PEPc kinase assay, so their kinase, which was activated by glutamine, may have been Ca^{2+}-dependent and therefore a separate kinase from the Ca^{2+}-independent kinase reported by Duff and Chollet (1995). Manh *et al.* (1993) also assayed for the activity of PEPc kinase by incubating extracts with the

Sorghum leaf dark-form (dephospho-) PEPc in the presence of ATP and subsequently assaying the activity of PEPc as an estimate of the degree of phosphorylation. Glutamine did not directly enhance the *in vitro* activity of wheat-leaf PEPc, but Manh *et al.* (1993) did not test the effect of glutamine on the activity of the *Sorghum* leaf PEPc used as the substrate in their kinase assays.

Isolated C_3 isoforms of PEPc were first shown to undergo phosphorylation *in vitro* using the purified enzyme from tobacco leaves, soybean nodules and the recombinant C_3-type PEPc from the C_4-plant *Sorghum vulgare* (Pacquit *et al.*, 1993; Schnabl *et al.*, 1992; Schuller and Werner, 1993; Wang and Chollet, 1993a). Wang and Chollet (1993) purified tobacco leaf PEPc approximately 1000-fold and demonstrated that it could be phosphorylated *in vitro* by both an illuminated tobacco leaf kinase and an illuminated maize leaf kinase (Wang and Chollet, 1993a). The PEPc kinase activity from illuminated tobacco leaves was partially purified and found to be Ca^{2+}-independent (Wang and Chollet, 1993a). Similarly , the recombinant C_3 isoform of PEPc from *Sorghum* was found to undergo regulatory phosphorylation by mammalian PKA, the C_4-PEPc kinase from *Sorghum* leaves and a novel C_3-PEPc kinase from *Sorghum* roots (Pacquit *et al.*, 1993). Phosphorylation of the recombinant C_3-type *Sorghum* PEPc by both PKA and a root PEPc kinase activity was accompanied by an increase in the $I_{0.5}$ for L-malate of the PEPc. This indicated that *in vitro* phosphorylation of C_3 PEPc mediated changes in the regulatory properties of the enzyme similar to those reported for the C_4 and CAM enzymes.

Detailed analysis of the regulatory properties of C_3-leaf PEPc in the dark and light has been performed using barley-leaf protoplasts. Kromer *et al.* (1996) demonstrated that barley-leaf PEPc can be phosphorylated *in vivo* and that this phosphorylation increases in response to light and decreases upon the return to darkness. Light-induced increases in PEPc phosphorylation were accompanied by an increase in PEPc activity and a decrease in PEPc malate sensitivity (Kromer *et al.*, 1996a). However, the inhibitory effect of malate was totally overcome by physiological concentrations of G6P and therefore considered to be of little importance *in vivo* (Kromer *et al.*, 1996b). In fact, Kromer *et al.* (1996b) considered that feedback regulation of PEPc by aspartate and glutamate is more important

in the C_3 system since they are present at *in vivo* concentrations much higher than that of malate. This data raised the possibility that metabolite regulation of C_3-leaf PEPc is more important than post-translational regulation. Leport *et al.* (1996) used an inhibitor based approach to show that pea leaf and root PEPc is not regulated by phosphorylation, but is instead allosterically controlled by changes in the relative concentration of malate, G6P and PEP. However, they did not determine whether the *in vivo* phosphorylation status of PEPc or the level of PEPc kinase activity varied in response to light and nitrate supply in their pea leaf and root system, but simply based their conclusions on the failure of protein kinase and phosphatase inhibitors to perturb changes in PEPc activity (Leport *et al.*, 1996).

The signal transduction cascade which regulates PEPc and PEPc kinase in response to light in C_3 leaves has been investigated in barley leaf mesophyll protoplasts (Smith *et al.*, 1996) and tobacco leaves (Li *et al.*, 1996). In barley protoplasts, light-mediated changes in the allosteric properties of PEPc and PEPc kinase activity were found to occur in the presence or absence of additional nitrogen (Smith *et al.*, 1996). The specific activity of barley protoplast PEPc did not change with dark to light transitions, but its sensitivity to inhibition by L-malate was significantly diminished following illumination. Protoplasts possessed PEPc kinase activity in the dark and this was enhanced by incubating the protoplasts in the light for 1-2 h. The light induction of PEPc kinase activity in barley mesophyll protoplasts was not affected by DCMU (a photosynthetic electron transport inhibitor) indicating that light activation of the barley leaf protoplast kinase does not require photosynthesis (Smith *et al.*, 1996). Nevertheless, DCMU did prevent the light activation of nitrate reductase in barley mesophyll protoplasts in concurrent work in the same laboratory indicating that the inhibitor was effective in this system (Lillo *et al.*, 1996a). Incubating the protoplasts in the presence of cycloheximide prevented the light-induced decrease in the malate sensitivity of PEPc and blocked the light-activation of PEPc kinase activity. However, approximately 50 % of the kinase activity in barley protoplasts was unaffected by prolonged treatment with cycloheximide indicating the presence of a basal level of PEPc kinase activity which is only slowly turned over (Smith *et al.*, 1996). Reducing the intercellular Ca^{2+} concentration of the protoplasts by supplying EGTA and

the calcium ionophore A23187 enhanced the light-mediated reduction in the malate sensitivity of PEPc, but had no effect on the PEPc kinase activity. This supports the idea of two different PEPc kinase activities in barley, one which is slowly turned over and present in the dark and a second that is light-induced and rapidly turned over.

In contrast to the findings of Smith *et al.* (1996) using barley mesophyll protoplasts, Li *et al.*. (1996) demonstrated that tobacco leaf PEPc kinase activity is absent in the dark and is reversibly light activated. The light activation of tobacco leaf PEPc kinase requires both photosynthesis and protein synthesis. Additionally, the light induction of PEPc kinase in tobacco leaves is blocked by inhibiting GS with methionine sulphoximine. This effect is partially, but specifically, reversed by feeding glutamine to the leaves. These findings supported the earlier work of Manh *et al.* (1993) by demonstrating the importance of glutamine as part of the C_3 PEPc kinase signal transduction cascade. The tobacco leaf PEPc kinase was found to be Ca^{2+}-independent confirming early findings for both the tobacco leaf enzyme and the wheat-leaf enzyme (Duff and Chollet, 1995; Li *et al.*, 1996; Wang and Chollet, 1993a). "In gel" kinase assays on the partially purified kinase from tobacco demonstrated that the light-induced, Ca^{2+}-independent PEPc kinase polypeptides in tobacco leaves have molecular weights of 30 and 37 kDa. These polypeptides have molecular weights which are identical to the corresponding polypeptides in maize leaves (Li *et al.*, 1996). However, unlike tobacco PEPc kinase, the light-induction of maize PEPc kinase was not influenced by the glutamine status of the leaf (Li *et al.*, 1996). It should be noted that the Ca^{2+}-independent PEPc kinase activity of one type of C_3 tissue, the aleurone layer of barley seeds, is present in dry seeds (Echevarria *et al.*, 1997). Similarly, the PEPc kinase activity in banana fruit is present throughout ripening (Law and Plaxton, 1997). Indirect evidence suggests that the phosphorylation state of wheat-seed PEPc increases during germination (Osuna *et al.*, 1996). When barley seeds were allowed to germinate in the presence of a wide range of inhibitors, which have previously been shown to prevent the light-induction of PEPc kinase in C_4 leaves, no effect on the *in situ* regulation of PEPc phosphorylation was detected (Echevarria *et al.*, 1997). Hence, it must

be remembered that C_3 PEPc kinase activity is not always induced, but can be constitutively expressed in certain tissues.

The roots of many Leguminous plants develop nitrogen-fixing nodules in a symbiotic association with bacteria. In these nodules, the host plant PEPc performs two specific roles. One is the supply of C_4 dicarboxylic acids (malate) to the bacteroid as a source of energy and the other is the provision of carbon skeletons for the assimilation of the NH_4^+, produced due to N_2 fixation in the bacteroid, into amino acids. PEPc constitutes 0.5 to 2 % of the soluble protein in alfalfa (Deroche and Carrayol, 1988) and soybean (Zhang *et al.*, 1995) nodules, and thus, is one of the most abundant plant enzymes in the nodule. Nodule PEPc was found to be regulated by a range of metabolites including G6P, L-malate and aspartate (Schuller *et al.*, 1990). *In vitro* phosphorylation of purified soybean nodule PEPc by an endogenous kinase increased its apparent K_i for malate from 0.35 to 1.24 mM (Schuller and Werner, 1993). Soybean nodule PEPc was also shown to undergo *in vivo* phosphorylation by incubating detached nodules with $^{32}P_i$ and subsequently immunoprecipitating the PEPc from crude extracts and separating it by SDS-PAGE followed by autoradiography (Zhang *et al.*, 1995). The malate sensitivity of nodule PEPc and the level of PEPc kinase activity seem to respond to the supply of photosynthate from the phloem. For example, in the light when sucrose is arriving in the nodule from the leaves, PEPc kinase activity is high and PEPc is relatively malate insensitive. After prolonged darkness, decapitation (shoot removal) or stem girdling, all of which stop the supply of photosynthate arriving in the phloem, PEPc is dephosphorylated and malate sensitive and PEPc kinase activity decreases (Wadham *et al.*, 1996; Zhang and Chollet, 1997; Zhang *et al.*, 1995). The soybean nodule PEPc kinase has been partially purified ~3000-fold and it is a Ca^{2+}-independent kinase which is inhibited by malate and has a native molecular weight of ~30 kDa (Zhang and Chollet, 1997). "In gel" kinase assays on the partially purified soybean nodule PEPc kinase revealed active polypeptides of ~32 and ~37 kDa (Zhang and Chollet, 1997). Hence, the properties of soybean nodule PEPc kinase seem to be virtually identical to those of PEPc kinase from tobacco, maize and *M. crystallinum* (Zhang and Chollet, 1997).

Finally, the 'C_3' isoform of PEPc found in stomatal guard cells has also been extensively studied. Stomatal aperture is regulated through changes in the osmotic pressure of the pair of guard cells that flank each stoma. During stomatal opening guard cells accumulate K^+ salts and this is accompanied by the pumping of H^+ out of the guard cells by the plasmalemma H^+-ATPase. As part of the mechanism of maintaining the intercellular pH during this proton extrusion, guard cells accumulate organic anions, mainly $malate^{2-}$ (Du *et al.*, 1997). During the accumulation of malate two protons are released to the cytosol, which replenishes the extruded protons. The increase in K^+ salts and organic anions decreases the guard cell osmolyte potential which causes osmotic water uptake and hence, guard cell swelling and stomatal opening. Thus, PEPc is a key enzyme in stomatal guard cells because it catalyses the first step in the synthesis of the malate. Guard cell PEPc has been shown to be significantly inhibited by 400 μM malate in the dark but not during opening (Zhang *et al.*, 1994). This change in malate sensitivity has recently been demonstrated to be correlated with an increase in the *in vivo* phosphorylation status of PEPc (Du *et al.*, 1997). Furthermore, the increase in the phosphorylation state of PEPc was promoted by fusicoccin (which promotes stomatal opening) and the effect of fusicoccin was reversed by abscisic acid (which causes stomatal closure) (Du *et al.*, 1997). However, *in vivo* phosphorylation of guard cell PEPc in *Commelina communis* guard cell protoplasts does not correlate with changes in the malate sensitivity of the enzyme, but does correlate with a drop in the K_m of the enzyme for PEP (Nelson, 1994). For example, treatment of *C. communis* guard cell protoplasts with either light or fusicoccin caused a significant increase in the phosphorylation state of the enzyme whilst the K_m of PEPc for PEP approximately halved (Nelson, 1994).

It is evident from the data described in this section that the 'C_3' isoform of PEPc is capable of undergoing phosphorylation both *in vivo* and *in vitro*. However, whilst in some cases the phosphorylation of C_3 PEPc can cause alterations in its regulatory properties, in particular its sensitivity to L-malate, there are others in which C_3 isoforms of PEPc are not regulated by phosphorylation. In particular, the light activation of some C_3 leaf isoforms of PEPc can be accounted for wholly by changes in the concentration of its effectors which

occur upon illumination (Leport *et al.*, 1996). Clearly, there are no fixed rules about the regulation of C_3 PEPc. Whilst the N-terminal phosphorylation motif with its target serine residue has been identified in all the published sequences of PEPc, including a gymnosperm PEPc, and PEPc kinase activity seems to have been detected in all plant tissues in which it has been sought, the mechanism of reversible phosphorylation may not always be central to the regulation of PEPc *in vivo*. It is perfectly possible that the phosphorylation motif and PEPc kinase activity are evolutionary relicts in some species. As more C_3 isoforms of PEPc are sequenced it will be interesting to note whether some actually lack the phosphorylation motif, as seems to be the case for another plant phosphoprotein which has an N-terminal phosphorylation motif (PEP carboxykinase), from some C_4 grasses (Walker and Leegood, 1996).

1.5. Circadian rhythms: their characteristics and regulation

1.5.1. Characteristics of circadian rhythms

A wide variety of biological functions occur rhythmically with a period of about 24 h. Such natural rhythms in biological systems are termed "circadian" (*circa* - about, *dies* - day). Circadian rhythms are a ubiquitous feature amongst living organisms (Anderson and Kay, 1996). For a rhythm to be truly circadian it must have certain characteristics. Firstly, the rhythm must persist in constant environmental conditions. Secondly, the rhythm should have a period of approximately 24 h, although few rhythms have a period of exactly 24 h, and finally, the period of oscillation must show significant temperature compensation (Wilkins, 1992). The ability of the circadian oscillator to compensate for fluctuations in temperature has led to it being called a 'biological clock'. Circadian rhythms are studied under constant environmental conditions of light (or dark), temperature, humidity, air composition etc. Rhythms are generally entrained by the daily light-dark cycle and persist under a range of constant conditions. The phase of the rhythm can be shifted by interrupting the constant conditions with specific environmental or chemical signals (Wilkins, 1992). The circadian oscillator which generates the circadian rhythm has been modelled as a negative feedback loop (see Anderson and Kay, 1996; Page, 1994). Biochemically, the oscillator may consist of the transcription, translation, post-translational

modification, and/or nuclear transport, of a clock protein that negatively autoregulates its own production (Anderson and Kay, 1996; Page, 1994).

Circadian rhythms have been studied in a wide variety of biological organisms including prokaryotes, fungi, plants, insects, reptiles and mammals, including man (Wilkins, 1992). The physiological characteristics of these rhythms are remarkably similar, perhaps indicating a conserved evolutionary origin (Wilkins, 1992). The following sections will give a brief overview of characteristics of circadian rhythms in a few well studied organisms.

1.5.2. The regulation of circadian rhythms in *Neurospora crassa* and *Drosophila melanogaster*

Some of the most detailed work on the molecular basis of the circadian clock has been performed using clock mutants in the fruit fly *D. melanogaster* and the filamentous fungus *N. crassa*. *D. melanogaster* displays physiological and behavioural rhythms and *N. crassa* displays rhythms in asexual spore production (conidiation). Screening mutant lines of both species for anomalies in these circadian rhythms led to the identification of the mutant loci *per* (period) in *D. melanogaster* and *frq* (frequency) in *N. crassa* respectively (Feldman and Hoyle, 1973; Konopka and Benzer, 1971). The mRNA and protein levels of *per* and *frq* display circadian oscillations (Aronson *et al.*, 1994; Hardin *et al.*, 1992; Hardin *et al.*, 1990). Both *per* and *frq* are elements of feedback loops in which their protein products (PER and FRQ) regulate the expression of their respective genes (Page, 1994). This feedback regulation is believed to be a central feature of the clock's loop (Aronson *et al.*, 1994). Independent single locus mutations of *per* and *frq* yield both long- and short-periods and arrhythmicity (Page, 1994).

The circadian regulation of conidiation by the *frq* locus in *N. crassa* has recently been the subject of intensive research. Following the peak in *frq* mRNA at the beginning of the day, two forms of FRQ appear some 4-6 h later as a result of the alternative use of the first and third in-frame AUG translation initiation sites (Garceau *et al.*, 1997). Negative feedback of FRQ to reduce its own transcript level is rapid and efficient, requiring only about 10 molecules of FRQ per nucleus, so that most of the day is devoted to

posttranscriptional and posttranslational processing of FRQ prior to its turnover (Merrow *et al.*, 1997). Both forms of FRQ are rapidly phosphorylated following translation and then progressively re-phosphorylated throughout the day, with the level of phosphorylation peaking prior to the turnover of FRQ. That *frq* transcript levels do not rise until the end of the night/beginning of the morning (when FRQ disappears) suggests that the various phosphorylated forms of FRQ are effective at maintaining depression of *frq* transcript levels throughout most of the day and night (Garceau *et al.*, 1997). Furthermore, the two forms of FRQ have been shown to vary in abundance according to temperature. Either form of FRQ is sufficient for a functional clock at some temperatures, but both are necessary for robust, temperature-compensated rhythmicity (Liu *et al.*, 1997). Temperature favours particular translation initiation sites such that expression solely from AUG#1 permitted rhythmicity at lower temperatures, but rhythmicity was lost above 27°C, whilst expression from AUG#3 permitted rhythmicity at higher temperatures but became arrhythmic at temperatures of 20°C and below. In wild type *N. crassa* the amounts of AUG#1 FRQ and AUG#3 FRQ are virtually equal at 18°C, but the relative proportion of AUG#1 FRQ increases with temperature such that at 29°C the ratio of AUG#1 FRQ/AUG#3 FRQ is above 2 compared to a ratio of 1 at 18°C (Liu *et al.*, 1997). Thus, it would seem that a threshold level of FRQ is necessary to achieve rhythmicity at low temperatures and that rhythmicity is maintained as temperature increases due to an increase in the relative translation from AUG#1 which largely accounts for an overall increase in FRQ amount (Liu *et al.*, 1997; Merrow *et al.*, 1997).

In *D. melanogaster* a second mutation, *timeless* (*tim*), has been isolated that abolishes both the circadian rhythms of behaviour, the rhythm of *per* mRNA abundance and the accumulation of several reporter-PER fusions in the nucleus (Sehgal *et al.*, 1994; Vosshall *et al.*, 1994). Both the *tim* and *per* genes are turned on in the morning, and their corresponding mRNAs accumulate during the day. After dusk the TIM and PER proteins peak and they interact, forming a heterodimeric complex (this process may be favoured by phosphorylation of PER) (Page, 1994; Zeng *et al.*, 1996). This complex moves to the nucleus to stop transcription of the *per* and *tim* genes (Vosshall *et al.*, 1994; Zeng *et al.*,

1996). Subsequently, *tim*, and then *per* mRNA levels fall and this is followed by a concomitant drop in their protein concentrations. By dawn, TIM and PER are at such low levels that they no longer inhibit transcription of their genes and the cycle restarts. TIM is destroyed by light which means that light mediates the dissociation of the PER-TIM complex. It would seem that light-mediated phase shifting of the *D. melanogaster* rhythm is caused by destruction of TIM. If flies are subjected to light in the early evening then TIM levels drop due to its destruction so it can no longer pair up with PER to move to the nucleus (Lee *et al.*, 1996a; Myers *et al.*, 1996; Zeng *et al.*, 1996). However, *tim* mRNA is still abundant in the early evening and TIM levels are soon restored so the clock is simply set back a few hours. When flies are subjected to light in the morning then the destroyed TIM cannot be replenished as *tim* mRNA is absent. This allows the *tim* and *per* genes to come on a few hours earlier in the morning, advancing the clock (Lee *et al.*, 1996a). In *N. crassa*, FRQ levels peak during the day rather than at night and it has been demonstrated that phase shifting by light pulses during the night is caused by an increase in transcription of the *frq* gene (Aronson *et al.*, 1994; Crosthwaite *et al.*, 1995). Hence, these two circadian clocks have similar characteristics, but whilst light-mediated phase shifting in *D. melanogaster* occurs due to the destruction of TIM, the equivalent process in *N. crassa* occurs due to increased transcription of *frq*.

Recently a candidate clock gene from mammals, named *clock*, has been cloned which may well be a functional homologue of *per*, *tim* and *frq*. The *clock* locus was identified in mice by mutagenizing ~300 gametes with N-ethyl-N-nitrosourea and then screening the second generation progeny for significant alterations in the period of the wheel running behavioural rhythm (see Reppert and Weaver, 1997). The mutated gene was cloned by both positional cloning and functional identification using a transgenic approach (Antoch *et al.*, 1997; King *et al.*, 1997). The sequence of *clock* contains a PAS domain which bears a striking resemblance to the basic-helix-loop-helix (bHLH)-PAS genes. The PAS domain is a protein dimerization domain which occurs in the sequence of PER and several bHLH transcription factors. The fact that *clock* is a member of the bHLH-PAS family of transcription factors suggests it is involved in the transcriptional regulation of a

basic clock mechanism. Of particular interest in this context is the discovery of two genes, *white collar* (*wc-1*) and *wc-2*, in *N. crassa* which are PAS-domain containing transcription factors thought to be responsible for controlling the expression of all light responsive genes in this fungus. New data suggests that in addition to their roles in photoperception and signalling, *wc-1* is a clock controlled gene and *wc-2* is a clock component (Crosthwaite *et al.*, 1997). WC-2 appears to be involved in promoting the expression of *frq* and is required for the normal operation of a functional circadian clock. WC-2 is essential for the oscillation in the level of *frq* transcript and protein in conditions of constant darkness indicating it is an oscillator component in addition to its role in photoperception and signalling. WC-2 is not involved in the light-induction of *frq* mRNA because the light-induction of *frq* mRNA occurs in *wc-2*$^-$ mutant strains. In the absence of WC-1, light-induced *frq* expression does not occur and the clock does not 'tick'. WC-1 is essential for the induction of *frq* by light and is therefore likely to be the link between photoreception and the oscillator which mediates light-dependent phase shifting of the *N. crassa* clock (Crosthwaite *et al.*, 1997; Crosthwaite *et al.*, 1995). However, in *wc-1*$^-$ mutants, inducing the expression of *frq* using a quinic acid inducible promoter was accompanied by an initial peak of frq mRNA and subsequently of FRQ which decayed away upon removal of the quinic acid. Unexpectedly, *frq* mRNA and FRQ levels rose again and completed a second circadian cycle in the absence of quinic acid (Crosthwaite *et al.*, 1997). This indicates that WC-1 is not a required component of the loop but probably is essential to drive the expression of genuine clock components such as *frq* and *wc-2*. In the light of the involvement of PAS-containing transcription factors or genes in the circadian oscillators of *D. melanogaster*, *N. crassa* and mice, it has been suggested that PAS-containing transcription factors may function as necessary components of circadian oscillators of a wide range of taxa (Crosthwaite *et al.*, 1997; Reppert and Weaver, 1997). Whether PAS-domains occur in all transcription-translation-based negative feedback loops that form the molecular basis of the circadian oscillator must await the cloning of circadian oscillator genes from other organisms such as plants and prokaryotes.

1.5.3. Circadian rhythms of bioluminescence in *Gonyaulax polyedra*

The marine alga *G. polyedra* possesses specialized organelles called scintillons which contain luciferase (LCF), its substrate luciferin (LH_2) and a luciferin binding protein (LBP) (Mittag and Hastings, 1996; Nicolas *et al.*, 1987). Scintillons extend from the cytoplasm/tonoplast into the vacuole as "bubbles" (Nicolas *et al.*, 1987). Luciferin is bound to LBP at cytosolic pH (~pH 7.5), but it is released due to a conformational change when the pH drops to ~6.5 due to protons being pumped into the scintillon (Morse *et al.*, 1989). The free luciferin is the substrate for the luciferase in the scintillon. Luciferase catalyses the oxidation of luciferin with O_2 to give a product in an electronically excited state which emits light upon decay (Mittag and Hastings, 1996).

The whole of this bioluminescent system is under circadian control. Up to 400 scintillons per cell occur at night whilst only ~40 per cell occur during the day . Levels of both luciferase and LBP peak at night and continue to peak in the subjective night under constant conditions (Mittag and Hastings, 1996). Luciferase and LBP mRNA levels remain constant over the diurnal cycle indicating they are not transcriptionally regulated by the circadian clock. In fact, they were found to be translationally regulated by the clock. A region of UG repeats that form a 14 nucleotide hairpin loop in the 3' untranslated region (UTR) of the *lbp* mRNA has been shown to bind a single protein (circadian controlled translational regulator, CCTR) from *G. polyedra* extracts, whilst no proteins were detected which bind to the 5' UTR. This protein is believed to mediate the translational regulation of *lbp* mRNA in response to the circadian clock (Mittag and Hastings, 1996). CCTR binds the *lbp* 3' UTR during the day, indicating that it represses *lbp* translation. Derepression occurs at night when the extent of binding of CCTR to the *lbp* 3' UTR diminishes. The binding activity of CCTR may be modulated between night and day by phosphorylation or possibly dimerization/multimerization (Mittag and Hastings, 1996). Daily fluctuations in the binding activity of CCTR persist under continuous dim light indicating that the regulation of the binding properties of CCTR is under circadian control.

Phase shifts in the rhythm of bioluminescence in *G. polyedra* occur in response to light. These phase shifts can be blocked by the protein kinase inhibitor 6-

dimethylaminopurine, but not the PP1 and PP2A inhibitors okadaic acid, calyculin A and cantharidin (Comolli *et al.*, 1996). However, okadaic acid did cause significant period lengthening, whereas calyculin A and cantharidin caused phase delays but had no effect on period. Thus, light phase shifting requires a protein kinase activity but not a phosphatase activity. This has been explained using a hypothesis in which light phase shifting requires the phosphorylation of an unknown protein (possibly CCTR) which is blocked by the kinase inhibitor, whereas the phosphatase inhibitors maintain the unknown protein in the phosphorylated state and enhance the response to light (Comolli *et al.*, 1996). Despite quite detailed knowledge of the regulation of some of the proteins which are controlled by the circadian clock in *G. polyedra,* such as luciferase and LBP, nothing is known about the functional homologue of *per*, *tim* and *frq* in this alga. Screening for mutants in circadian bioluminescence should prove a powerful route to isolating the circadian oscillator in *G. polyedra*.

1.5.4. Circadian rhythms in plants

A wide variety of physiological processes, enzymes and genes have been found to be regulated by the circadian clock in higher plants. These include leaf movement, stomatal conductance, photosynthesis, CO_2 assimilation (see section 1.3.2), PEPc and PEPc kinase activity (see section 1.4.3), nitrate reductase activity, sucrose phosphate synthase activity and the transcription of the genes for the chlorophyll a/b binding protein (*cab*), the small subunit of Rubisco (*rbcS*) and catalase (*cat2*, *cat3*) (Boldt and Scandalios, 1995; Carter *et al.*, 1991; Giuliano *et al.*, 1988; Hennessey *et al.*, 1993; Jones and Ort, 1997; Kloppstech, 1985; Lillo *et al.*, 1996b; Nimmo *et al.*, 1987b; Pilgrim and McClung, 1993; Satter *et al.*, 1988; Wilkins, 1959; Zhong *et al.*, 1994). Whilst knowledge of the circadian regulation of all these diverse output processes is relatively detailed, very little is known about the underlying circadian oscillator in plants (Anderson and Kay, 1997). Such an oscillator would be the functional homologue of the *D. melanogaster* genes *tim* and *per* or the *N. crassa* gene *frq,* but no homologous sequences to these genes have been detected in *Arabidopsis* (Anderson and Kay, 1996).

However, a genetic approach has allowed the identification of circadian clock mutants in *Arabidopsis* (Millar *et al.*, 1995a). A reporter gene construct consisting of the promoter of the circadian-regulated *CAB2* gene fused to the firefly *luciferase* (*luc*) coding region was used to generate transgenic *Arabidopsis* plants (*cab2-luc*). These plants display circadian oscillations of bioluminescence that indicate periods when the *CAB2* promoter is transcriptionally active. When transgenic plants were grown for 5 days in 12 h light: 12 h dark (LD) cycles and transferred to constant white light (LL) the mean period of bioluminescence driven by transcription of the *CAB2* promoter was 24.7 h. When the same plants were transferred to constant darkness (DD) the oscillations rapidly damped and displayed a period of 30 to 36 h (Millar *et al.*, 1995b). This rapid damping is thought to be due to the decline in photoreceptor activation in DD conditions. Seed from *cab2-luc* plants was mutagenized using ethylmethane sulphonate (EMS) and the resultant seed was screened for aberrant patterns of cyclic bioluminescence. A total of 26 'timing of CAB' (toc) lines were identified under LL conditions, encompassing both long (26-28 h) and short (21-22.5 h) periods and a single line with wild type period but reduced amplitude (Millar *et al.*, 1995a).

One particular short period line (*toc1,* mean period of 20.9 h) was analyzed in detail. Light or dark grown *toc1* seedlings were morphologically indistinguishable from wild type. The *toc1* gene segregated away from the transgene, indicating that the mutation was independent of the reporter gene, and was mapped to the lower arm of chromosome 5 (Millar *et al.*, 1995a). *toc1* was found to be a semi-dominant mutation as has been found for *per* and *frq*. The period of the circadian rhythm of leaf movement was also shorter in *toc1* plants (23.3 h compared to 25.2 h in the wild type) indicating that *toc1* is a common element in the circadian regulation of both *CAB* expression and leaf movement in *Arabidopsis*. The cloning of *toc1* is currently underway and analysis of the expression of the cloned gene and its corresponding protein will demonstrate whether *toc1* is a component of the oscillator mechanism or a component of an input pathway upstream of the oscillator (Anderson and Kay, 1996; Millar *et al.*, 1995a). Of particular interest will be the determination of whether the *toc1* gene possesses a PAS protein dimerization domain

in its sequence as has been found for the *D. melanogaster* clock protein *PER*, the *N. crassa* clock component *wc-2* and clock-associated gene *wc-1*, and the recently isolated mouse gene *clock* (Crosthwaite *et al.*, 1997; King *et al.*, 1997; Page, 1994).

Transgenic *cab2-luc Arabidopsis* plants have also been used to study the involvement of phototransduction pathways in the regulation of circadian period. Both constant red and blue light shorten the length of the period to 25 h (compared to 30-36 h in the wild type under DD). This indicates the involvement of phytochrome and the blue-light photoreceptor in the input pathway of the circadian rhythm of *CAB2* expression. When *cab2-luc* was crossed into the *hy1-100* mutant (which lacks spectrophotometrically detectable phytochrome) the length of the period in red light increased to 26.5 h. However, this is still much shorter than the wild type in DD (30-36 h) indicating that *hy1* plants contain sufficient phytochrome to allow phytochrome-mediated shortening of the DD period in the presence of red light. The *cab2-luc* transgene was also crossed into the *det1* (de-etiolated) mutant background. This mutant exhibits characteristics of light-grown plants even when grown under constant darkness. For example, CAB genes are expressed in *det1* despite the absence of a light signal to induce their expression. In this *det1* mutant background the period of expression of the *cab2-luc* transgene in DD was shortened to about 18 h and slightly longer (19-20 h) in blue, red and LL. Furthermore, crossing *cab2-luc* into another *det* mutant (*det2*) and a *cop* mutant (*cop1*, constitutively photomorphogenic) shortened the DD period to approximately 29 and 23 h respectively, compared to 30-36 h for the wild type (Millar *et al.*, 1995b). Collectively, these results indicate that the phototransduction pathways which are constitutively activated in *det* and *cop* mutants are involved in the control of both the amplitude (difference between maximum and minimum *CAB2* levels) and period of the circadian oscillations in *CAB2* gene expression. It is likely that DET1, DET2 and COP1 function upstream of the circadian clock because some signal transduction pathways from the phytochromes and the blue-light photoreceptors converge at or before the regulatory steps defined by *det* and *cop* (Anderson and Kay, 1996).

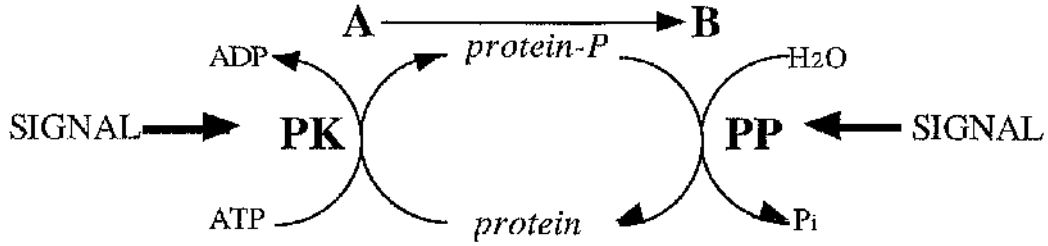
One obvious way in which the circadian oscillator in plants could communicate with its output pathways in a co-ordinate manner is via calcium signals. Expression of a transgene for the calcium-sensitive luminescent protein apoaequorin in tobacco and *Arabidopsis* revealed circadian oscillations in cytosolic and chloroplastic free calcium (Johnson *et al.*, 1995). The circadian oscillations in the cytosolic calcium concentration were most marked in constant light and tended to damp out upon transfer to constant darkness, but low amplitude oscillations were occasionally detectable in constant darkness as well. Constant red light restored the circadian oscillations of cytosolic free-calcium following periods of constant darkness. This implicates a role for phytochrome in the reinitiation and maintenance of calcium rhythms in plants. Chloroplastic free-calcium was relatively constant throughout periods of constant light, but upon transfer to constant darkness there was a massive peak in luminescence representing an increase from a basal level of 150 nM to 5 to 10 μM calcium. Thereafter, in constant darkness, damped oscillations of chloroplastic luminescence with a circadian period were detected (Johnson *et al.*, 1995). The rapid and large increase in chloroplast free-calcium following the transition from light to dark suggests that this calcium spike may be part of the plant's recognition of lights-off. Johnson *et al.* (1995) speculated that the circadian oscillations in free-calcium which they were able to detect could account for circadian oscillations in the function of many cellular systems. Examples include the movements of stems, petioles and changes in stomatal aperture, which are regulated by changes in turgor pressure due to the movement of potassium ions across the plasma membrane through a Ca^{2+}-gated potassium channel. In addition, growth and mitosis, protein kinases and the expression of CAB genes are known to be regulated by cytosolic calcium concentration and the circadian clock (Johnson *et al.*, 1995). Thus it would seem likely that one mechanism for co-ordinating a wide range of cellular processes in response to the circadian clock in plants is via oscillations in the free-calcium concentration. However, discovery of whether calcium is an essential component of the plant circadian oscillator itself or simply part of the input and output pathways must await the cloning and analysis of plant circadian pacemakers.

One possible lead in the hunt for plant circadian oscillator genes is the fact that a number of the cloned circadian clock genes contain the PAS protein dimerization sequence (Crosthwaite *et al.*, 1997). Phytochromes, which play a key role in the regulation of the circadian clock in plants, possess PAS-domains (Johnson *et al.*, 1995; Millar *et al.*, 1995b; Wilkins, 1992). Thus, homology based cloning of other plant PAS-domain containing genes in plants may fortuitously yield plant circadian oscillator components. Additionally, cloning and sequence analysis of the *Arabidopsis toc1* locus is eagerly awaited because this gene may not only represent a component of the plant circadian clock but could possibly contain a PAS-domain (Anderson and Kay, 1997; Millar and Kay, 1997). The next few years should be extremely exciting time in the study of the plant circadian clock.

1.6. Reversible protein phosphorylation in plants

1.6.1. Plant protein kinases

Protein phosphorylation performs a wide variety of functions in living organisms. Proteins can be phosphorylated on serine, threonine, tyrosine and histidine residues and the phosphorylation of these residues generally mediates conformational changes in the protein which alter its properties. Phosphorylation is performed by protein kinases (**PK**) and dephosphorylation is performed by phosphoprotein phosphatases (**PP**) as outlined below:



The SIGNAL can affect either the activity of the **PK** or the **PP**. Either way, the phosphorylation state of the *protein* is increased (increased proportion of *protein-P* compared to *protein*) which in turn favours the conversion of **A** to **B**. The active form of the *protein* could also be the dephosphorylated form (*protein*). The factors **A** and **B** could be the substrate and product of an activated metabolic enzyme (*protein-P*). Likewise, *protein-P* could be an activated protein kinase (or an inactivated phosphoprotein phosphatase) such that **A** could be an inactive second kinase and **B** is the active form,

which subsequently passes on the signal to the next level of a signal transduction cascade. Protein kinases are commonly activated by a second messenger such as calcium, cyclic AMP or mitogen. However, the plant protein kinase which phosphorylates and activates phosphoenolpyruvate carboxylase is uniquely activated via *de novo* protein synthesis (see section 1.4.3). Protein phosphorylation cascade systems are extremely efficient methods of transducing signals within the living cell and are widely employed for this purpose. Although protein phosphorylation has been most intensively studied in animals and yeast (particularly *Saccharomyces cerevisiae*), the understanding of plant protein phosphorylation cascades and plant PKs and PPs has increased significantly over the last decade.

Protein kinases are a large superfamily of enzymes (Hanks *et al.*, 1988). Estimates have suggested that between 1 and 3% of functional eukaryotic genes encode protein kinases (Stone and Walker, 1995). Apart from the histidine kinases, which belong to a family of genes distinct from the eukaryotic protein kinase superfamily, all the sequenced eukaryotic protein kinases contain a catalytic domain which possesses 11 highly conserved domains interspersed by much more divergent regions (Hanks *et al.*, 1988). This has meant that a large number of the cloned plant protein kinases have been isolated by a homology based approach using these conserved domains as probes or PCR primers. A number of other plant protein kinase cDNAs have been cloned due to their ability to complement yeast mutants that are lacking particular proteins kinase activities (e.g. plant SNF1 homologues) (Alderson *et al.*, 1991). A small number of plant protein kinases have been cloned as the knocked out genes in plant mutants (e.g. the Raf homologue *CTR1*, which is involved in the ethylene response and the novel serine/threonine kinase *Tsl*, which is involved in plant morphological development) (Kieber *et al.*, 1993; Roe *et al.*, 1993). Whilst these diverse approaches have proved a very powerful approach for isolating plant protein kinase cDNAs, the characteristics of the proteins encoded by the cloned genes are largely unknown. In particular the substrate of cloned plant kinases has rarely been identified. This means that there are only a few examples where a plant protein kinase with a known substrate and function has been cloned. One particular example is the plant

homologue of sucrose non-fermenting 1 (SNF1) kinase and this will be discussed in section 1.6.4.

The eukaryotic protein kinase superfamily has been subdivided into five main groups (Hanks and Hunter, 1995): (a) the "AGC" group consisting of the cyclic nucleotide-dependent family (PKA and PKG), the PKC (protein kinase C) family, and the ribosomal S6 kinase family; (b) the "CaMK" group, consisting of calcium-/calmodulin-dependent kinases (CaMKs) and the SNF1/AMP-activated protein kinase; (c) the "CMGC" group, containing the CDK (cyclin-dependent kinase), the MAPK (mitogen activated protein kinase), GSK-3 (glycogen synthase kinase), and CKII (casein kinase II) families; (d) the "conventional PTK" group, and (e) the "other" group. Plant members of every group except the protein tyrosine kinase (PTK) group have been identified (Hardie, 1996; Stone and Walker, 1995). Although no plant homologues of conventional isoforms of protein kinase C (PKC) have been cloned, biochemical evidence is beginning to accumulate to support the existence of a kinase resembling PKC in plants (Nanmori *et al.*, 1994; Subramaniam *et al.*, 1997; Xing *et al.*, 1996). In particular, plant homologues of PKC seem to participate in elicitor-induced defence responses and it has been speculated that one PKC homologue may in fact be directly activated by the elicitor arachidonic acid in potato tubers (Subramaniam *et al.*, 1997; Xing *et al.*, 1996). Additionally, many plant calcium-dependent protein kinases (CDPKs, "CaMK" group) have been cloned. The majority of the CDPKs in plants do not require calmodulin, phospholipids, or diacylglycerol which makes them different from both the CaMK and PKC families prevalent in mammals (Roberts and Harmon, 1992). A number of unique plant CDPKs have been cloned which possess an integral calmodulin-like domain at their C-terminus and a protein kinase domain with homology to the protein kinase catalytic domains of the CaMK family at their N-terminus (Harper *et al.*, 1991).

Despite the fact that a large number of these CDPKs have been cloned, their physiological roles are little understood. However, recent work has identified one possible target of plant CDPKs, namely serine acetyltransferase (SAT), a key enzyme in the cysteine synthesis pathway (Lee *et al.*, 1997). When soybean SAT was expressed in

bacteria it could be phosphorylated by three different soybean CDPKs. The resultant phosphorylated form of SAT was not subject to feedback inhibition by cysteine whilst the dephospho-form of SAT was inhibited (Lee *et al.*, 1997). This demonstrates that SAT is not only a substrate for CDPKs, but also that phosphorylation of SAT by CDPKs causes physiologically significant changes in its regulatory properties. CDPKs have also been shown to phosphorylate the root nodule symbiosome membrane protein nodulin 26 (Weaver and Roberts, 1992). Nodulin 26 is a membrane channel protein which is a major protein component of the symbiosome membrane. Nodulin 26 is specifically phosphorylated on serine 262 by a CDPK and this alters the voltage-dependent channel gating by the protein (Roberts, 1996; Weaver and Roberts, 1992). A third possible target which has been identified for the plant CDPKs is the plasma membrane proton pump (H^+-ATPase). The H^+-ATPase can be phosphorylated by a CDPK from oat root plasma membrane vesicles and a recombinant *Arabidopsis thaliana* CDPK (Sussman, 1993). In yeast, glucose alters the phosphorylation state of the plasma membrane proton pump and this is thought to be at least partially responsible for the rapid activation of the proton pump in response to glucose (Chang and Slayman, 1991). In plants, there is no data concerning the effects of phosphorylation on the H^+-ATPase, but simply evidence that phosphorylation can be performed by CDPKs (Sussman, 1993). However, Xing *et al.* (1996) demonstrated that dephosphorylation of the tomato plasma membrane H^+-ATPase occurred in response to fungal elicitor treatment. In this case, the re-phosphorylation of the H^+-ATPase was performed in the first hour by a PKC-like kinase activity and in the second hour by a CaMKII-like protein kinase rather than a CDPK activity (Xing *et al.*, 1996).

Another group of kinases which have been widely identified in plants are the mitogen activated protein kinases (MAPKs), which are members of the "CMGC" group of kinases. This group of kinases are serine/threonine kinases which link extracellular signals perceived at the cell surface to intracellular events controlling growth and differentiation. In vertebrates MAPKs are typically activated by various mitogenic agents such as growth factors and hormones and they subsequently phosphorylate other kinases and transcription

factors such as c-Myc and c-Jun. MAPK's are themselves activated due to phosphorylation on tyrosine and threonine residues by a single dual-specific activator, MAP kinase kinase (MAPKK). This latter kinase is itself activated via phosphorylation on a serine residue by MAP kinase kinase kinase (MAPKKK) and in some cases MAPKKK's are themselves activated by protein kinase C. Thus, the MAPK signal cascade employs a whole series of phosphorylation steps to transduce signals from outside the cell to particular intracellular targets. In plants, the MAPK signalling pathway has been proposed to be involved in environmental stress (in particular cold, drought and wounding), auxin and ethylene responses and plant homologues of MAPK, MAPKK and MAPKKK have been cloned (Bogre *et al.*, 1997; Mizoguchi *et al.*, 1993; Shinozaki *et al.*, 1997; Tregear *et al.*, 1996). Some workers have shown that wounding causes an increase in the steady state transcript levels for MAP kinase (Seo *et al.*, 1995; Usami *et al.*, 1995), whilst others have demonstrated that wounding mediates a transient activation of MAP kinase which does not require transcription and translation (Bogre *et al.*, 1997). Thus, it seems likely that more than one MAP kinase signalling cascade exists in plants and that each pathway can have a separate mode of regulation. Some MAP kinases are induced via transcription and translation whilst others are induced by post-translational modification of pre-existing MAP kinase. It is clear from the currently published data that MAP kinase cascades play a central role in some of the major plant signal transduction pathways and their further dissection will be vital to a thorough understanding of these signalling pathways.

One fascinating group of plant protein kinases that belong to the "other" group in the kinase superfamily classification are the receptor-like protein kinases (RLKs) (Walker *et al.*, 1996). The RLK's have structural features similar to the receptor tyrosine kinases (RTKs), which are transmembrane proteins that have an extracellular domain which recognises a polypeptide ligand. Recognition of this ligand leads to autophosphorylation of the cytoplasmic kinase domain which can then propagate the signal within the cell. However, plant RLKs have kinase catalytic domains which are serine and threonine specific rather than tyrosine specific. RLKs have been identified from a number of plants and whilst their functions a largely unknown, a number of recent studies have revealed that

some RLKs function in plant development and disease resistance (Becraft *et al.*, 1996; Clark *et al.*, 1997; Lee *et al.*, 1996b; Song *et al.*, 1995; Torii *et al.*, 1996). In addition, the self-incompatibility locus (S-locus) in *Brassica* is tightly linked with two genes, the S-locus glycoprotein (SLG) and the S-locus receptor kinase (SRK). Mutations in the SRK abolish the self-incompatibility response indicating the essential role of this particular RLK in the *Brassica* self-incompatibility signalling pathway (Goring *et al.*, 1993). Two proteins that interact with SRK were cloned using the yeast two-hybrid system (Bower *et al.*, 1996). Both these proteins were found to be members of the thioredoxin-h family and the fact that they interact with SRK demonstrates that they could be responsible for propagating the signal to reject incompatible pollen (Bower *et al.*, 1996).

A third protein that interacts with the catalytic domain of a plant RLK has been cloned using an interaction cloning technique. *KAPP* (*k*inase *a*ssociated *p*rotein *p*hosphatase) interacts only with the autophosphorylated form of the kinase catalytic domain of the *A. thaliana* RLK5 gene (Horn and Walker, 1994; Stone *et al.*, 1994). *KAPP* has three functional domains, an N-terminal region characteristic of a type one signal anchor which localizes proteins to the cytoplasmic surface of a membrane, a central kinase interaction (KI) domain responsible for phosphorylation-dependent interaction with RLK5, and a C-terminal region with sequence homology to, and activity characteristic of, a type 2C protein phosphatase (Stone *et al.*, 1994). The KI domain is conserved between monocots and dicots and interacts with a subset of the known plant RLKs (Braun *et al.*, 1997). This indicates that KAPP may be involved in transducing signals from multiple RLKs, but that it is not a component of all RLK signalling pathways. However, if KAPP is responsible for transducing the signal from multiple RLKs, then there must be a mechanism to permit temporal specificity of KAPP for particular receptors. The nature of this mechanism and the role of KAPP and RLK5 in *A. thaliana* cell signalling are unknown at present.

1.6.2. Plant phosphoprotein phosphatases

Research on plant phosphoprotein phosphatases (PPs) has tended to lag behind that into protein kinases because the protein kinases have generally been found to be the highly regulated, and therefore most interesting, partner in most plant protein phosphorylation events. However, a number of different protein phosphatase activities have been detected in plant extracts which are homologous to mammalian type-1, -2A and -2C protein serine/threonine phosphatases (MacKintosh *et al.*, 1991; MacKintosh and Cohen, 1989; Smith and Walker, 1996). These protein phosphatase activities are found in a number of the subcellular compartments of the plant cell, including the cytosol and nucleus (MacKintosh *et al.*, 1991; Smith and Walker, 1996).

The classification of protein phosphatases into type-1 and type-2 is based on their unique substrate specificities and sensitivities to various inhibitors. Type 1 protein phosphatases preferentially dephosphorylate the ß-subunit of mammalian phosphorylase kinase and are selectively inhibited by two heat stable proteins, termed inhibitor-1 and inhibitor-2. Type 2 protein phosphatases prefer the α–subunit of phosphorylase kinase as a substrate and are resistant to inhibitors -1 and -2. Type 2 protein phosphatases are further categorized into PP2A, PP2B and PP2C according to their subunit structure, divalent cation requirements and substrate specificities (Cohen, 1989). PP2A is a heterotrimer of a catalytic C-subunit and two different regulatory subunits, termed A and B. PP2B is activated by calcium ions and exists as a heterodimer containing a catalytic A-subunit and a regulatory B-subunit that possesses an EF-hand motif which is found in some calcium-binding proteins. PP2C exists as a monomer that requires Mg^{2+} for activity. In terms of sequence, PP2C is clearly in a different family to the rest of the protein phosphatases which are related. In mammals, the catalytic subunits of all type 1 and 2 protein phosphatases are encoded by separate genes.

A great deal of information concerning the involvement of protein phosphatases in signalling processes in plants has been obtained by using specific inhibitors of protein phosphatases. For example, the diarrhetic shellfish poison okadaic acid is a polyether carboxylic acid that inhibits both PP1 and PP2A, but it inhibits PP2A ($I_{0.5} \approx 0.1$-1.0 nM)

much more potently than PP1 ($I_{0.5} \approx$ 10-100 nM) (MacKintosh and MacKintosh, 1994). Many of the PP inhibitors are readily taken up by plant cells and have therefore been used for the *in vivo* analysis of the involvement of PPs in plant signalling processes (Christie and Jenkins, 1996; MacKintosh and MacKintosh, 1994; Sheen, 1993). The use of PP inhibitors, and in particular combinations of inhibitors with different specificities, allows differentiation between the involvement of PP1 and PP2A in a particular signal cascade (Sheen, 1993). For example, greening of etiolated maize leaves in response to light requires protein phosphatase activity (Sheen, 1993). Okadaic acid blocked chlorophyll accumulation only when applied at a concentration of 1 µM. Furthermore, by using a protoplast transient expression system, Sheen (1993) demonstrated that transcription of light-induced genes is prevented by pretreatment with both 1 µM okadaic acid and 10-100 nM calyculin A. At a concentration of 100 nM, okadaic acid was ineffective at preventing the light-induced transcription from the C_4 *ppdk* promoter and the *rbcS* promoter, but it did block transcription at 1 µM. Thus, this evidence suggests that PP1 activity is necessary for the light-induction of *ppdk* and *rbcS* in etiolated maize leaves (Sheen, 1993).

The induction of pathogenesis-related (PR) proteins by salicylic acid (SA) in tobacco leaves has recently been demonstrated to involve protein dephosphorylation by using a range of protein phosphatase inhibitors (Conrath *et al.*, 1997). Both okadaic acid and calyculin A blocked the SA-mediated induction of *PR-1* steady state mRNA levels and protein, whilst protein kinase inhibitors induced *PR-1* expression in the absence of SA. Thus, blocking protein kinase activity favours the dephosphorylation of some protein or proteins involved in the induction of *PR-1*. Interestingly, the protein kinase inhibitor K-252a stimulated the production of SA which suggests that a phosphoprotein acts upstream of SA as well as downstream, as demonstrated by the fact that PP inhibitors block SA induction of *PR-1* (Conrath *et al.*, 1997). Protein phosphatase inhibitors have been used to demonstrate that protein dephosphorylation by serine/threonine phosphatases is required for proper functioning of the circadian clock in *G. polyedra* (Comolli *et al.*, 1996). Okadaic acid and cantharidin block the UV-B and UV-A/blue light induction of chalcone synthase steady state mRNA levels in a photomixotrophic *Arabidopsis* cell suspension

culture (Christie and Jenkins, 1996) and okadaic acid blocks the gibberellic acid (GA)-induced changes in calcium concentration, gene expression and cell death in wheat aleurone cells (Kuo *et al.*, 1996). These are just a few from a growing plethora of examples which, by the use of specific inhibitors, demonstrate the involvement of protein dephosphorylation in a wide range of signalling processes in plants and other photosynthetic organisms. Initial confirmation of the requirement for protein phosphatase activity in a particular signal transduction pathway using inhibitors can then be followed up by identifying protein phosphatases which are active or activated during the induced (light, SA, GA, etc.) conditions for a particular signalling pathway.

Protein phosphatase inhibitors have also been used to demonstrate that particular phosphorylated plant proteins require dephosphorylation for activation/inactivation. Okadaic acid blocks the light-activation (dephosphorylation) of SPS and NR (Huber *et al.*, 1992; Huber and Huber, 1990). SPS-phosphatase undergoes a rapid dimer-trimer conversion upon illumination or feeding mannose to leaves in order to deplete metabolic phosphate pools in the cytoplasm. This light/mannose activation requires *de novo* protein synthesis indicating that the additional subunit in the trimeric form may be newly synthesized in response to light (Weiner, 1997). The trimeric form of SPS-phosphatase is the active form and this mediates the light-activation of SPS by dephosphorylating it. Okadaic acid also blocks the *in vitro* dephosphorylation and concomitant increase in the L-malate sensitivity of PEPc from the C_4 plant maize and the CAM plant *B. fedtschenkoi* by inhibiting both a rabbit skeletal muscle PP2A and a partially purified *B. fedtschenkoi* leaf PP2A (Carter *et al.*, 1990; McNaughton *et al.*, 1991). Furthermore, supplying the PP1 and PP2A inhibitor microcystin-LR to detached maize leaves had no effect on the light-induced decrease in the L-malate sensitivity of PEPc and overcame the effect of cycloheximide which blocks the light decrease when supplied alone (Jiao *et al.*, 1991a). Another plant metabolic enzyme which can undergo phosphorylation is phosphoenolpyruvate carboxykinase (PEPCK). This enzyme can be dephosphorylated *in vitro* by a mammalian PP2A, and again this is blocked by microcystin-LR (Walker and Leegood, 1995). However, unlike the situation for SPS, NR and PEPc, it is not yet known how

phosphorylation/dephosphorylation influences PEPCK activity (Walker and Leegood, 1995; Walker and Leegood, 1996).

Plant protein phosphatases, along with their accompanying kinases, seem to be involved in virtually every signal transduction pathway that has been analysed in this respect. The use of highly specific protein phosphatase inhibitors has greatly facilitated the elucidation of the fundamental importance of protein phosphorylation/dephosphorylation in plant signalling. Hopefully in the ensuing years this solid background of inhibitor based information will be expanded by the identification of many of the protein kinases and phosphatases that are vital to each individual plant signal transduction pathway and probably to the numerous points of cross talk between many of the plant signal transduction pathways.

1.6.3. The regulation of plant metabolism by protein phosphorylation

As already alluded to on a number of occasions in the preceding sections, a number of key enzymes in plant metabolism have their activity tightly regulated by reversible protein phosphorylation. PEPc is one of the most thoroughly studied plant proteins which undergoes regulation via reversible phosphorylation and this is described in detail in section 1.4. Other metabolic enzymes which undergo reversible phosphorylation in plants include SPS (sucrose biosynthesis), sucrose synthase (SuSy, sucrose breakdown)), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG CoA reductase, sterol biosynthesis), NR (nitrate assimilation), the mitochondrial pyruvate dehydrogenase complex (mtPDC, provision of acetyl CoA for the Krebs cycle and NADH for oxidative phosphorylation), and possibly PEPCK (gluconeogenesis and decarboxylation in some C_4 and CAM plants) and malate synthase (MS, glyoxylate cycle in fatty seeds) (Hardie *et al.*, 1997; Huber and Huber, 1996; Huber *et al.*, 1996a; Huber *et al.*, 1996b; MacKintosh *et al.*, 1996; Randall *et al.*, 1996; Yang *et al.*, 1988). Of these enzymes, SPS, NR and HMG CoA reductase have been studied in most detail and the kinases involved in the phosphorylation of these enzymes may be closely related. Thus, the regulation of these three plant enzymes by phosphorylation will be discussed below in section 1.6.4. The regulation of a few of the remainder of the enzymes mentioned above will be briefly discussed in this section.

PDCs are large multi-enzyme complexes which catalyse the oxidative decarboxylation of pyruvate to yield acetyl-CoA, CO_2 and NADH. The multi-enzyme complex is made up of pyruvate dehydrogenase (E1), dihydrolipoyl transacetylase (E2) and dihydrolipoyl dehydrogenase (E3). Plants have PDCs in both their mitochondria and their chloroplasts, but only the mitochondrial PDC undergoes regulatory phosphorylation (Randall *et al.*, 1996). Phosphorylation of mtPDC on up to three serine residues of the E1 subunit inactivates the complex and is catalysed by PDH kinase. Dephosphorylation is catalysed by PDH-P phosphatase and this activates the complex. The reversible phosphorylation of the plant mtPDC acts as an on-off switch with phosphorylation/inactivation being promoted by light (Randall *et al.*, 1996). The light-inactivation of mtPDC results from ATP production during photorespiratory glycine metabolism and can be mimicked by supplying darkened leaf strips with glycine (Gemel and Randall, 1992). During photorespiration NH_4^+ is produced in the mitochondria due to glycine oxidation (see section 1.1.2). This NH_4^+ activates the PDH kinase and in so doing promotes the inactivation of the mtPDC (Schuller *et al.*, 1993). Thus, as photosynthesis increases in response to illumination so photorespiration will increase due to the oxygenase activity of Rubisco, and it seems that the portion of photorespiration which occurs in the mitochondrion inactivates the mtPDC complex preventing mitochondrial respiration in the light.

Sucrose synthase (SuSy) catalyses the breakdown of sucrose using UDP to yield UDP-glucose and fructose. The reaction is readily reversible, but is believed to go in the direction of sucrose breakdown *in vivo* and consequently the highest SuSy activity is found in heterotrophic sink tissues that are actively growing or accumulating starch (Geigenberger and Stitt, 1993; Kruger, 1990; Sung *et al.*, 1994; Sung *et al.*, 1989). Plants possess two major isoforms of SuSy, termed SS1 and SS2, and these proteins are encoded by the *Sh1* and *Sus1* genes respectively in maize (Echt and Chourey, 1985). A possible mode of regulation of SuSy, which may account for it predominantly performing sucrose breakdown *in vivo,* is protein phosphorylation (Huber *et al.*, 1996a). In developing maize leaves SuSy activity is highest in the elongation zone which is an actively growing sink

(Kalt-Torres and Huber, 1987). The predominant form of SuSy in the elongation zone is the SS2 protein (Nguyen-Quoc *et al.*, 1990). When detached maize shoots were supplied with [^{32}P]orthophosphate both SS1 and SS2 became phosphorylated (Huber *et al.*, 1996a). Phosphorylation occurs on serine-15 of the maize leaf enzyme. A Ca^{2+}-dependent protein kinase that phosphorylated SuSy *in vitro* was identified and partially purified from maize leaves. Phosphorylation was accompanied by an increase in the affinity of the enzyme for its substrates sucrose and UDP indicating that sucrose breakdown is promoted by phosphorylation of SuSy. Thus, a physiologically relevant alteration in the kinetic properties of SuSy is concomitant with phosphorylation and this may account for the *in vivo* flux through SuSy predominantly occurring in the direction of sucrose breakdown. The phosphorylation site in maize leaf SuSy was found to be conserved in the sequences of all plant SuSys for which sequence data exists, indicating that phosphorylation may be a ubiquitous mechanism for favouring the breakdown of sucrose by SuSy (Huber *et al.*, 1996a).

Phosphoenolpyruvate carboxykinase (PEPCK) catalyses the ATP-dependent decarboxylation of oxaloacetate to form phosphoenolpyruvate. In some CAM and C_4 plants this is a key step in photosynthetic carbon dioxide fixation (see section 1.2 and 1.3). PEPCK is also a key enzyme in gluconeogenesis during the germination of fat-storing seeds such as cucumber (Leegood and ap Rees, 1978). Only recently has an unproteolysed form of PEPCK been purified from a plant tissue (Walker *et al.*, 1995). It transpired that only the intact form of the purified PEPCK (74 kDa form) from cucumber cotyledons could be phosphorylated *in vitro* by both a partially purified maize PEPc kinase preparation and mammalian cAMP-dependent protein kinase (Walker and Leegood, 1995). The proteolysed, 62 kDa form of PEPCK could not be phosphorylated and proteolysis was accompanied by loss of the ^{32}P-label from the phosphorylated 74 kDa form of PEPCK (Walker and Leegood, 1995). Feeding [^{32}P]P_i to cucumber cotyledons led to the labelling of PEPCK in the dark, but not in the light (Walker and Leegood, 1995). Walker and Leegood (1996) went on to demonstrate that PEPCK can undergo *in vivo* phosphorylation in a wide variety gluconeogenic seedlings, C_4 and CAM plants. In the leaves of C_4 plants,

which had a 71 kDa isoform of PEPCK, phosphorylation was highest in the dark and greatly reduced by illumination, as had been found in the cucumber seedlings (Walker and Leegood, 1996). However, the PEPCK in some of the C_4 species analysed had a molecular weight around 67-69 kDa and this form of the enzyme was not subject to phosphorylation. This is thought to be due to these smaller isoforms of PEPCK lacking the N-terminal extension which contains the putative phosphorylation site. In the leaves of CAM plants, PEPCK had a molecular weight of 74-78 kDa and was subject to *in vivo* phosphorylation in all the species examined (Walker and Leegood, 1996). In the CAM plant *Tillandsia fasciculata,* PEPCK (78 kDa) was most highly phosphorylated towards the middle of the day, but then became dephosphorylated in the late afternoon. The level of phosphorylation began to increase again upon the return to darkness. In CAM plants, both PEPc and PEPCK occur together in the cytoplasm. Thus, futile cycling of carboxylation and decarboxylation steps could easily occur. Phosphorylation of PEPc at night in CAM plants makes the enzyme physiologically active (see section 1.4). It therefore follows that the phosphorylation of PEPCK at night and into the middle of the day may represent evidence that phosphorylation inactivates PEPCK preventing futile cycling. However, this hypothesis is dependent on phosphorylation causing inactivation of PEPCK and the effect of phosphorylation on the enzyme has yet to be determined (Walker and Leegood, 1996). In C_4 plants, carboxylation of PEP by PEPc occurs in the mesophyll cells and decarboxylation of oxaloacetate by PEPCK occurs in the bundle-sheath cells. This spatial separation of PEPc and PEPCK will in itself overcome the possibility of futile cycling between carboxylation and decarboxylation. Hence, the phosphorylation domain may have been lost in the evolution of some C_4 grasses because there was no longer a requirement to regulate flux through PEPCK tightly.

1.6.4. The post-translational regulation of sucrose phosphate synthase, nitrate reductase and HMG-CoA reductase in plants

In higher plant tissues, SPS, NR and HMG CoA reductase are all inactivated by protein phosphorylation on serine residues. SPS is a key enzyme in the pathway of sucrose biosynthesis and catalyses the conversion of UDP-glucose and fructose-6-phosphate to sucrose-6'-phosphate, UDP and H^+. *In vivo* the sucrose-phosphate is rapidly removed by sucrose phosphatase and this effectively makes the reaction irreversible. SPS activity is greatest in source leaves where sucrose is actively synthesised from the products of photosynthesis, but it is also found in sink tissues where it seems to be involved in a futile cycle of sucrose turnover (Geigenberger and Stitt, 1991; Reimholz *et al.*, 1994). SPS is regulated by metabolites and reversible protein phosphorylation. It is activated by glucose-6-phosphate and inhibited by P_i. SPS is more sensitive to inhibition by P_i at night than during the day (Stitt *et al.*, 1988). A number of groups discovered that this alteration in the kinetic properties of SPS is due to reversible protein phosphorylation (Huber *et al.*, 1989; Siegl *et al.*, 1990; Walker and Huber, 1989). SPS undergoes phosphorylation at a number of sites, but regulatory phosphorylation seems to occur at serine 158 of the spinach leaf enzyme and serine 162 of the maize leaf enzyme (Huber and Huber, 1996; McMichael *et al.*, 1993). Phosphorylation of SPS inactivates the enzyme and in leaves of spinach and maize this process occurs in the dark. Activation of phosphorylated SPS occurs in response to illumination and is performed by a light-activated PP2A (Weiner *et al.*, 1993). The light-activation of SPS-PP2A occurs via a dimer-trimer conversion and requires protein synthesis (see section 1.6.2) (Weiner, 1997; Weiner *et al.*, 1993). SPS kinase has been partially purified from a number of species. In spinach leaves, two SPS protein kinases have been resolved with apparent molecular masses of 45 (peak I) and 150 kDa (peak III) (McMichael *et al.*, 1995). The peak I kinase is Ca^{2+}-dependent whilst the peak III kinase, which has a tendency to copurify with SPS, is Ca^{2+}-independent. By contrast only a single form of SPS kinase has been identified in maize leaves (Huber and Huber, 1996).

In darkened spinach leaves SPS is phosphorylated and inactivated via phosphorylation of ser-158. However, the SPS in darkened leaves can be partially activated

in the dark in response to osmotic stress. This osmotic stress-induced activation of SPS has been demonstrated to be due to phosphorylation of a separate site, ser-424 (Toroser and Huber, 1997). Phosphorylation of ser-424 in the dark seems to reactivate SPS which has already been inactivated by phosphorylation of ser-158. A novel Ca^{2+}-dependent, 150 kDa protein kinase (peak IV) was partially purified and found to phosphorylate and activate phosphoserine-158 SPS *in vitro*. Hence, it would appear that SPS can undergo two separate phosphorylation events. One inactivates it in response to darkness (low G6P, F6P and high P_i), and a second activates the enzyme in response to osmotic stress (Toroser and Huber, 1997).

Nitrate reductase (NR) catalyses the reduction of nitrate to nitrite using NAD(P)H as the electron donor and this is considered the rate limiting step in nitrogen assimilation in plants. NR transcript levels are induced by nitrate and this is concomitant with an increase in NR activity (Campbell, 1996). NR activity is also regulated post-translationally in response to light/dark transitions and other environmental signals. NR is rapidly converted to a low activity, magnesium-inhibited form whenever the rate of photosynthesis is reduced (Huber *et al.*, 1992; MacKintosh, 1992; MacKintosh *et al.*, 1996). Inactivation of NR is caused by phosphorylation of the enzyme on serine-543 of the spinach leaf enzyme (Douglas *et al.*, 1995; Bachmann *et al.*, 1996b). Dephosphorylation and activation of NR is catalysed by a microcystin-, calyculin A-, cantharidin- and okadaic-acid sensitive protein phosphatase and this occurs in response to illumination (Kaiser and Huber, 1994; MacKintosh, 1992). The phosphorylated residue, serine-543, is located in the hinge 1 region that connects the cytochrome b domain with the molybdenum-pterin cofactor binding domain of NR (Bachmann *et al.*, 1996b; Su *et al.*, 1996). However, phosphorylation alone does not inactivate NR, inactivation also requires the binding of an inhibitor protein (NIP) to the phosphorylated form of NR (Glaab and Kaiser, 1995; Glaab and Kaiser, 1996; MacKintosh *et al.*, 1995; Spill and Kaiser, 1994).

This inhibitor protein has recently been purified and was found to be a 14-3-3 protein (Bachmann *et al.*, 1996a; Moorhead *et al.*, 1996). 14-3-3 proteins constitute a large family of proteins which participate in a wide array of signal transduction pathways (Ferl,

1996). Their exact mode of action is still largely unknown, but in some cases they act as adapters which bring specific target proteins together or to particular locations, as solubility factors or as enzyme inhibitors (Ferl, 1996). The isolation of the NIP 14-3-3 has assisted the purification of various kinases capable of phosphorylating NR because a reconstituted assay can be set up containing NR and NIP and the NR is then simply incubated with the kinase and assayed for sensitivity to inhibition by Mg^{2+}. McMichael *et al.* (1995) isolated three peaks of kinase activity from spinach leaves variously capable of phosphorylating NR and/or SPS. Their peak I enzyme had an apparent molecular weight of 45 kDa and could phosphorylate and inactivate both NR and SPS, whilst the peak II kinase (60 kDa) was active against NR and the peak III kinase (150 kDa) was active against SPS (McMichael *et al.*, 1995). Bachmann *et al.* (1995, 1996) also purified the peak I and peak II (45 and 60 kDa) kinases capable of phosphorylating NR and further characterized the peak II kinase because it solely phosphorylated NR. Both the peak I and II kinases were Ca^{2+}-dependent and this raised the possibility that they may be CDPKs (Bachmann *et al.*, 1996b). Both kinases were inhibited by K252a (a CDPK and PKC inhibitor), but not H-7 and H-8 (cyclic-nucleotide dependent protein kinase and PKC inhibitors). However, only the peak II enzyme cross-reacted with a mixture of monoclonal antibodies specific to the catalytic domain of soybean cell CDPKα (Bachmann *et al.*, 1996b).

Douglas *et al.* (1997) identified three peaks of kinase activity from spinach leaf extracts which phosphorylated and inactivated NR in the presence of NIP 14-3-3 (Douglas *et al.*, 1997; Moorhead *et al.*, 1997). The major NR kinase activity was peak I which had a molecular mass of 45 kDa. Interestingly, this peak I kinase was Ca^{2+}-dependent when prepared in the absence of protein phosphatase inhibitors, and largely Ca^{2+}-independent when prepared in the presence of protein phosphatase inhibitors. The peak I kinase itself seems to be regulated by two functionally distinct phosphorylation events (Douglas *et al.*, 1997; Moorhead *et al.*, 1997). The peak II kinase was Ca^{2+}-dependent under all tested conditions whilst the peak III enzyme (~140 kDa) was Ca^{2+}-independent and inactivated by incubating it with PP2A or PP2C. This suggests that the peak III kinase is also regulated by phosphorylation. The peak III kinase was also very active in phosphorylating and

inactivating *Arabidopsis* HMG CoA reductase at the same site which is phosphorylated by HMG CoA reductase kinase A (HRK-A) from cauliflower (Douglas *et al.*, 1997; Moorhead *et al.*, 1997). HRK-A has been demonstrated to be a SNF-1 like kinase and western blotting of the peak III kinase with two separate antisera specific to plant SNF-1 homologues revealed that the peak III kinase is a plant SNF-1 homologue (Ball, 1996; Douglas *et al.*, 1997; MacKintosh *et al.*, 1992; Moorhead *et al.*, 1997).

The yeast *SNF1* protein kinase is an integral part of the carbon catabolite signal transduction pathway which senses cellular glucose levels and links them to the repression and derepression of glucose-repressible genes such as the invertase gene *SUC2* (Celenza and Carlson, 1986). A mammalian gene which has high homology to *SNF1* is the gene for AMP-activated protein kinase (AMPK) (Carling *et al.*, 1994). AMPK inactivates both acetyl CoA carboxylase and HMG CoA reductase by phosphorylating them, and this regulates lipid metabolism (Hardie, 1992). A number of plant homologues of the *SNF1* related protein kinases have been cloned by both complementation of the yeast *snf1* mutant and homology based methods (Alderson *et al.*, 1991; Halford *et al.*, 1996). The rye endosperm gene RKIN1 was the first plant *SNF1* related cDNA to be cloned. The Rkin1 protein was able to complement the yeast *snf1* mutant by restoring growth on a glycerol minimal medium (Alderson *et al.*, 1991). This indicates that the rye RKIN1 gene encodes a functional *SNF1*-related protein kinase. Subsequently a number of plant homologues of RKIN1/*SNF1* have been isolated by homology based cloning methods. These include *SNF1* homologues from *Arabidopsis* (Le Guen *et al.*, 1992), *M. crystallinum* (Baur *et al.*, 1994), barley (Halford *et al.*, 1992), and tobacco (Muranaka *et al.*, 1994).

Biochemical evidence for the existence of SNF1 homologues in plants has come from the analysis of HMG-CoA reductase kinase (HRK) activity in plants. The development of a peptide based kinase assay for AMPK/SNF1-like activity has greatly assisted attempts to purify plant HRK activities. The SAMS peptide has a sequence based on the major phosphorylation site of rat acetyl-CoA carboxylase and is phosphorylated readily and specifically by mammalian AMPK and yeast SNF1 (Ball, 1996). MacKintosh *et al.* (1992) partially purified SAMS peptide kinase activity from cauliflower, pea, oilseed

rape, potato and wheat. These kinase activities could also phosphorylate mammalian HMG-CoA reductase, mammalian acetyl-CoA carboxylase and plant HMG-CoA reductase, but not plant acetyl-CoA carboxylase (Ball *et al.*, 1994; MacKintosh *et al.*, 1992). Two HRK activities from cauliflower florets were resolved by column chromatography, termed HRKA and HRKB, but HRKA was the major activity and had a subunit molecular weight characteristic of plant SNF1 homologues (58 kDa) (Ball *et al.*, 1994). However, both the plant HRK activities and the yeast SNF1 kinase have not been found to require AMP for activity, unlike mammalian AMPK (Ball, 1996). An activity equivalent to cauliflower HRKA has also been isolated from barley endosperm (Barker *et al.*, 1996). This latter kinase activity could phosphorylate *Arabidopsis* HMG-CoA reductase and the SAMS peptide, and had a subunit molecular weight of 58 kDa which is identical to the predicted size of plant SNF1-homologues. However, the most convincing evidence that the barley HRK activity was a plant SNF1-related kinase was obtained by immunological methods. Two antisera were raised, one against both an overexpressed portion of the rye RKIN1 gene and a second against a conserved peptide designed by aligning the sequences of all plant SNF1-homologues. Both antisera recognized a 60 kDa polypeptide in the purified barley endosperm HRK preparation. Furthermore, both antisera also recognize cauliflower HRK-A (Ball *et al.*, 1995). This confirms that plant HRK is a SNF1 homologue (Ball *et al.*, 1995; Barker *et al.*, 1996).

Two isoforms of HRK, HRK-A and HRK-C have been isolated from spinach leaves. Both enzymes inactivate *A. thaliana* HMG-CoA reductase by phosphorylating it at ser-577 (Hardie *et al.*, 1997). They also phosphorylate and inactivate spinach SPS and NR. Both kinases were inactivated by dephosphorylation and can be reactivated by phosphorylation with mammalian AMPK kinase. They have native molecular masses of ~160 kDa and contain 58 kDa polypeptides that are recognized by the RKIN1 antiserum. Thus, it is becoming increasing likely that plant HRK, SPS-kinase and NR-kinase are closely related members of the SNF-1 related protein kinase family. They seem to play a central role in sensing the supply of carbohydrate from photosynthesis or energy reserves and in response they can switch off major biosynthetic pathways by rapidly

phosphorylating enzymes such as SPS, NR and HMG-CoA reductase. They may also regulate gene expression (Halford, 1997). It would seem highly likely from the evidence currently available that plant SNF1 homologues, like their mammalian and yeast counterparts, play a central role in the regulation of carbon metabolism. Manipulation of the expression of specific plant SNF-1 homologues by antisense techniques may permit the redirection of carbon into particular pathways which could, for example, allow the starch composition of potato tubers and cereal grains to be manipulated for economic gains.

1.7 Objectives

Higher plant phosphoenolpyruvate carboxylase is responsible for primary CO_2 fixation in C_4 and CAM plants and a range of housekeeping functions in all plants. Flux through PEPc can be regulated by changes in its allosteric properties. The allosteric properties of plant PEPc are controlled by reversible protein phosphorylation. The phosphorylation state of PEPc is largely a result of the level of PEPc kinase activity. PEPc kinase is induced by light in C_3 and C_4 plants and the circadian clock in CAM plants. Light- and circadian clock-mediated activation of PEPc kinase activity has been shown to involve *de novo* protein synthesis (Carter *et al.*, 1991; Jiao *et al.*, 1991a; Li *et al.*, 1996). The nature of the protein which must be synthesized *de novo* is currently unknown. However, it has been speculated that it could be the PEPc kinase protein itself or a secondary component that is required for the activation of PEPc kinase. Numerous attempts to purify PEPc kinase to homogeneity have failed, and thus, there are no antibodies or cDNA probes available for PEPc kinase. The major aim of this work was to overcome this shortfall in the understanding of PEPc and PEPc kinase regulation by studying the regulation of PEPc kinase translatable mRNA levels in plants. At the outset, a novel assay was proposed which would permit analysis of PEPc kinase translatable mRNA in which isolated plant RNA would be translated into proteins using an *in vitro* translation system and the translation products would then be assayed directly for PEPc kinase activity. The initial objective was to determine if this assay would work and if so was it a valid assay for PEPc kinase mRNA. If the assay was to permit the analysis of PEPc kinase

mRNA levels then the signal transduction cascades involved in regulating PEPc kinase mRNA and activity were to be dissected. It was also hoped that the novel assay would provide a method for cloning the PEPc kinase cDNA based on the reaction catalysed by the kinase rather than homology-based cloning which requires prior knowledge of the sequence of the desired gene.

Chapter 2

Materials and Methods

2.1. Materials

Acetic acid (glacial), acetone, boric acid, chloroform, disodium hydrogen phosphate, ethylene diamine tetra-acetic acid (EDTA, disodium salt), glycine, Hepes, hydrochloric acid (HCl), magnesium chloride($MgCl_2$), methanol, potassium chloride (KCl), sodium acetate, sodium chloride (NaCl), sodium hydrogen carbonate ($NaHCO_3$) and sodium hydroxide (NaOH) were from Fisher Scientific (U.K.) Ltd., Loughborough, U.K.

Actinomycin D, antipain hydrochloride, benzamidine hydrochloride, Bis-Tris, bovine serum albumin (BSA), Bromophenol Blue, cantharidin, casein enzymatic hydrolysate, chymostatin, Coomassie Brilliant Blue G250, cordycepin, cycloheximide, diethyl pyrocarbonate (DEPC), dimethyl sulphoxide (DMSO), formaldehyde, formamide, glucose 6-phosphate (G6P, monosodium salt), hexadecyltrimethylammonium bromide (CTAB), leupeptin (hemisulphate salt), lithium chloride, L-malic acid, octanol, phosphoenolpyruvate (PEP, monosodium salt), polyethylene glycol (PEG, MW 15000-20000), polyvinylpolypyrrolidone (PVPP or insoluble PVP), puromycin, sodium azide, spermidine hydrochloride and N,N,N',N'-tetramethylethylenediamine (TEMED) were from Sigma (London) Chemical Co., Poole, Dorset, U.K.

Adenosine 5'-triphosphate (ATP, disodium salt), nicotinamide adenine dinucleotide reduced form (NADH, disodium salt) and pig heart malate dehydrogenase (MDH) were from Boehringer Mannheim Corp. (London) Ltd., Lewes, Sussex, U.K.

[γ-^{32}P] ATP (triethylammonium salt, 3000 Ci mmol^{-1}), [α-^{32}P] dCTP (3000 Ci mmol^{-1}), Hybond N blotting membrane, rabbit reticulocyte lysate *in vitro* translation kit and Redivue^{TM} ^{35}S-methionine (1000 Ci mmol^{-1}) were from Amersham International, Bucks., U.K.

Agar, tryptone (peptone from casein) and yeast extract were from Merck, Darmstadt, Germany.

Agarose and 1 Kb DNA ladder were from Gibco BRL, Life Technologies Ltd., Paisley, Scotland.

Ammonium peroxodisulphate, ethanol (100 % v/v), hydrogen peroxide, 2-mercaptoethanol, potassium dihydrogen orthophosphate, sodium dodecyl sulphate (SDS), and trichloroacetic acid were 'AnalaR' grade from BDH Chemicals, Poole Dorset, U.K.

Bay K8644, staurosporin and W7-HCl were from Calbiochem-Novabiochem (U.K.) Ltd., Nottingham, U.K.

Dithiothreitol (DTT) was from Alexis Corporation (U.K.) Ltd., Nottingham, U.K.

Dynabeads Oligo (dT)25 were from Dynal (U.K.) Ltd., Merseyside, U.K.

Hydroxylapatite (Bio-gel HTP) and 37.5:1 acrylamide:bis-acrylamide solution were from Bio-Rad Laboratories (England) Ltd., Bramley, Kent, U.K.

mMessage mMachine T3 *in vitro* transcription kit was from Ambion Inc., AMS Biotechnology (U.K.) Ltd., U.K.

Nucleon Easi-RNA kit was from Scotlab Biosciences, Coatbridge, Scotland, U.K.

Oligo (dT)-cellulose redi column, Sephadex G25 (medium), Sephadex G50 and FPLC Superose 6 pre-packed column were from Pharmacia, Milton Keynes, Bucks., U.K.

Okadaic acid was from Moana Bioproducts.

Poly ATract mRNA isolation system, RNA size markers (0.28 - 6.6 Kb), Taq DNA polymerase, all DNA restriction enzymes and the Wizard DNA clean-up system were from Promega (U.K.) Ltd., Southampton, U.K.

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

Tris was from ICN Biomedicals, Ohio, U.S.A.

2.2. Plant material

2.2.1. *Bryophyllum (Kalanchoë) fedtschenkoi*

Bryophyllum (Kalanchoë) 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 h 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 h 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 100 W tungsten lamps, giving a radiant fluence rate of 20 W m^{-2} s^{-1}. 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.

2.2.2. Maize

Maize (*Zea mays*, cv. Jubilee F1) were grown from seed in potting and bedding compost (William Sinclair Horticulture Ltd., Lincoln, U.K.) in a greenhouse under a 16 h photoperiod supplemented with mercury-vapour lamps. Two to three week old plants were transferred to a controlled environment growth chamber (Fi-Totron model PG1700, Sanyo-Gallenkamp, Loughborough, U.K.) and grown under a 12 h photoperiod provided by eight 250 W halogen and eight 60 W tungsten lamps to give a light intensity of 700 μmoles m^{-2} s^{-1} at plant height. The temperature was 27 °C during the photoperiod and 18 °C in the dark. The relative humidity was 60 % in the photoperiod and 80 % during the dark. Plants were used from the age of six weeks.

2.2.3. Barley

Spring barley (*Hordeum vulgare* L. var. Hart) (Plant Breeding International, Cambridge, U.K.) were grown from seed under a 12 h photoperiod of 300 μmol m^{-2} s^{-1} using warm white fluorescent tubes, tungsten lamps and constant temperature (20°C) in vermiculite moistened with nutrient solution containing 15 mM NO_3^- (Hoagland and Arnon, 1950) in a Fitotron TM 600 growth chamber (Fisons, Loughborough, U.K.). The plants were resupplied with the above nutrient solution 5 d and 7 d after sowing. The primary leaf of each plant was harvested 10 days after sowing and, after either 10 h of darkness or 3 h of light, used to isolate RNA.

2.2.4. *Kalanchoë daigremontiana*

Plants of *Kalanchoë daigremontiana* Hamet et Perrier, which were approximately one year old and growing in 100-mm diameter pots, were acclimated in the growth chamber for 4 weeks prior to experimentation. All measurements were conducted on the fourth leaf pair from the growing tip.

The plants were acclimated in a Fitotron growth chamber (Sanyo Gallenkamp, Leicester, UK) which was programmed to provide gradual changes in temperature, humidity and photon flux density (PFD) at the start and end of the photoperiod in an attempt to mimic conditions found naturally at dawn and dusk. From 08:30 until 12:00, PFD increased to a maximum of 530 μmol m^{-2} s^{-1} at leaf height, temperature increased from 19°C to 27°C and relative humidity decreased from 80% to 60% (vapour pressure deficit (VPD) increased from 1.8-2.9kPa). These conditions were maintained until 16:00 when PFD decreased gradually until lights off at 19:30, temperature decreased to 19°C and relative humidity increased to 80% (VPD 1.8kPa). Over the 13h dark period, temperature (19°C) and relative humidity (80%) remained constant.

2.3. General biochemical methods

2.3.1. pH calibrations were performed using a Russel pH probe connected to an EDT instruments 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 were cleaned in "Haemo-sol" solution (Alfred Cox (Surgical) Ltd., UK), rinsed in distilled water and oven dried.

2.3.3. Chromatographic materials. Sephadex G-25M, DEAE-cellulose and hydroxylapatite were swollen and packed according to the manufacturer's protocols. Sephadex G-25M was stored in 0.02 % (w/v) sodium azide. Hydroxylapatite and DEAE-cellulose retained plant pigments and were therefore only used once.

2.3.4. Concentration of protein samples was performed using Centricon 30 microconcentrators (Amicon Ltd., Stonehouse, Glos., UK) according to the manufacturer's protocols.

2.3.5. Spectrophotometric assays were carried out in semi-micro quartz cuvettes or plastic disposable cuvettes (1 cm path length, 1 ml volume). A Philips PU 8700 series UV/Vis scanning spectrophotometer was used for all enzyme assays and the determination of the concentration of DNA/RNA solutions.

2.3.6. Centrifugation was performed in a Beckmann model J2-21 centrifuge using either a JA20 or JS13.1 rotor. Samples less than 1.5 ml were centrifuged in Eppendorf tubes using a benchtop microfuge (MSE Micro Centaur or Eppendorf 5415 C).

2.3.7. Micropipetting (0.5 µl-1 ml) was performed using adjustable Finnpipettes (Labsystems, Helsinki, Finland).

2.4. Enzyme extraction and purification

2.4.1. Purification of phosphoenolpyruvate carboxylase from *Bryophyllum (Kalanchoë) fedtschenkoi*

All buffers are defined in section 2.3.2. All steps were performed at 4°C in a cold room except for the Superose 6 (FPLC) column which was at room temperature. Day form (dephosphorylated) PEPc was extracted by taking 40 g of leaves between 09.00 and 11.00 h and homogenizing them at low speed in a Waring blender for a total of 30 seconds with 30 ml of buffer 1, 1.5 g of insoluble polyvinyl pyrrolidone, 2.0 g Sodium Bicarbonate and a few drops of octanol. The homogenate was filtered through two layers of muslin and centrifuged for 15 minutes at 5000 rpm. The supernatant was filtered again through two layers of muslin prior to loading the entire sample onto a 15 cm x 4.5 cm (200 ml) bed volume Sephadex G-25 M column pre-equilibrated with buffer 2. The extract was washed through the column with buffer 2 at a flow rate of 5 ml/min and 5 ml fractions were collected with a fraction collector. The $A_{280\ nm}$ of the fractions was determined in order to identify the peak of protein. The peak fractions were pooled and mixed with 10 ml of hydroxylapatite (HAP) pre-equilibrated with buffer 2. The mixture was swirled gently on ice for 10 minutes and then spun briefly to 5000 rpm to rapidly pellet the HAP. The supernatant was poured off and the pellet resuspended and used to repour the HAP column. The column bed was allowed to settle and the eluate discarded. The column was then

washed in about 30 mls of buffer 2. The bulk of the protein was then eluted with buffer 3 until the $A_{280\ nm}$ of the fractions was the same as the $A_{280\ nm}$ of the buffer. The PEPc was then eluted from the HAP column with buffer 4. The flow rate was 2 ml/min and 1 min fractions were collected. Again the peak fractions were pooled and desalted on a 55 cm x 2.2 cm (200 ml) Sephadex G-25 M column pre-equilibrated with buffer 5. The flow rate was 7 ml/min and 0.5 min fractions were collected. The peak fractions were pooled to a maximum of 30 ml and slowly (no faster than 1 ml / 2 minutes) loaded onto a 1.2 cm x 1 cm (1 ml) DEAE-cellulose column pre-equilibrated with buffer 5. The column was washed with buffer 5 until the $A_{280\ nm}$ of the washings decreased to that of the buffer. The PEPc was eluted from the DEAE column with buffer 6. 500 µl fractions were collected and checked for PEPc activity. The peak fractions were pooled, filtered and then concentrated in Centricon-30 tubes at 5000 rpm using a 8 X 50 ml angled-rotor (JA-20) in a Beckman centrifuge (Model J2-21) for 40-80 minutes until the volume was reduced to below 500 µl. The concentrated sample was loaded onto a 30 cm x 1 cm Pharmacia (FPLC) Superose 6 column pre-equilibrated with buffer 7. The flow rate on the FPLC was set at 0.3 ml/minute and three 10 minute fractions were collected followed by 1 minute fractions. The PEPc eluted at fractions 20-25. The fractions were checked for activity and the activity peak was pooled and dialysed overnight into dialysis buffer at 4°C. The dialysed, purified PEPc was then aliquoted into separate tubes at 0.3 units per tube (enough for 10 PEPc kinase assays) and stored at -70°C.

2.4.2. Buffers used in the purification of PEPc

Buffer 1

100 mM Tris -HCl (pH 8.0) containing 2 mM EDTA, 10 mM L-malate, 2% (w/v) polyethylene glycol 15-20, 000, 1 mm DTT, 1 mM benzamidine hydrochloride.

Buffer 2 (desalting Sephadex $G25_f$ and HAP)

100 mM Tris-HCl (pH 7.5) containing 2 mM EDTA, 10 mM L-malate, 1 mM DTT, 1 mM benzamidine hydrochloride.

Buffer 3 (Hydroxylapatite wash)

100 mM Tris-HCl (pH 7.5) containing 0.1 mM EDTA, 10 mM L-malate, 40 mM potassium phosphate, 1 mM DTT, 1 mM benzamidine hydrochloride.

Buffer 4 (PEPc elution from HAP)

100 mM Tris-HCl (pH 7.5) containing 0.1 mM EDTA, 10 mM L-malate, 150 mM potassium phosphate, 1 mM DTT, 1 mM benzamidine hydrochloride.

Buffer 5 (desalting Sephadex $G25_{II}$ and DEAE-cellulose)

50 mM Tris -HCl (pH 7.5) containing 0.1 mM EDTA, 1 mM DTT, 1 mM benzamidine hydrochloride.

Buffer 6 (Elute PEPc from DEAE-cellulose)

50 mM Tris-HCl (pH 7.5) containing 0.1 mM EDTA, 100 mM NaCl, 1 mM DTT, 1 mM benzamidine hydrochloride.

Buffer 7 (Superose 6 column on Waters FPLC)

50 mM Bis-tris Propane (pH 7.5) containing 0.1 mM EDTA, 50 mM potassium chloride, 1 mM DTT, 1 mM benzamidine hydrochloride.

Dialysis Buffer

50 mM Bis-tris Propane (pH 7.5) containing 0.1 mM EDTA, 1 mM DTT, 1 mM benzamidine hydrochloride and 50% (v/v) glycerol.

2.4.3. Preparation of rapidly desalted extracts

(i) ***B. fedtschenkoi*** **and** ***K. daigremontiana*** Leaves were ground in extraction buffer (100 mM Tris-HCl, pH 8.0, 2 mM EDTA, 10 mM L-malate, 1 mM benzamidine hydrochloride, 1 mM dithiothreitol, 2% (w/v) polyethylene glycol 20000) (2 ml/g fresh tissue) with 100 mg sodium bicarbonate/g and 200 mg insoluble polyvinylpyrrolidine/g in a mortar and pestle at 4°C (Carter *et al.*, 1991). The homogenate was filtered through two layers of muslin and centrifuged for 30 s in a microfuge at full speed to remove particulate material. Prior to assays of PEPc and PEPc kinase, extracts were desalted into 50 mM Tris-HCl, pH 7.8, 1 mM benzamidine hydrochloride, 1 mM dithiothreitol , 5% (v/v) glycerol using a pre-equilibrated 12 cm x 1 cm (10 ml) Sephadex G25 M column.

(ii) Maize. Leaves were ground in extraction buffer (100 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM EDTA, 10 µg/ml chymostatin) (4 ml/g fresh tissue) with 40 mg insoluble polyvinylpyrrolidine/g and 0.5 % (w/w) acid-washed sand in a mortar and pestle at 4°C. The homogenate was filtered through two layers of muslin and centrifuged for 30 s at full speed in a microfuge to remove particulate material. Extracts were then desalted into 50 mM Hepes-KOH, pH 7.3, 5 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM EDTA, 10 µg/ml chymostatin, 20% (v/v) glycerol using a 12 cm x 1 cm (10 ml) Sephadex G25 M column.

2.5. Enzyme Assays

2.5.1. Estimation of PEPc activity and the apparent K_i of PEPc for L-malate

The apparent K_i for L-malate and the activity of PEPc in desalted extracts of *B. fedtschenkoi* leaves were determined using a spectrophotometric assay. The standard 1 ml assay cocktail contained 50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 2 mM PEP, 0.2 mM NADH, 10 mM $NaHCO_3^-$, 5 units of MDH and the enzyme sample. The oxidation of NADH by the coupling enzyme MDH was monitored as the decrease in the $A_{340\ nm}$ at 25°C. This decrease was dependent on the PEPc concentration in the enzyme sample (Nimmo *et al.*, 1984). The malate sensitivity of PEPc was ascertained by adding an appropriate range of concentrations of L-malate to the standard assay cocktail. The concentration of malate needed to produce 50 % inhibition the initial PEPc activity (apparent K_i) was determined using a plot of percentage inhibition of the rate minus L-malate versus malate concentration.

For maize the assay buffer consisted of 50 mM Hepes-KOH, pH 7.3, 0.5 mM phosphoenolpyruvate, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 0.2 mM NADH, 5 units of malate dehydrogenase, 5 mM glucose-6-phosphate and the enzyme sample. The apparent K_i of the PEPc for L-malate was determined as for *B. fedtschenkoi* (Nimmo *et al.*, 1987a).

One unit of enzyme activity is the amount required to catalyse the formation of 1 µmol of product per min.

2.5.2. PEPc kinase activity in rapidly desalted extracts

PEPc kinase activity in desalted extracts was assayed using an *in vitro* assay (Carter *et al.*, 1991). Desalted extract containing 0.002 units of PEPc activity was incubated in 50 mM Tris-HCl, pH 7.8 containing 1 mM benzamidine hydrochloride, 10 μg antipain/ml, 10 μg leupeptin/ml, 5 nM okadaic acid, 5 mM $MgCl_2$, 0.01 mM[γ-^{32}P] ATP (1 μCi) and 0.03 units of purified, dephosphorylated PEPc from *B. fedtschenkoi*, in a total volume of 25 μl. Assays were initiated by adding the radioactive ATP. Incubations were at 30°C for 30 min. Assays were stopped by the addition of one fifth volume of 5X SDS sample buffer (see 2.7.1) and heating in a boiling water bath for 4 min. Samples were then separated by SDS-PAGE (see 2.7.1) (Laemmli, 1970). Dried radioactive gels were routinely phosphoimaged and autoradiographed (see 2.7.3).

2.5.3. PEPc kinase activity in *in vitro* translation products

PEPc kinase activity in *in vitro* translation products was assayed by incubating 5 μl of translation products (which contain unlabelled ATP) with 50 mM Tris-HCl, pH 7.8 containing 3 mm $MgCl_2$, 1 mM benzamidine hydrochloride, 10 μg antipain/ml, 10 μg leupeptin/ml, 5 nM okadaic acid, 0.03 units of purified, dephosphorylated PEPc from *B. fedtschenkoi* and 10 μCi [γ-^{32}P] ATP. Incubations (30 min at 30°C) were terminated by the addition of 10 μl of polyclonal rabbit anti-*Bryophyllum* PEPc antibody (Nimmo *et al.*, 1986), followed by incubation on ice for 1 h. Immunoprecipitated PEPc was pelleted by centrifugation in a microfuge at full speed for 7 min. The supernatant was removed and the pellet washed in 200 μl of 1.5 M NaCl, 2 mM EDTA (pH 7.0). The PEPc was repelleted by microfuging at full speed for 7 min and the pellet resuspended in 30 μl of 5X SDS sample buffer (see 2.7.1), followed by heating to 100°C for 4 min in a boiling water bath. Incorporation of ^{32}P into PEPc was monitored by SDS-PAGE (see 2.7.1) (Laemmli, 1970) and phospho-imaging or autoradiography (see 2.7.3).

2.6. Manipulation of Crassulacean acid metabolism

As a means of inhibiting C_4 carboxylation at night, individual leaves of intact *K. daigremontiana* plants were enclosed in an atmosphere of N_2 overnight, thereby preventing access to external CO_2 and inhibiting the release of internal (respiratory) sources of CO_2 (full N_2). Some leaves were enclosed in an atmosphere of N_2 for the first half of the dark period and then exposed to ambient air for the remainder of the dark period (half N_2). Control leaves were exposed to the ambient atmosphere in the growth chamber.

A batch of plants, half of which were maintained in ambient air (control), half with leaves enclosed in an atmosphere of N_2 (half N_2) were subjected to an increase in temperature from 19°C to 27°C in the middle of the dark period (02:30-03:00). The leaves enclosed in N_2 were subsequently exposed to ambient air from 03:00 onwards with temperature maintained at 27°C and relative humidity at 70%.

2.7. Gas exchange measurements

For experiments on *K. daigremontiana,* rates of net CO_2 assimilation were measured continuously on the same leaf over 24h with 3 separate runs made for each treatment. The leaf was enclosed in a porometer head 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 a Binos gas analyser. Gas exchange parameters were calculated using DIAGAS software supplied by Walz.

2.8. Measurement of total leaf malate content

For experiments on *K. daigremontiana*, discs were punched from leaves subjected to the various treatments at intervals over the dark and light periods and immediately plunged into hot (80°C) methanol (80% v/v). The methanol extracts were heated for 1 h at 70°C before being evaporated to dryness, taken up in 100 mM bicine, pH 7.8 and malate

content determined enzymatically using malate dehydrogenase in a spectrophotometric assay at 340 nm by monitoring the oxidation of NADH.

2.9. RNA isolation

2.9.1. Isolation of total RNA

For all three plants, total RNA was isolated according to the protocol of Chang *et. al.* (1993), with slight modifications. All solutions, except for ones containing Tris, were treated overnight with 0.5 % (v/v) diethylpyrocarbonate (DEPC) to destroy RNase activity and subsequently autoclaved for at least 20 min to destroy the DEPC (it breaks down into ethanol and carbon dioxide upon autoclaving). Tris containing solutions were prepared using autoclaved, DEPC-treated, distilled water and subsequently autoclaved. All plastic tubes and pipette tips were autoclaved prior to use. Gloves were worn at all stages of the procedure to reduce the risk of contaminating samples with hand-borne RNases. Every possible precaution was taken to keep RNA samples free from RNase and samples were kept on ice at all times, except when the samples required heating, to minimise degradation.

In a fume hood, 2-3 g of liquid nitrogen-frozen leaf tissue were ground to a fine powder in liquid nitrogen using a pestle and mortar. The fine powder was quickly added to a 30 ml plastic centrifuge tube (Sardstedt, U.K.) containing 10 ml of extraction buffer (2 % (w/v) hexadecyltrimethylammonium bromide, 2 % (w/v) polyvinylpyrrollidone 40, 100 mM Tris-HCl, pH 8.0, 25 mM EDTA, 2 M NaCl, 0.5 g/l spermidine-HCl and 2 % (v/v) ß-mercaptoethanol (added after autoclaving)), which was preheated in a 65°C water bath. The tube was quickly inverted a few times to mix the frozen material into the hot extraction buffer and returned to the 65°C water bath whilst further samples were processed. Tubes were shaken at regular intervals to ensure thorough mixing of the tissue in the extraction buffer. Next, 10 ml of chloroform was added to each tube, the tubes were shaken to mix the two phases and the tubes were centrifuged for 10 min at 9500 rpm, 4°C using a 6 X 50 ml swing-out rotor (JS 13.1) in a Beckman centrifuge (Model J2-21). The upper aqueous phase was removed to a fresh 30 ml centrifuge tube on ice, using a

disposable plastic transfer pipette, and a further 10 ml of chloroform was added followed by thorough mixing. The tubes were centrifuged for 10 min at 5000 rpm, 4°C and the upper, aqueous layer removed to a fresh tube. 3 ml of ice-cold 10 M lithium chloride was added to each tube and the tubes were shaken to mix the contents. Samples were then placed at 4°C in a fridge overnight to allow the RNA to precipitate. Tubes were then centrifuged at 9500 rpm for 20 min at 4°C to pellet the RNA. The supernatant was removed and discarded using a disposable, plastic transfer pipette. The pellets were allowed to dry for 5 min by inverting the tubes onto clean, dry tissues. Next, the pellets were resuspended in 500 µl of ice-cold 10 mM Tris-HCl, pH 7.5 containing 1 mM EDTA (TE) and transferred to 1.5 ml microfuge tubes. 500 µl of chloroform was added to each tube and the contents mixed. Tubes were then centrifuged for 10 min at full speed in a microfuge in the cold room. The upper aqueous phase was transferred to a fresh microfuge tube and 50 µl of 3 M sodium acetate, pH 5.2 and 2.5 volumes of ice cold ethanol were added. The tubes were thoroughly mixed and placed at -70°C for at least an hour to allow the RNA to precipitate. The tubes were then centrifuged at full speed in a microfuge in the cold room for 20 min. The supernatant was removed and discarded, and the pellets dried using a Speed Vac Plus (Savant, model SC 110A) connected to a vacuum pump (Vacuubrand Gmbh and Co., Wertheim, Germany). The dried pellets were redissolved in a minimal volume of sterile, DEPC-treated, distilled water and stored at -70°C.

The quantity and purity of the RNA was determined spectrophotometrically by monitoring the absorbance at 260 nm and comparing it to the absorbance at 280 nm. For RNA an $A_{260\ nm}$ of 1 is equivalent to a concentration of 40 µg/ml. An $A_{260/280}$ ratio of between 1.8 and 2.0 indicates 'clean' RNA, relatively free from protein contamination. $A_{260/280}$ ratios below 1.8 indicate significant contamination with protein. Intactness of the RNA was determined by agarose gel electrophoresis; the integrity of the ribosomal RNA bands was checked.

2.9.2. Isolation of poly $(A)^+$ RNA

Poly $(A)^+$ RNA was obtained from isolated total RNA using either oligo dT-cellulose column chromatography (Pharmacia, U.K.), Dynabeads oligo dT (Dynal, Oslo, Norway) or Promega's poly ATract system (Promega, U.K.). All systems were used according to the manufacturers' protocols.

2.10. *In vitro* translation

The rabbit reticulocyte lysate system (Amersham, U.K.) possessed no detectable PEPc kinase activity and was used for *in vitro* translation of isolated RNA. 5 µg of total RNA, or the poly $(A)^+$ RNA from 20 µg total RNA in the case of maize, 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 unless stated otherwise. The incorporation of [^{35}S]-Met into protein was measured by precipitation with trichloroacetic acid according to the manufacturer's protocols to allow standardisation of the subsequent PEPc kinase assays.

2.11. Gel electrophoresis techniques

2.11.1. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins

Proteins were separated by discontinuous SDS-PAGE according to the method originally outlined by Laemmli, (1970). All SDS-PAGE gels used in this work contained an 8 % polyacrylamide separating gel and a 3 % stacking gel. Samples were denatured by the addition of one fifth volume of 5 X concentration sample buffer (125 mM Tris-HCl, pH 6.8, 2.5 % (w/v) SDS, 25 % (v/v) glycerol, 0.05 % Bromophenol Blue and 2.5 % (v/v) 2-mercaptoethanol) to achieve a final concentration of 1 X, and heating in a boiling water bath for 4 min. Samples were then loaded onto the gel and electrophoresed at 60-70 mA for 2-2.5 h until the tracking dye had left the bottom of the separating gel.

2.11.2. Staining SDS-PAGE gels

The stacking gel was removed and discarded and the separating gel was stained for 30 min at 37 °C in 0.1 % Coomassie Brilliant Blue G250, 50 % (v/v) methanol, 10 % (v/v) glacial acetic acid and destained in several changes of 10 % (v/v) methanol, 10 % (v/v) glacial acetic acid at 37 °C.

2.11.3. Drying, autoradiography and phospho-imaging of SDS-PAGE gels

SDS-PAGE gels were dried onto Whatman 3MM chromatography paper using a Biorad Laboratories Gel Drier (model 1125) connected to an Aquavac Junior multi-purpose vacuum unit (Uniscience Ltd., London). Dried radioactive gels and blots were exposed onto X-ray film (Fuji RX) using two intensifying screens at -70 °C for 1-3 days. X-ray film was developed using a Kodak X-OMAT Processor (model ME-3). Radioactive gels and blots were also routinely phospho-imaged using a Fuji Bio-Imaging Analyser (Fuji Photo Film Co. Ltd., Japan) by exposing the gel to a pre-blanked imaging plate for 1-24 h in a cassette at room temperature. 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)

2.11.4. Agarose gel electrophoresis of RNA and DNA

Samples of purified DNA and RNA were routinely checked for integrity and molecular weight distribution using horizontal agarose gel electrophoresis. 1 % (w/v) agarose gels were prepared and run in 0.5 X TBE (45 mM Tris-borate, pH 8.0, 1 mM EDTA) buffer containing 0.25 μg/ml ethidium bromide. Samples of DNA or RNA were mixed with 4 X loading buffer (0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol and 30 % glycerol (v/v) in water) to facilitate loading into the gel, and electrophoresed at 5 V/cm for 1-2 h depending on the dimensions of the gel. For DNA samples, 1 Kb ladder (Gibco BRL, U.K.) was run alongside the samples to allow the subsequent estimation of the molecular weight of the sample DNA.

2.11.5. Denaturing agarose gel electrophoresis of RNA

RNA samples to be blotted onto nitro-cellulose for Northern hybridization were separated by electrophoresis through denaturing formaldehyde/Mops agarose gels. This method ensures accurate separation of RNA according to size as the denaturing conditions remove secondary structure that can cause RNA to run at aberrant molecular weights in non-denaturing agarose gels.

Gels (0.8-1.3 % (w/v) agarose) were prepared in 1 X Mops buffer, pH 8.0 (10 X Mops buffer: 200 mM Mops, pH 8.0, 50 mM sodium acetate and 10 mM EDTA) and 10 % (v/v) formaldehyde. This was achieved by dissolving the agarose in water by microwaving it until it just reached boiling point. This agarose solution was swirled until all the agarose had dissolved and left to cool to approximately 60°C. Then, in a fume hood, the formaldehyde and 10 X Mops were added and mixed in thoroughly. The gel was poured and left to set in the fume hood. Gels were run in 1 X Mops buffer, pH 7.0 (10 X stock as above but with the pH adjusted to 7.0). Samples were prepared by combining 8 µl of RNA (5-20 µg, but constant for all samples on a single gel) with 6 µl of sample buffer (72 µg/ml ethidium bromide, 2 X Mops, pH 8.0, 6 % (v/v) formaldehyde and 70 % (v/v) formamide) and heating to 65°C for 2.5 min. 1-2 µg of RNA size markers (Promega, UK) were treated in the same manner, and run alongside the samples to allow the later estimation of the molecular weight of RNA bands of interest. Samples were then snap cooled on ice for 5 min and loaded onto the gel. Gels were run at 5 V/cm for 2-4 h.

2.12. Northern blotting

2.12.1. Transfer of RNA from denaturing agarose gels onto nitro-cellulose membranes

RNA was blotted onto Hybond N nitro-cellulose membrane using 20 X SSC (3 M NaCl and 0.3 M sodium citrate, pH 7.0) as the transfer buffer. A tray was filled with 20 X SSC and a platform big enough to support the gel placed within it. The platform was covered with a wick made from a sheet of Whatman 3MM filter paper, saturated with blotting buffer. The gel was placed face down on the wick and any air bubbles removed by

rolling them out with a sterile pipette. The gel was surrounded with cling film to prevent the blotting buffer short-circuiting directly into the paper towels above. Then, a piece of Hybond-N membrane was cut to the exact size of the gel, pre-wet in distilled water and placed on the gel. Again, air bubbles were rolled out with a sterile pipette. Three sheets of Whatman 3MM were cut to the size of the gel, soaked in transfer buffer and placed on top of the Hybond-N membrane. Air bubbles were rolled out again and a stack of paper towels were placed on top of the Whatman paper (approximately 5 cm high). Finally a glass plate was placed on top of the paper towels and a 0.75-1 Kg weight placed on the plate. Transfer was then allowed to proceed overnight. After transfer, the apparatus was dismantled down to the gel which was removed with the membrane attached and the membrane was marked with a biro to orientate it relative to the gel. The gel and membrane were briefly examined on a UV transilluminator to check that all the RNA had transferred to the membrane prior to separating the membrane from the gel. The membrane was briefly dipped in distilled water to remove any excess salt deposits left by the transfer buffer and placed RNA side-up on a sheet of Whatman 3MM in a UV crosslinker (UVP Ultraviolet Crosslinker). The RNA was then crosslinked to the membrane by exposing it to a set dose (120000 $\mu J\ cm^{-2}$) of UV irradiation. The blot was then left to dry in air and stored between two pieces of Whatman 3MM wrapped in aluminium foil.

2.12.2. Hybridization of radiolabelled cDNA probes to Northern blots

All hybridization steps were performed in an hybridization oven (Techne Hybridiser HB-1D). To prepare blots for hybridization with cDNA probes they were first prehybridized at 55°C for 1-2 h in 0.5 M Na_2HPO_4, pH 7.2, 7 % (w/v) SDS, 10 mg/ml BSA. Following this, the radioactive cDNA probe (see section 2.11.2) was added to the prehybridization solution and the blot left to hybridize at 55°C overnight. Then the hybridization buffer containing the remaining radioactive probe was poured off into a tube and stored for future use. The blot was routinely washed in 2 X SSC / 1 % SDS at 55°C for 10 min. This would be followed by a number of other possible higher stringency

washes depending on how radioactive the blot was, for example, 2 X SSC / 1 % SDS at 65°C for 10 min, 1 X SSC / 1 % SSC at 65°C for 10 min, 0.5 X SSC / 0.1 % SDS at 65°C for 10 min and so on, until the signal to background ratio was acceptable. Then the blot was heat sealed into plastic and exposed onto X-ray film or phospho-imaged.

2.13. Culture and transformation of *Eschericia coli*

2.13.1. Production of competent cells of *E. coli*

Competent cells were produced by the calcium chloride method. 5 ml of LB broth (1% (w/v) Bacto tryptone, 0.5 % (w/v) Bacto yeast extract, 1 % (w/v) NaCl, pH 7.5) was inoculated with a single colony of XL1 Blue MRF' *E. coli* and grown at 37°C with shaking overnight. This culture was diluted 1 in 100 into fresh LB broth and grown at 37°C with shaking for 3-4 h until the optical density at 600 nm was between 0.4 and 0.6. The cells were then centrifuged at 3000 rpm, 4°C for 10 min and the supernatant decanted off. The pellet was gently resuspended in a half volume of ice-cold 50 mM $CaCl_2$ and left on ice for 1 h. Again the cells were centrifuged at 3000 rpm, 4°C for 10 min and the supernatant decanted off. The pellet was then resuspended in one tenth of the original volume of ice-cold 50 mM $CaCl_2$/20 % glycerol. The cells were then aliquoted into 1.5 ml Eppendorf tubes, frozen in liquid nitrogen and stored at -70°C.

2.13.2. Transformation of *E. coli* with plasmid DNA

Calcium chloride competent cells of *E. coli* XL1 Blue MRF' were used for transformation. An aliquot of cells were thawed on ice and 10-100 ng of plasmid DNA added to 100 µl of competent cells. These were mixed gently by flicking the tube. This mixture was incubated on ice for 30 min, followed by incubating at 42°C for 60 s. Then 1 ml of LB broth was added and the cells incubated for 60 min at 37°C with shaking. The cells were then spun down gently in a microfuge for 5 min and gently resuspended in 100 µl of LB broth. 10 and 90 µl of these resuspended cells were plated out onto LB broth plates (as for LB broth plus 1.5 % (w/v) Bacto agar) containing 100 µg/ml ampicillin

(added before pouring the plates when the temperature of the LB broth is approximately 45°C). These plates were incubated overnight at 37°C.

2.13.3. Culture of *E. coli* strains possessing plasmids containing cloned cDNA inserts

Single colonies were selected from the selection plates detailed in section 2.10 using a sterile yellow micropipette tip and the whole tip was ejected into 5 ml of LB broth containing 100 µg/ml ampicillin. The culture was grown overnight at 37°C with shaking. Glycerol stocks were generated by mixing 850 µl of this overnight culture with 150 µl of sterile glycerol. The mixture was vortexed to mix and frozen in liquid nitrogen before being stored at -70°C. To grow cells from a glycerol stock the surface of the frozen glycerol stock was scratched with an inoculating ring and the attached cells spread out onto selection plates. The plates were grown overnight at 37°C and then individual colonies were picked and used to inoculate 5 ml overnight cultures as described above.

2.14 Plasmid DNA isolation

Plasmid DNA was isolated from 5 ml overnight cultures of the desired strain of *E. coli* containing the plasmid of interest using the QIA-prep spin plasmid miniprep kit (Qiagen, UK) according to the manufacturer's protocols.

2.15 cDNA isolation and generation of radiolabelled cDNA probes for Northern blotting

2.15.1. Excision of cDNA inserts from isolated plasmids

cDNA inserts were excised from plasmids by performing a restriction digest. Restriction enzymes which would cut the plasmid DNA at sites flanking the cDNA insert were chosen by studying the plasmid map. These restriction enzymes were then used according to the manufacturers' protocols to excise the cDNA fragment from the plasmid. The digested DNA was separated using a 1 % (w/v) agarose gel and the agarose containing the cDNA fragment was excised from the gel with a sterile scalpel. The cDNA was

extracted from the agarose using the QIA-quick gel extraction kit according to the manufacturer's protocols.

2.15.2. Random priming of cDNAs to synthesize radioactive probes

cDNA inserts excised from plasmids and purified as described in section 2.11.1 were radiolabelled for use as probes on Northern blots by the method of random priming using the Klenow fragment of DNA polymerase I. 2.5-25 ng of DNA were labelled with the *redi*prime DNA labelling system (Amersham, UK) according to the manufacturer's protocol, using 50 µCi of Redivue [^{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 2.8.2.

2.16 *In vitro* transcription

In vitro transcription was performed using the T3 mMESSAGE mMACHINE large scale *in vitro* transcription kit for the synthesis of capped RNAs according to the manufacturer's protocol (Ambion, UK). Template DNA consisted of isolated plasmid DNA linearized by performing a restriction digest with Xho 1. The linearized DNA was purified from the restriction digest using the Wizard DNA clean up kit (Promega, UK) before being used for *in vitro* transcription.

2.17 Size fractionation of mRNA

Size fractionation of mRNA was performed using 10 µg of poly (A)$^+$ RNA isolated from *B. fedtschenkoi* leaves sampled at 00.00 h. This mRNA was separated using a sterile 1 % (w/v) agarose gel in 1 X TAE (50 X TAE: 2 M Tris-HCl, pH 8.0, 3 % (v/v) glacial acetic acid, 0.1 mM EDTA). An aliquot of RNA size markers (Promega, UK) was run either side of the mRNA. The gel was run for 4-5 h at 5 V/cm, 4°C in a cold room. The RNA size marker lanes were excised and stained in 1 X TAE/1 µg/ml ethidium bromide for 30 min. They were then realigned with the mRNA containing lane to allow size selection of the mRNA lane. The mRNA containing lane was cut into fourteen 0.5 cm

segments between approximately 300 bp and 8 Kb to size fractionate the RNA. The mRNA was recovered from each 0.5 cm segment of agarose using the Nucleon Easi-RNA kit (Scotlab Biosciences, UK) according to the manufacturer's protocol. The recovered mRNA from each segment was used to prime the *in vitro* translation assay for PEPc kinase, to identify which size fraction of mRNA contained PEPc kinase mRNA.

Chapter 3

Development and validation of an assay for PEPc kinase translatable mRNA

3.1 Introduction

The phosphorylation state of PEPc is largely regulated by the activity of its kinase, PEPc kinase (Carter *et al.*, 1991). PEPc phosphatase is a protein phosphatase type 2A (Carter *et al.*, 1990) and its activity seems to be constant throughout the diurnal cycle (Carter *et al.*, 1991). The discovery that it is the activity of PEPc kinase which controls the phosphorylation state of PEPc was made possible by the development of an *in vitro* assay for PEPc kinase activity which could be used on crude extracts (Carter *et al.*, 1991; Echevarria *et al.*, 1990; Jiao and Chollet, 1989; McNaughton *et al.*, 1991). This also led to the elucidation of some of the ways in which PEPc kinase activity is regulated *in vivo*. For example, the CAM enzyme is regulated by a circadian clock and appears at night whilst the C_4 and C_3 enzymes are light-induced (Carter *et al.*, 1991; Echevarria *et al.*, 1990; Li *et al.*, 1996; McNaughton *et al.*, 1991; Smith *et al.*, 1996). Furthermore, experiments involving the use of protein and RNA synthesis inhibitors have shown that kinase activity is regulated by a process that involves protein synthesis/degradation (Carter *et al.*, 1996; Carter *et al.*, 1991; Jiao *et al.*, 1991a; Li *et al.*, 1996; Nimmo, 1993; Smith *et al.*, 1996). The ability to assay PEPc kinase activity also allowed a number of workers to attempt to purify the enzyme from CAM and C_4 tissues (G.A. Nimmo, unpublished) (Carter *et al.*, 1991; Li and Chollet, 1994; Wang and Chollet, 1993b). However, though purification factors of 3500 to 4000 (Li and Chollet, 1994; Wang and Chollet, 1993b) or considerably greater than this (G.A. Nimmo, unpublished) have been achieved, there have been no reports of the isolation of antibodies to the enzyme, nor has its gene been cloned (Vidal and Chollet, 1997). It has therefore not been possible to ascertain whether the component which must be synthesized for kinase activity to appear is the kinase protein itself or another component that activates the kinase.

With this shortfall in the understanding of PEPc kinase regulation in higher plants in mind, a novel assay for monitoring the level of PEPc kinase mRNA in plant tissues was

developed. Isolated RNA is translated *in vitro* using rabbit reticulocyte lysate and the translation products are assayed directly for PEPc kinase activity. A wide variety of experiments were performed to test the validity of this assay, and size fractionation of mRNA allowed the determination of the approximate length of PEPc kinase mRNA.

3.2 Results

3.2.1 The *in vitro* translation of isolated total or mRNA produces active PEPc kinase

To determine whether PEPc kinase activity could be detected in the *in vitro* translation products from isolated plant total RNA, total RNA was isolated from *B. fedtschenkoi* leaves harvested in the light and dark periods of the diurnal cycle. Previous work had shown that PEPc kinase activity is readily detectable in leaf extracts prepared during the middle of the dark period but not those prepared during the light period (Carter *et al.*, 1991). The *in vitro* translations were carried out using [^{35}S]-Met and the results of a typical experiment are shown in Figure 3.1. Panel A shows that the RNA samples from leaves harvested during the light and dark periods direct the synthesis of a somewhat different range of proteins. However, the total amounts of protein synthesized in the two cases were similar, as judged by the incorporation of ^{35}S-radioactivity into trichloroacetic acid-precipitable protein. No protein was synthesised in incubations without added RNA. Samples of the translation products were assayed for PEPc kinase activity *in vitro* using exogenous, dephosphorylated PEPc as substrate. The PEPc was isolated by immunoprecipitation and analysed by SDS gel electrophoresis as shown in Panel B. The control, with the products from a translation with no added RNA, gave some ^{32}P-labelling of PEPc. This was caused by the presence of trace amounts of PEPc kinase activity in the exogenous PEPc, not in the translation mix (data not shown). However, the RNA isolated from leaves harvested during the dark period directed the appearance of much more PEPc kinase activity than did the sample isolated from leaves in the light (compare lane 2 with lane 3). *B. fedtschenkoi* PEPc contains two related subunits with M_r values of 112,000 and 123,000 in a ratio of some 10:1 (Nimmo *et al.*, 1986). Both bands were phosphorylated, as can be seen in Panel B lane 2. The ^{32}P-radioactivity in both PEPc bands shown in Panel B was assessed by phosphoimaging. Subtraction of the background (lane 1) indicated that the

RNA sample isolated from leaves in darkness contained some 20-fold more translatable PEPc kinase mRNA than the sample isolated from leaves in the light. The relative band intensities quoted in this and other experiments were calculated by correcting the ^{32}P-radioactivity in each band for the efficiency of translation in the different samples as revealed by the use of [^{35}S]Met (see Materials and Methods).

3.2.2 *In vitro* translated PEPc kinase activity is Ca^{2+}-independent

In an attempt to establish that the PEPc kinase activity generated by the *in vitro* translation of *B. fedtschenkoi* RNA was representative of the 'real' PEPc kinase, experiments were performed which included EGTA in the kinase assay. The PEPc kinase responsible for phosphorylating PEPc in *B. fedtschenkoi* has previously been demonstrated to be Ca^{2+}-independent (Carter *et al.*, 1991). This has also been found to be the case for the C_4 and C_3 enzymes (Li and Chollet, 1993; Li *et al.*, 1996; Wang and Chollet, 1993b). The inclusion of EGTA in the kinase assay chelates all available Ca^{2+} ions rendering them unavailable for use by Ca^{2+}-dependent enzymes such as Ca^{2+}-dependent protein kinases (at least one of which is capable of correctly phosphorylating PEPc) (Bakrim *et al.*, 1992; Li and Chollet, 1993; Ogawa *et al.*, 1992). Thus, if the PEPc kinase activity generated in the translation products was Ca^{2+}-dependent, the inclusion of EGTA in the kinase assay should completely inhibit the kinase. The translation mix itself contains EGTA in excess over Ca^{2+} ((Pelham and Jackson, 1976); see also protocol for the use of the rabbit reticulocyte lysate system, RPN 3150 and RPN 3151, Amersham International plc), but additional EGTA (0.25 mM) was added to ensure that all Ca^{2+} ions were rendered unavailable to the kinase. Figure 3.2 shows that the PEPc kinase activity generated in the *in vitro* translation products of *B. fedtschenkoi* RNA was only slightly less in the presence of 0.25 mM EGTA than in the absence of EGTA. This was the case for the translation products from both 'dark' and 'light' RNA. Thus the PEPc kinase synthesized by the *in vitro* translation of 'dark' and 'light' RNA is Ca^{2+}-independent like the kinase detected in desalted extracts (Carter *et al.*, 1991).

The omission of radioactive ATP from some of the kinase assays allowed the level of *de novo* synthesized, ^{35}S-Met labelled PEPc in each translation to be determined. For the

'dark' translation products, this was less than the amount of radioactive labelling detected in the presence of radioactive ATP (figure 3.2). However, the 'light' translation products contained levels of ^{35}S-Met labelled PEPc similar to the total level of labelling detected in the plus ^{32}P-ATP assays. This suggests that there is little if any PEPc kinase activity produced by the 'light' translation products. Thus, the detection of much greater PEPc kinase activity in the 'dark' translation products than in the 'light' translation products (see figure 3.1) is not due to ^{35}S-Met labelling of PEPc in the translations. This also holds true in the case of maize and barley where the 'light' translation products possess much greater PEPc kinase activity than the 'dark' translations products (see chapter 6, figure 6.2). Collectively, this data demonstrates that, in *B. fedtschenkoi*, the mRNA for the Ca^{2+}-independent PEPc kinase is absent in the 'light' and that this level increases very significantly in the 'dark', whilst in maize and barley the reverse is true. However, barley does possess very low levels of PEPc kinase mRNA in the dark (see figure 6.2).

3.2.3 The validity of the PEPc kinase translatable mRNA assay

The validity of any assay depends on the satisfaction of several criteria. In this case, for the PEPc kinase translatable mRNA assay to be a valid assay for the level of PEPc kinase mRNA in a plant leaf it had to show certain basic traits. The degree of phosphorylation of the PEPc in kinase assays on translation products should increase linearly in response to increasing the volume of translation products, the time of translation and the amount of RNA translated.

Figure 3.3 shows that the extent of phosphorylation of PEPc is proportional to the input of translation products. Since the non-radioactive ATP in the assay is derived from the translation mix (see Materials and Methods), this experiment was carried out by mixing the translation products from RNA isolated from leaves in darkness with products from a control translation with no added RNA (e.g. to assay 1 µl of translation products, 1 µl of a 'dark' *B. fedtschenkoi* RNA translation products was mixed with 4 µl of 'no RNA' translation products).

Figure 3.4 shows that the amount of kinase activity present in translation products and the amount of total protein synthesized during the translation increased in parallel with the

amount of RNA translated. This relationship holds up to 10 μg of total RNA; in routine assays, 5 μg of RNA were used.

The amount of kinase activity present in the translation products increased with time after a short lag (0-5 min) for up to 40-50 min. One representative experiment is shown in figure 3.5. The same ratio of translatable kinase mRNA in RNA samples from leaves in darkness to that in RNA samples from leaves in light was obtained when translations were carried out for 20 or 60 min (figure 3.6).

3.2.4 Size fractionation of *B. fedtschenkoi* mRNA yields a single mRNA fraction possessing PEPc kinase translatable mRNA

In order to determine the approximate length of *B. fedtschenkoi* PEPc kinase mRNA, a sample of mRNA was size fractionated. The mRNA sample used was isolated from *B. fedtschenkoi* leaves sampled at 00.00 h and therefore represented an mRNA sample containing high levels of PEPc kinase mRNA (see figure 3.1). The mRNA was separated through a non-denaturing agarose gel. Non-denaturing conditions were used because the formaldehyde and formamide in a denaturing gel interfered with the subsequent translation of any RNA recovered from the gel. However, the estimate of the size of PEPc kinase mRNA could be erroneous if the mRNA adopts any secondary structure in the gel.

Significant PEPc kinase activity was detected in the translation products of only a single fraction of the mRNA. This fraction corresponded to the mRNAs having lengths between approximately 0.9 - 1.3 Kb (figure 3.7). It must be noted that this size fraction of the mRNA also gave the greatest incorporation of ^{35}S-Met into TCA-precipitable protein. However, this is not surprising, as the majority of mRNAs would be expected to fall within this size range. The fractions above and below this fraction gave only slightly less incorporation of ^{35}S-Met and yet produced virtually no detectable PEPc kinase activity.

3.3 Discussion

The data presented in this chapter demonstrate that a valid assay has been developed which allows the accurate determination of the level of translatable mRNA for PEPc kinase in isolated RNA samples from plant tissues. The assay clearly differentiates

between samples which possess high levels of kinase mRNA and samples with low levels of kinase mRNA (see figure 3.1). However, it was vital for the subsequent application of the assay that the kinase activity produced by the *in vitro* translation should possess the same properties as the PEPc kinase already characterized from desalted extracts of plant tissues. It was demonstrated that the *in vitro* translated PEPc kinase activity was largely Ca^{2+}-independent, as had been found for the enzyme in plant extracts (figure 3.2)(Bakrim *et al.*, 1992; Carter *et al.*, 1991; Li and Chollet, 1993; Li and Chollet, 1994; Vidal and Chollet, 1997; Wang and Chollet, 1993b). If the *in vitro* translation assay produced a Ca^{2+}-dependent PEPc kinase it would have been impossible to justify any data obtained using the assay as being physiologically relevant with regard to the kinase found *in planta*. However, the data indicates that the *in vitro* translation assay produces a kinase with the same properties as the PEPc kinase activity detected *in planta*. Obviously, the only property tested was the Ca^{2+}-independence of the *in vitro* translated kinase, but this is widely accepted as the most diagnostic feature of the *bona fide* PEPc kinase.

Having established that the assay produces a physiologically relevant PEPc kinase activity it was important to ascertain whether the assay provides a valid estimate of the level of PEPc kinase mRNA. To test the validity of the assay a number of the assay's parameters were varied. Increasing the duration of translation, the volume of translation products and the amount of RNA translated, all caused a linear increase in the degree of phosphorylation of the PEPc in kinase assays on the translation products (figures 3.3, 3.4, and 3.5). Hence, if equal amounts of RNA are translated for the same amount of time and equal volumes of the translation products are assayed for PEPc kinase, the resulting phosphorylation of PEPc will be directly proportional to the amount of PEPc kinase mRNA in each of the RNA samples. This means that RNA samples from leaves treated with any number of experimental conditions can be directly compared to one another with regard to their PEPc kinase mRNA levels determined using this assay.

Another important opportunity raised by the development of a valid assay for PEPc kinase mRNA was the size fractionation of a sample of RNA. This allowed the determination of the length of PEPc kinase mRNA. A sample of mRNA isolated from *B.*

fedtschenkoi leaves collected in the middle of the night was size fractionated using a non-denaturing agarose gel. Only mRNA having a length between 0.9 and 1.3 Kb produced PEPc kinase activity upon *in vitro* translation, indicating that PEPc kinase mRNA falls within this size range. This estimation of the length of PEPc kinase mRNA corresponds well with the predicted length of the mRNA estimated from the size of the partially purified candidate protein. For example, assuming the mean molecular weight of amino acid residue is about 110, the ~32 kDa and ~39 kDa Ca^{2+}-independent PEPc kinase polypeptides reported to be salt-induced in the facultative CAM plant *Mesembryanthemum crystallinum* would have predicted mRNAs of approximately 0.9 and 1.1 Kb respectively (Li and Chollet, 1994). Both of these lengths fall well within the approximate length range estimated in this study by size fractionation of mRNA from *B. fedtschenkoi*.

Another interesting point from the results of the size fractionation of mRNA is that only a single fraction possessed PEPc kinase mRNA. It was possible that, following size fractionation and translation, none of the fractions would produce PEPc kinase activity or that more than one fraction would possess PEPc kinase mRNA. The fact that only one fraction contained PEPc kinase mRNA indicates either that there is only one PEPc kinase and it falls within this length range or that, if there is more than one PEPc kinase mRNA then they all fall within this length range. Also, if the kinase has to be activated by a secondary component, both the kinase and the secondary component must fall within this length range.

Figure 3.1. PEPc kinase translatable mRNA levels are highest during the dark period in *B. fedtschenkoi*. Proteins were separated on 8% SDS-polyacrylamide gels. The figure shows phosphoimages of the dried gels.

(**A**) Total *in vitro* translation products from isolated RNA.

Lane 1, control - 5 µl of translation products from no added RNA.

Lane 2, 5 µl of translation products from 5 µg of total RNA from leaves in the middle of the dark period.

Lane 3, 5 µl of translation products from 5 µg of total RNA from leaves in the middle of the light period.

(**B**) PEPc kinase assays on 5 µl of the translation products shown in (A).

Lane 1, no RNA translation products.

Lane 2, 'dark' RNA translation products.

Lane 3, 'light' RNA translation products.

The major immunoprecipitated ^{32}P-labelled PEPc band is arrowed. The numbers below each lane represent the relative intensities of the PEPc bands.

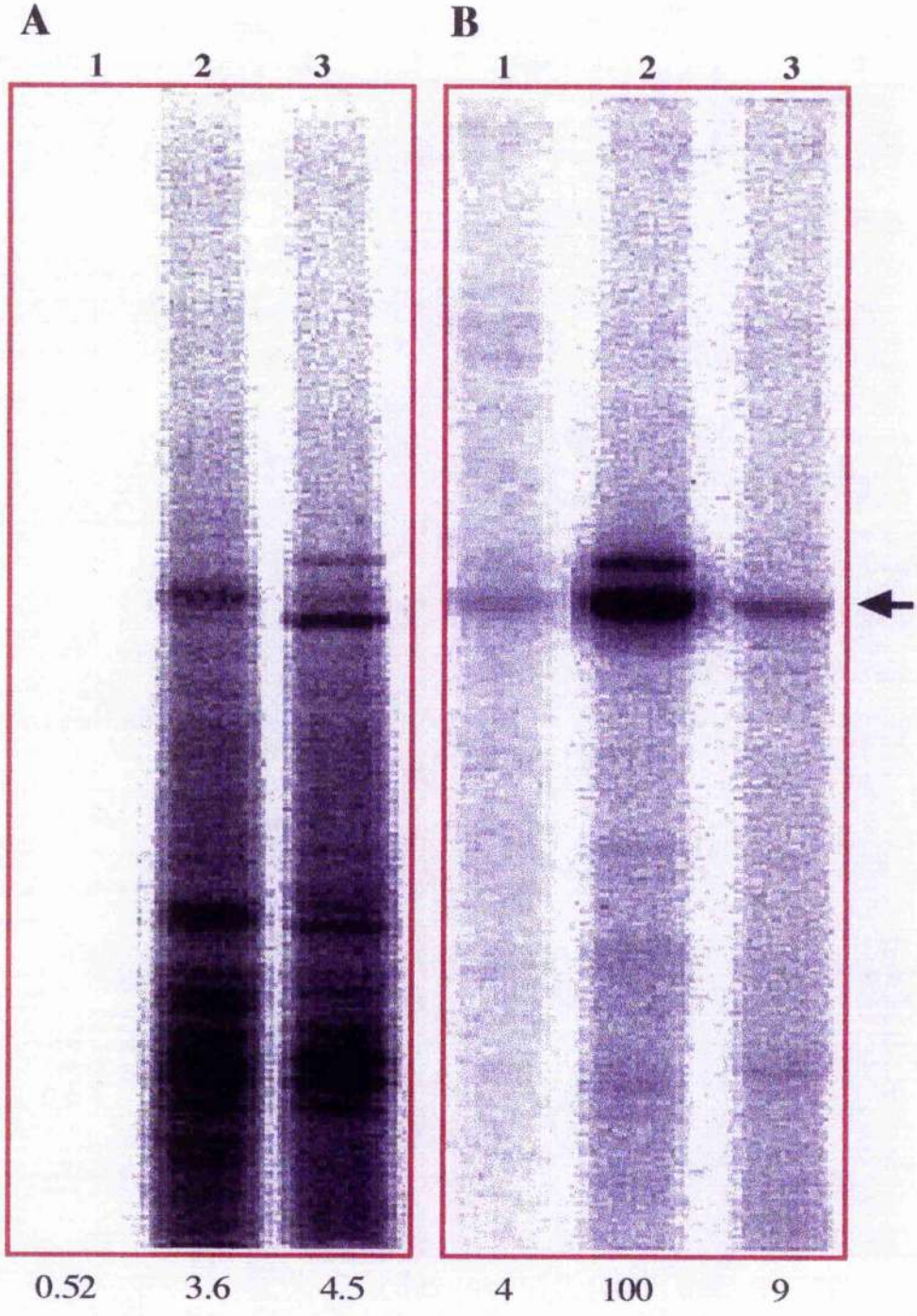

A
B
1
2
3
1
2
3
0.52
3.6
4.5
4
100
9
% incorporation of [35S]Met into TCA precipitable material
Relative intensity of the PEPc doublet

Figure 3.2. The PEPc kinase activity synthesized during the *in vitro* translation of *B. fedtschenkoi* total RNA is Ca^{2+}-independent and the labelling detected in the PEPc kinase assays is not a result of [^{35}S]Met labelling of *de novo* synthesized PEPc in the translation products.

Samples of *B. fedtschenkoi* total RNA from leaves taken at 21.00 h (dark) and 12.00 h (light) were translated *in vitro* and the translation products assayed for PEPc kinase activity in the presence or absence of 0.25 mM EGTA. In addition assays was performed in the absence of ^{32}P-ATP to allow the determination of the amount of [^{35}S]Met labelled PEPc in the translation products.

(A) PEPc kinase assays on *in vitro* translation products.

Lane 1, dark translation products plus EGTA. Lane 2, dark translation products minus EGTA. Lane 3, dark translation products assayed in the absence of ^{32}P-ATP. Lane 4, light translation products plus EGTA. Lane 5, light translation products minus EGTA. Lane 6, light translation products assayed in the absence of ^{32}P-ATP.

The numbers below each lane represent the relative intensities of the PEPc bands.

(B) Total *in vitro* translation products.

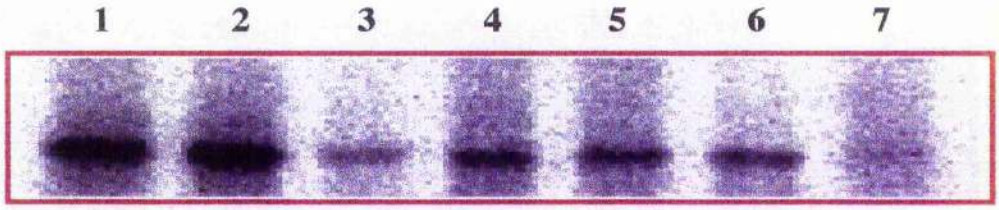
Lane 1, total *B. fedtschenkoi* RNA isolated from leaves sampled at 21.00 h (dark).

Lane 2, total *B. fedtschenkoi* RNA isolated from leaves sampled at 12.00 h (light).

Lane 3, control no RNA.

The numbers below each lane represent the percentage incorporation of ^{35}S-Met into TCA precipitable material by each RNA sample.

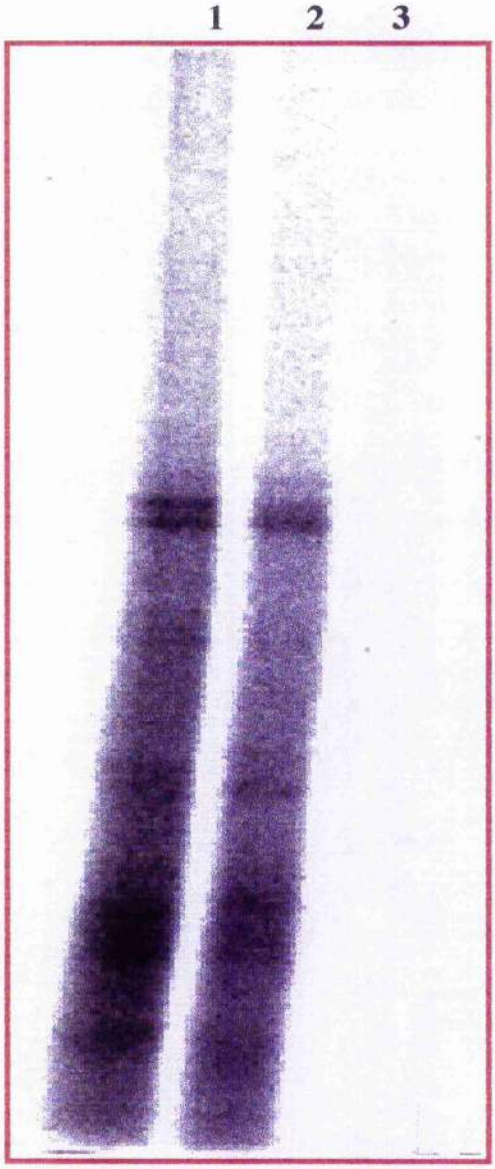
A



96 100 10 11 12 11 0

Relative intensity

B



% incorporation of [^{35}S]Met into TCA precipitable material

2.3 2.4 0.22

Figure 3.3. Increasing the volume of translation products increases the signal from the translatable mRNA assay.

Different volumes of translation products from *B. fedtschenkoi* 'dark' total RNA were mixed with translation products from a "no RNA" translation to give a fixed volume of 5 µl of translation products in each assay. Values represent the relative ^{32}P-labelling of the exogenous PEPc on a phosphoimage of the dried SDS-gel and are corrected for background labelling of the PEPc.

(**A**) Phosphoimage of the gel of the kinase assays. The volume of translation products assayed for PEPc kinase activity is given above each lane.

(**B**) Graphical representation of the relative ^{32}P-labelling of the PEPc doublet in the kinase assays shown in (A).

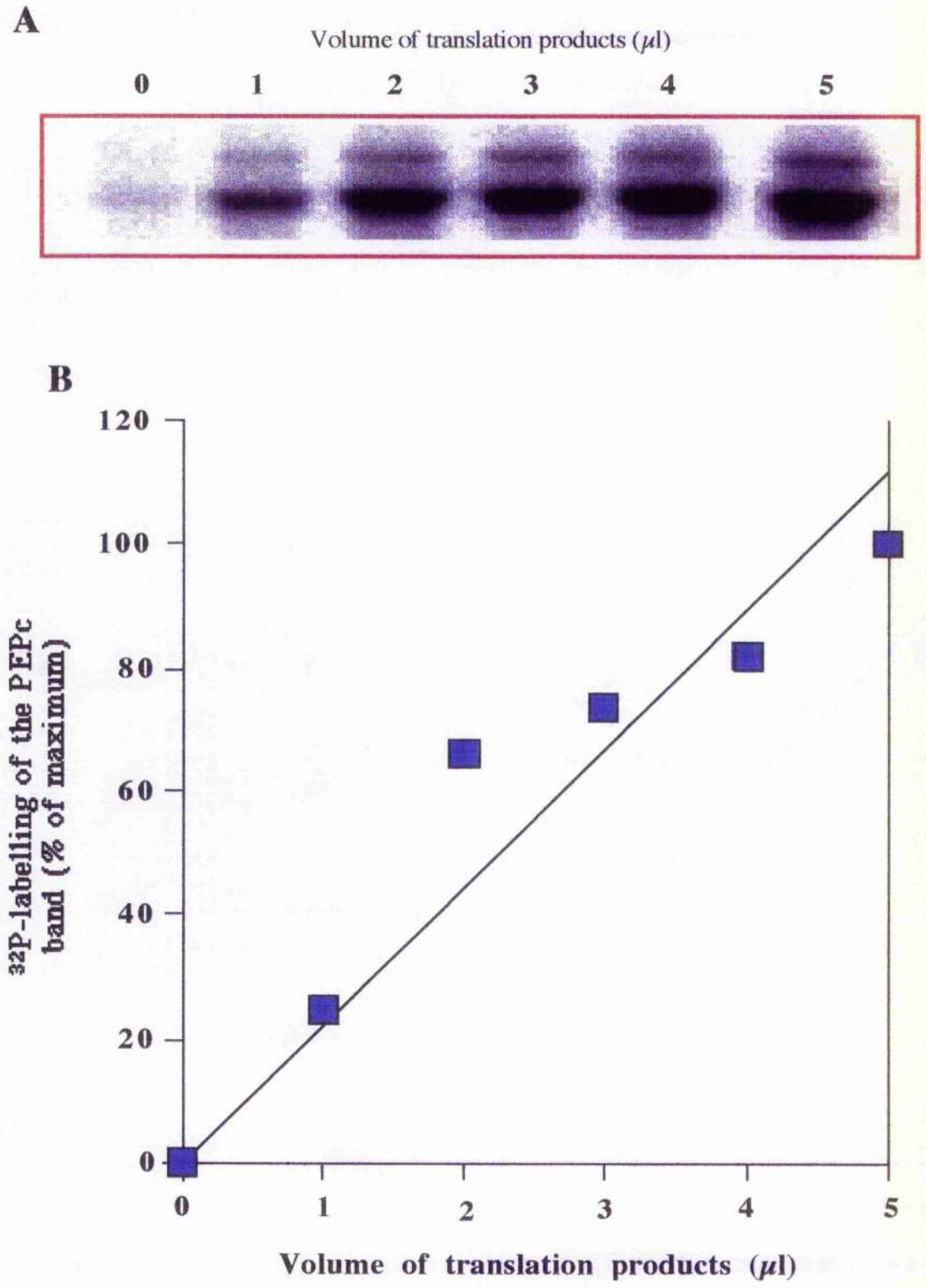

A
Volume of translation products (μl)
0
1
2
3
4
5
B
120
100
80
60
40
20
0
32P-labelling of the PEPc band (% of maximum)
0
1
2
3
4
5
Volume of translation products (μl)

Figure 3.4. The response of the translatable mRNA assay to increasing amounts of RNA.

Different amounts of *B. fedtschenkoi* total RNA (isolated in the middle of the dark period) were translated for 45 min and assayed for PEPc kinase activity.

(**A**) Phosphoimage showing the PEPc kinase activity in translation products from 0 to 10 μg of total RNA. The amount of RNA translated is given above each lane.

(**B**) Graphical representation of the amount of total protein synthesized (□) and the amount of kinase activity in the translation products (○), corrected for the values obtained in a control with no added RNA, and expressed as a percentage of the values obtained with 10 μg RNA. The incorporation of [^{35}S]Met into trichloroacetic acid-precipitable protein represented 0.2 and 3 % of the total [^{35}S]Met with 0 and 10 μg RNA, respectively.

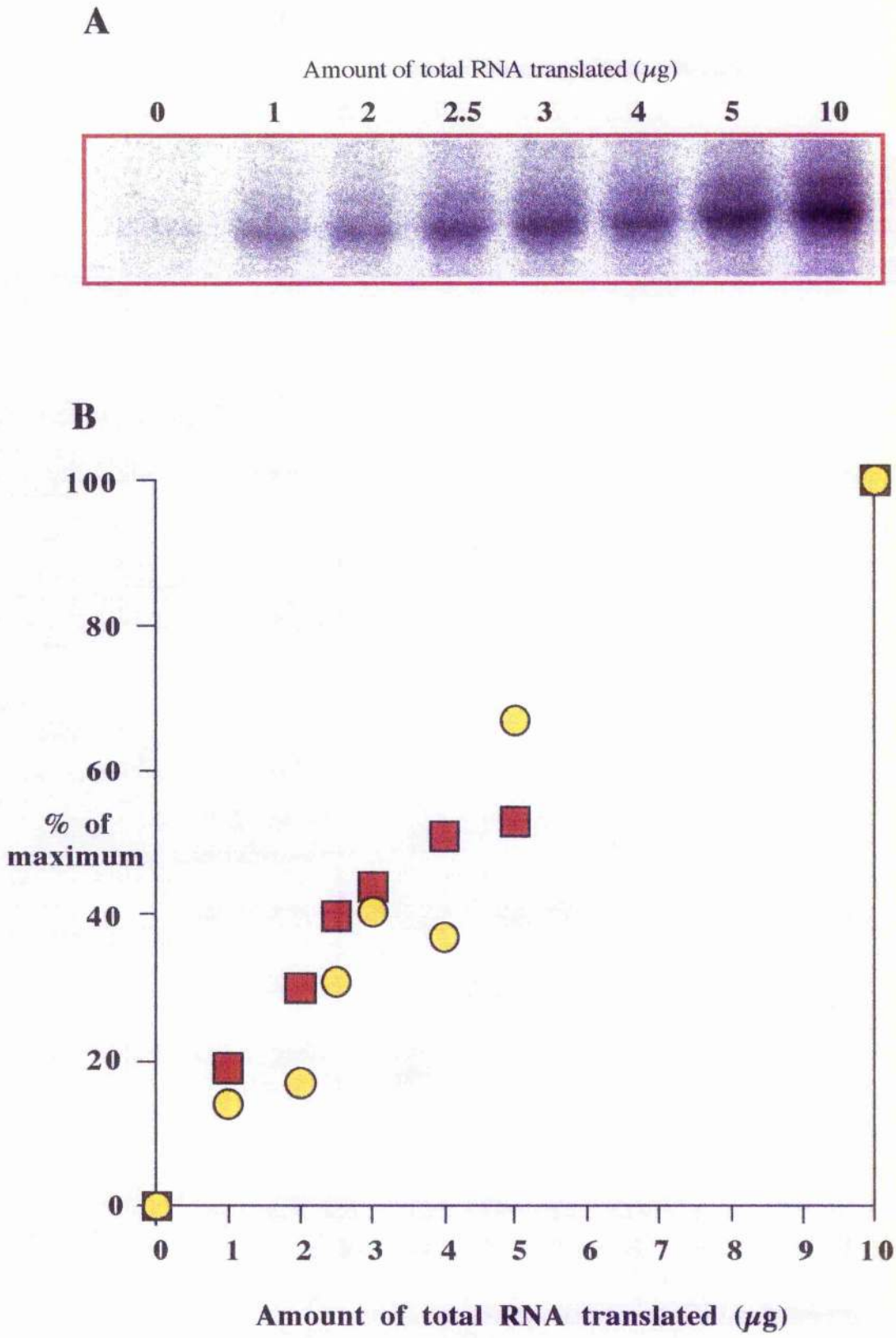

A
Amount of total RNA translated (μg)
0
1
2
2.5
3
4
5
10
B
100
80
60
% of maximum
40
20
0
0
1
2
3
4
5
6
7
8
9
10
Amount of total RNA translated (μg)

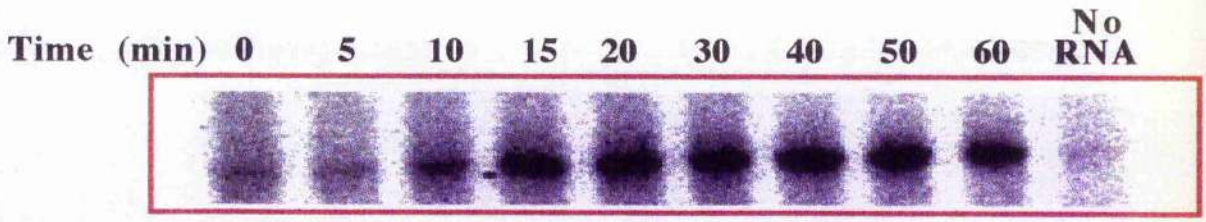
Figure 3.5 The appearance of PEPc kinase activity with time during an *in vitro* translation.

A large scale translation was set up using 5 µg of *B. fedtschenkoi* 'dark' total RNA and 5 µl aliquots were removed at the times indicated and assayed for PEPc kinase activity.

(**A**) Phosphoimage of the kinase assays with the time indicated above each lane.

(**B**) Graphical representation of the relative ^{32}P-labelling of the PEPc bands shown in (A).

A


Time (min) 0 5 10 15 20 30 40 50 60 No RNA

B

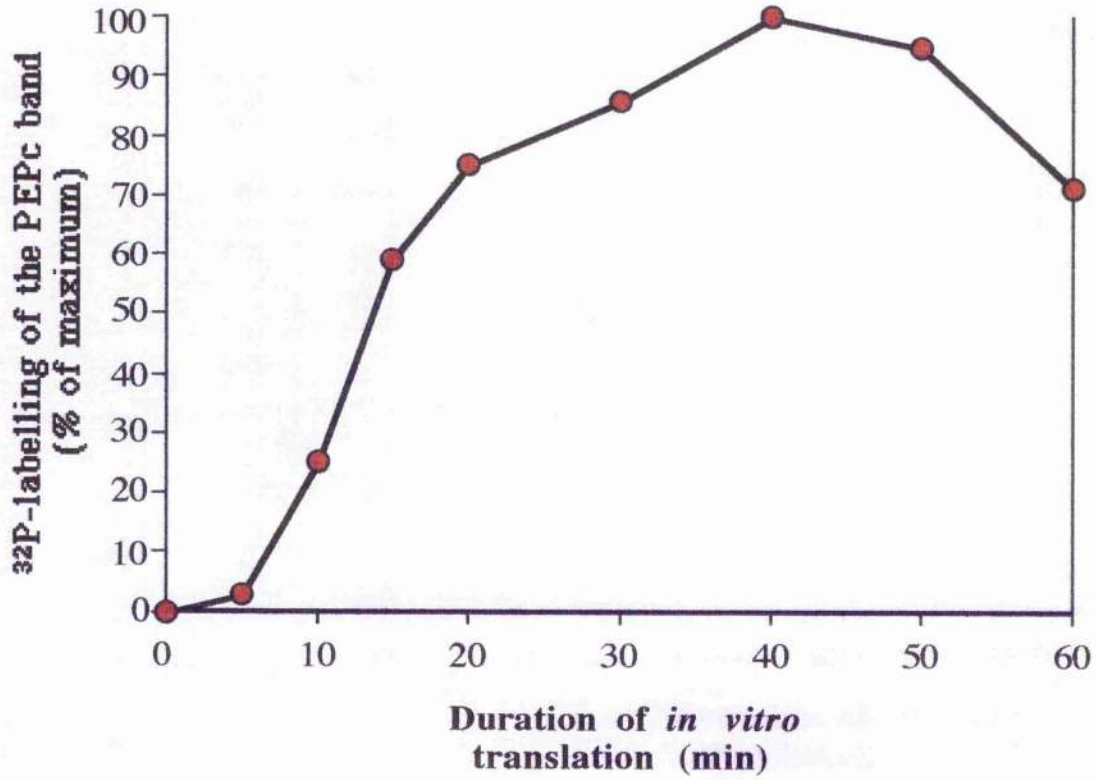

32P-labelling of the PEPc band (% of maximum)
100
90
80
70
60
50
40
30
20
10
0
0 10 20 30 40 50 60
Duration of in vitro translation (min)

Figure 3.6 The relative PEPc kinase activity in the translation products from different *B. fedtschenkoi* total RNA samples remains constant irrespective of whether translations are incubated for 60 or 20 min.

B. fedtschenkoi total RNA from three different time points in the diurnal cycle was translated for either 60 or 20 min and the translation products assayed for PEPc kinase activity. Phosphoimages are shown of the dried SDS-gels. The relative intensity of the PEPc doublet is given below each lane.

(**A**) Total RNA from 12.00, 21.00 and 24.00 h was translated for 60 min and then 5 µl of each translation was assayed for PEPc kinase activity.

Lane 1, 12.00 h total RNA.

Lane 2, 21.00 h total RNA.

Lane 3, 24.00 h total RNA.

Lane 4, no RNA control.

(**B**) Total RNA from 12.00, 21.00 and 24.00 h was translated for 20 min and then 5 µl of each translation was assayed for PEPc kinase activity.

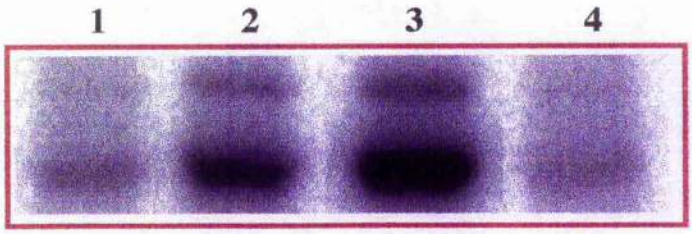
Lane 1, 12.00 h total RNA.

Lane 2, 21.00 h total RNA.

Lane 3, 24.00 h total RNA.

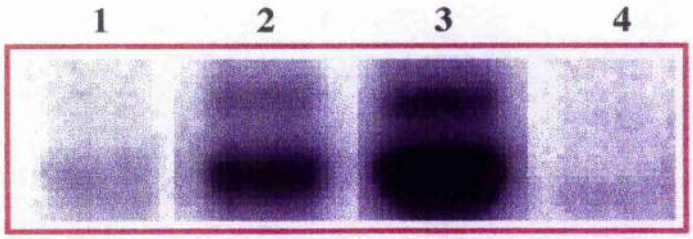
Lane 4, no RNA control.

A



Relative intensity 1 42 100 0

B



Relative intensity 1 42 100 0

Figure 3.7 Size fractionation of *B. fedtschenkoi* 'dark' mRNA yields a single fraction possessing PEPc kinase translatable mRNA.

10 μg of *B. fedtschenkoi* 'dark' poly $(A)^+$ RNA was size fractionated using a non-denaturing agarose gel at 4°C. The mRNA containing portion of the gel was 4 cm long and covered mRNAs from 0.3 to 3.1 Kb in length. This portion of the gel was divided into 5 mm segments and the RNA was recovered from each segment using the Scotlab Nucleon Easi RNA kit.

(**A**) Kinase activity in translation products from mRNA size fractions between 0.3 and 0.9 Kb.

Lane 1, total mRNA control; lane 2, 0.3-0.4 Kb mRNAs; lane 3, 0.4-0.5 Kb mRNAs; lane 4, 0.5-0.6 Kb mRNAs; lane 5, 0.6-0.8 Kb mRNAs; lane 6, no RNA control.

(**B**) Kinase activity in translation products from mRNA size fractions between 0.9 and 3 Kb.

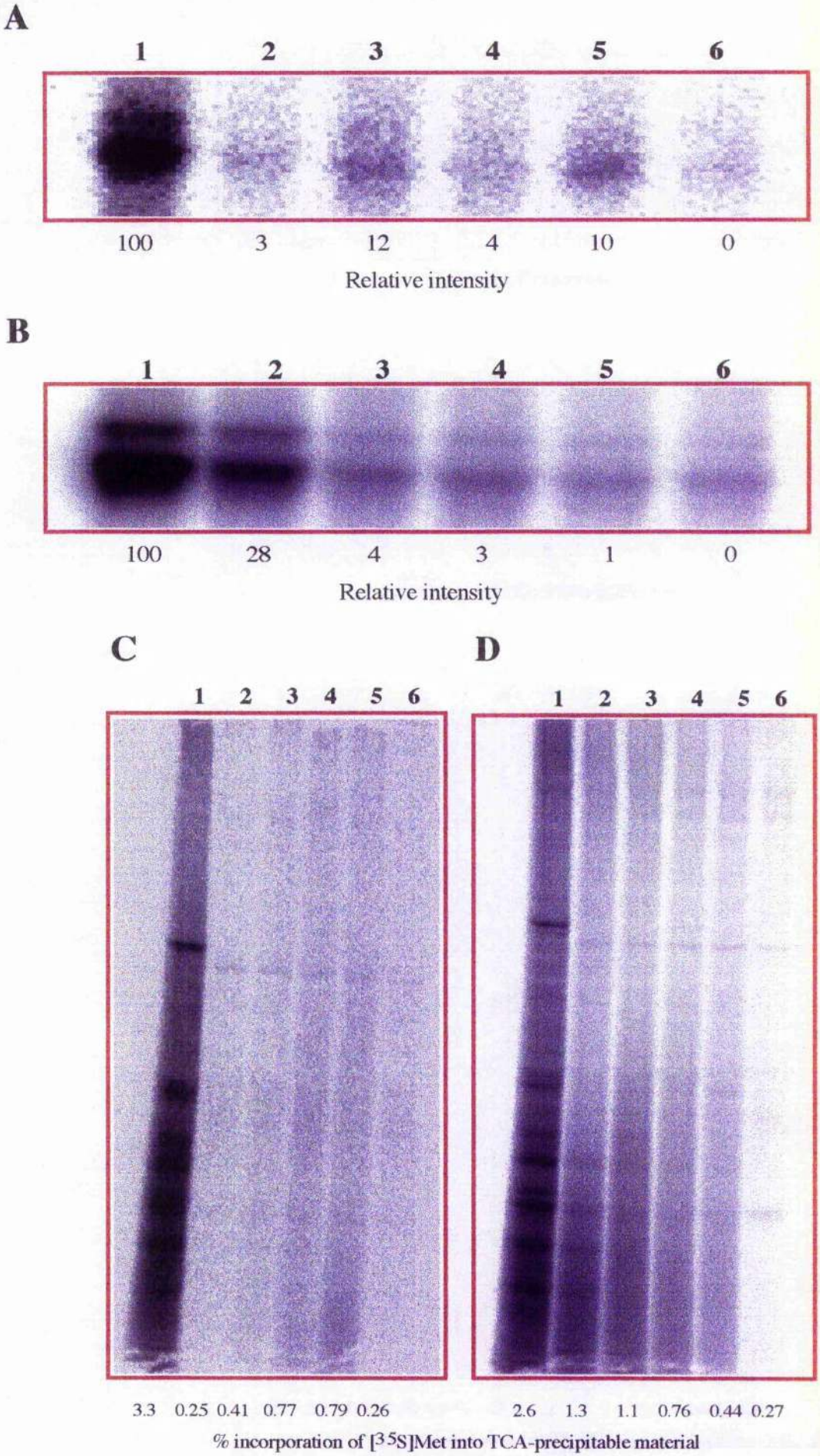
Lane 1, total mRNA control; lane 2, 0.8-1.4 Kb mRNAs; lane 3, 1.4-1.9 Kb mRNAs; lane 4, 1.9-2.5 Kb mRNAs; lane 5, 2.5-3.1 Kb mRNAs; lane 6, no RNA control.

For panels (A) and (B) the relative intensity of the PEPc bands is given below each lane.

(**C**) Translation products from mRNA size fractions between 0.3 and 0.9 Kb. The lanes correspond to those in panel (A).

(**D**) Translation products from mRNA size fractions between 0.9 and 3.1 Kb. The lanes correspond to those in panel (B).

For panels (C) and (D) the percentage incorporation of [^{35}S]Met into TCA precipitable material is given below each lane.


A
1 2 3 4 5 6
100 3 12 4 10 0
Relative intensity
B
1 2 3 4 5 6
100 28 4 3 1 0
Relative intensity
C
D
1 2 3 4 5 6
1 2 3 4 5 6
3.3 0.25 0.41 0.77 0.79 0.26
2.6 1.3 1.1 0.76 0.44 0.27
% incorporation of [35S]Met into TCA-precipitable material

Chapter 4

The regulation of PEPc kinase mRNA in *Bryophyllum (Kalanchoë) fedtschenkoi*

4.1 Introduction

Bryophyllum (Kalanchoë) fedtschenkoi Hamet et Perrier is a Crassulacean acid metabolism (CAM) plant. In the characteristic diurnal pattern of CAM, flux through PEPc at night leads to the formation of malic acid, which is stored in the vacuole. During the following day, malate released from the vacuole is decarboxylated, and the resulting CO_2 is fixed in the Calvin cycle (Osmond and Holtum, 1981). Thus, in order to avoid the futile cycling of CO_2 through PEPc, CAM plants must have a mechanism which allows flux through PEPc at night and reduces or eliminates this flux during the day. CAM leaf PEPc was found to be significantly more sensitive to inhibition by malate during the day than at night (Winter, 1982). The molecular mechanism responsible for this change in the malate sensitivity of PEPc was found to be protein phosphorylation (Nimmo *et al.*, 1984; Nimmo *et al.*, 1986). In the CAM leaf, PEPc is phosphorylated at night and less sensitive to malate. During the day, PEPc is dephosphorylated and more sensitive to malate inhibition (Nimmo *et al.*, 1986). It was established that the nocturnal increase in the phosphorylation state of PEPc is mediated through an increase in the activity of PEPc kinase whilst PEPc phosphatase activity remains relatively constant throughout the day and night (Carter *et al.*, 1990; Carter *et al.*, 1991).

B. fedtschenkoi exhibits circadian rhythms of CO_2 metabolism that arise through circadian control of the flux through PEPc (Wilkins, 1992). This circadian control of flux through PEPc was found to be largely due to circadian rhythms in the activity of PEPc kinase, which cause concomitant rhythms in the phosphorylation state and the L-malate sensitivity of PEPc (Carter *et al.*, 1991). It was found that the circadian increase in PEPc kinase activity requires protein and RNA synthesis (Carter *et al.*, 1996; Carter *et al.*, 1991). This could represent evidence that the circadian clock itself requires protein and RNA synthesis to function or that either PEPc kinase itself or a secondary component must

undergo *de novo* synthesis to achieve the observed circadian increase in PEPc kinase activity.

The signal transduction cascade which regulates PEPc kinase activity in *B. fedtschenkoi* is poorly understood. In contrast, a number of the events involved in the light-mediated increase in PEPc kinase activity in the leaves of C_4 plants have been determined. These events include photosynthesis in the bundle sheath cells, alkalinisation of the mesophyll cytosol, an efflux of vacuolar Ca^{2+} into the mesophyll cytosol, an unknown Ca^{2+}/calmodulin-dependent protein kinase and protein synthesis (Giglioli-Guivarc'h *et al.*, 1996; Vidal and Chollet, 1997). For *B. fedtschenkoi*, protein synthesis and RNA synthesis are required. It has been reported that, in a C_4 plant, RNA synthesis is not required, but this is discussed further in chapter 6.

In the present study the novel method described in the previous chapter was used to examine the regulation of the level of PEPc kinase translatable mRNA in leaves of *B. fedtschenkoi*. This method overcomes the fact that there are no antibodies or cDNA clones available for PEPc kinase by allowing a study of the regulation of this kinase at the molecular level. Experiments were performed to determine whether diurnal and circadian changes in kinase activity and the malate sensitivity of PEPc are accompanied by changes in kinase mRNA. The involvement of protein and RNA synthesis in the circadian induction of PEPc kinase was reassessed by measuring kinase mRNA levels. Furthermore, the signalling cascade which mediates changes in the level of kinase mRNA was dissected using both a pharmacological approach and experimental manipulations of the environment. All these experiments were performed with a view to deciphering precisely how PEPc kinase is regulated in *B. fedtschenkoi*.

4.2 Results

4.2.1 PEPc kinase translatable mRNA fluctuates in concert with PEPc kinase activity and the malate sensitivity of PEPc in *B. fedtschenkoi*

Initial experiments, in which it was established that PEPc kinase mRNA is significantly higher during the night than in the day in *B. fedtschenkoi* leaves, have already been described (see chapter 3, figure 3.1). PEPc kinase mRNA was readily detectable in an

RNA sample from leaves in the middle of the night, but there was no PEPc kinase mRNA present during the day (see figures 3.1 and 3.2). It was therefore important to examine how PEPc kinase mRNA levels fluctuate during the normal diurnal cycle.

Pairs of leaves were sampled from *B. fedtschenkoi* plants at regular intervals throughout a 24 h period. One leaf was used to isolate RNA, which allowed the subsequent analysis of the level of PEPc kinase mRNA, and the second leaf was used to make a rapidly desalted extract so that the malate sensitivity of PEPc and the PEPc kinase activity could be determined. Figure 4.1 shows how the levels of PEPc kinase translatable mRNA and PEPc kinase activity fluctuate throughout the normal diurnal cycle in *B. fedtschenkoi.* The apparent K_i of PEPc for malate, which reflects the phosphorylation state of PEPc (Carter *et al.*, 1991), was measured in the same extracts that were assayed for PEPc kinase activity. The level of PEPc kinase mRNA reaches its peaks and troughs before those in both the kinase activity and the apparent K_i of PEPc for malate. This is most clearly seen in the decrease in the level of PEPc kinase translatable mRNA between 24.00 h and 03.00 h, some 5 to 8 h before the onset of light. The level of PEPc kinase activity begins to decrease between 03.00 h and 05.00 h, 3 to 5 h before the onset of light. The level of translatable PEPc kinase mRNA peaks in the middle of the dark period (24.00 h) whilst the level of the kinase protein and the apparent K_i for malate of PEPc peak at 03.00 h. Neither kinase activity nor translatable mRNA increase within the first hour of the dark period; the major changes occur 3-5 h after the start of the dark period.

4.2.2 Circadian control of PEPc kinase translatable mRNA in *B. fedtschenkoi*

The discovery that the level of PEPc kinase mRNA increases and decreases entirely within the dark period of the diurnal cycle (figure 4.1) indicated that it may be regulated by a circadian clock. To investigate this possibility further, detached *Bryophyllum* leaves were placed in constant conditions (darkness and CO_2-free air at 15°C) for 48 h. RNA samples were prepared from leaves at intervals during this period. Figure 4.2 shows the level of PEPc kinase translatable mRNA at four time points throughout the 48 h period. As can be seen, the amount of PEPc kinase translatable mRNA did indeed oscillate in constant conditions, confirming that its level is controlled by a circadian clock. However, the

magnitude of the oscillations in the level of kinase mRNA diminishes rapidly within 48 h whilst the circadian rhythm of CO_2-output in leaves subjected to the same constant conditions persists for about four days (see Introduction). The possible reasons for this difference will be explored in the discussion.

4.2.3 Perturbation of the circadian decrease in PEPc kinase translatable mRNA by temperature

Temperature has been shown to influence the disappearance of PEPc kinase activity that normally occurs towards the end of the dark period in *B. fedtschenkoi* (Carter *et al.*, 1995a). These effects were re-examined at the translatable mRNA level (Figure 4.3). Treatments began at midnight, 8 h before the end of the dark period. At 4°C PEPc kinase activity and translatable mRNA are stabilised over 6 h whilst at 15°C both disappear over this period. At 30°C both the kinase activity and translatable mRNA disappear rapidly within 2 h whilst after 2 h at 15°C both still remain. This suggests that a temperature of 4°C causes PEPc kinase mRNA and hence PEPc kinase activity levels to be stabilised. PEPc remains phosphorylated and its apparent K_i for malate stays high. At 30°C the PEPc kinase mRNA and hence activity disappear rapidly and PEPc becomes dephosphorylated giving a concomitant decrease in its apparent K_i for malate. Hence, the circadian control of PEPc kinase mRNA can be affected by temperature changes in such a way that the mRNA will persist at 4°C and disappear rapidly at 30°C. This is consistent with observations on the effect of temperature on the rhythms in CO_2 metabolism and PEPc kinase activity in *B. fedtschenkoi* (Carter *et al.*, 1995a; Carter *et al.*, 1995b). It has been suggested that the stabilisation of PEPc kinase activity and the phosphorylation state of PEPc at 4°C may contribute to the ability of CAM plants to fix CO_2 during cold nights (Carter *et al.*, 1995a).

4.2.4 The circadian increase in PEPc kinase translatable mRNA requires both protein and RNA synthesis

The circadian increase in PEPc kinase activity can be disrupted by the prior supply of protein and RNA synthesis inhibitors to detached leaves (Carter *et al.*, 1996; Carter *et al.*, 1991; Nimmo, 1993). The effects of these treatments were examined at the translatable mRNA level and the results for PEPc kinase mRNA levels with two types of both classes

of inhibitor are shown in Figure 4.4. The results demonstrate that virtually no PEPc kinase mRNA appears in leaves supplied with either type of inhibitor, whilst in the control leaves supplied with distilled water (or the solvent the inhibitor was dissolved in) kinase mRNA appears. Similar results were obtained for PEPc kinase activity in duplicate leaves supplied with the same inhibitor solutions (figure 4.4).

4.2.5 Some signalling elements involved in the circadian increase in PEPc kinase translatable mRNA

Having established that the circadian increase in PEPc kinase mRNA requires both protein and RNA synthesis, it was important to see if any further steps in the signal transduction cascade could be elucidated. A range of inhibitors of specific signal transduction events were supplied to detached *B. fedtschenkoi* leaves at the beginning of the light period and samples were collected in the middle of the subsequent dark period. Duplicate leaves were treated with each inhibitor, one for RNA isolation and the other for the determination of the malate sensitivity of PEPc and the PEPc kinase activity. The effect of a calcium channel blocker (Bay K8644), a protein kinase inhibitor (staurosporin), a Ca^{2+}/calmodulin antagonist (W7) and a protein phosphatase inhibitor (cantharidin) on the nocturnal decrease in the L-malate sensitivity of PEPc is shown in figure 4.5. Staurosporin and Bay K8644 have no effect on the change in the L-malate sensitivity of PEPc, whilst cantharidin almost completely inhibits the change in the L-malate sensitivity of PEPc. Cycloheximide was included as a positive control because it had already been shown to block the change in the L-malate sensitivity of PEPc. Cantharidin is almost as effective as cycloheximide at preventing the circadian decrease in the malate sensitivity of PEPc. W7 seems to cause a slight inhibition of the decrease in the L-malate sensitivity of PEPc when used at 500 μM. W7 also caused a slight inhibition of the circadian increase in PEPc kinase translatable mRNA (figure 4.6). Cantharidin caused the complete abolition of the increase in PEPc kinase mRNA, whilst staurosporin and Bay K8644 had no detectable effect at the concentrations used (figure 4.6). When W7 was supplied to detached *B. fedtschenkoi* leaves at 1 mM, it caused significant inhibition of the change in the L-malate sensitivity of PEPc and the increase in the level of PEPc kinase mRNA and activity (figure

Cycloheximide was again included as a positive control, and W7 was not as effective as cycloheximide at blocking the changes in the three parameters measured. This may be due to poor uptake of W7 by detached *B. fedtschenkoi* leaves as suggested by the need for such a high concentration to achieve significant inhibition.

4.3 Discussion

Previous work had established that PEPc kinase activity is regulated by a circadian clock in *B. fedtschenkoi* and that circadian oscillations in PEPc kinase activity require protein and RNA synthesis (Carter *et al.*, 1996; Carter *et al.*, 1991; Nimmo, 1993). However, this earlier work did not reveal whether the component which must be synthesized for PEPc kinase activity to appear is the kinase protein itself or another protein that activates the kinase. Attempts to solve this problem have been impaired because PEPc kinase is an extremely low abundance enzyme, making it a very difficult protein to purify to homogeneity. Thus, there are no antibodies or cDNA clones available that are specific to PEPc kinase.

This shortfall was circumvented in the present work by using a novel assay for PEPc kinase mRNA (see chapter 3). The data presented in this chapter demonstrate that PEPc kinase is regulated at the translatable mRNA level in the CAM plant *B. fedtschenkoi*. There is an excellent correlation between the amount of PEPc kinase activity generated in the *in vitro* translation products, the amount of PEPc kinase activity in *B. fedtschenkoi* leaf extracts and the phosphorylation state of PEPc, as judged by its apparent K_i for malate (see figure 4.1). This correlation holds not only during the diurnal cycle in *B. fedtschenkoi*, but also during temperature and inhibitor treatments that affect the phosphorylation state of PEPc and the fixation of CO_2 (Carter *et al.*, 1991; Carter *et al.*, 1995a).

The oscillations in the level of PEPc kinase mRNA under constant conditions of 15 °C, CO_2-free air and constant darkness (figure 4.2) are evidence that kinase mRNA levels are controlled by the circadian clock. However, the magnitude of these oscillations rapidly damps out when compared to the oscillations in CO_2 output under the same constant conditions (cf. figure 1.6). This may represent evidence that the circadian rhythm of CO_2

output is not only controlled by oscillations in PEPc kinase mRNA and activity, but also by oscillations in the cytosolic malate concentration as proposed by Wilkins (1984).

The nocturnal increase in PEPc kinase mRNA in *B. fedtschenkoi* was blocked by two protein synthesis inhibitors and two RNA synthesis inhibitors, each having different modes of action. Cycloheximide inhibits the peptidyl transferase activity of the 60 S ribosomal subunit whilst puromycin causes premature chain termination by acting as an analogue of aminoacyl-tRNA. Actinomycin D intercalates specifically into double-helical DNA and prevents it from being an effective template for RNA synthesis. Cordycepin prevents the completion of RNA synthesis by inhibiting the poly A polymerase responsible for the 3'-polyadenylation of eukaryotic mRNAs. It is particularly interesting that actinomycin D blocked the increase in PEPc kinase mRNA in *B. fedtschenkoi* because it acts directly on the DNA. Hence, its effect indicates that transcription and translation must occur in their entirety from the DNA level through to the completed polypeptide chain. It is possible that the transcription step is part of the operation of the circadian clock, or the transcription of the PEPc kinase gene itself, or both. Assessment of exactly where transcription occurs in the signalling pathway must await the cloning and expression analysis of the PEPc kinase gene.

The circadian appearance of PEPc kinase activity in *B. fedtschenkoi* requires protein synthesis (Carter *et al.*, 1991). The data presented here extend that observation by showing that protein synthesis inhibitors block the appearance of PEPc kinase translatable mRNA. Since the level of PEPc kinase translatable mRNA oscillates both in the normal diurnal cycle and in constant conditions, this implies that at least two separate protein synthesis steps are required for the appearance of the kinase. One is in the chain of events leading to appearance of kinase translatable mRNA, for example the operation of the circadian clock itself. The other is the translation of PEPc kinase mRNA into protein.

PEPc kinase activity declines rapidly some 3 h after PEPc kinase translatable mRNA declines in *B. fedtschenkoi* (figure 4.1). This indicates that kinase protein is turned over rapidly. Carter *et. al.* (1995a) showed that the circadian disappearance of PEPc kinase activity is delayed by low temperature and accelerated by high temperature. This could

reflect control of either synthesis or destruction of the kinase. The present results show that these temperature changes alter the amounts of translatable kinase mRNA and kinase activity in parallel. Hence, there is no direct evidence that the rate of destruction of PEPc kinase is itself regulated, though any such effects cannot be ruled out at present.

The circadian increase in PEPc kinase mRNA was found to require protein phosphatase activity and a Ca^{2+}/calmodulin interaction through the use of specific inhibitors. However, a calcium channel blocker and a protein kinase inhibitor had no detectable effect at the concentrations applied. Precisely where the protein phosphatase activity and Ca^{2+}/calmodulin interaction are required in the signal transduction cascade is unclear from this data. Both events could be involved in the functioning of the circadian clock itself and/or the signal pathway between the clock and PEPc kinase mRNA. For example, one possible explanation of their effect would be that the protein phosphatase dephosphorylates a calcium-dependent protein kinase (CDPK), and activates it, and then the activated CDPK phosphorylates a transcription factor which binds to the PEPc kinase promoter and mediates transcription of the gene. Obviously this is only one of many explanations which could be proposed for the effects of these inhibitors. The exact sequence of events will only be elucidated by cloning the individual genes involved.

Figure 4.1. Diurnal regulation of the apparent K_i of PEPc for L-malate, PEPc kinase activity and translatable PEPc kinase mRNA in leaves of *B. fedtschenkoi*.

The photoperiod was 08.00 h to 16.00 h. Samples for PEPc and PEPc kinase assays, and RNA isolation were taken simultaneously. Kinase activity and translatable mRNA are expressed as a percentage of the maximum reached during the 24 h period. Δ, Apparent K_i of PEPc for L-malate; O, PEPc kinase activity; ◇, PEPc kinase translatable mRNA.

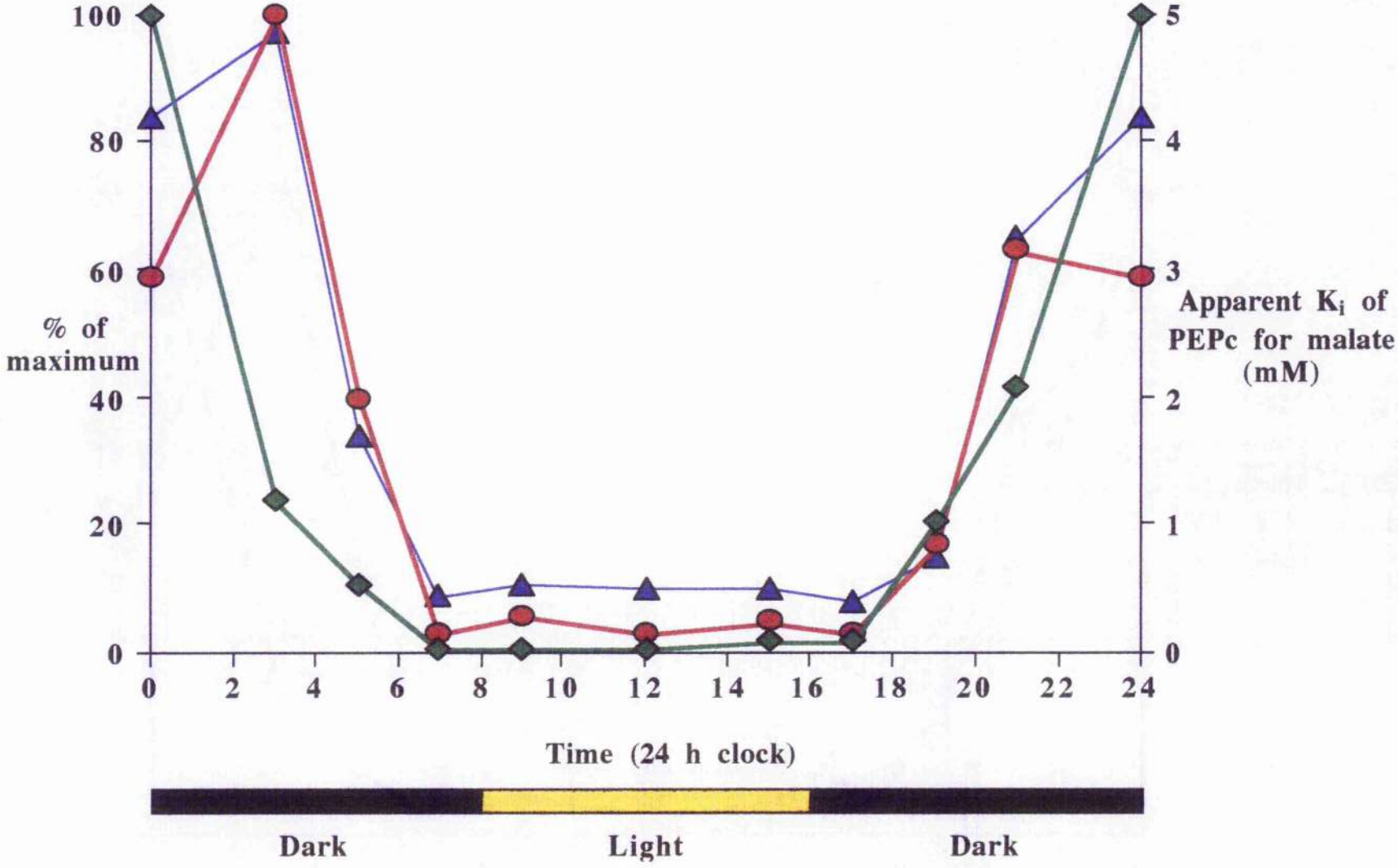

100
80
60
40
20
0
% of maximum
5
4
3
2
1
0
Apparent K_i of PEPc for malate (mM)
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (24 h clock)
Dark
Light
Dark

Figure 4.2. Circadian regulation of PEPc kinase translatable mRNA levels in *B. fedtschenkoi*.

Leaves were detached at 16.00 h and placed in constant darkness and CO_2-free air at 15°C. Samples were collected at intervals throughout the following 48 h. PEPc kinase translatable mRNA was assayed as described in Materials and Methods and quantified by phosphoimaging the dried SDS-gel.

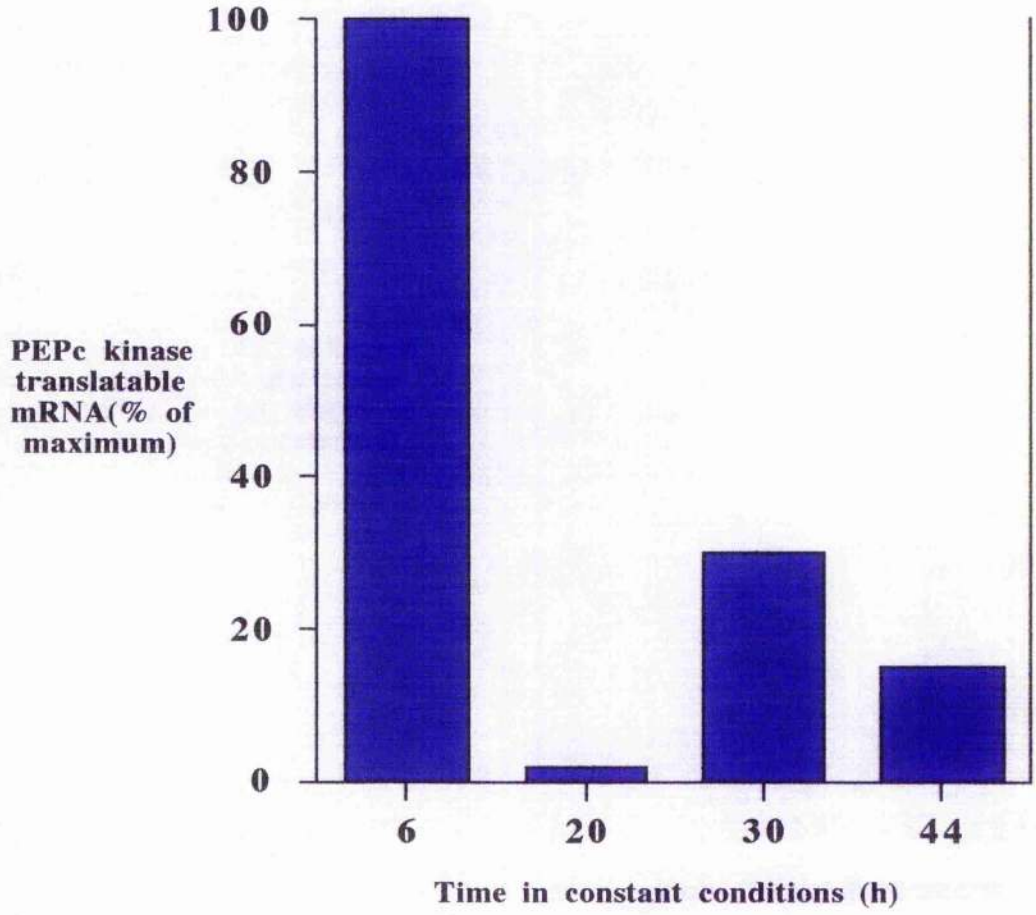

100
80
60
40
20
0
PEPc kinase translatable mRNA(% of maximum)
6
20
30
44
Time in constant conditions (h)

Figure 4.3. The influence of temperature on the disappearance of PEPc kinase in detached leaves of *B. fedtschenkoi*.

Leaves were detached in the middle of the 16 h dark period and placed in constant darkness and normal air at 4°C, 30°C or 15°C (control). Leaves were sampled 2 h later for the 30°C treatment and 6 h later for the 4°C treatment; a control leaf kept at 15°C was taken at both time points. The figure shows phosphoimages of the ^{32}P-labelled PEPc bands following gel electrophoresis. The relative intensity of each PEPc doublet on the phosphoimages is shown below each track.

(**A**) Desalted extracts were assayed for PEPc kinase activity and the apparent K_i for malate of the PEPc in the extracts was measured.

Lane 1, 30°C treatment for 2 h.

Lane 2, 15°C treatment for 2 h.

Lane 3, 4°C treatment for 6 h.

Lane 4, 15°C treatment for 6 h.

(**B**) *In vitro* translation products from isolated total RNA were assayed for PEPc kinase activity.

Lane 1, control, no RNA.

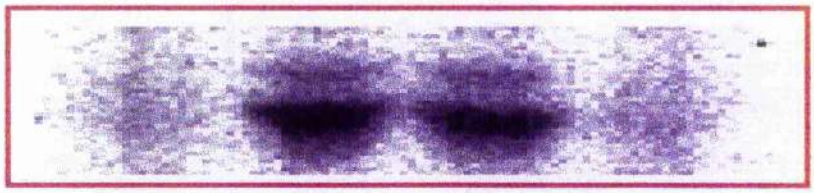
Lane 2, 30°C treatment for 2 h.

Lane 3, 15°C treatment for 2 h.

Lane 4, 4°C treatment for 6 h.

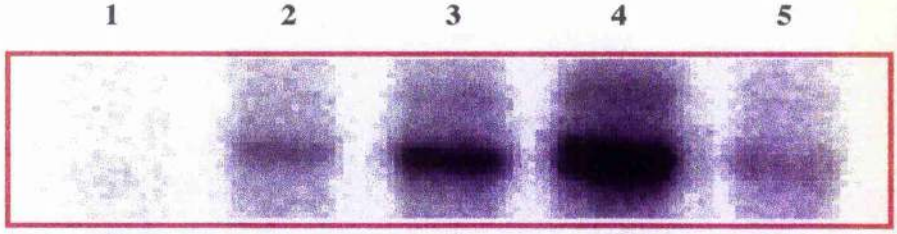
Lane 5, 15°C treatment for 6 h.

A



	1	2	3	4
Relative intensity	17	100	88	16
Apparent K_i (mM)	0.6	5.5	5.2	0.7

B



	1	2	3	4	5
Relative intensity	0	9	69	100	27

Fig. 4.4. Effects of protein and RNA synthesis inhibitors on the appearance of PEPc kinase translatable mRNA and activity in detached leaves of *B. fedtschenkoi.*

Leaves were detached and placed with their petioles in inhibitor solution at the beginning of the 8 h photoperiod. In the middle of the subsequent dark period, desalted extracts and total RNA were prepared from separate leaves. The figure shows phosphoimages of the ^{32}P-labelled PEPc bands following gel electrophoresis.

(**A**) Desalted extracts were assayed for PEPc kinase activity and the apparent K_i for malate of the PEPc in the extracts was also measured.

Lane 1, control leaf placed in distilled water.

Lane 2, leaf placed in 1 mM cordycepin.

Lane 3, leaf placed in 1 mM puromycin.

(**B**) PEPc kinase activity in *in vitro* translation products.

Lane 1, control, no RNA.

Lane 2, RNA from a leaf placed in distilled water.

Lane 3, RNA from a leaf placed in 1 mM cordycepin.

Lane 4, RNA from a leaf placed in 1 mM puromycin.

Lane 5, RNA from a control leaf in the light (7 h into the photoperiod).

(**C**) Desalted extracts were assayed for PEPc kinase activity and the malate sensitivity of the PEPc in the extracts was also measured.

Lane 1, control leaf placed in 13 % methanol.

Lane 2, control leaf placed in 1% ethanol.

Lane 3, leaf placed in 500 µM actinomycin D.

Lane 4, leaf placed in 1 mM cycloheximide.

(**D**) PEPc kinase activity in *in vitro* translation products.

Lane 1, control, no RNA.

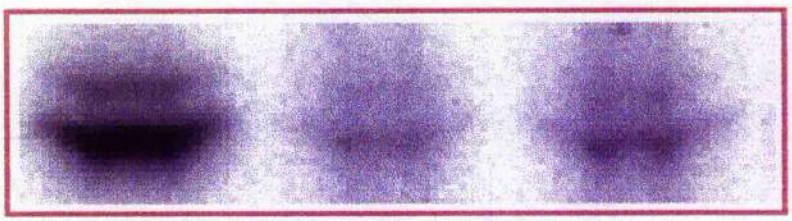
Lane 2, RNA from a leaf placed in 13 % methanol.

Lane 3, RNA from a leaf placed in 1 % ethanol.

Lane 4, RNA from a leaf placed in 500 µM actinomycin D.

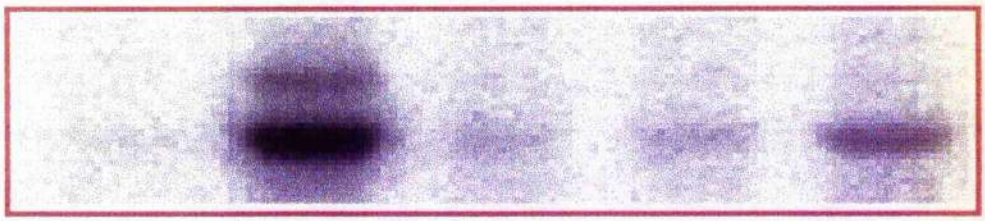
Lane 5, RNA from a leaf placed in 1 mM cycloheximide.

A



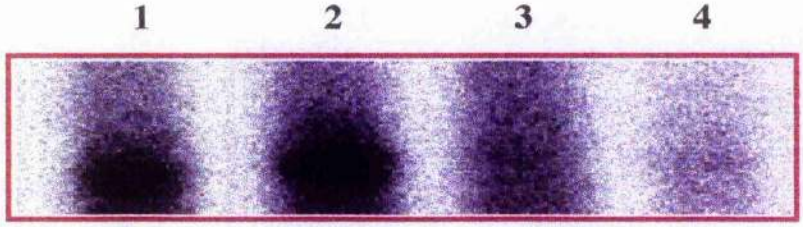
	1	2	3
Relative intensity	100	18	27
Apparent K_i of PEPc for L-malate (mM)	5	0.6	0.7

B



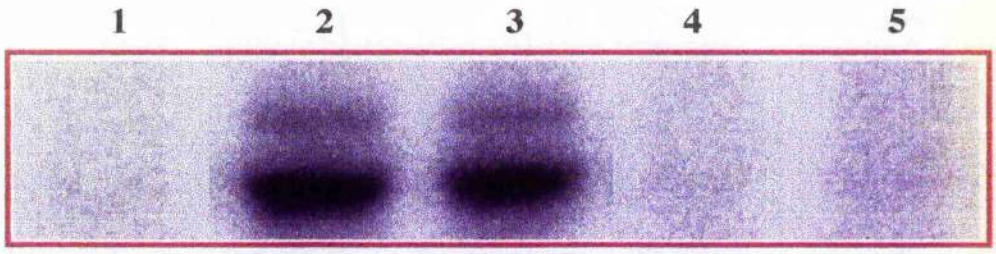
	1	2	3	4	5
Relative intensity	4	100	8	5	13

C



	1	2	3	4
Relative intensity	87	100	3	5
% inhibition by 3 mM L-malate	71	69	91	92

D



	1	2	3	4	5
Relative intensity	0	100	79	3	2

Figure 4.5. Influence of a range of pharmacological agents on the circadian decrease in the L-malate sensitivity of PEPc in *B. fedtschenkoi*.

Leaves were detached at 16.00 h and placed with their petiole in the appropriate inhibitor solution. Controls treatments involved placing leaves in the appropriate concentration of ethanol, methanol, DMSO or water because the inhibitors were variously dissolved in these solvents. Leaves were sampled at 24.00 in the middle of the subsequent dark period and desalted extracts were assayed for the malate sensitivity of PEPc. The solvent control for each inhibitor precedes the inhibitor(s) dissolved in it.

1, distilled water control.

2, 500 μM W7.

3, 1 % ethanol control.

4, 1 mM cycloheximide.

5, 13 % methanol control.

6, 500 μM actinomycin D.

7, 10 μM staurosporin.

8, 100 μM Bay K8644.

9, 1 % DMSO control.

10, 1 mM cantharidin.

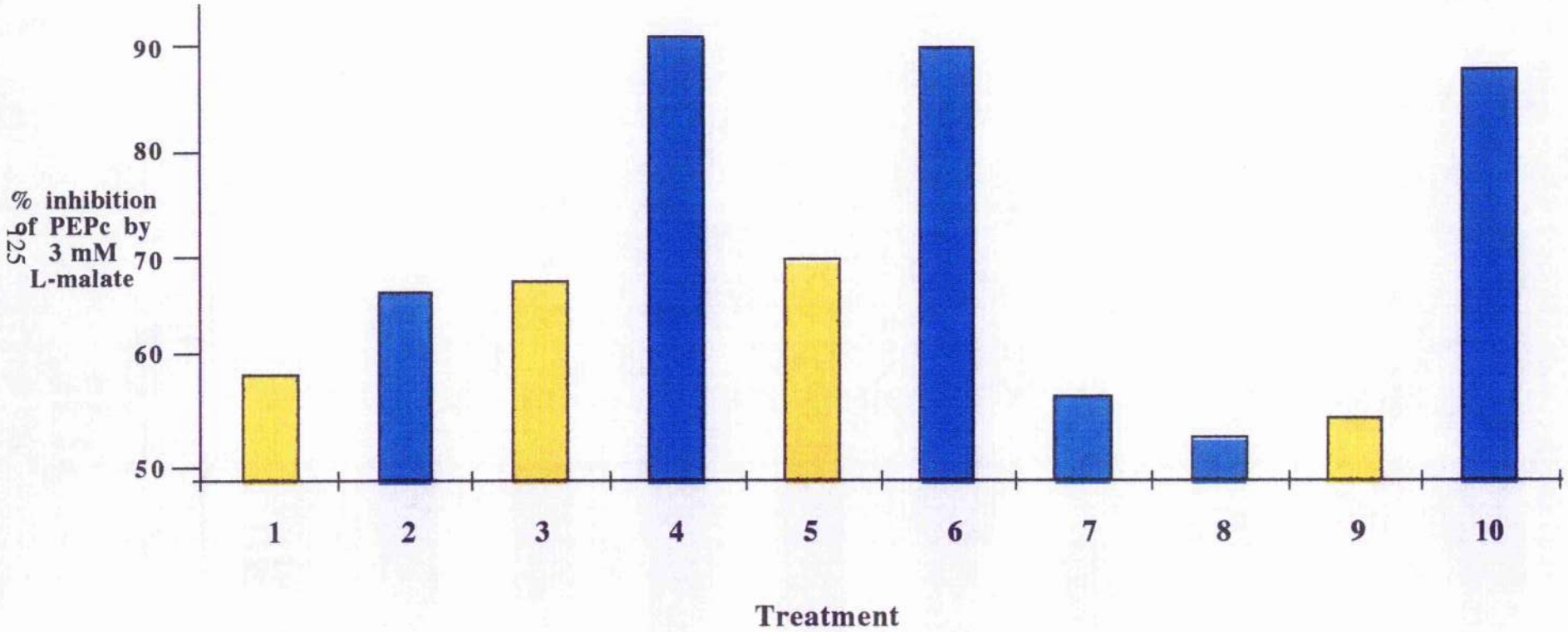

90
80
% inhibition of PEPc by 3 mM L-malate
70
60
50
1
2
3
4
5
6
7
8
9
10
Treatment

Figure 4.6 Pharmacological characterization of the circadian increase in translatable PEPc kinase mRNA in *B. fedtschenkoi.*

Duplicate leaves were treated as described in figure 4.5 with a variety of pharmacological agents as a means to characterize some of the signal transduction events involved in the circadian increase in PEPc kinase mRNA.

(**A**) Effect of W7 on the nocturnal appearance of PEPc kinase translatable mRNA.

Lane 1, distilled water control.

Lane 2, 500 μM W7.

(**B**) Effect of cycloheximide on the nocturnal appearance of PEPc kinase translatable mRNA (positive control for inhibitor feeding experiments).

Lane 1, 1 % ethanol control.

Lane 2, 1 mM cycloheximide.

(**C**) Effect of actinomycin D, staurosporin and Bay K8644 on the nocturnal appearance of PEPc kinase translatable mRNA.

Lane 1, 13 % methanol control.

Lane 2, 500 μM actinomycin D.

Lane 3, 10 μM staurosporin.

Lane 4, 100 μM Bay K8644.

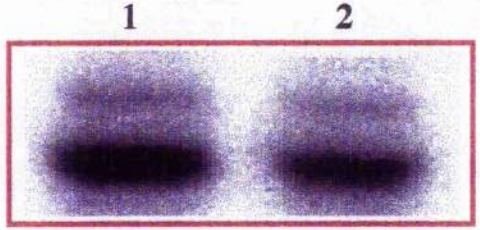
(**D**) Effect of cantharidin on the nocturnal appearance of PEPc kinase translatable mRNA.

Lane 1, 1 % DMSO control.

Lane 2, 1 mM cantharidin.

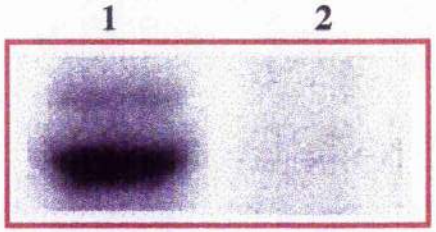
Lane 3, no RNA control.

A



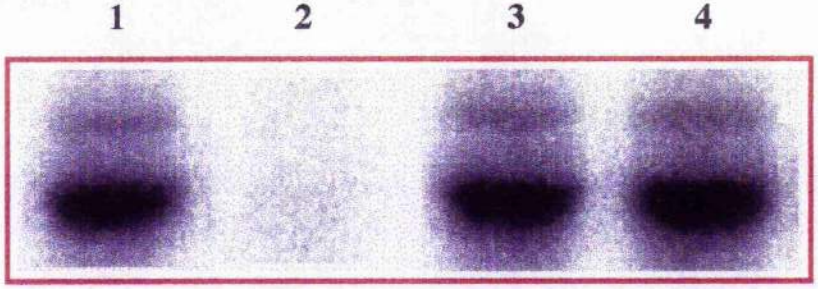
Relative intensity 100 69

B



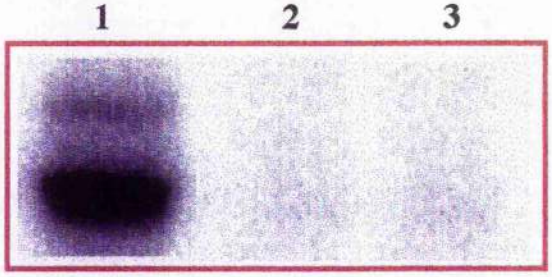
86 2

C



Relative intensity 68 3 78 75

D



85 1 0

Figure 4.7 A calcium/calmodulin interaction is required for the nocturnal appearance of PEPc kinase mRNA and activity in *B. fedtschenkoi*.

B. fedtschenkoi leaves were detached at 16.00 h and placed with their petiole in a solution of distilled water or the calmodulin inhibitor W7. Duplicate leaves were then collected at 24.00 h in the middle of the subsequent dark period. One leaf was used to prepare a desalted extract to allow the determination of the PEPc kinase activity and the L-malate sensitivity of PEPc and the other leaf was used to isolate total RNA.

(**A**) PEPc kinase activity and malate sensitivity of PEPc.

Lane 1, distilled water control.

Lane 2, 100 μM cycloheximide (positive control).

Lane 3, 1 mM W7.

The relative intensity of the PEPc bands is given below each lane and along with the corresponding malate sensitivity of PEPc.

(**B**) PEPc kinase translatable mRNA.

Lane 1, distilled water control.

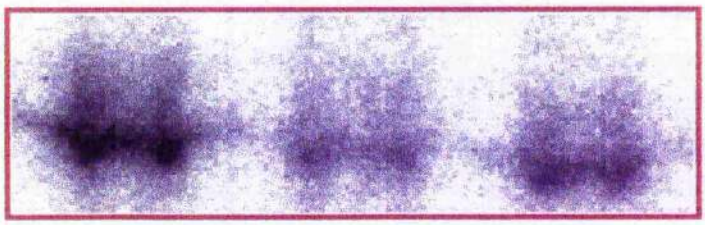
Lane 2, 100 μM cycloheximide (positive control).

Lane 3, 1 mM W7.

Lane 4, no RNA control.

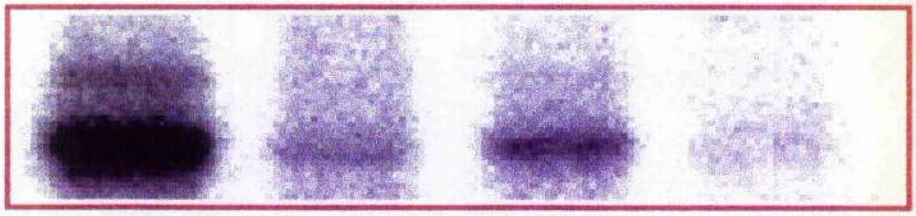
The relative intensity of the PEPc bands each shown below each lane.

A



	1	2	3
Relative intensity	100	31	45
% inhibition of PEPc by 3 mM L-malate	73	86	83

B



	1	2	3	4
Relative intensity	100	8	23	0

Chapter 5

The regulation of PEPc kinase translatable mRNA under anaerobic conditions in *Kalanchoë daigremontiana*

5.1 Introduction

When the leaves of the Crassulacean acid metabolism plant *K. fedtschenkoi* are detached and placed in constant conditions they display a circadian rhythm of CO_2 metabolism. This rhythm varies with the conditions. For example, when leaves are placed in CO_2-free air at 15°C in the dark they show a rhythm of CO_2 output which lasts for about four days and which has a period of about 23 h. Leaves placed in continuous light and normal air at 15°C show a rhythm of CO_2 uptake with a period of about 16 h which persists for up to 10 days (Wilkins, 1992). Wilkins (1983) proposed that these rhythms are controlled by the concentration and distribution of malate within each compartment of the cell. In particular it was postulated that movement of malate in and out of the vacuole combined with its periodic breakdown in the cytoplasm could maintain the rhythm (Wilkins, 1983). It has been hypothesized that both light and high temperature treatments open 'gates' in the tonoplast and thus allow malate to move down a concentration gradient from the vacuole to the cytoplasm (Wilkins, 1983; Wilkins, 1989). This hypothesis can account successfully for the properties of the rhythms and phase re-setting by light and temperature changes and places malate as a pivotal signalling metabolite in the generation and perturbation of the circadian rhythm of CO_2 metabolism in *K. fedtschenkoi*.

At the biochemical level, it was found that the rhythm in CO_2 fixation is generated by periodic flux through PEPc which is regulated by changes in the malate sensitivity of the enzyme (Nimmo *et al.*, 1984; Nimmo *et al.*, 1987b). As described in chapter 4, PEPc is phosphorylated and malate-insensitive at night and dephosphorylated and malate-sensitive in the day. The changes in the phosphorylation state of PEPc are regulated by the nocturnal induction of PEPc kinase mRNA and activity by the circadian oscillator (see chapter 4) (Carter *et al.*, 1991). Hence, when malate is released from the vacuole during the day, PEPc kinase mRNA and activity are absent, and the dephosphorylated form of PEPc is inhibited by the malate and rendered inactive. At night, PEPc kinase is induced and active

and the phosphorylated form of PEPc can maintain CO_2 fixation despite the accumulation of malate generated as a result of the flux through PEPc. The flux through PEPc is further assisted by pumping of the malate into the vacuole at night.

Whilst the results in chapter 4 demonstrate a strict correlation between the level of PEPc kinase translatable mRNA, the level of kinase activity and the K_i of PEPc for malate, the majority of the experiments (except for the results in figure 4.1) were performed with detached leaves of *K. fedtschenkoi* rather than leaves which remained attached to a healthy plant. For the work described in this chapter, a collaboration was established with Dr. A.M. Borland of the University of Newcastle with a view to examining whether changes in PEPc kinase mRNA and activity are physiologically relevant in the intact CAM plant. In particular, it was important to manipulate the malate content of intact leaves during the night to determine whether this might influence the level of PEPc kinase mRNA and activity.

Dr. Borland had previously developed an experimental system in which nocturnal CO_2 fixation by leaves of intact *K. daigremontiana* plants could be prevented by placing them in an atmosphere of pure nitrogen (anaerobic conditions) throughout the night (Borland and Griffiths, 1997). Such leaves showed no nocturnal malate accumulation when compared to the control leaves kept in normal air. However, malate did accumulate at the beginning of the photoperiod, when N_2-treated leaves were returned to normal air, at a time when the total malate content of control leaves was decreasing. This increase in leaf malate content correlated well with a sharp increase in the rate of CO_2 assimilation and an elevated K_i of PEPc for malate which remained high for 3-4 h during phase II. However, despite this period of CO_2 fixation by PEPc during phase II the leaf malate content achieved much less than half that in the control leaves. This was most probably due to decarboxylation accompanying carboxylation (Borland and Griffiths, 1997).

One of the most interesting points about these experiments in the context of the present work was that, upon the return to normal air, the K_i of PEPc for malate in N_2-treated leaves initially increased slightly, achieved an higher value than the control leaves and remained above the K_i in control leaves for some 4 h into the light period (phase II).

This suggested that PEPc kinase might remain active well into the light period in N_2-treated leaves whilst in control leaves it appeared to be inactive within 30 minutes of the start of the photoperiod. Thus, in N_2-treated leaves, some factor overcame the circadian-regulated destruction of PEPc kinase upon the return to normal air and light. One of the most obvious candidates for this factor was the leaf malate content. In control leaves, the commencement of the photoperiod, with its accompanying increase in temperature, is accompanied by diffusion of malate from the vacuole into the cytoplasm whereas in N_2-treated leaves there is no accumulated malate. The possibility of metabolite regulation of PEPc kinase mRNA and activity in intact plants was therefore investigated in the present study by subjecting leaves of *K. daigremontiana* to various periods of N_2 encapsulation. The rate of CO_2 assimilation, the total leaf malate concentration, the K_i of PEPc for L-malate, the PEPc kinase activity and the level of PEPc kinase translatable mRNA both during and after N_2-treatment were examined. These experiments were planned jointly with Dr. Borland. The plants were grown and manipulated at the University of Newcastle by Dr. Borland who also determined the rate of CO_2 assimilation, the total leaf malate concentration and the apparent K_i of PEPc for malate. Frozen samples of the same leaves were brought to Glasgow where PEPc kinase activity and mRNA levels were determined. The results demonstrate a fascinating complexity of control of PEPc kinase mRNA and activity in response to the metabolite status of the cell in addition to the underlying circadian regulation.

5.2 Results

5.2.1 Physiology of CAM and manipulation by N_2

Figure 5.1a illustrates how the dark/light pattern of net CO_2 uptake, which may be dissected into four phases (Osmond, 1978), was modulated in response to anaerobic conditions which were imposed for part or all of the dark period. Inhibiting CO_2 uptake over the first half of the dark period by enclosing leaves in an atmosphere of N_2 for 7.5 h, resulted in a substantial increase in rates of net CO_2 assimilation when leaves were removed from N_2 and transferred to ambient air (half-N_2) compared to control plants which were exposed to ambient air throughout the night. The malate content of the half-

N_2-treated leaves increased rapidly when leaves were transferred to ambient air (Figure 5.1b). After only 3 h in ambient air, the malate content in half-N_2 leaves was higher than the malate in control leaves which had accumulated over 9 h. At the end of the dark period, the malate content of half-N_2 leaves was approximately 25 % higher than that measured in control leaves.

In half-N_2 leaves at the start of the photoperiod, both the amplitude and the duration of phase II net CO_2 uptake were stimulated compared to control plants (Figure 5.1a). This was accompanied by a short delay (~30 min) in the net breakdown of malate compared to controls (Figure 5.1b). However, in full N_2 leaves, transfer to ambient air at the start of the photoperiod resulted in a substantial increase in the rate of net CO_2 assimilation over both control and half N_2 leaves during phase II and stomatal closure was delayed by about 2 h compared to controls (Figure 5.1a). Moreover, after transfer to ambient air at the start of the photoperiod, the full N_2 leaves accumulated roughly 60 mol m^{-2} malate over the first 2.5 h of the photoperiod, indicating that PEPc was still active at a time when net breakdown of malate was occurring in control and half N_2 leaves (Figure 5.1b). Despite this day-time accumulation of malate in full N_2 leaves, the malate content only attained 50% of that measured in control leaves and the majority of decarboxylation was accomplished within 2 h. Consequently, stomata remained closed for only 2 h in full N_2 leaves compared to 5 h in control leaves (Figure 5.1a).

5.2.2 PEPc kinase translatable mRNA and activity in N_2-treated leaves

The variation in PEPc kinase activity and translatable mRNA in control and full N_2 leaves throughout the dark period is shown in figure 5.2. In control leaves, PEPc kinase activity increased over the first part of the dark period, peaking after 8.5 h in darkness (Figure 5.2a). For leaves maintained in N_2 during the dark period, PEPc kinase activity increased steadily over the course of the dark period and was substantially higher than that measured in control leaves at comparable stages throughout the night. However, the results in figure 5.2c and 5.2d indicate that the level of kinase translatable mRNA in control and full N_2 leaves was similar for the first 8.5 h of the dark period. Subsequently, the level of

translatable mRNA in full N_2-leaves was higher than that measured in control leaves over the last 3 h of the dark period.

The time course of changes in PEPc kinase activity and translatable mRNA, together with changes in the apparent K_i of PEPc for malate for control and full N_2 leaves are illustrated graphically in figure 5.3. Changes in the apparent K_i of PEPc for malate, which reflects the phosphorylation state of PEPc (Carter *et al.*, 1991) closely followed changes in PEPc kinase activity; the increased PEPc kinase activity measured in full N_2 compared to controls was reflected by a decreased malate sensitivity of PEPc in full N_2 leaves. The marked increase in the level of PEPc kinase translatable mRNA from 00:00 to 04:00 h in control leaves is accompanied by an increase in PEPc kinase activity. In full N_2 leaves, the peak in kinase mRNA levels occurred at 06:00 h with levels of mRNA substantially higher than those measured in control leaves at this time. In both control and full N_2 leaves, levels of translatable mRNA declined over the last 2 h of the photoperiod.

Figure 5.4 illustrates changes in the apparent K_i of PEPc for malate, PEPc kinase activity and translatable mRNA which occurred when half-N_2 leaves were subsequently transferred to ambient air for the remainder of the night. Again, changes in the apparent K_i of PEPc for malate closely mirror the change in PEPc kinase activity in half-N_2 leaves. The apparent K_i of PEPc for malate, the level of kinase mRNA and the PEPc kinase activity were approximately 2-fold higher than controls in N_2-treated leaves immediately after removal from N_2. In the 2 h following transfer to ambient air, levels of translatable kinase mRNA rose sharply and rates of net CO_2 uptake reached a maximum (figure 5.1a). By 06:00 h, when malate content peaked (figure 5.1b), kinase mRNA had dropped to a level comparable to that measured in control leaves. The peak in kinase mRNA (at 04:00 h) preceded the peak in PEPc kinase activity in half N_2 leaves at 06:00 h when the malate content reached a maximum (Figure 5.1b).

Figure 5.5 compares changes in the above components which occur at the start of the photoperiod in control and full-N_2 leaves. In control leaves, PEPc is rapidly down-regulated due to a decrease in PEPc kinase activity with the concomitant increase in the sensitivity of PEPc to malate inhibition over the first hour of the photoperiod. This down-

regulation of PEPc is accompanied by closure of the stomata and the commencement of malate breakdown (Figure 5.1). In the same leaves, the already low levels of kinase mRNA declined to levels below the limits of detection after 2 h in the light. In full-N_2 leaves, the apparent K_i of PEPc for malate, PEPc kinase activity and mRNA at the start of the photoperiod were substantially higher than those measured in control leaves and remained so for the next 2-3 h as net CO_2 uptake continued and malate was accumulated (Figure 5.1). As found with leaves removed from N_2 in the middle of the dark period (Figure 5.4), levels of kinase mRNA rose sharply during the first hour after transfer to ambient air in the light. However, unlike the situation in half-N_2 leaves in the dark (Figure 5.4), this peak in kinase mRNA level in the light was not followed by an increase in PEPc kinase activity (Figure 5.5), which may reflect an effect of the increase in cytosolic malate content which would not have occurred in the dark.

5.2.3 Physiological aspects of temperature manipulations

It has been suggested that disruption of the circadian oscillator in CAM plants by high temperature may be a consequence of increased efflux of malate from the vacuole to the cytosol, the site of PEPc activity (Grams *et al.*, 1997; Wilkins, 1983). Figure 5.6 illustrates the physiological consequences of exposing both control and half-N_2 leaves to an 8°C increase in temperature in the middle of the night (from 02:30-03:00 h). In control leaves there was a rapid drop in the rate of net CO_2 assimilation as temperature increased from 19-27°C (Figure 5.6a). The sharp increase in malate content over the 30 min rise in temperature may be attributed to an increase in refixation of respiratory CO_2. Overall, maximum net assimilation rate at 27°C was < 50 % of maximum net assimilation rate measured at 19°C in control leaves. Despite the continued net uptake of CO_2 by control leaves, the malate content dropped slightly over the first few hours of exposure to the higher temperature. This may represent utilization of the malate as a respiratory metabolite in the mitochondria as the rate of respiration will be significantly higher at 27°C. However, marked breakdown of malate was observed over the last hour of the dark period when net CO_2 assimilation had virtually ceased. This could be due to earlier activation of decarboxylation enzymes in addition to the high rates of respiration. Rates of net CO_2

assimilation in leaves removed from N_2 immediately after the temperature had increased to 27°C were approximately 5-fold higher than those measured in control leaves at this time. Net assimilation rates dropped sharply during the first 1.5 h at the higher temperature in N_2-treated leaves, reached a plateau for 3h and then decreased over the last hour of the dark period. The malate content in the N_2-treated leaves showed a marked increase over the first 2 hours at the higher temperature and a more gradual increase over the remaining 3.5 h. By contrast to control leaves, net breakdown of malate in N_2-treated leaves did not commence until the start of the photoperiod, as was found for control and half-N_2-leaves which had not been subjected to the 8°C rise in temperature in the middle of the night.

5.2.4 Modulation of PEPc kinase activity and translatable mRNA by temperature

Figure 5.7 shows that in leaves prevented from accumulating malate over the first half of the dark period, an 8°C rise in temperature resulted in a marked increase in PEPc kinase activity and kinase translatable mRNA compared to the decrease in these components in control leaves. However, after the N_2-treated leaves were transferred to ambient air at 27°C, the level of PEPc kinase translatable mRNA, kinase activity and the apparent K_i of PEPc for malate all decreased markedly within 2 h at the higher temperature. These changes occurred at a time when malate accumulated (figure 5.6b), presumably in the cytosol. For the remainder of the dark period, the apparent K_i of PEPc for malate and the level of kinase activity and mRNA were marginally higher in N_2-treated leaves, which continued to accumulate malate, compared to controls in which malate content declined (Figure 5.6b). For example in the half-N_2 leaves at 05:00 h, it appears that an apparent K_i of 2.5 mM measured *in vitro* can sustain PEPc in an active state in the presence of around 80 mmol m^{-2} malate, assuming the malate is equilibrated equally between the tonoplast and cytosol at 27°C. However, in the control leaves at 05:00 h, the apparent K_i of PEPc for malate is 0.5 mM which evidently does not sustain PEPc in an active state in the presence of ~130 mmol m^{-2} malate.

5.3 Discussion

For the work described in this chapter, intact plants were manipulated to control the magnitude of dark CO_2 uptake and malate accumulation, and monitored for the effects of these manipulations on the levels of PEPc kinase mRNA and activity. The results allow a number of conclusions about the control of PEPc kinase to be drawn. First, the data clearly demonstrate the physiological significance of PEPc phosphorylation, as is shown by the close correlation between the activity *in vitro* of PEPc kinase, net CO_2 uptake by PEPc and malate accumulation *in vivo* under ambient air and after transfer from anaerobic conditions to ambient air. For example, leaves prevented from accumulating malate overnight in an atmosphere of N_2 exhibited an extended period of CO_2 uptake by PEPc for 2-3 hours at the start of the photoperiod under ambient air (Borland and Griffiths, 1997). Under these conditions, kinase activity remained detectable and PEPc remained phosphorylated for several hours into the photoperiod (figure 5.5). In leaves moved from N_2 to ambient air mid-way through the dark period, malate accumulated significantly faster thereafter, PEPc kinase activity was higher and PEPc was more highly phosphorylated (as judged by its malate sensitivity) than in control leaves (figures 5.1 and 5.4). The data presented here support and extend those in the previous chapter on *K. fedtschenkoi* in showing that these physiologically significant changes in PEPc kinase activity usually reflect changes in the translatable mRNA for this protein. There is one exception to the general rule that kinase activity tracks kinase mRNA. In leaves that had been exposed to ambient air at 08:30 after a night in N_2, there is a marked decline in kinase activity between 08:30 and 11:00 despite a marked increase in kinase mRNA between 08:30 and 09:30. Possible reasons for this are discussed further below.

Previous work has demonstrated clearly that PEPc kinase mRNA and activity, and the phosphorylation state of PEPc, are under the control of the circadian oscillator (Carter *et al.*, 1991; Nimmo *et al.*, 1987b). These effects contribute to the well-established circadian control of CO_2 fixation in CAM plants (Wilkins, 1992). A second conclusion about the control of PEPc kinase is that the circadian control of kinase mRNA and activity can be influenced by metabolite levels. In leaves that cannot accumulate malate, PEPc

kinase activity is significantly higher than in control leaves, even though PEPc kinase mRNA levels are comparable. This suggests that some metabolite, present at a higher concentration in control leaves, either reduces translation of the mRNA or increases the rate of destruction of the kinase protein. One attractive candidate metabolite is the cytosolic malate concentration. This factor might also affect transcription of the PEPc kinase gene, or stability of PEPc kinase mRNA, so that a high malate concentration leads to a low level of PEPc kinase mRNA. Such an effect could account for the effect of an increase in temperature from 19°C to 27°C on PEPc kinase mRNA levels. This temperature jump reduced PEPc kinase mRNA in control leaves, but actually increased it in leaves in which malate accumulation had been prevented (fig. 5.7). Equally, a metabolite present at a lower concentration in control than N_2-treated leaves might enhance translation of kinase mRNA or reduce the rate of destruction of the kinase protein.

The effect of increased temperature on circadian rhythms of CO_2 fixation has been ascribed to increased permeability of the tonoplast membrane to malate (Wilkins, 1983; Wilkins, 1992), and there is some direct experimental support for this hypothesis (Fricmert *et al.*, 1988). The sharp reduction in PEPc kinase mRNA seen during the temperature increase in control leaves (fig. 5.7) could reflect a large increase in cytosolic malate, resulting from efflux from the vacuole, that was not possible in the N_2-treated leaves. If this hypothesis is correct, the cytosolic malate level in control leaves must be insufficient to reduce the accumulation of PEPc kinase mRNA during the first 10 h of darkness. PEPc kinase mRNA starts to decline later in control leaves than in half-N_2 leaves, at 06:00 and 04:00 respectively. It is worth noting that the total leaf malate contents are actually similar at these times in the two treatments. Hence there may be a threshold level of total malate, at about 120 mol m^{-2}, above which the cytosolic malate is sufficient to reduce PEPc kinase mRNA. In leaves treated with full N_2, the decline in PEPc kinase mRNA started when the total malate content had only reached some 70 mol m^{-2}. However, it must be borne in mind that this decline occurred after the start of the photoperiod, at a time when the temperature had reached 27°C, and a much higher proportion of the total malate was presumably in the cytosol than would be the case in darkness.

There were sharp increases in the level of PEPc kinase mRNA when N_2-treated leaves were transferred to ambient air in the dark (fig. 5.4) or the light (fig. 5.5). These results imply that kinase gene expression might also be modulated by a factor related to the rate of respiration. The increase in mRNA in the dark is followed, after a lag, by an increase in kinase activity (fig. 5.4). However, as noted above, the increase in kinase mRNA in the light occurs during a decrease in kinase activity (fig. 5.5). There are several possible reasons for this. One is that the rapid increase in total malate content, under conditions in which malate is not stored in the vacuole, could lead to a high cytosolic malate concentration, and stimulation of the rate of destruction of the kinase (or reduction in the translation of kinase mRNA, see above). Other possibilities are that the start of the photoperiod might lead to formation of a tight-binding inhibitor of the kinase, or that light itself decouples transcription of the kinase gene from translation.

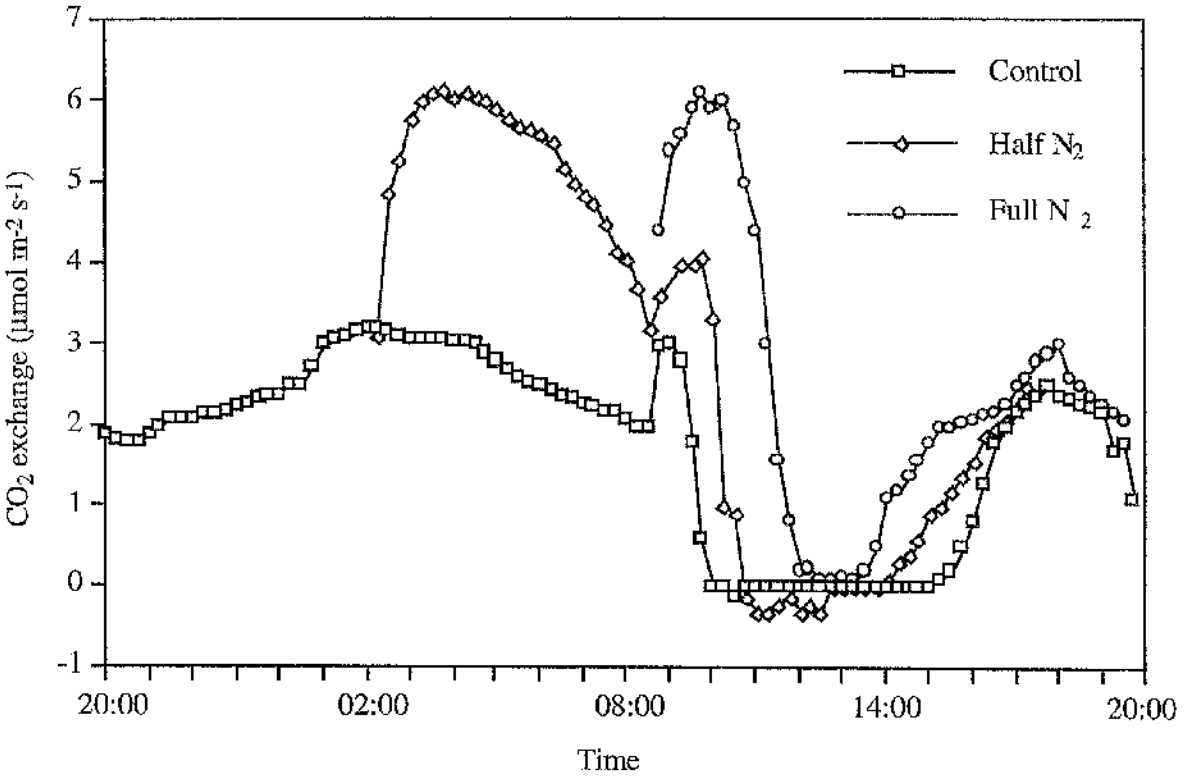
Overall, the control of flux through PEPc is clearly multi-layered. Fine control is achieved by changes in cytosolic levels of opposing metabolic effectors such as malate (negative) and glucose 6-phosphate (positive). The phosphorylation of PEPc represents a means for course control of flux through this enzyme. Timing of the phosphorylation is set by a circadian oscillator. The data in this chapter show that circadian control can be over-ridden by metabolite control, probably in various ways. It would seem highly likely that a metabolite, possibly cytosolic malate, can affect PEPc kinase gene expression or mRNA stability, or the stability of the kinase itself. Respiratory metabolism may also affect these parameters. Such metabolite effects may influence entrainment of the circadian rhythm to environmental conditions which, in turn, support photosynthetic flexibility and survival through temporarily optimizing CO_2 uptake. Identification of the metabolites responsible will require careful measurement of metabolite pools in particular compartments of the cell.

Figure 5.1. Rates of net CO_2 uptake and the concomitant malate content in leaves of *K. daigremontiana* exposed to anaerobic conditions for either half or the whole of the dark period.

(**A**). Leaves were enclosed in an atmosphere of N_2 for the first half (half N_2) or entire duration (full N_2) of the dark period before transfer to ambient air and rates of net CO_2 assimilation were measured. Control leaves were exposed to the ambient atmosphere in the growth chamber. Each gas exchange curve is representative of 3 replicate runs with SE < 10% of mean.

(**B**). Malate content was measured in leaves subjected to the above treatments with each point the mean of 3 replicates with SE < 10% of mean. The solid bar on the x-axis represents the period of darkness.

A


Control
Half N_2
Full N_2
CO_2 exchange (µmol m^{-2} s^{-1})
7
6
5
4
3
2
1
0
-1
20:00
02:00
08:00
14:00
20:00
Time

B

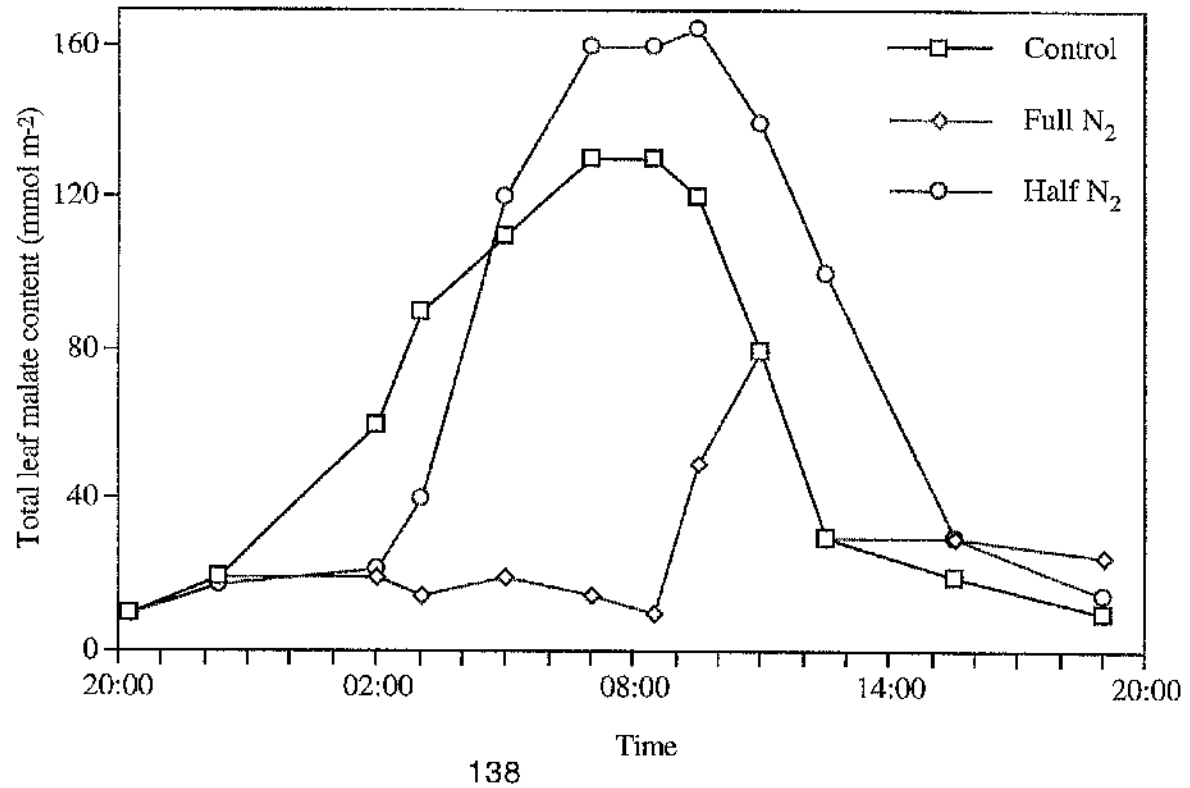

Control
Full N_2
Half N_2
Total leaf malate content (mmol m^{-2})
160
120
80
40
0
20:00
02:00
08:00
14:00
20:00
Time

Figure 5.2. The effect of anaerobic conditions on the level of PEPc kinase translatable mRNA and activity during the dark period in *K. daigremontiana* leaves .

Leaves were enclosed in an atmosphere of N_2 overnight, to prevent malate accumulation, or maintained in ambient air. Samples for PEPc kinase activity and RNA isolation were taken simultaneously from the same leaves at intervals over the 13 h dark period. The figure shows autoradiographs of the ^{32}P-labelled PEPc bands following gel electrophoresis. The relative intensity of each PEPc band, shown below each track, was determined by phosphoimaging. Total *in vitro* translation products appeared similar for RNA isolated from control and N_2 treated leaves (data not shown).

(**A**) PEPc kinase activity in control leaves kept in normal air.

Lane 1, 0.75 h into the dark period.

Lane 2, 2.5 h into the dark period.

Lane 3, 4.5 h into the dark period.

Lane 4, 6.5 h into the dark period.

Lane 5, 8.5 h into the dark period.

Lane 6, 10.5 h into the dark period.

Lane 7, 13 h into the dark period.

(**B**) PEPc kinase activity in leaves enclosed in nitrogen (anaerobic conditions).

Lane 1, 2.5 h into the dark period.

Lane 2, 4.5 h into the dark period.

Lane 3, 6.5 h into the dark period.

Lane 4, 8.5 h into the dark period.

Lane 5, 10.5 h into the dark period.

Lane 6, 13 h into the dark period.

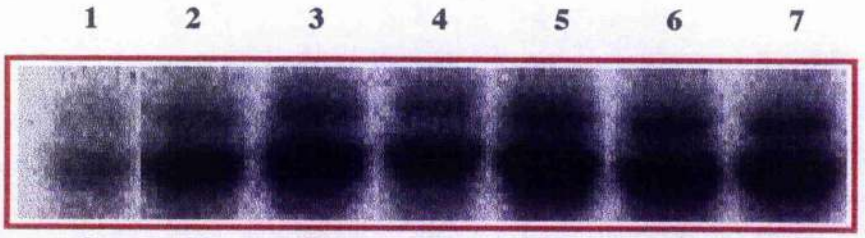
(**C**) PEPc kinase translatable mRNA levels in control leaves kept in normal air.

Lanes correspond to those listed for panel (A) above.

(**D**) PEPc kinase translatable mRNA levels in leaves enclosed in nitrogen (anaerobic conditions).

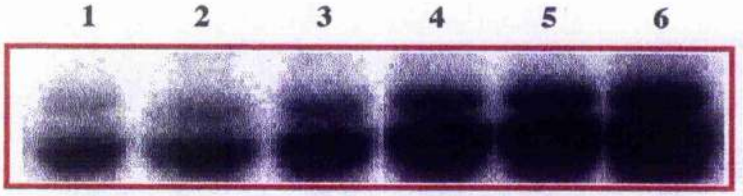
Lanes correspond to those listed for panel (B) above.

A



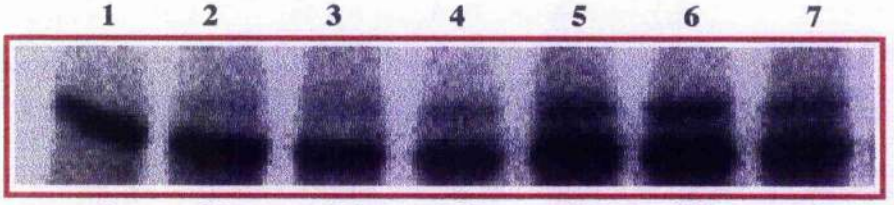
	1	2	3	4	5	6	7
Relative intensity	9	19	29	29	49	40	46

B



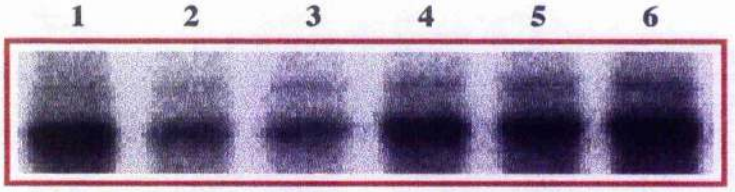
	1	2	3	4	5	6
Relative intensity	38	42	63	79	90	100

C



	1	2	3	4	5	6	7
Relative intensity	9	33	28	57	90	75	43

D



	1	2	3	4	5	6
Relative intensity	30	29	68	64	100	70

Figure 5.3. Time course of apparent K_i of PEPc for L-malate, PEPc kinase activity and translatable mRNA under ambient and anaerobic conditions during the dark period.

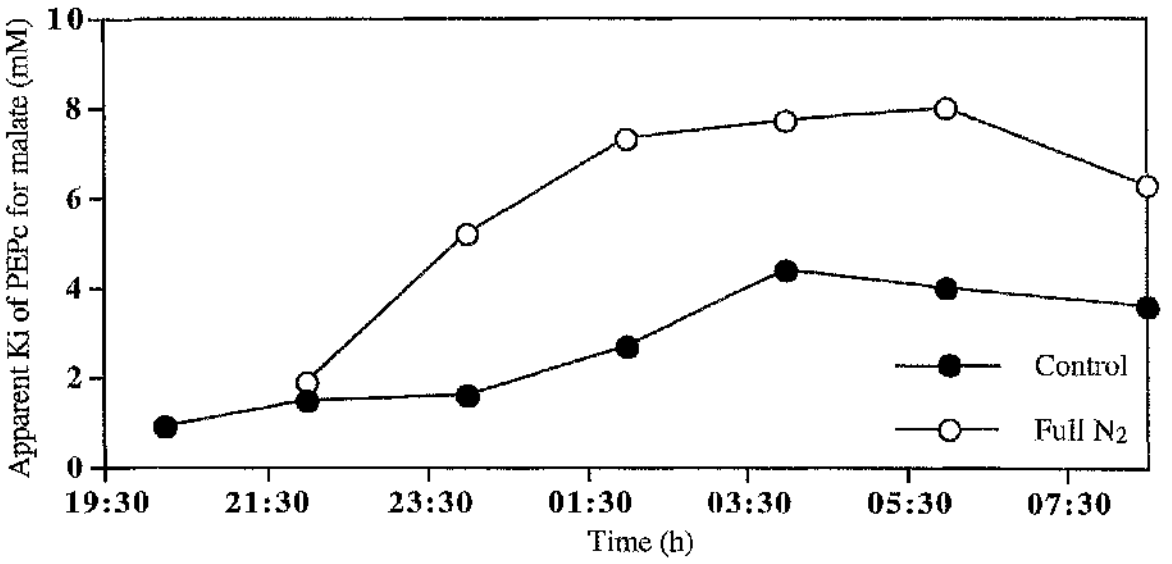
Leaves were enclosed in an atmosphere of N_2 overnight, to prevent malate accumulation, or maintained in ambient air. Samples for PEPc and PEPc kinase assays, and RNA isolation were taken simultaneously from the same leaves at intervals throughout the 13 h dark period. Kinase activity and translatable mRNA values are expressed as a percentage of the maximum reached during the 13 h dark period.

(**A**) Variation in the apparent K_i of PEPc for L-malate in rapidly desalted extracts prepared from control and full N_2-treated leaves.

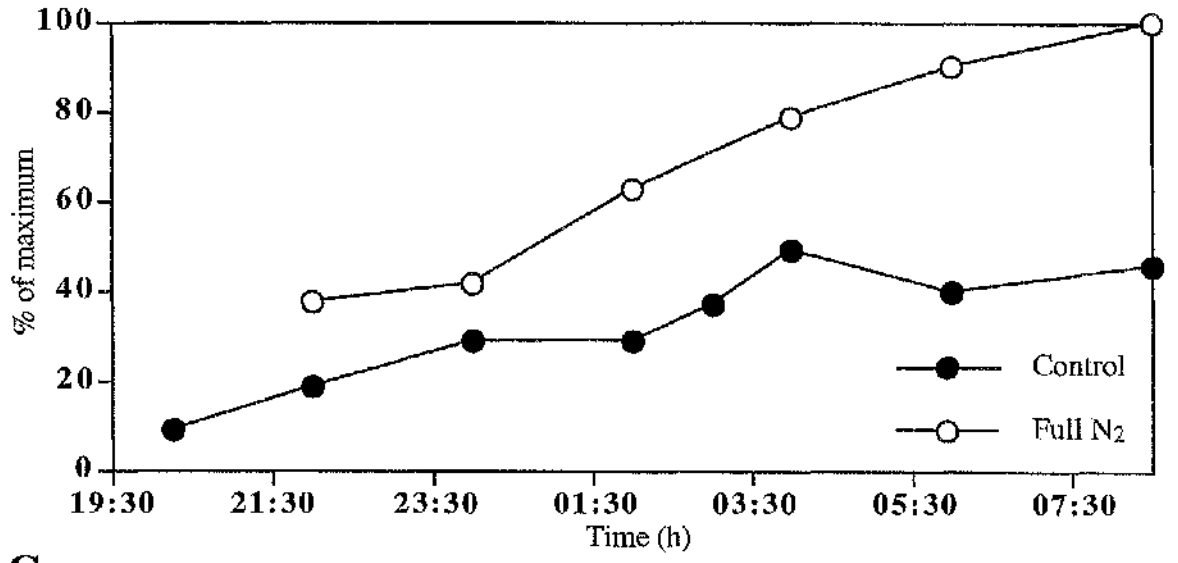
(**B**) Variation in PEPc kinase activity in rapidly desalted extracts prepared from control and full N_2-treated leaves.

(**C**) Variation in PEPc kinase translatable mRNA levels in control and full N_2-treated leaves.

A


Apparent Ki of PEPc for malate (mM)
10
8
6
4
2
0
19:30
21:30
23:30
01:30
03:30
05:30
07:30
Time (h)
Control
Full N2

B


% of maximum
100
80
60
40
20
0
19:30
21:30
23:30
01:30
03:30
05:30
07:30
Time (h)
Control
Full N2

C

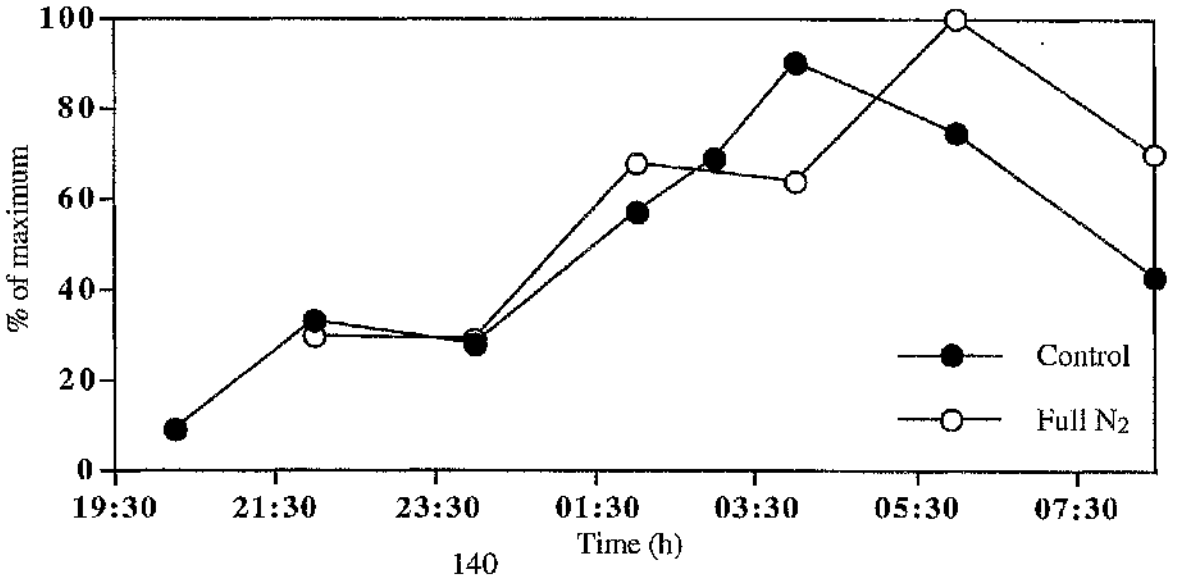

% of maximum
100
80
60
40
20
0
19:30
21:30
23:30
01:30
03:30
05:30
07:30
Time (h)
Control
Full N2

Figure 5.4. Apparent K_i of PEPc for L-malate, PEPc kinase activity and translatable mRNA after transfer from anaerobic conditions to ambient air in the dark period.

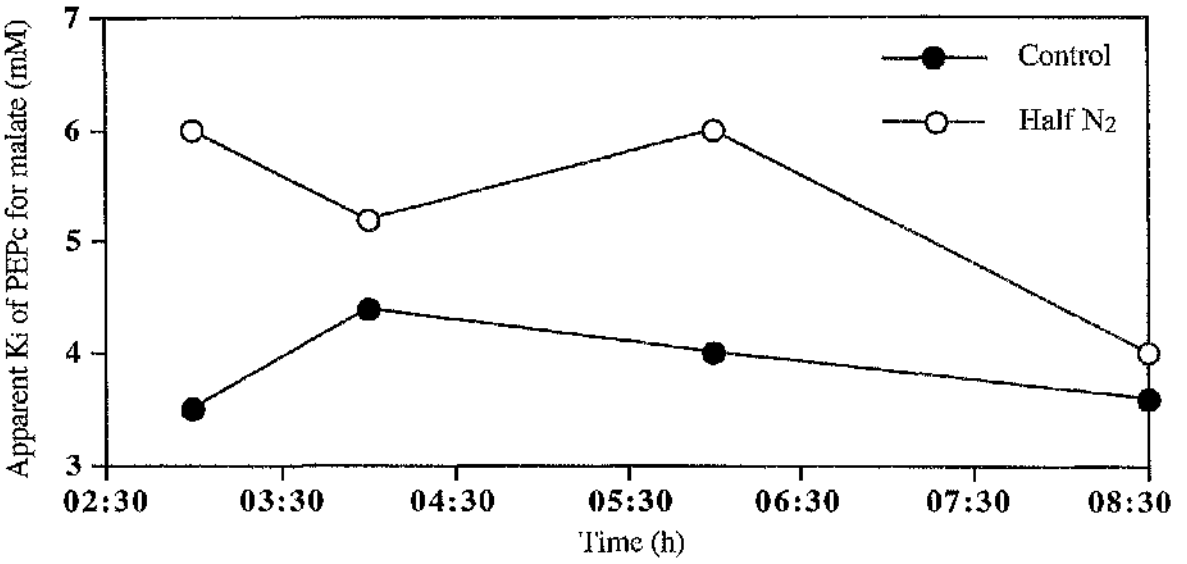
Leaves were enclosed in an atmosphere of N_2 to prevent malate accumulation for the first half of the dark period before transfer to ambient air. Control leaves were maintained in ambient air throughout the night. Samples for PEPc and PEPc kinase assays, and RNA isolation were taken simultaneously from the same leaves at intervals over the dark period. Kinase activity and translatable mRNA values are expressed as a percentage of the maximum reached during the 13 h dark period.

(**A**) Variation in the apparent K_i of PEPc for L-malate in rapidly desalted extracts prepared from control and half N_2-treated leaves.

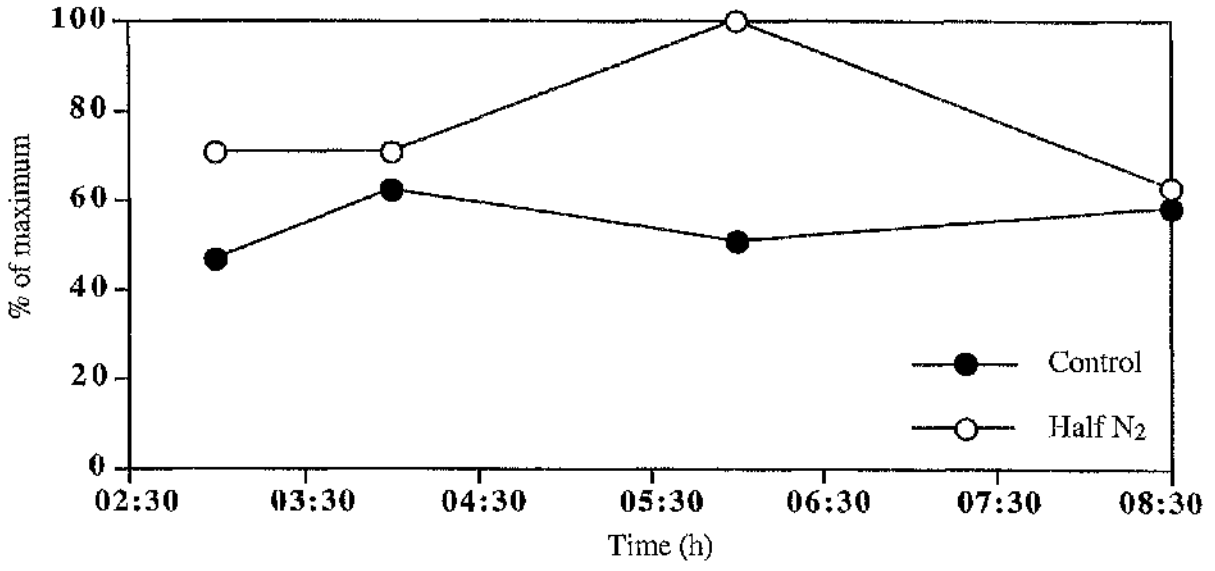
(**B**) Variation in PEPc kinase activity in rapidly desalted extracts prepared from control and half N_2-treated leaves.

(**C**) Variation in PEPc kinase translatable mRNA levels in control and half N_2-treated leaves.

A


Apparent Ki of PEPc for malate (mM)
7
6
5
4
3
Control
Half N2
02:30
03:30
04:30
05:30
06:30
07:30
08:30
Time (h)

B


% of maximum
100
80
60
40
20
0
Control
Half N2
02:30
03:30
04:30
05:30
06:30
07:30
08:30
Time (h)

C

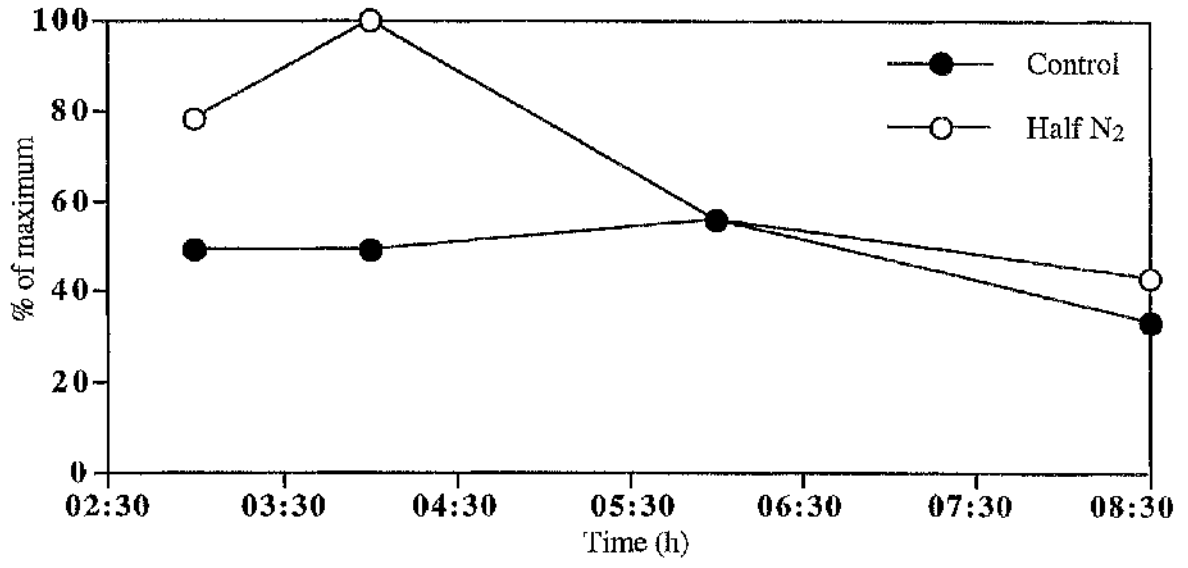

% of maximum
100
80
60
40
20
0
Control
Half N2
02:30
03:30
04:30
05:30
06:30
07:30
08:30
Time (h)

Figure 5.5. Down regulation of the apparent K_i of PEPc for malate and PEPc kinase activity and translatable mRNA at the start of the photoperiod following a night in ambient or anaerobic conditions.

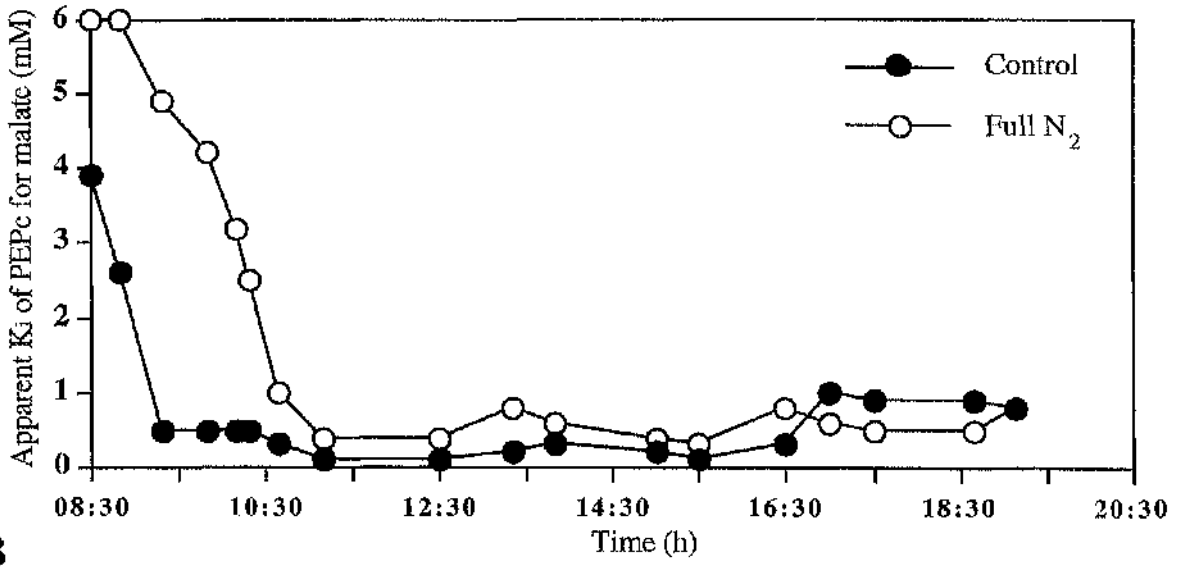
Leaves which had been maintained in an atmosphere of N_2 overnight to prevent malate accumulation were transferred to ambient air at the start of the photoperiod. Control leaves were maintained in ambient air throughout. Samples for PEPc and PEPc kinase assays, and RNA isolation were taken simultaneously from the same leaves at intervals during the first few hours of the photoperiod. Kinase activity and translatable mRNA values are expressed as a percentage of the maximum reached during the photoperiod.

(**A**) Variation in the apparent K_i of PEPc for L-malate in rapidly desalted extracts prepared from control and full N_2-treated leaves.

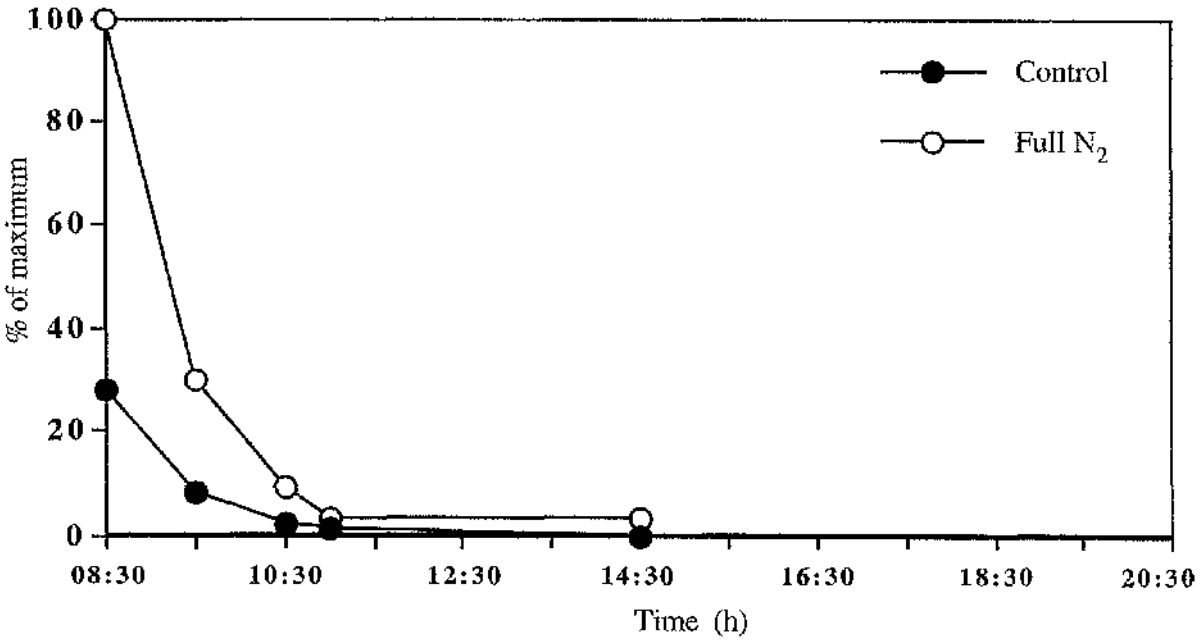
(**B**) Variation in PEPc kinase activity in rapidly desalted extracts prepared from control and full N_2-treated leaves.

(**C**) Variation in PEPc kinase translatable mRNA levels in control and full N_2-treated leaves.

A


Apparent Ki of PEPc for malate (mM)
0
1
2
3
4
5
6
08:30
10:30
12:30
14:30
16:30
18:30
20:30
Time (h)
Control
Full N2

B


% of maximum
0
20
40
60
80
100
08:30
10:30
12:30
14:30
16:30
18:30
20:30
Time (h)
Control
Full N2

C

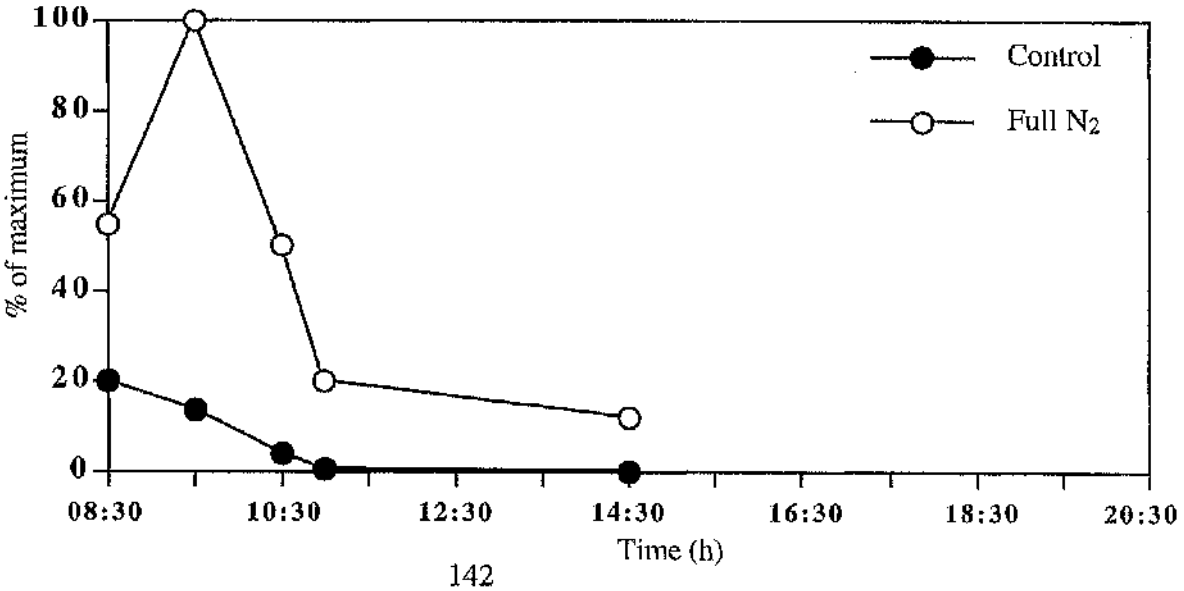

% of maximum
0
20
40
60
80
100
08:30
10:30
12:30
14:30
16:30
18:30
20:30
Time (h)
Control
Full N2

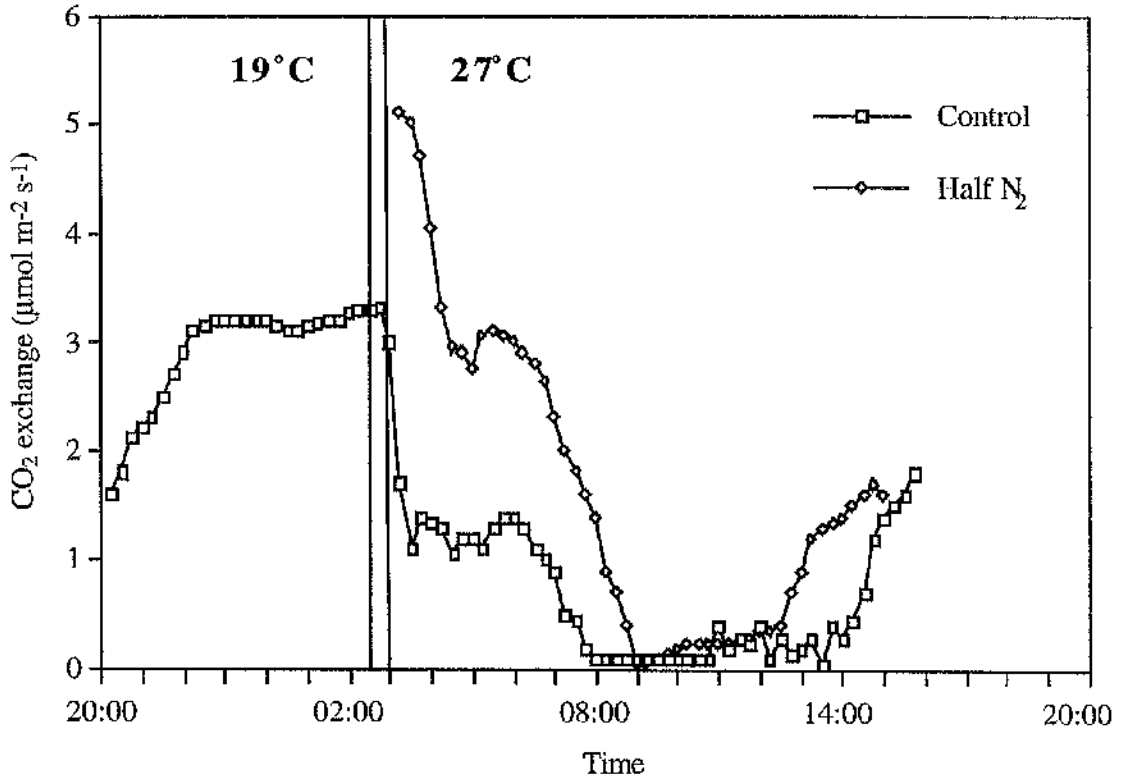
Figure 5.6. Modulation of net CO_2 assimilation rates and malate accumulation by a nocturnal temperature increase.

Leaves were exposed to ambient air (control) or enclosed in an atmosphere of N_2 for the first half of the dark period to prevent malate accumulation (half N_2) and subjected to an 8°C rise in temperature (19-27°C) between 02:30-03:00 h. The N_2 treated leaves were subsequently exposed to ambient air and the temperature remained at 27°C for the duration of the dark and subsequent photoperiod.

(**A**) Rates of net CO_2 uptake in leaves under the two treatments with each gas exchange curve representative of 3 replicate runs with SE < 10% of mean.

(**B**) Malate content was measured in leaves subjected to the above treatments with each point the mean of 3 replicates with SE < 10% of mean. The solid bar on the x-axis represents the period of darkness.

A


19°C
27°C
Control
Half N_2
CO_2 exchange (µmol m^{-2} s^{-1})
0
1
2
3
4
5
6
20:00
02:00
08:00
14:00
20:00
Time

B

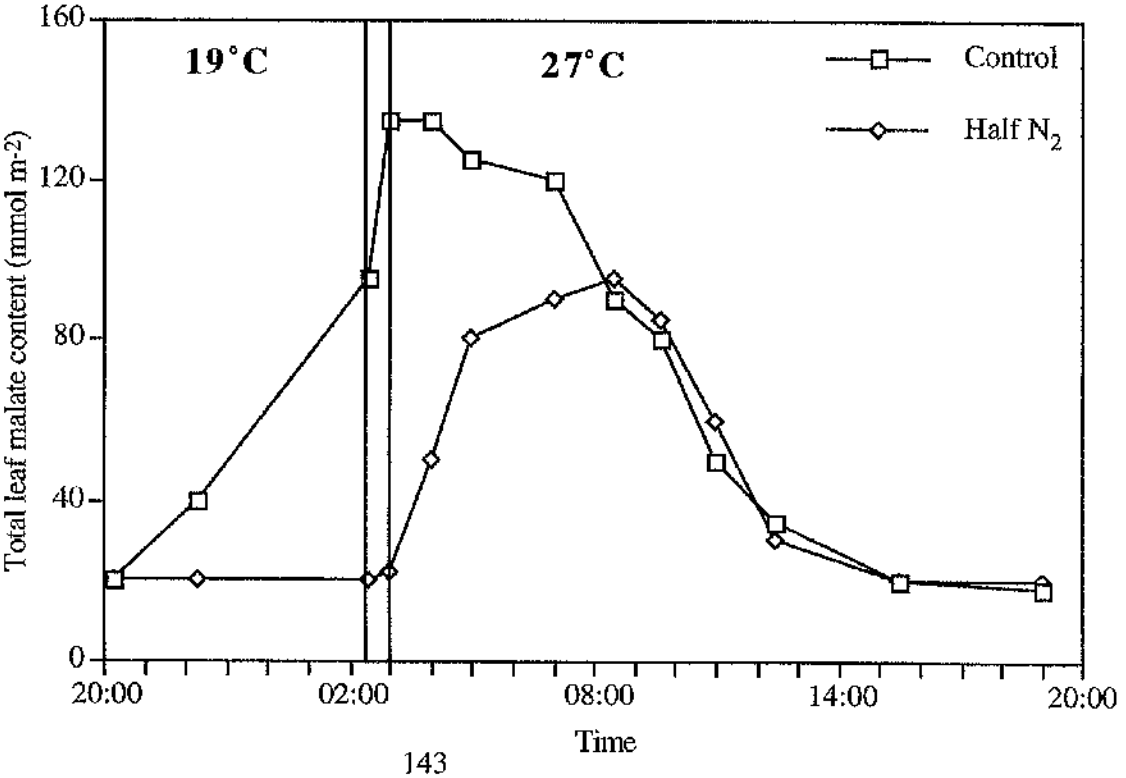

19°C
27°C
Control
Half N_2
Total leaf malate content (mmol m^{-2})
0
40
80
120
160
20:00
02:00
08:00
14:00
20:00
Time

Figure 5.7. Modulation of apparent K_i of PEPc for L-malate, PEPc kinase activity and translatable mRNA by an increase in temperature at night.

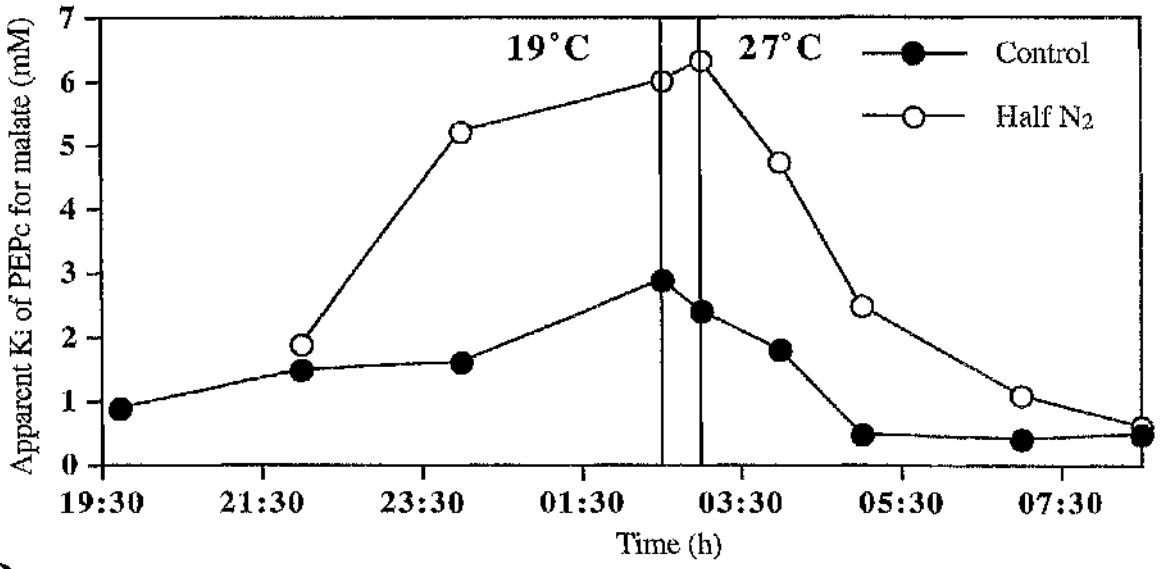
Leaves which were maintained in ambient air or in an atmosphere of N_2 to prevent malate accumulation were subjected to an 8°C rise in temperature (19-27°C) in the middle of the dark period (from 02:30-03:00h). The temperature remained at 27°C for the remainder of the dark period and subsequent photoperiod. The N_2 treated leaves were transferred to ambient air at 03:00 h, immediately following the temperature increase and samples for PEPc and PEPc kinase assays, and RNA isolation were taken simultaneously from the same leaves at intervals over the dark period. Kinase activity and translatable mRNA values are expressed as a percentage of the maximum reached during the 13 h dark period

(**A**) Variation in the apparent K_i of PEPc for L-malate in rapidly desalted extracts prepared from control and half N_2-treated leaves.

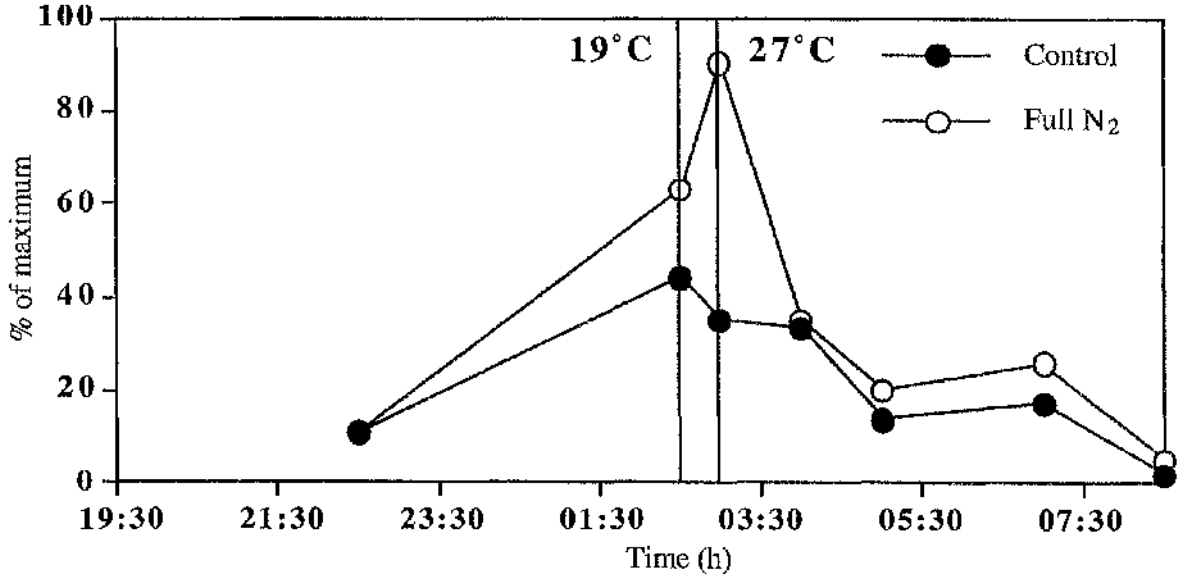
(**B**) Variation in PEPc kinase activity in rapidly desalted extracts prepared from control and half N_2-treated leaves.

(**C**) Variation in PEPc kinase translatable mRNA levels in control and half N_2-treated leaves.

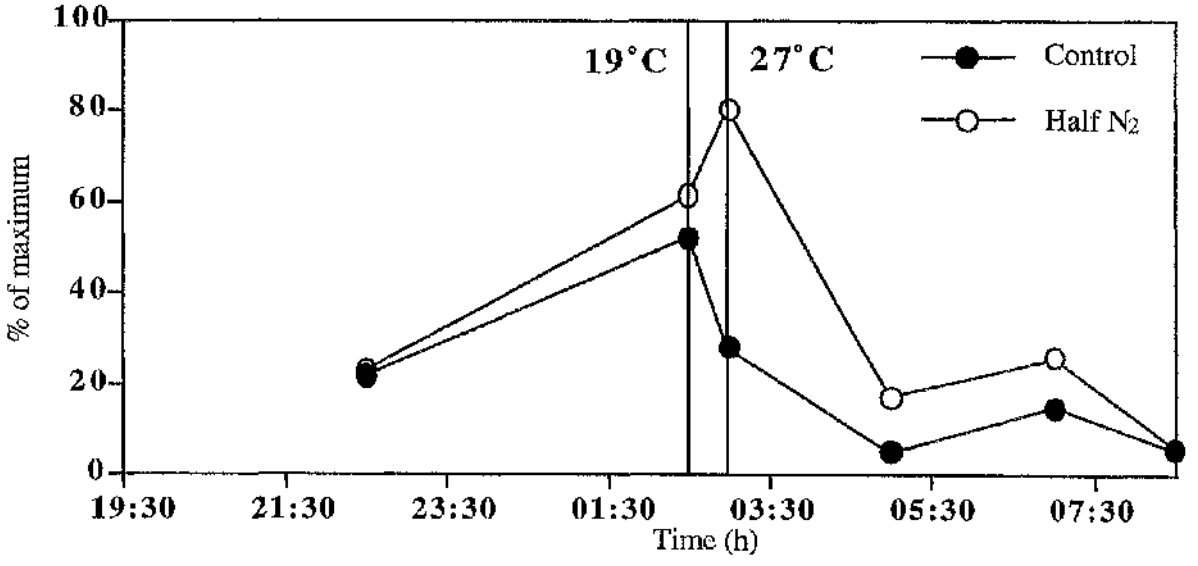
A


19°C
27°C
Control
Half N2
Apparent Ki of PEPc for malate (mM)
0
1
2
3
4
5
6
7
19:30
21:30
23:30
01:30
03:30
05:30
07:30
Time (h)

B


19°C
27°C
Control
Full N2
% of maximum
0
20
40
60
80
100
19:30
21:30
23:30
01:30
03:30
05:30
07:30
Time (h)

C


19°C
27°C
Control
Half N2
% of maximum
0
20
40
60
80
100
19:30
21:30
23:30
01:30
03:30
05:30
07:30
Time (h)

Chapter 6

The regulation of PEPc kinase translatable mRNA by light in maize and barley

6.1 Introduction

In the leaves of C_4 plants, primary CO_2 fixation occurs in the mesophyll cells and is catalysed by a specific isoform of PEPc. The malate which is subsequently formed is transported to the bundle-sheath cells where it is decarboxylated. The CO_2 is then refixed by Rubisco in the Calvin cycle which is powered by photosynthetic energy. In C_3 plants, PEPc performs a range of housekeeping functions. Specific isoforms are involved in the anaplerotic provision of TCA cycle intermediates for amino acid biosynthesis, the regulation of stomatal aperture, seed formation and germination, fruit ripening, and C_4-acid formation in the nitrogen-fixing nodules of legume roots (Chollet *et al.*, 1996; Du *et al.*, 1997; Osuna *et al.*, 1996; Zhang *et al.*, 1995). All of the C_4 and C_3 isoforms of PEPc which have been sequenced possess the N-terminal serine that is the target of PEPc kinase (Vidal and Chollet, 1997). Additionally, PEPc kinase activity has been detected in desalted extracts from the leaves of at least four species of C_4 plant, plus *Sorghum* roots, and the leaves of at least three species of C_3 plant (Duff and Chollet, 1995; Giglioli-Guivarc'h *et al.*, 1996; Jiao *et al.*, 1991a; Li *et al.*, 1996; Pacquit *et al.*, 1993; Smith *et al.*, 1996). PEPc kinase has also been detected in *Vicia faba* and *Comellina communis* guard cells, soybean root nodules, banana fruits and wheat seeds (Du *et al.*, 1997; Law and Plaxton, 1997; Nelson, 1994; Osuna *et al.*, 1996; Zhang *et al.*, 1995).

In contrast to the circadian-regulation of PEPc kinase activity in CAM plants which has already been described in chapters 3, 4 and 5, the equivalent enzyme in C_4 and C_3 leaf tissue is induced by light (Chollet *et al.*, 1996). The C_3 enzyme has also been shown to be induced by nitrate re-supply in nitrogen-limited wheat leaves (Duff and Chollet, 1995).

The light induction of PEPc kinase in C_4 plants has been the subject of intensive research over the last ten years. Both calcium-dependent and independent kinases capable of phosphorylating C_4-PEPc have been described. It is now widely accepted that it is the calcium-independent kinase which is induced in response to light and is responsible for

altering the K_i of PEPc for malate (Vidal and Chollet, 1997). The light-mediated increase in the activity of this calcium-independent PEPc kinase requires protein synthesis (Jiao *et al.*, 1991a). This implies that either the kinase itself, or a secondary component, must be synthesized *de novo*. A number of other steps in the C_4 signal transduction cascade have been determined using a variety of specific inhibitors of cellular processes. These steps include: the movement of a photosynthetically derived signal (possibly 3-PGA) from the bundle sheath cells to the mesophyll, which is thought to cause the alkalinisation of the mesophyll cytosol; an increase in cytosolic Ca^{2+} concentration, probably due to its release from the vacuole; stimulation of a Ca^{2+}/calmodulin-dependent protein kinase; and protein synthesis (Bakrim *et al.*, 1992; Giglioli-Guivarc'h *et al.*, 1996; Pierre *et al.*, 1992; Vidal and Chollet, 1997). The exact order of these events and the number of times each one occurs is unknown. Despite all this detailed knowledge of possible upstream events, no evidence exists to demonstrate whether the increase in the activity of PEPc kinase is caused by an alteration in its transcription, translation, turnover or post-translational state.

In C_3 plants, PEPc kinase activity is induced by light and/or the re-supply of nitrate (Duff and Chollet, 1995; Li *et al.*, 1996; Smith *et al.*, 1996). The kinase is calcium-independent and its induction by light is blocked by protein synthesis, glutamine synthetase and photosynthesis inhibitors (Li *et al.*, 1996). As with the C_4 system, it is not known how PEPc kinase activity is increased *in vivo*.

It was therefore vital to investigate the regulation of PEPc kinase mRNA levels in a C_4 and a C_3 plant. Maize and barley were used as representative C_4 and C_3 plants respectively. Light mediated changes in the level of kinase mRNA were investigated in these plants. Inhibitors of protein and RNA synthesis were employed to ascertain whether transcription and/or translation are necessary for the observed changes in kinase mRNA. Other pharmacological agents were utilised to dissect the signalling cascade involved in the light-induction of PEPc kinase mRNA levels.

6.2 Results

6.2.1 Regulation of PEPc kinase translatable mRNA by light in maize and barley

Preliminary experiments were carried out to ascertain whether light has any effect on the level of PEPc kinase translatable mRNA in maize and barley. For both plants, the *in vitro* translation assay was used to compare the kinase activity in translation products from samples of isolated 'dark' and 'light' RNA. The purified, dephosphorylated PEPc from *B. fedtschenkoi* can be phosphorylated by the light-activated PEPc kinase from maize and barley and was therefore used as the substrate in kinase assays. Translations were performed using total RNA for barley, but, in initial experiments, the efficiency of translation with total maize RNA was very low (this was later overcome by using very high concentrations (800 μg/ml) of total RNA). However, maize poly $(A)^+$ RNA directed protein synthesis efficiently. Figure 6.1 shows that for both maize and barley, mRNA coding for PEPc kinase is significantly increased in the light compared to the dark. Parts C and D of figure 6.1 show that there are only minor differences between the *in vitro* synthesized proteins produced by samples of 'light' and 'dark' RNA from maize and barley. However, for both maize and barley, the total amounts of protein synthesized in the 'light' and 'dark' were similar, as judged by the incorporation of ^{35}S-radioactivity into trichloroacetic acid-precipitable protein.

As demonstrated previously for *B. fedtschenkoi*, the kinase activity generated in the translations is Ca^{2+}-independent (figure 6.2). Kinase activity in 'dark' and 'light' translation products was consistently slightly higher in the presence of EGTA, emphasising that the signal is produced by a calcium-independent kinase. The omission of radioactive ATP from some of the kinase assays allowed the degree of ^{35}S-Met labelling of newly synthesised PEPc in the translations to be ascertained. Barley RNA synthesised no detectable ^{35}S-Met labelled PEPc (figure 6.2). However, maize mRNA directed the synthesis of ^{35}S-Met labelled PEPc from both the 'light' and 'dark' mRNA samples. In fact, the labelling of *de novo* synthesised, ^{35}S-Met labelled PEPc in maize 'dark' mRNA translations was equivalent to the total labelling detected in kinase assays on the same translation products which contained radioactive ATP (figure 6.2). This indicates that

PEPc kinase mRNA is not detectable in the dark in maize because the observed labelling is accounted for entirely by the ^{35}S-Met portion of the signal. However, in the light, the labelling of PEPc in the plus radioactive ATP assays is tenfold higher than that in the absence of radioactive ATP.

The abundance of PEPc kinase mRNA, as judged by this assay, is lower in barley than in either maize, *B. fedtschenkoi* or *K. daigremontiana*. The amount of kinase mRNA approximately doubled in the light in barley, and increased from zero (infinitely) in the light in maize. Thus the data indicate that the light-induced increase in PEPc kinase activity in both C_4 and C_3 plants is due, at least in part, to a light-induced increase in the level of PEPc kinase translatable mRNA.

6.2.2 The light induction of PEPc kinase mRNA in maize and barley requires transcription but not translation

Having established that the level of translatable mRNA for PEPc kinase increases in response to light in maize and barley it was logical to investigate how this increase in translatable mRNA is mediated. For example, it was important to determine whether the observed increase in kinase mRNA requires transcription and/or translation, as has been established for *B. fedtschenkoi*. Initially, the influence of two types of RNA and protein synthesis inhibitors on the light-induced change in the L-malate sensitivity of PEPc was examined. A range of concentrations of all four inhibitors were supplied to detached maize leaves in the dark and the leaves were then subjected to 3 h of illumination. The percentage inhibition, by 1 mM L-malate, of the PEPc in rapidly desalted extracts made from these treated leaves was determined (figure 6.3). This allowed the establishment of whether each inhibitor had any effect on the decrease in the malate sensitivity of PEPc and, if so, the minimum effective concentration of each inhibitor. These minimum concentrations were then used to investigate the effect of each type of inhibitor on the level of PEPc kinase translatable mRNA in leaves illuminated for 3 h. Both 500 μM actinomycin D and 500 μM cordycepin (RNA synthesis inhibitors) partially blocked the light-induction of PEPc kinase mRNA in detached maize leaves. This concentration also inhibited the decrease in the L-

malate sensitivity of PEPc (figure 6.4). However, at a concentration of 50 μM both RNA synthesis inhibitors had no detectable effect.

Interestingly, whilst 100 μM cycloheximide and 1 mM puromycin (protein synthesis inhibitors) blocked the decrease in the L-malate sensitivity of PEPc, they both caused an accumulation of PEPc kinase translatable mRNA over and above the light control level (Figure 6.5). This data demonstrates that both types of protein synthesis inhibitor block the translation of PEPc kinase mRNA into PEPc kinase *in vivo*, which prevents the kinase from changing the malate sensitivity of PEPc. This also causes an accumulation of the mRNA. The possible reasons for this observed accumulation will be covered in the discussion.

As a control for these inhibitor treatments Northern analysis was carried out on the same RNA samples in order to ascertain whether the steady-state expression of other C_4 photosynthetic genes was also perturbed in a similar manner. The expression of malic enzyme (ME) and pyruvate: orthophosphate di-kinase (PPDK) was examined (figs. 6.4 and 6.5). Light induces both genes and this induction is diminished by 500 μM, but not 50 μM, actinomycin D and cordycepin. This mirrors the situation for PEPc kinase mRNA (figure 6.4). However, both of the protein synthesis inhibitors block the increase in the steady-state level of ME and PPDK mRNA whilst they cause PEPc kinase mRNA to accumulate (figure 6.5). Hence, the inhibition of light-induced increases in the mRNA levels for other C_4 photosynthetic genes does not disrupt the light-induction, and excess accumulation, of PEPc kinase mRNA.

The same inhibitors were applied to detached barley leaves to investigate how they influence the light induction of PEPc kinase translatable mRNA in a C_3 plant. As observed with maize, RNA synthesis inhibitors block the light-induction of PEPc kinase mRNA in barley, but protein synthesis inhibitors cause kinase mRNA to accumulate above light control levels (figure 6.6). Northern analysis on the level of expression of the small subunit of Rubisco (rbcS) in the same RNA samples shows that this gene is induced by light and that the light induction is blocked by both RNA and protein synthesis inhibitors (figure 6.6). Again it seems that, whilst protein synthesis inhibitors block the light-

induction of the rbcS gene, they cause the light-induced accumulation of PEPc kinase translatable mRNA.

It is therefore evident that, for both maize and barley, the light-induction of PEPc kinase translatable mRNA requires a transcription event, whilst translation is not necessary. However, translation is required for the increase in both kinase activity and the K_i of PEPc for L-malate, and the transcript levels of ME and PPDK. This data strongly suggests that the light-induction of PEPc kinase activity in both C_4 and C_3 plants requires transcription of the PEPc kinase gene itself. Complete confirmation of this and analysis of the extent to which transcription is involved in the *in vivo* regulation of the enzyme awaits the cloning of the PEPc kinase cDNA.

6.2.3 Some possible elements in the signal transduction cascade between light and PEPc kinase translatable mRNA in maize

In an attempt to ascertain some of the other elements in the light signal transduction cascade which mediates an increase in the level of PEPc kinase translatable mRNA, a variety of inhibitors were supplied to detached maize leaves. A range of concentrations were tested for each inhibitor in order to determine whether they had any effect on the induction of PEPc kinase and if so what the minimum effective concentration was. Neither staurosporin, an inhibitor of protein kinases nor the calcium channel blocker Bay K8644 had any detectable effect on the light-induced decrease in the L-malate sensitivity of PEPc at the concentrations used (figure 6.7). However, the calcium-calmodulin antagonist W7 and the protein phosphatase inhibitors okadaic acid and cantharidin were all found to inhibit the light-induced increase in PEPc kinase translatable mRNA (figure 6.8). W7 also inhibited the decrease in the L-malate sensitivity of PEPc whilst cantharidin did not. In fact, cantharidin caused the L-malate sensitivity of PEPc to decrease more than in light controls (figure 6.8). Thus conditions were discovered where, after 3 h of illumination, the PEPc in the cantharidin treated leaves was in a highly phosphorylated, malate insensitive form and yet the level of PEPc kinase translatable mRNA was at dark control levels.

This appeared to be anomalous because under all previously examined conditions PEPc kinase mRNA was high when the PEPc was in its malate insensitive, phosphorylated

form. However, a possible explanation for this anomaly concerns the time course of changes in kinase mRNA. The mRNA may have risen early during the 3 hour period of illumination but subsequently decreased to the level detected at 3 hours. This would mean that PEPc kinase was active during the early part of the illumination period. PEPc would become phosphorylated and then be maintained in a highly phosphorylated, malate insensitive state due to inhibition by cantharidin of the type 2A protein phosphatase responsible for dephosphorylating PEPc *in vivo*.

The data in figure 6.8 indicate the involvement of a calcium-calmodulin interaction, possibly mediating its effect through a calcium-dependent protein kinase (CDPK), and a protein dephosphorylation event upstream of the increase in the level of PEPc kinase mRNA. These events are in addition to, and probably upstream of, the transcription step already mentioned. A protein kinase inhibitor and a calcium channel agonist had no effect at the concentrations tested. However, this does not rule out the involvement of protein phosphorylation and Ca^{2+} movements in the signal cascade, as no controls were performed to demonstrate that these inhibitors had actually inhibited their target processes.

6.3 Discussion

The results obtained in this part of the study, for the C_4 plant maize and the C_3 plant barley, further demonstrate that the *in vitro* translation assay provides valid data over a whole range of higher plant taxa. This includes representatives of all 3 major forms of photosynthetic metabolism in which PEPc plays diverse roles. This emphasises the extreme sensitivity of the assay and its ability to detect accurately when PEPc kinase mRNA is present or absent, regardless of the plant system under examination. As already described for the CAM system, the level of kinase mRNA in C_4 and C_3 plants changes in concert with changes in kinase activity and the K_i of PEPc for malate. In C_4 and C_3 plants all three parameters increase in response to light and not the circadian clock. However, the light increase can be blocked by a number of inhibitors of specific cellular processes. This provides evidence about the signal transduction cascade which transmits the light signal through the cell and ultimately causes an increase in kinase translatable mRNA.

Work published during the course of this study indicated that RNA synthesis is not necessary to obtain a light- and weak base-dependent increase in PEPc kinase activity, and a concomitant decrease in the L-malate sensitivity of PEPc, in mesophyll protoplasts from the C_4 plant *Digitaria sanguinalis* (Giglioli-Guivarc'h *et al.*, 1996). This result implies that the increase in PEPc kinase activity in *D. sanguinalis* protoplasts may result from increased translation of pre-existing mRNA. The translation assay employed in the present study involves isolated total or mRNA which is heated to 67°C for 10 min immediately before translation. Hence, if the increases in PEPc kinase translatable mRNA in maize shown in the figures of this chapter are due to increased translatability, this effect must be mediated by modification of the RNA, rather than by protein/RNA interactions or effects on the secondary structure of the RNA. However, it must be noted that Giglioli-Guivarc'h *et. al.* (1996) included no positive control to demonstrate the effectiveness of the RNA synthesis inhibitors used in their study. Furthermore, in the present study the use of one of the inhibitors (actinomycin D) at the same concentration used by Giglioli-Guivarc'h *et. al.* (1996) had no detectable effect, whereas this inhibitor was effective in preventing increases in PEPc kinase mRNA and activity at higher concentrations (see figures 6.3, 6.4 and 6.5). Admittedly, in the present study, the inhibitor was supplied to intact, detached leaves and not isolated protoplasts so it is understandable that a significantly higher concentration was necessary to cause inhibition. However, without positive controls it is difficult to interpret anything from the negative result obtained by Giglioli-Guivarc'h *et. al.* (1996) because the inhibitors may not have been inhibitory to RNA synthesis in their protoplasts at the concentration used.

The data obtained in this study using RNA and protein synthesis inhibitors on the leaves of a C_4 and a C_3 plant suggest the clear possibility that PEPc kinase is transcriptionally regulated in response to light. Unlike the circadian clock-controlled CAM system, protein synthesis is not required for the increase in kinase mRNA whilst RNA synthesis is. It remains possible that the *in vitro* translation system translates both a putative kinase activator and PEPc kinase itself and therefore reconstitutes the events that are blocked *in vivo* by protein synthesis inhibitors. This would mean that the *in vitro*

translation of RNA from light-induced, protein synthesis inhibitor-treated leaves would generate both the activator and the kinase and hence active kinase, overcoming the blockage caused by the inhibitor *in vivo*. However, this scenario is very unlikely, because attempts to increase the activity of PEPc kinase in extracts by mixing experiments and/or addition of potential co-factors (e.g. mixing extracts of *B. fedtschenkoi* or maize leaves prepared during the day and night, plus and minus ATP) have proved negative (P.J. Carter, G.A. Nimmo and H.G. Nimmo, unpublished). Moreover, the appearance of PEPc kinase activity during *in vitro* translation is approximately linear with time for 40-60 min, after a short lag that probably results from the time required to translate mRNAs. A linear increase would not be expected if there were a cascade system in which PEPc kinase is activated by an additional component. Thus, the most feasible and likely explanation of the results obtained in this study is that PEPc kinase is regulated transcriptionally in response to light in C_4 and C_3 plants.

That protein synthesis inhibitors cause PEPc kinase to accumulate above control levels in response to light is an interesting effect in itself. All evidence suggests that PEPc kinase mRNA and activity are rapidly turned over *in vivo*. The exact level of kinase mRNA detected in a given RNA sample is a result of the balance between its rate of synthesis and its rate of degradation. For example, in maize following illumination the level of PEPc kinase mRNA may increase due to an increase in the rate of its transcription whilst the rate of degradation remains unchanged or vice versa. Thus, for the level of kinase mRNA to accumulate above control levels in protein synthesis inhibitor treated leaves, the rate of transcription must increase or the rate of degradation must decrease. If, for example, the rate of transcription of the gene increases, this could be due to disruption by the protein synthesis inhibitor of the transmission of a signal which controls the rate of transcription. If the rate of degradation of the kinase mRNA is diminished, this could be because the protein synthesis inhibitor blocks the synthesis of a specific RNase. Whether either of these possible hypotheses are correct awaits the results of future experiments.

Some of the elements in the light-induced signal transduction cascade that instigates the increase in PEPc kinase in C_4 plants have previously been dissected using both intact leaves, and isolated protoplasts and cells, from a range of C_4 species. However, the location of these processes in the signalling cascade relative to the increase in the level of PEPc kinase mRNA was not investigated due to the inability of earlier workers to measure the mRNA level. Thus, the effect of a variety of inhibitors of processes previously reported to be involved in the cascade were re-examined in the present study. W7, a calcium-calmodulin antagonist which had previously been found to block the light-induction of PEPc kinase activity was also found to inhibit the light-induction of PEPc kinase mRNA. Also two protein phosphatase inhibitors were found to inhibit the light-induction of PEPc kinase mRNA whilst a protein kinase inhibitor and a calcium-channel blocker were both found to have no effect at the concentrations used. This is the first evidence suggesting the involvement of a dephosphorylation event in the signalling cascade. Protein kinase inhibitors and vacuolar calcium channel blockers have previously been found to block the light-induction of PEPc kinase in another C_4 system (Giglioli-Guivarc'h *et al.*, 1996). Although it is possible that the protein kinase and calcium channel steps are downstream of the level of PEPc kinase mRNA, it is more likely that the inhibitors used in this study were simply ineffective at the concentrations used. This is probably due to the inhibitors failing to reach the target mesophyll cells at high enough concentrations. This problem may be overcome by employing either isolated mesophyll protoplasts or a cell culture approach. Other inhibitors which merit future investigation include the calcium channel agonists TMB8, nifedipine and diltiazem, and the photosynthesis inhibitors DCMU, methyl viologen, isocil, DL-glyceraldehyde and gramicidin. The use of these inhibitors should allow the dissection of which type of calcium channel and which parts of photosynthesis may be involved in the light-induction of PEPc kinase mRNA in maize. Similar inhibitor experiments should also be performed on the C_3 leaf system with the addition of feeding glutamine, and the glutamine synthetase inhibitors methionine sulphoximine and phosphoinothricine. These latter treatments should allow dissection of the involvement of glutamine as a signal metabolite in the C_3 signal

transduction network, and thus shed light on the cross-talk between light and nitrate supply in the regulation of PEPc kinase mRNA.

Figure 6.1. Effect of light on PEPc kinase translatable mRNA in maize and barley.

The figure shows phosphoimages of PEPc phosphorylated *in vitro* by translation products from maize poly $(A)^+$ RNA and barley total RNA. The figures given below each band represent the relative intensity of each band.

(**A**) PEPc kinase activity (^{32}P-labelling of PEPc) using the *in vitro* translation products from maize poly $(A)^+$ RNA purified from 20 μg of total RNA.

Lane 1, no RNA control.

Lane 2, poly $(A)^+$ RNA isolated from mature leaves illuminated for 3 h.

Lane 3, poly $(A)^+$ RNA isolated from mature leaves darkened for 3 h.

(**B**) PEPc kinase activity (^{32}P-labelling of PEPc) using the *in vitro* translation products from barley total RNA.

Lane 1, no RNA control.

Lane 2, barley total RNA isolated from leaves illuminated for 3 h.

Lane 3, barley total RNA isolated from leaves darkened for 10 h.

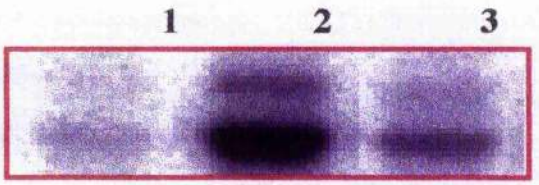
(**C**) *In vitro* translation products ([^{35}S]Met labelled) corresponding to panel (A).

The lanes correspond to those listed above for panel (A).

(**D**) *In vitro* translation products ([^{35}S]Met labelled) corresponding to panel (B).

The lanes correspond to those listed above for panel (B).

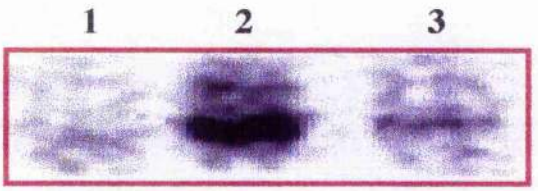
A



PEPc kinase translatable mRNA

Relative intensity 7 100 22

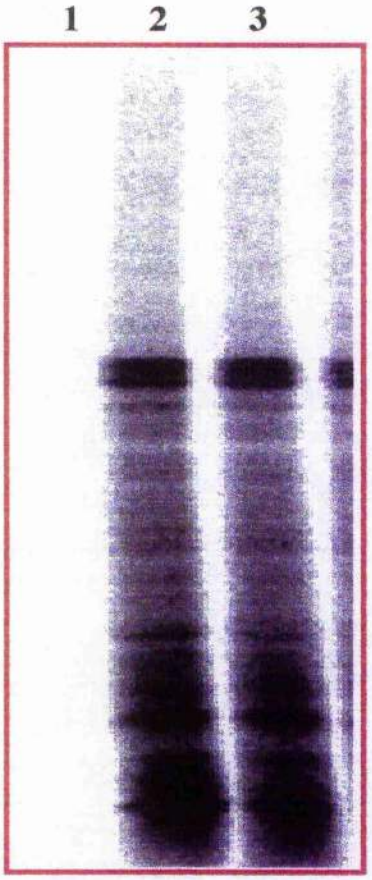
B



PEPc kinase translatable mRNA

Relative intensity 0 100 57

C



D

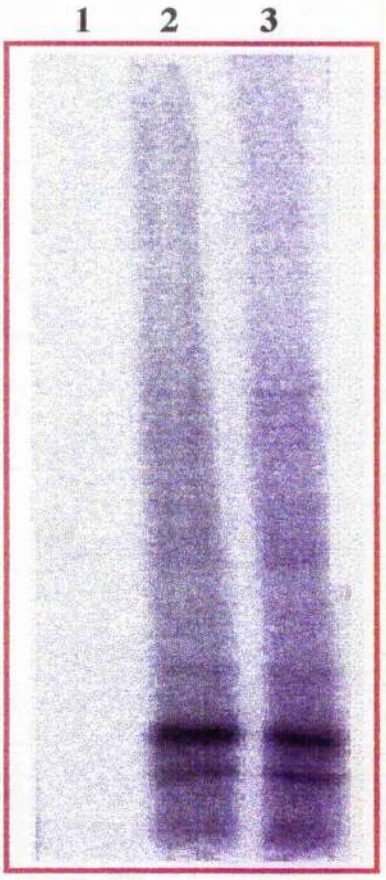


Figure 6.2. The PEPc kinase activity synthesized during the *in vitro* translation of maize poly (A)$^+$ RNA and barley total RNA is Ca^{2+}-independent and the labelling detected in the PEPc kinase assays is not a result of ^{35}S-Met labelling of *de novo* synthesized PEPc in the translation products.

The figure shows phosphoimages of PEPc phosphorylated *in vitro* by translation products from maize poly (A)$^+$ RNA and barley total RNA.

(**A**) PEPc kinase activity (^{32}P-labelling of PEPc) using the *in vitro* translation products from maize poly (A)$^+$ RNA purified from 20 µg of total RNA.

Lane 1, poly (A)$^+$ RNA isolated from mature leaves illuminated for 3 h plus 0.25 mM EGTA.

Lane 2, poly (A)$^+$ RNA isolated from mature leaves illuminated for 3 h minus 0.25 mM EGTA.

Lane 3, poly (A)$^+$ RNA isolated from mature leaves illuminated for 3 h assayed in the absence of ^{32}P-ATP .

Lane 4, poly (A)$^+$ RNA isolated from mature leaves darkened for 3 h plus 0.25 mM EGTA.

Lane 5, poly (A)$^+$ RNA isolated from mature leaves darkened for 3 h minus 0.25 mM EGTA.

Lane 6, poly (A)$^+$ RNA isolated from mature leaves darkened for 3 h assayed minus ^{32}P-ATP.

(**B**) PEPc kinase activity (^{32}P-labelling of PEPc) using the *in vitro* translation products from barley total RNA.

Lane 1, barley total RNA isolated from leaves illuminated for 3 h plus 0.25 mM EGTA.

Lane 2, barley total RNA isolated from leaves illuminated for 3 h minus 0.25 mM EGTA.

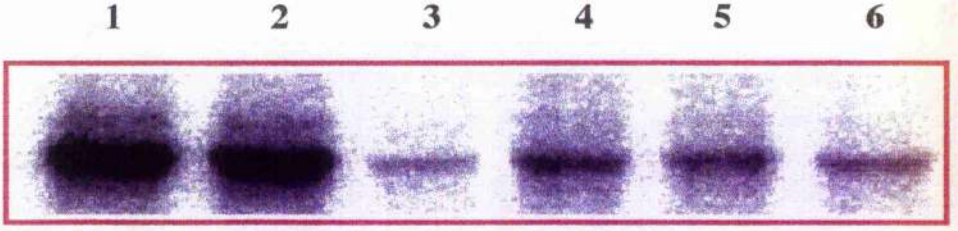
Lane 3, barley total RNA isolated from leaves illuminated for 3 h assayed minus ^{32}P-ATP.

Lane 4, barley total RNA isolated from leaves darkened for 10 h plus 0.25 mM EGTA.

Lane 5, barley total RNA isolated from leaves darkened for 10 h minus 0.25 mM EGTA.

Lane 6, barley total RNA isolated from leaves darkened for 10 h assayed minus ^{32}P-ATP.

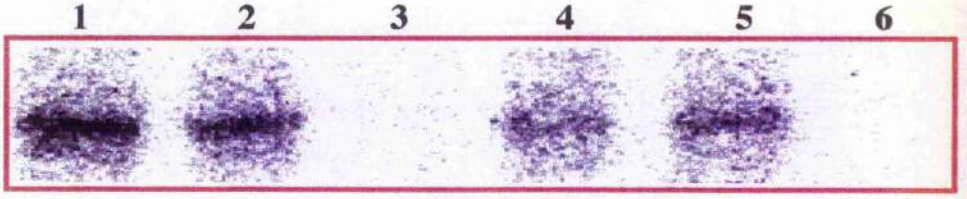
A. Maize



100 83 10 16 12 17

Relative intensity

B. Barley



100 82 0 57 67 0

Relative intensity

Figure 6.3. Influence of protein and RNA synthesis inhibitors on the light-mediated decrease in the L-malate sensitivity of PEPc in desalted extracts from maize leaves. Fully expanded maize leaves were detached from the plant and placed in the indicated concentrations of control solutions or inhibitors in darkness for 3 h. Next, dark controls were sampled immediately and frozen in liquid nitrogen. The remainder of the leaves were illuminated (~700 μmoles m^{-2} s^{-1}) for 3 h prior to sampling. Rapidly desalted extracts were prepared from the leaves and assayed for the L-malate sensitivity of PEPc. The light regime is indicated by the shaded bar along the top of the graph and the mean percentage inhibition of PEPc by 1 mM L-malate in the dark and light controls is indicated to assist in visualizing the effect of each inhibitor.

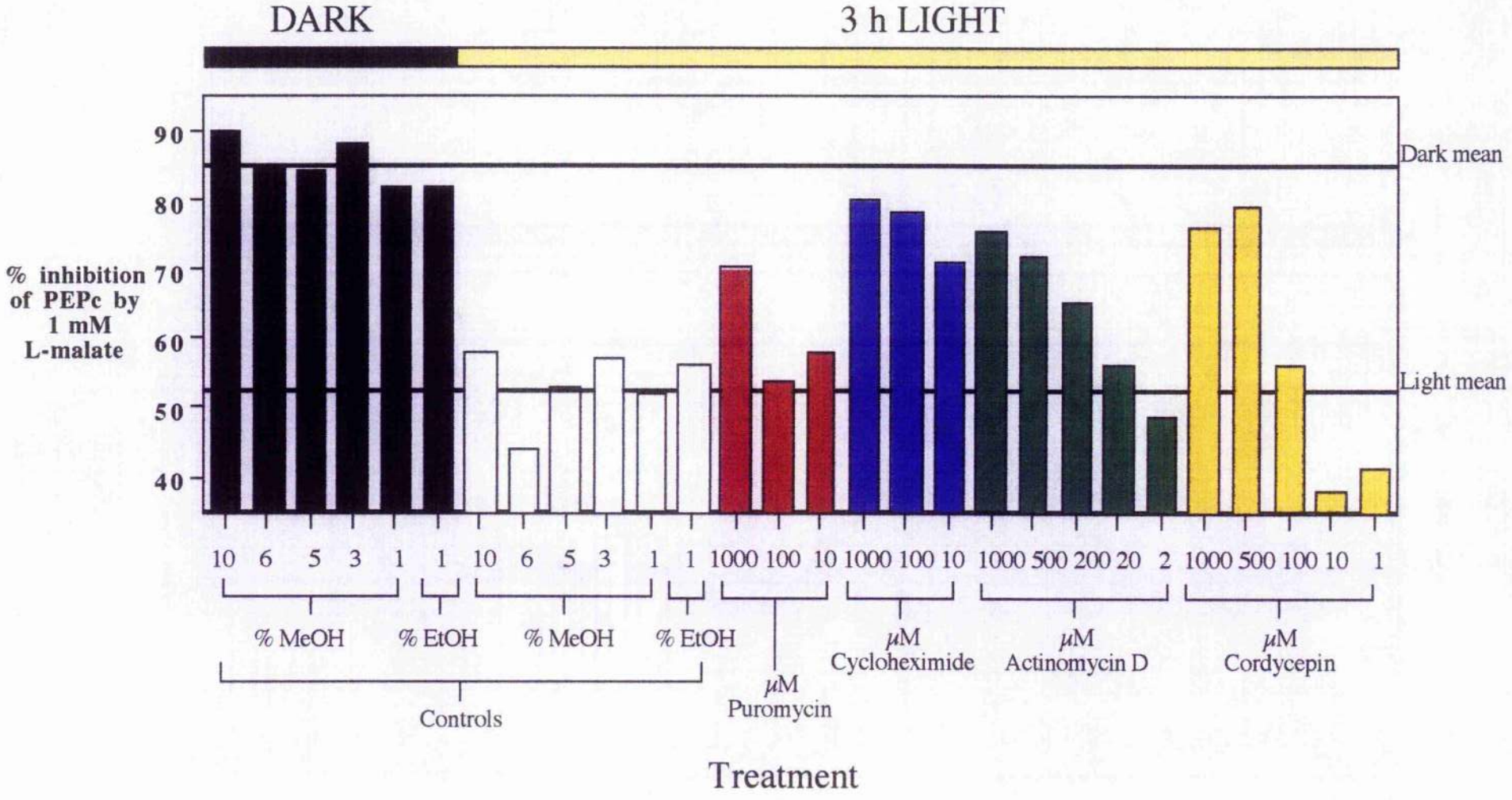

DARK
3 h LIGHT
% inhibition of PEPc by 1 mM L-malate
90
80
70
60
50
40
Dark mean
Light mean
10 6 5 3 1 1 10 6 5 3 1 1 1000 100 10 1000 100 10 1000 500 200 20 2 1000 500 100 10 1
% MeOH
% EtOH
% MeOH
% EtOH
Controls
μM Puromycin
μM Cycloheximide
μM Actinomycin D
μM Cordycepin
Treatment

Figure 6.4. RNA synthesis is required for the light-induction of PEPc kinase translatable mRNA in maize.

Duplicate detached maize leaves were treated with RNA synthesis inhibitors or control solutions as described for figure 6.3 and sampled for RNA isolation. For each sample, 20 μg of total RNA was *in vitro* translated and the translation products were assayed for PEPc kinase activity. As a control for the inhibitor treatments, 10 μg of the same RNA samples were analysed by Northern blotting for the steady state transcript levels of the light-inducible C_4 photosynthesis genes *me* and *ppdk*.

(**A**) PEPc kinase translatable mRNA levels in light-induced leaves supplied with RNA synthesis inhibitors.

Lane 1, 3 h dark plus 10 % methanol control.

Lane 2, 3 h light plus 10 % methanol control.

Lane 3, 3 h light plus 50 μM actinomycin D.

Lane 4, 3 h light plus 500 μM actinomycin D.

Lane 5, 3 h light plus 50 μM cordycepin.

Lane 6, 3 h light plus 500 μM cordycepin.

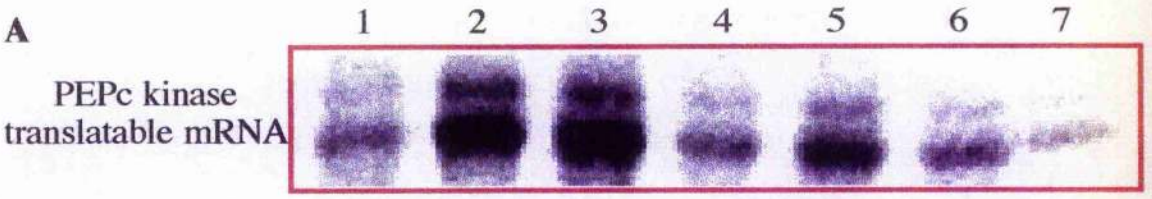
Lane 7, no RNA control.

(**B**) Northern blot probed using *me*.

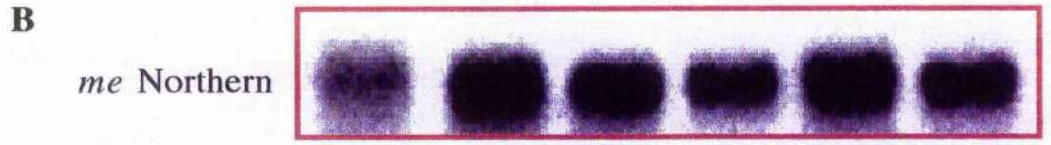
Lanes correspond exactly to those above in panel (A) except that lane 7 is absent.

(**C**) Northern blot probed using *ppdk*.

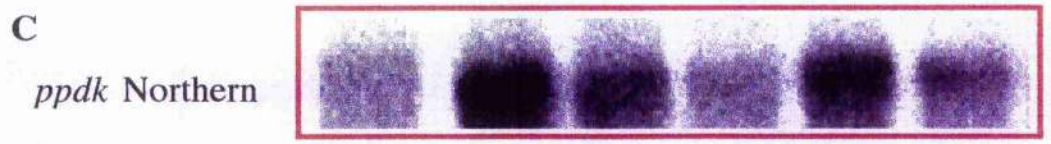
Lanes correspond exactly with those above in panels (A) and (B) except that lane 7 is absent.



Relative intensity	34	100	111	50	54	34	0
Percentage inhibition of PEPc by 1 mM L-malate	85	42	44	68	47	72	



Relative intensity	43	100	94	63	107	76



Relative intensity	27	100	57	33	69	45

Figure 6.5. Protein synthesis is not required for the light-induction of PEPc kinase translatable mRNA in maize.

Duplicate detached maize leaves were treated with protein synthesis inhibitors or control solutions as described for figure 6.3 and sampled for RNA isolation. For each sample, 20 μg of total RNA was *in vitro* translated and the translation products were assayed for PEPc kinase activity. As a control for the inhibitor treatments, 10 μg of the same RNA samples were analysed by Northern blotting to determine the steady state transcript levels of the light-inducible C_4 photosynthesis genes *me* and *ppdk*.

(**A**) PEPc kinase translatable mRNA levels in light-induced leaves supplied with protein synthesis inhibitors.

Lane 1, 3 h dark plus 1 % ethanol control.

Lane 2, 3 h light plus 1 % ethanol control.

Lane 3, 3 h light plus 1 mM puromycin.

Lane 4, 3 h light plus 100 μM cycloheximide.

Lane 5, no RNA control.

(**B**) Northern blot probed using *me*.

Lanes correspond with those above in panel (A) except that lane 5 is absent.

(**C**) Northern blot probed using *ppdk*.

Lanes correspond with those above in panels (A) and (B) except that lane 5 is absent.

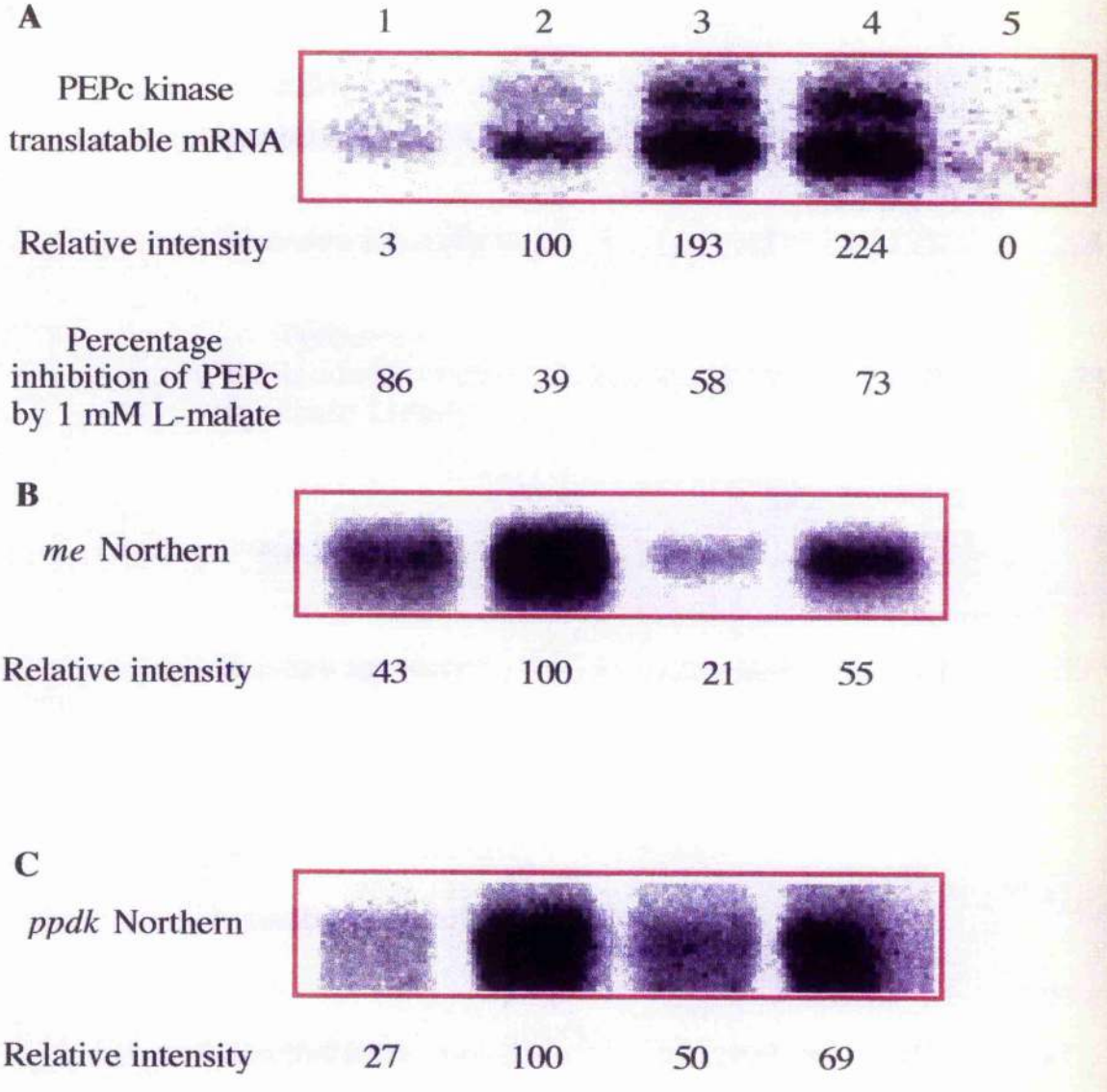

A
1
2
3
4
5
PEPc kinase translatable mRNA
Relative intensity
3
100
193
224
0
Percentage inhibition of PEPc by 1 mM L-malate
86
39
58
73
B
me Northern
Relative intensity
43
100
21
55
C
ppdk Northern
Relative intensity
27
100
50
69

Figure 6.6. The light-induction of PEPc kinase translatable mRNA in barley leaves requires RNA but not protein synthesis.

Detached barley leaves (10 days old) were placed in the appropriate control solution or solutions of protein or RNA synthesis inhibitors and kept in the dark for 3 h. The leaves were then either sampled immediately as dark controls or illuminated (~700 μmoles m^{-2} s^{-1}) for 3 h. Total RNA was isolated from the leaf samples and used to prime the PEPc kinase translatable mRNA assay. As a control for the inhibitor treatments, 10 μg of each RNA sample was also analysed by Northern blotting to determine the steady state transcript levels of the light-inducible *rbcS* gene.

(**A**) PEPc kinase translatable mRNA in light-induced barley leaves which had been pre-treated with protein and RNA synthesis inhibitors.

Lane 1, dark control.

Lane 2, 3 h light control.

Lane 3, 3 h light plus 50 μM actinomycin D.

Lane 4, 3 h light plus 50 μM cordycepin.

Lane 5, 3 h light plus 5 μM cycloheximide.

Lane 6, 3 h light plus 50 μM cycloheximide.

Lane 7, no RNA control.

(**B**) Northern blot probed with *rbcS*.

Lanes correspond with those above in panel (A) except that lane 7 is absent.

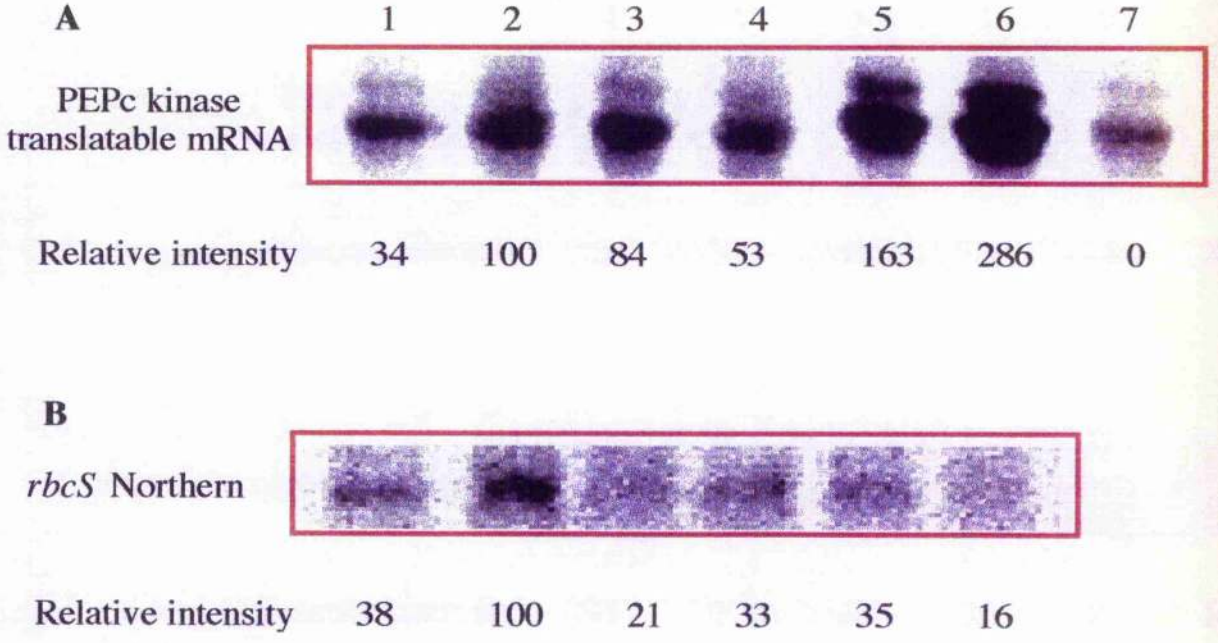

A
1 2 3 4 5 6 7
PEPc kinase translatable mRNA
Relative intensity 34 100 84 53 163 286 0
B
rbcS Northern
Relative intensity 38 100 21 33 35 16

Figure 6.7. The influence of a range of pharmacological agents on the light-mediated decrease in the L-malate sensitivity of PEPc in maize leaves.

Fully expanded maize leaves were detached from the plant and placed in the indicated concentrations of control solutions or inhibitors in darkness for 3 h. Next, dark controls were sampled immediately and frozen in liquid nitrogen. The remainder of the leaves were illuminated (~700 μmoles m^{-2} s^{-1}) for 3 h prior to sampling. Rapidly desalted extracts were prepared from the leaves and assayed for the L-malate sensitivity of PEPc. The light regime is indicated by the shaded bar along the top of the graph and the mean percentage inhibition of PEPc by 1 mM L-malate in the dark and light controls is indicated to assist in visualizing the effect of each inhibitor.

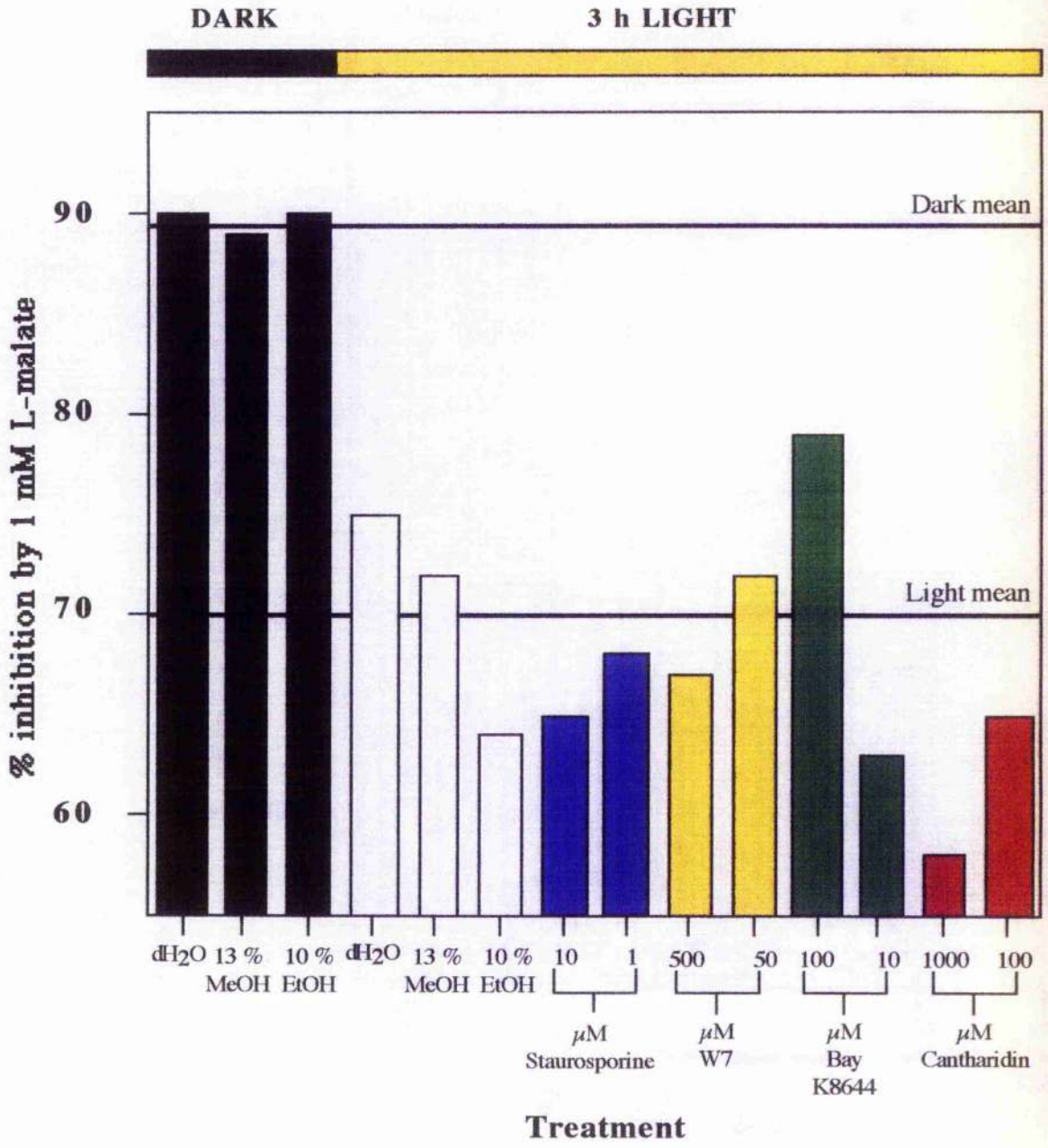

DARK
3 h LIGHT
Dark mean
Light mean
90
80
70
60
% inhibition by 1 mM L-malate
dH2O
13 % MeOH
10 % EtOH
dH2O
13 % MeOH
10 % EtOH
10
1
500
50
100
10
1000
100
μM Staurosporine
μM W7
μM Bay K8644
μM Cantharidin
Treatment

Figure 6.8. The influence of a range of pharmacological agents on the light-induction of PEPc kinase translatable mRNA in maize leaves.

Duplicate detached maize leaves were treated as described in figure 6.7, but samples were used for RNA isolation. For each sample, 20 μg of total RNA was *in vitro* translated and the translation products were assayed for PEPc kinase activity. The figures show phosphoimages of the ^{32}P-labelled PEPc bands. The relative intensity of the PEPc bands is given below each lane.

(**A**) The effect of the type 1 and type 2A protein phosphatase inhibitor okadaic acid and the calmodulin inhibitor W7 on the light-induction of PEPc kinase translatable mRNA and the decrease in the L-malate sensitivity of PEPc in maize leaves.

Lane 1, dark control.

Lane 2 dark plus 250 nM okadaic acid.

Lane 3, 3 h light control.

Lane 4, 3 h light plus 250 nM okadaic acid.

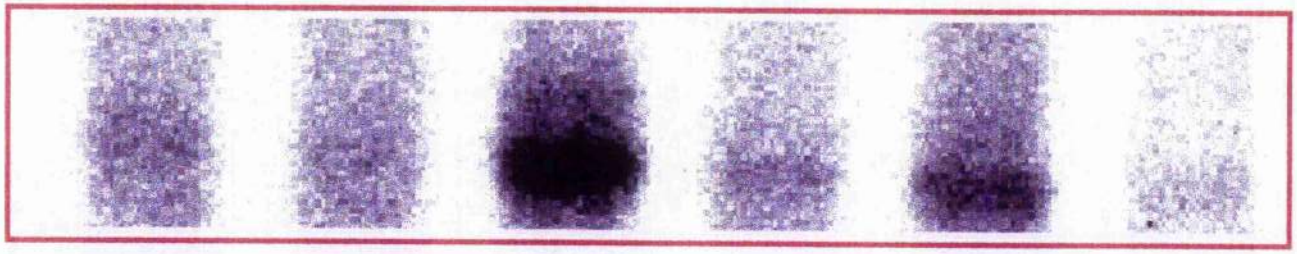
Lane 5, 3 h light plus 250 μM W7.

Lane 6, no RNA control.

(**B**) The effect of protein kinase (staurosporin), calmodulin (W7), calcium channel (Bay K8644) and protein phosphatase (cantharidin) inhibitors on the light-induction of PEPc kinase translatable mRNA and the decrease in the L-malate sensitivity of PEPc in maize leaves.

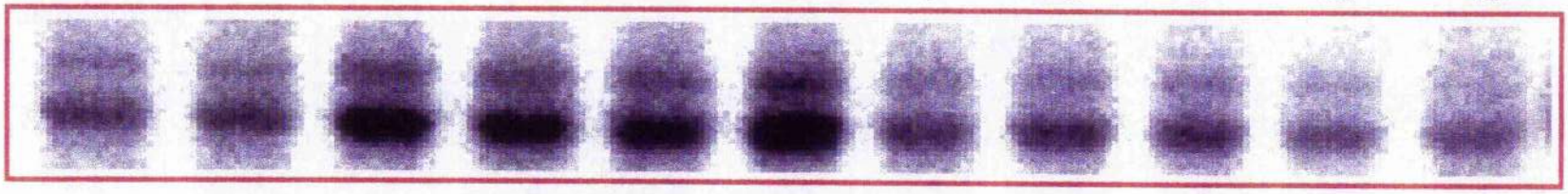
Lane 1, dark plus distilled water control. Lane 2, dark plus 13 % methanol control. Lane 3, dark plus 10 % ethanol control. Lane 4, 3 h light plus distilled water control. Lane 5, 3 h light plus 13 % methanol control. Lane 6, 3 h light plus 10 % ethanol control. Lane 7, 3 h light plus 10 μM staurosporin. Lane 8, 3 h light plus 500 μM W7. Lane 9, 3 h light plus 100 μM Bay K8644. Lane 10, 3 h light plus 1 mM cantharidin. Lane 11, no RNA control.

A



	1	2	3	4	5	6
Relative intensity	25	30	100	36	51	0
% inhibition by 1 mM L-malate	90	n.d.	48	66	n.d.	

B



	1	2	3	4	5	6	7	8	9	10	11
Relative intensity	21	21	59	100	40	59	21	21	9	0	0
% inhibition by 1 mM L-malate	90	89	90	75	72	64	65	67	79	58	

Chapter 7

General Discussion and Future Research

The regulation of higher plant PEPc by protein phosphorylation has been the subject of intensive research since its first discovery some 13 years ago (Nimmo *et al.*, 1984; Nimmo *et al.*, 1996; Vidal and Chollet, 1997). Major developments have occurred since the initial reports which have established PEPc as one of the most thoroughly understood phosphorylated plant proteins. These developments include the discovery that it is the activity of PEPc kinase, rather than PEPc phosphatase, which is regulated in response to light and the circadian clock and that the highly regulated PEPc kinase is Ca^{2+}-independent (Carter *et al.*, 1991; Li and Chollet, 1993; Li and Chollet, 1994; Li *et al.*, 1996; Wang and Chollet, 1993b). Also, the finding that all plant PEPc sequences contain the N-terminal phosphorylation site suggests that protein phosphorylation may be involved in the regulation of all the plant isoforms of PEPc, including the gymnosperm form (Lepiniec *et al.*, 1994; Relle and Wild, 1996; Vidal and Chollet, 1997). Significant insights have been made into the light signal transduction cascade which regulates PEPc kinase in C_4 plants whilst understanding of the CAM signalling processes has lagged behind (Giglioli-Guivarc'h *et al.*, 1996; Vidal and Chollet, 1997). One fundamental finding has been the discovery that the induction of PEPc kinase requires *de novo* protein synthesis in the leaves of C_3, C_4 and CAM plants (Carter *et al.*, 1991; Jiao *et al.*, 1991a; Li *et al.*, 1996). However, further analysis of this requirement for protein synthesis has been hampered by difficulties with purifying PEPc kinase to homogeneity and/or cloning the PEPc kinase gene.

Major efforts have been directed towards the purification of PEPc kinase from C_4, CAM and C_3 plants, but still there are no reports of protein sequence from these purified preparations nor has the gene been cloned, nor antibodies raised (Law and Plaxton, 1997; Li and Chollet, 1993; Li and Chollet, 1994; Li *et al.*, 1996; Wang and Chollet, 1993b; Zhang and Chollet, 1997). This has left a large gap in the understanding of PEPc kinase regulation because it is not known whether the factor which must undergo *de novo* protein synthesis is the kinase itself or a secondary component which is required for kinase

activation. One report has suggested that the light-induction of PEPc kinase in C_4 plants does not involve transcription because RNA synthesis inhibitors fail to block the light-induced decrease in the malate sensitivity of PEPc (Giglioli-Guivarc'h *et al.*, 1996). However, no evidence was included to demonstrate the effectiveness of the RNA synthesis inhibitors used and so the involvement of transcription could not be ruled out. By contrast, RNA synthesis inhibitors were found to block the circadian increase in kinase activity in a CAM plant, implicating a transcription event upstream of the *de novo* protein synthesis step (Carter *et al.*, 1996).

The aim of the work described in this thesis was to circumvent this shortfall in the understanding of PEPc kinase regulation in plants by developing a novel assay for PEPc kinase mRNA which does not require a complementary nucleic acid probe. This was achieved by translating isolated plant RNA into protein *in vitro* using a rabbit reticulocyte lysate system that possessed no detectable endogenous PEPc kinase activity. The translated proteins are assayed *in vitro* for PEPc kinase activity using the purified dephosphorylated form of PEPc as a substrate and [γ-^{32}P]ATP as the label. A series of control experiments established that the resultant labelling of the exogenous PEPc reflects the level of PEPc kinase mRNA in each RNA sample (see chapter 3). Furthermore, the kinase activity synthesized was Ca^{2+}-independent like the activity detected *in planta,* and the RNA could be size fractionated to a single size range of 0.9-1.3 Kb. This assay was used to examine the regulation of PEPc kinase mRNA levels in C_3, C_4 and CAM plants. In general, changes in the level of PEPc kinase mRNA were found to underlie changes in both PEPc kinase activity and the malate sensitivity of PEPc, and in some cases the malate content and net CO_2 assimilation of leaves. In addition, a number of interesting exceptions to this rule were discovered which increase our understanding of the complex regulatory machinery which controls PEPc in plants.

The results in chapters 4 and 6 demonstrate that transcription is required for the increase in PEPc kinase mRNA in all three photosynthetic classes, but whilst protein synthesis is also required in CAM plants, it is not necessary in C_3 and C_4 plants. The requirement for both transcription and translation for the circadian increase in kinase

mRNA in CAM plants could simply be due to inhibition of the underlying circadian oscillator. At the molecular level circadian oscillators have been found to involve an autoregulatory feedback loop in which a clock protein inhibits the transcription of its own clock gene (Page, 1994). Thus, both protein and RNA synthesis inhibitors will prevent the operation of the circadian oscillator itself. However, the involvement of transcription of the PEPc kinase gene itself cannot be ruled out for the CAM system at this stage. The fact that these inhibitors prevent the nocturnal increase in PEPc kinase mRNA in the CAM system also indicates that at least two protein synthesis events are required for the increase in kinase mRNA. One is the translation of PEPc kinase mRNA into active PEPc kinase protein. The other could be in the operation of the circadian oscillator itself and/or the translation of a component of the clock output pathway which is required to convey the signal from the level of the circadian clock through to the level of PEPc kinase mRNA.

In C_3 and C_4 plants there is only a single protein synthesis event in the light-induction of PEPc kinase activity. This can be concluded because, whilst RNA synthesis inhibitors block the light-induction of kinase mRNA and the decrease in the malate sensitivity of PEPc, protein synthesis inhibitors only blocked the latter effect. Furthermore, protein synthesis inhibitors not only did not block the light-induction of PEPc kinase translatable mRNA levels but actually caused the mRNA to accumulate to over twice the light control level. This increased accumulation of kinase mRNA suggests that, in the presence of protein synthesis inhibitors, the signal to increase the level of PEPc kinase translatable mRNA is transmitted, but some other signal which controls the maximum level of kinase mRNA is blocked. There are two possible explanations for this large accumulation of kinase mRNA. One is that, in the absence of protein synthesis inhibitors, a specific RNase is synthesized which turns over a proportion of the kinase mRNA. The second explanation is that, in addition to the feedforward signal from light which induces kinase mRNA, there is a feedback signal, possibly from a metabolite such as malate, which accumulates as a result of flux through PEPc. If the feedback signalling pathway requires *de novo* protein synthesis to function then the presence of protein synthesis inhibitors would block the feedback pathway and thus prevent any moderation of kinase mRNA

levels. Fixation of CO_2 by PEPc and the formation of malate may mediate this feedback signalling pathway because protein synthesis inhibitors block the light-induced phosphorylation and activation of PEPc.

Another important conclusion which can be drawn from the effects of protein and RNA synthesis inhibitors on PEPc kinase mRNA levels is that the PEPc kinase gene is probably transcriptionally regulated. Whilst this conclusion would be a rather ambitious one to draw from the effects of RNA synthesis inhibitors on the circadian induction of PEPc kinase mRNA in CAM plants, because it is likely that transcription is required for the functioning of the clock itself, the results with C_3 and C_4 plants provide much stronger support for this hypothesis. As mentioned above, transcription is required for the increase in kinase mRNA in C_3 and C_4 plants, but translation is not necessary. The simplest explanation of this result is that the transcription of the PEPc kinase gene itself and/or reduced destruction of kinase mRNA is necessary to produce the observed increase in the level of kinase translatable mRNA. This explanation is supported by the fact that protein synthesis is not required for the increase in kinase mRNA, whilst it is required for the decrease in the malate sensitivity of PEPc. However, a more complicated explanation, which cannot be ruled out, is that a secondary component undergoes transcriptional regulation and that the *in vitro* translation system translates this secondary component, allowing it to activate the translation of pre-existing PEPc kinase mRNA. This would mean that the translation system reconstitutes the last two steps of the signal transduction cascade which were blocked *in planta* by the RNA synthesis inhibitor. Although this latter hypothesis is more complicated, it is supported to some extent by the conclusions of Giglioli-Guivarc'h *et al.* (1996) that the light-induction of PEPc kinase activity in the C_4 grass *Digitaria sanguinalis* is the result of increased translation of pre-existing mRNA. However, Giglioli-Guivarc'h *et al.* (1996) drew this conclusion from a failure of RNA synthesis inhibitors to block the light- and weak base-induced decrease in the malate sensitivity of PEPc in isolated mesophyll protoplasts of *D. sanguinalis*. In the present work, RNA synthesis inhibitors did block the light-induced decrease in the malate sensitivity of PEPc when supplied to detached whole maize leaves (see chapter 6). This

conflict between results is difficult to explain, but Giglioli-Guivarc'h *et al.* (1996) did not display any results which demonstrated that the RNA synthesis inhibitors they applied to their protoplasts prevented *de novo* transcription in the protoplasts. It therefore remains possible that transcription could still occur in the protoplasts and thus, kinase mRNA and activity could increase in response to the light and weak base signal. Unfortunately, unequivocal proof of whether or not transcription of the PEPc kinase gene itself is involved in the induction of PEPc kinase activity in plants must await the cloning and expression analysis of the PEPc kinase gene. However, the results presented in this thesis provide significant new impetus for such studies to be performed as soon as possible, because they provide strong evidence to support the transcriptional regulation of PEPc kinase. This would be a novel and exciting finding for a protein kinase gene.

A number of separate lines of evidence also support the hypothesis that PEPc kinase is not regulated by a secondary component. As mentioned in chapter 6, mixing experiments in which dark and light extracts of *B. fedtschenkoi* or maize were mixed in the presence of potential co-factors to investigate whether, for example, a component in a light extract of maize could activate an inactive PEPc kinase in a dark extract, have proved negative (P.J. Carter, G.A. Nimmo and H.G. Nimmo, unpublished) . Such results suggest that PEPc kinase is not a pre-existing protein that is activated by a *de novo* synthesized secondary component but is itself synthesized *de novo* in response to the clock or light. Furthermore, the appearance of PEPc kinase activity in an *in vitro* translation is linear with time, which would be unlikely if a cascade system had to be reconstituted in which PEPc kinase is activated by a secondary component which is also translated by the rabbit reticulocyte lysate. In this latter case one might expect that the appearance of active PEPc kinase during *in vitro* translation would show a lag followed by an exponential increase with time. This would be caused by the requirement for a second component to be synthesized and then either activate PEPc kinase or activate the translation of pre-existing PEPc kinase mRNA. Such a secondary component could be a positive translation factor which, once synthesized, mediates the specific translation of PEPc kinase mRNA. Another possibility is that samples of RNA which produce low PEPc kinase activity upon *in vitro*

translation may contain high levels of mRNA for a specific PEPc kinase RNase which, when translated *in vitro,* destroys pre-existing kinase mRNA before it can itself be translated. Despite these perfectly feasible alternative hypotheses, the simplest explanation of the results in this thesis still remains that the level of PEPc kinase mRNA in isolated RNA samples reflects the level of transcription of the PEPc kinase gene at the time of sampling.

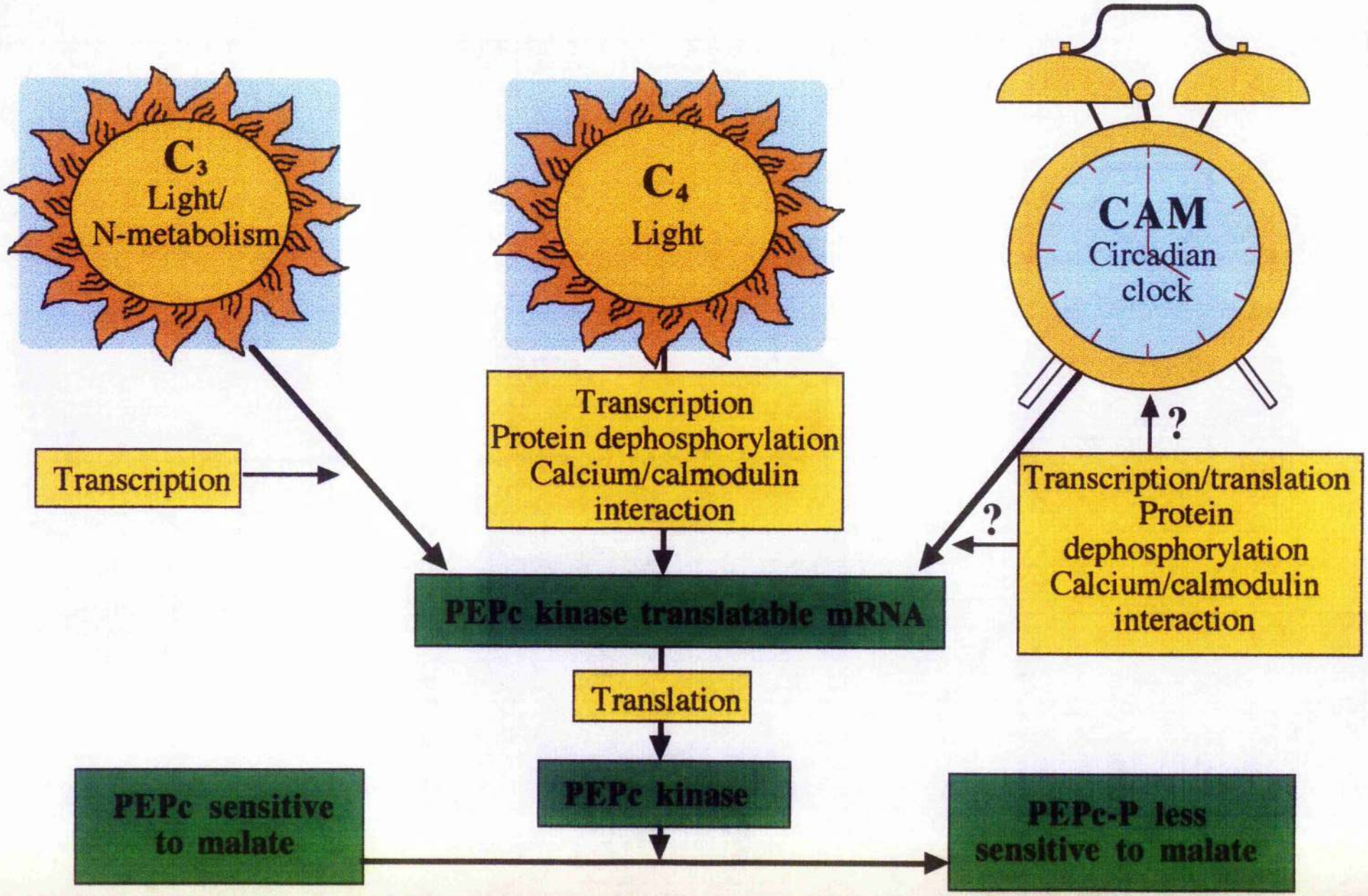
In addition to the requirement for transcription in the induction of PEPc kinase mRNA and activity in maize and *B. fedtschenkoi* both protein dephosphorylation and calmodulin are involved in the induction of the kinase. This conclusion is drawn from the effects of specific inhibitors of PP1 and PP2A and calmodulin. Other inhibitors of protein kinases and calcium channels were found to have no effect at the concentrations applied. However, this does not preclude the involvement of an additional protein phosphorylation event and a flux of calcium through calcium channels because the effectiveness of the inhibitors on their target proteins was not determined. Furthermore, the effects of phosphatase and calmodulin inhibitors must be interpreted with an element of caution due to the high concentrations of inhibitors required to achieve inhibition. This requirement for high concentrations of the inhibitors to achieve inhibition is probably due to the mode of application of the inhibitors to the leaves. Detaching leaves from their parent plant is an inherently stressful process which will induce a wounding response that is likely to be accompanied by the induction of a range of genes involved in the defence response. However, short of cutting leaf discs or even isolating protoplasts, which are both much more invasive, it is the simplest method of supplying inhibitors to leaves. The assumption is that the inhibitor will be taken up through the petiole and will eventually reach the target mesophyll cells via the transpiration stream. Problems with uptake and cell to cell movement will all decrease the final concentration of inhibitor which reaches the target cells. This may mean that, the concentration of inhibitor which reaches the target mesophyll cells could be several orders of magnitude lower than the concentration supplied. This is why significant attempts were made in this study to define the minimum effective concentration of each inhibitor. In addition to problems with uptake, a further

problem is that the inhibitor may have an effect due to it inhibiting a step in a closely related pathway which impinges on the pathway of interest. In some cases inhibitors can also inhibit processes quite distant to their target which impinge on the whole metabolism of the cell. For example, protein phosphatase inhibitors can block mammalian protein synthesis due to the requirement for dephosphorylation for continued initiation (eIF-2) and elongation (eEF-2) factor activity (Redpath and Proud, 1989). Protein phosphatase inhibitors have also been found to inhibit the incorporation of ^{35}S-Met into total soluble protein in maize leaves (Redinbaugh *et al.*, 1996). Hence, inhibitor experiments must be interpreted with an element of caution. However, with this caveat in mind, valuable insights into some of the possible elements in the signal transduction cascades which regulate PEPc kinase mRNA and activity levels have been made in this study. These possible signal elements are indicated in figure 7.1 which summarizes the data on the regulation of PEPc kinase in this thesis.

Although PEPc kinase activity in the leaves of C_3, C_4 and CAM plants has been found to be induced by light or the circadian clock, examples have recently appeared which demonstrate that in other systems, such as seeds and fruits, PEPc kinase is pre-existing and its activity does not vary throughout the diurnal cycle (Echevarria *et al.*, 1997; Law and Plaxton, 1997). It will be interesting to examine whether PEPc kinase mRNA is present in such tissues. This would allow the determination of whether kinase mRNA is also permanently present in seeds and fruits which might suggest that the RNA and activity are constantly turned over and therefore require continuous synthesis to maintain a high level of activity, or whether the kinase activity is stable and does not require *de novo* synthesis to maintain a high level. The level of PEPc kinase in the seeds of barley is unaffected by cycloheximide, which indicates that in this system the mRNA could well be absent (Echevarria *et al.*, 1997). Analysis of such systems with the PEPc kinase translatable mRNA assay would prove valuable because it may provide evidence of other conditions where the level of kinase mRNA does not always mirror the level of kinase activity. Such conditions have been identified in this study in the CAM plant *K. daigremontiana* during

Figure 7.1. Summary of the signalling events involved in regulating the level of PEPc kinase translatable mRNA and activity in C_3, C_4 and CAM plants

The diagram represents an overview of the results presented in this thesis concerning the signal transduction cascades which regulate PEPc kinase, and hence PEPc itself, in the three major photosynthetic classes of higher plants. Question marks (?) are used to indicate steps which could occur at one or more points in the signalling cascade.


C3
Light/
N-metabolism
C4
Light
CAM
Circadian
clock
Transcription
Transcription
Protein dephosphorylation
Calcium/calmodulin
interaction
?
?
Transcription/translation
Protein
dephosphorylation
Calcium/calmodulin
interaction
PEPc kinase translatable mRNA
Translation
PEPc kinase
PEPc sensitive
to malate
PEPc-P less
sensitive to malate

daigremontiana during and after a night in anaerobic conditions and when the leaves of C_3 and C_4 plants are supplied with protein synthesis inhibitors.

As described in chapter 5 the level of PEPc kinase mRNA generally underlies changes in PEPc kinase activity in *K. daigremontiana*. However, when leaves are encapsulated with pure nitrogen during the dark period, kinase mRNA levels remain close to control levels until the latter part of the night, whilst kinase activity is higher than controls and increases throughout the night at a greater rate than controls. Furthermore, when leaves were removed from nitrogen at the beginning of the photoperiod a sharp peak in kinase mRNA was detected which was not followed by a peak in kinase activity. However, when leaves were removed from nitrogen in the middle of the night the peak in kinase mRNA was followed by a peak in kinase activity. These results demonstrate that circadian clock control of PEPc kinase interacts with metabolite and possibly light control of the kinase. Malate is one metabolite which may interact with clock regulation of PEPc kinase mRNA and activity. For example, when malate is low due to nitrogen encapsulation a rapid increase in temperature from 19 to 27°C causes an increase in kinase mRNA and activity whilst in control leaves, where the temperature rise will cause accumulated malate to diffuse out of the tonoplast, kinase mRNA and activity drop over the same period. Light may prevent the translation of PEPc kinase mRNA or cause the appearance of a tight-binding inhibitor of PEPc kinase which prevents detection of PEPc kinase activity. In addition, the return of nitrogen encapsulated leaves to normal air in the middle of the dark period results in a peak in kinase mRNA and activity at a time when control levels have plateaued in response to the clock. This suggests that respiration and/or the commencement of flux through PEPc can stimulate PEPc kinase production. One possible explanation of this effect is that the resupply of air permits aerobic respiration and thus ATP will begin to become more readily available. Also PEP may have accumulated during the period of anaerobic conditions due to the inhibition of flux through PEPc so the resupply of CO_2 will cause high flux through PEPc leading to malate formation. It is clear from this study that metabolites play a key role in the regulation of PEPc kinase in CAM plants in addition to the circadian clock.

In vitro translation products have only rarely been assayed successfully for enzyme activity (Hruby and Ball, 1981; Raj and Pitha, 1977). The novel method described here for measuring the mRNA encoding a protein kinase is of great sensitivity, because the specific activity of the ATP can be very high. It is complementary to estimates of mRNA abundance that are based on hybridization, and may be useful in cases of translational control. The method cannot of course discriminate between mRNAs for isoenzymes that catalyse the same reaction. The method should prove generally applicable for measuring the mRNAs for any protein kinase for which both a specific substrate and an antibody to that substrate are available. It may also be applicable to mRNA prepared from cDNA cloned into a vector with an appropriate adjacent promoter. Analysis of such libraries could provide a simple way of cloning protein kinase cDNAs, based on the reactions catalysed by particular kinases rather than on conserved sequences within the protein kinase family.

The possibility that the PEPc kinase translatable mRNA assay may be a powerful method of cloning the PEPc kinase cDNA was explored in this study. A cDNA library was synthesized from a sample of poly $(A)^+$ RNA prepared from *B. fedtschenkoi* leaves sampled in the middle of the dark period. This RNA was used because it possessed maximum levels of PEPc kinase translatable mRNA. Mass excision of the plasmids from the phage of this cDNA library allowed the production of a plasmid library containing cDNA inserts representing all the expressed genes in the original RNA sample in the pBluescript plasmid. Plasmids were isolated from a representative sample of the plasmid library and linearized using the Xho 1 restriction enzyme. The linearized plasmid was used as the template for *in vitro* transcription reactions. This was possible because the multiple cloning site of pBluescript, in which the cDNA inserts reside, is flanked by the T3 and T7 bacterial promoters. T3 RNA polymerase was used to synthesize RNA from the cDNA because the original cDNA was obtained by unidirectional cloning which meant that reading from the T3 promoter would generate sense mRNA. The *in vitro* transcribed RNA was isolated and used to prime the *in vitro* translation assay for PEPc kinase activity.

Figure 7.2 shows that *in vitro* transcribed RNA from the *B. fedtschenkoi* dark cDNA library does direct the synthesis of detectable amounts of PEPc kinase activity. This

Figure 7.2. Evidence that transcription and translation of a *B. fedtschenkoi* 'dark' plasmid cDNA library can yield active PEPc kinase

In vitro transcribed RNA synthesized using linearized *B. fedtschenkoi* 'dark' cDNA library plasmid as a template was translated using the rabbit reticulocyte lysate *in vitro* translation system and the translation products were assayed for PEPc kinase activity.

(**A**) PEPc kinase activity synthesized by various amounts of *in vitro* transcribed RNA

Lane 1, 2.5 μg of *in vitro* transcribed RNA

Lane 2, 6.3 μg of *in vitro* transcribed RNA

Lane 3, 12.5 μg of *in vitro* transcribed RNA

Lane 4, 5 μg of *B. fedtschenkoi* 'dark' control total RNA

Lane 5, no RNA control

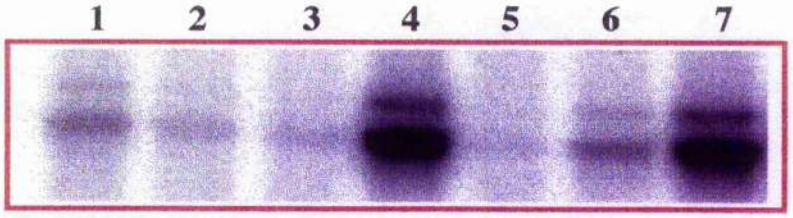
Lane 6, 1.25 μg of *in vitro* transcribed RNA

Lane 7, 7.5 μg of *B. fedtschenkoi* 'dark' control total RNA

(**B**) *In vitro* translation products synthesized by various amounts of *in vitro* transcribed RNA

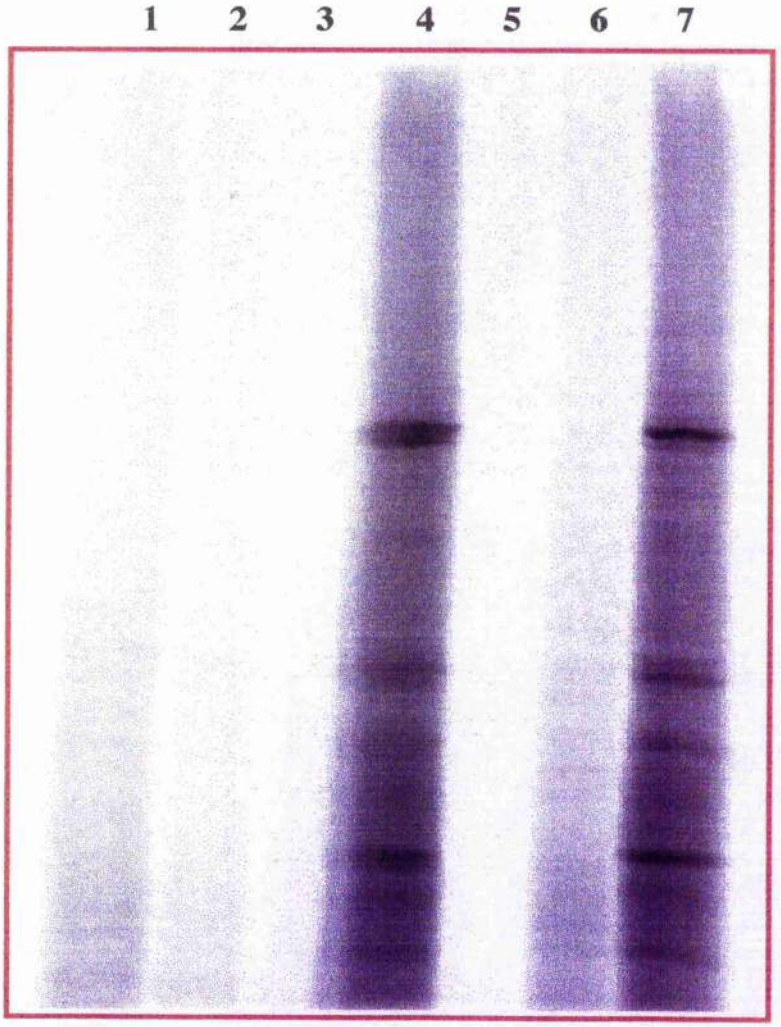
Lanes are as described for panel (A)

A



	1	2	3	4	5	6	7
Relative intensity	60	1	0	954	0	100	1035

B



	1	2	3	4	5	6	7
% incorporation of [^{35}S]Met into TCA precipitable material	0.55	0.41	0.23	4.2	0.44	1.1	6.7

indicates that this basic assay can be used to isolate the PEPc kinase cDNA. This task would be approached by separating the plasmid library into pools, isolating the plasmid from each pool and performing the transcription/translation assay for PEPc kinase cDNA inserts in the plasmids. The pool of plasmid DNA possessing the peak amount of PEPc kinase cDNA would be retransformed into *E. coli*, grown up to multiply each plasmid and the pooling process repeated. Numerous rounds of this pooling should eventually lead to the isolation of a single population of plasmids which contain cDNA inserts which encode kinases capable of phosphorylating PEPc. Presumably one of these kinase genes would be the *bona fide* Ca^{2+}-independent PEPc kinase. Isolation of the PEPc kinase cDNA would open the way for a wide array of exciting new research. For example, expression analysis of the kinase gene could be performed using Northern blotting and this should finally resolve the question of whether PEPc kinase is transcriptionally regulated in response to light or the circadian clock. Also, the genomic clone for PEPc kinase could be isolated and the promoter analysed. Isolation of the promoter should allow cloning of transcription factors which regulate the PEPc kinase gene and this may in turn permit the isolation of earlier steps in the signalling pathway such as protein kinases or phosphatases that control the transcription factors. The promoter could also be placed in front of a reporter gene such as ß-glucuronidase or green flourescent protein to permit the analysis of the *in vivo* expression of PEPc kinase in transgenic plants. Also, introducing the PEPc kinase gene into transgenic plants in the antisense orientation may permit analysis of precisely how crucial PEPc kinase is to the circadian regulation of CO_2 assimilation in CAM plants and the maintenance of high rates of photosynthesis in C_4 plants. These and many other exciting aspects of research on the regulation of PEPc in higher plants must await the cloning of the PEPc kinase gene. Hopefully, this will be achieved within the coming year and research into PEPc and PEPc kinase regulation can enter this exciting new phase as we enter the new millennium.

Chapter 8

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