Cloning of cDNAs for glyoxysomal malate dehydrogenase and for phospho*enol*pyruvate carboxykinase of cucumber (*Cucumis sativus* L.) and gene expression in cotyledons during development

by

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FOR

EUI-HYUN

I declare that this thesis was composed by myself, and that the work contained within is my own, unless otherwise stated.

DAE-JAE KIM

19 APRIL 1994

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Abstract

In higher plants a massive conversion of the storage lipid to carbohydrate takes place in germinating seedlings. The conversion involves β-oxidation, the glyoxylate cycle and gluconeogenesis. The key glyoxylate cycle enzymes isocitrate lyase (ICL) and malate synthase (MS) are coordinately synthesised to high levels during germination and decline thereafter to undetectable levels. The enzymes reappear at the final stage of the growth, in senescent tissues. In this study the synthesis of other enzymes involved in the conversion of lipid to carbohydrate was investigated to determine if they are coordinately synthesised with ICL and MS, and whether the genes encoding them are subject to the same control of expression.

Full length cDNA clones encoding glyoxysomal malate dehydrogenase (gMDH) and phospho*enol*pyruvate carboxykinase (PEPCK) have been isolated from a *Cucumis sativus* senescent cotyledon cDNA library. gMDH is one of the glyoxylate cycle enzymes, and PEPCK is a key enzyme for gluconeogenesis, which had not previously been cloned from plants. The cDNA and predicted amino acid sequence of gMDH show very high homology (94% and 97% respectively) with watermelon counterparts. The amino acid sequence deduced from the cDNA encoding PEPCK shows 43 to 57% identity with bacterial, yeast and trypanosome enzymes, and includes a conserved ATP-binding domain. The sequence of a full length cDNA predicts a polypeptide of 74,397 Da. The cDNA was expressed in *Escherichia coli* and antibodies raised to the resultant protein.

The cucumber genome was shown to contain single genes encoding gMDH and PEPCK, the expression of which was investigated by northern and western blotting. In the seven day period following seed imbibition, the amounts of gMDH and PEPCK mRNAs (and PEPCK protein) increase significantly in cotyledons and then decrease. Both mRNAs accumulate again to a low level in senescing cotyledons. In these respects the pattern of expression of these genes is similar to that of ICL and MS. However, unlike the ICL and MS transcripts, gMDH mRNA has another peak in green cotyledons, and a low level of PEPCK mRNA and protein is detected in green tissues. Genes encoding ICL and MS are activated by starvation and are subject to metabolic regulation. The expression of genes encoding gMDH and PEPCK was therefore investigated in detached cotyledons incubated in the light and dark, and in the presence or absence of sucrose. These genes showed no starvation or metabolic responses similar to those of ICL and MS. The functions of gMDH and PEPCK, and the control of their synthesis in plant development is discussed.

Contents

Chapter One: Introduction

Chapter Two: Materials and Methods

Chapter Three: Glyoxysomal NAD-malate dehydrogenase gene expression

Chapter Four: Differential screening for cDNA clone(s) of mRNAs coordinately expressed during germination and senescence in cotyledons

Chapter Five: Identification and characterisation of the phospho*enol*pyruvate carboxykinase (PEPCK) cDNA clone and gene expression in cucumber during development

Chapter Six: Potential metabolic regulation of gMDH and PEPCK synthesis in cucumber cotyledons

Chapter Seven: General Discussion

Abbreviations

A	adenine
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
bp	base pair
C	cytosine
cAMP	cyclic 3', 5'-cyclic monophosphate
cDNA	complementary DNA
Ci	Curie
dATP	2' deoxyadenosine 5' triphosphate
dCTP	2' deoxycytidine 5' triphosphate
dGTP	2' deoxyguanosine 5' triphosphate
DNA	deoxyribonucleic acid
DNaseI	deoxyribonuclease
DTT	dithiothreitol
dTTP	2' deoxythimine 5' triphosphate
EDTA	ethylenediaminetetraacetate
G	guanine
g	gram
g	relative centrifugal force
GUS	β-glucuronidase
IgG	immunoglobulin G
kbp	kilo base pair
kDa	kilo Dalton
mRNA	messenger RNA
NAD^+	nicotinamide adenine dinucleotide (oxidised)
NADH	nicotinamide adenine dinucleotide (reduced)
NADP	nicotinamide adenine dinucleotide phosphate (oxidised)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
nm	nano metre
PMSF	phenylmethyl sulfonyl fluoride
poly(a)	polyadenylation
p.s.i.	lbs $inch^{-2}$
RNA	ribonucleic acid
RNaseA	ribonuclease A
rRNA	ribosomal RNA

SDS	sodium dodecyl sulphate
T	thymine
TBS	tris buffered saline
TEMED	N, N, N', N',-tetramethylethylenediamine
Tris	tris-(hydroxymethyl)-methylamine
Tween-20	polyoxyethylene (20)-sorbitanmonolaurate
UV	ultra violet
v/v	volume:volume ratio
w/v	weight:volume ratio

DEPC	diethyl pyrocarbonate
MOPS	3-[*N*-morpholino]propanesulfonic acid
SSPE	sodium chloride, sodium phosphate EDTA buffer, defined in section 2.2.5.2
TBE	tris-borate EDTA buffer, defined in section 2.2.3.1
TE	tris-EDTA buffer, defined in section 2.2.2.5

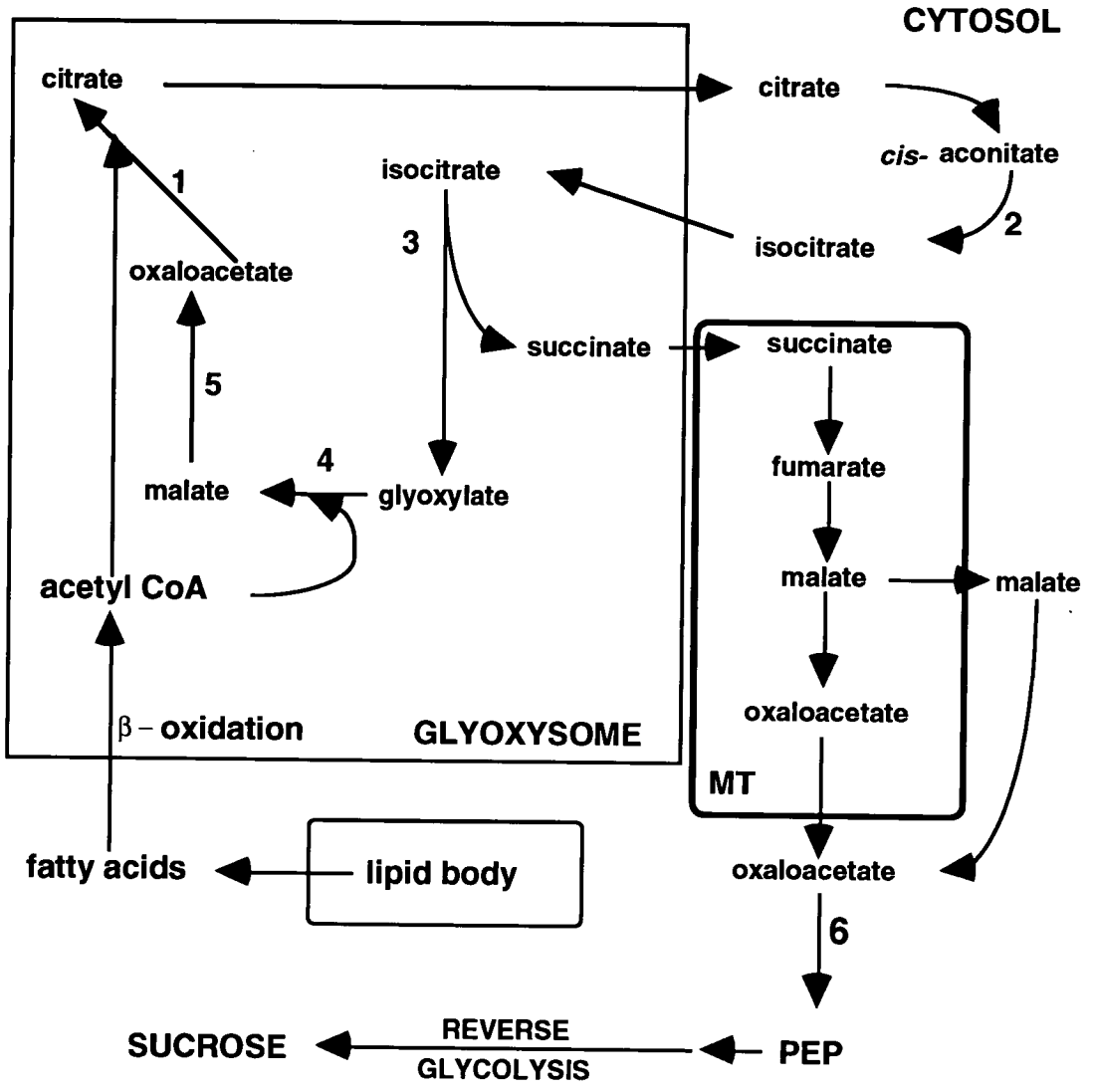
Chapter One: Introduction

1. 1. Lipid mobilisation, glyoxylate cycle and gluconeogenesis in plants

1. 1. 1. Storage lipid breakdown in oil-seed plants during early germination

In many plants lipid is the major carbon reserve which is stored in the endosperm tissue surrounding the embryo (*eg*, castor bean), or in the cotyledons (*eg*, cucurbits). Upon germination, such seedlings show a distinctive metabolic conversion of fat to carbohydrate. Glyoxysomes are involved in storage lipid conversion into sugars during this stage of growth (Trelease and Doman, 1984). They participate in reactions of the glyoxylate cycle; a pathway responsible for the net conversion of two molecules of acetyl coenzyme A (acetyl-CoA) into succinate. As can be seen from Figure 1. 1, the pathway involves four cellular compartments; spherosome (lipid body), glyoxysome, mitochondrion and cytosol. Briefly, the fatty acids are liberated from triacylglycerols in the lipid bodies, then are oxidized via glyoxysomal β-oxidation producing acetyl-CoA, which is the first input into the glyoxylate cycle. The acetyl-CoA is metabolised via the glyoxylate cycle, within the glyoxysome. The product succinate moves out from the glyoxysome and enters the mitochondrion, then is further oxidised to oxaloacetate by enzymes of the tricarboxylic acid (TCA) cycle. The last step of the gluconeogenic pathway happens in the cytosol. The oxaloacetate from the mitochondrion, is decarboxylated to form phospho*enol*pyruvate (PEP) which is finally converted to hexose via reverse glycolysis (Beevers, 1961).

Figure 1. 1. Simplified pathway of storage lipid mobilisation. Four cellular compartments are involved in the conversion. The initial breakdown of lipid (triacylglycerol) to fatty acids is carried out by lipases present in the lipid body and the fatty acids are transported to glyoxysome for further breakdown to acetyl-CoA via the β-oxidation spiral. The acetyl-CoA enters the glyoxylate cycle and this results in the export of succinate to mitochondria. The oxaloacetate produced is converted to PEP by PEPCK. Finally, the PEP is converted in the cytosol to hexose via reverse glycolysis and thence to sucrose. MT, mitochondrion; PEP, phospho*enol*pyruvate. 1, citrate synthase; 2, aconitase; 3, ICL; 4, MS ; 5, gMDH; 6, PEPCK. Other enzymes involved are described in Figures 1. 2. and 1. 3.


CYTOSOL
citrate
citrate
cis- aconitate
isocitrate
isocitrate
1
2
3
oxaloacetate
succinate
succinate
5
fumarate
4
malate
glyoxylate
malate
malate
acetyl CoA
oxaloacetate
β – oxidation
GLYOXYSOME
MT
fatty acids
lipid body
oxaloacetate
6
SUCROSE
REVERSE
GLYCOLYSIS
PEP

1. 1. 2. Hydrolysis of triacylglycerols and β–oxidation; the first step

The gluconeogenic pathway begins with hydrolysis of stored lipid in germinating oil-seed plants. Triacylglycerols stored in the lipid bodies are firstly hydrolysed to fatty acids by lipases, which are associated with the membrane of the storage lipid bodies (spherosomes). Then monoacylglycerol and two fatty acids are transported into the glyoxysomes and further hydrolysed within the glyoxysome by alkaline lipase. Then, the β-oxidation pathway produces acetyl-CoA within the glyoxysome (Huang *et al.*, 1983) (Figure 1. 2).

First, β-oxidation requires ATP and CoA for fatty acid activation. Primarily, the β-oxidation substrate fatty acyl-CoA is formed by a fatty acyl-CoA synthetase from fatty acid in the presence of CoA and ATP. Degradation advances by oxidation in the β–position to the carboxyl group and sequential removal of 2C units. The first reaction of a four enzyme sequence in β-oxidation is performed by fatty acyl-CoA oxidase that utilises oxygen as the direct electron acceptor to produce H_2O_2, which is apparently decomposed by catalase within the glyoxysome. The second and third reactions are catalysed by enoyl-CoA hydratase and β-hydroxyacyl-CoA dehydrogenase respectively. The last enzyme is acetyl-CoA acetyltransferase (thiolase). These enzymes have been thought to be exclusively located in glyoxysomes of oil-seeds but the presence of β-oxidation enzymes in mitochondria was also reported (Thomas and Wood, 1986). In mammalian cells both fatty-acyl-CoA synthetase and the β-oxidation enzymes are found in mitochondrion and peroxisome.

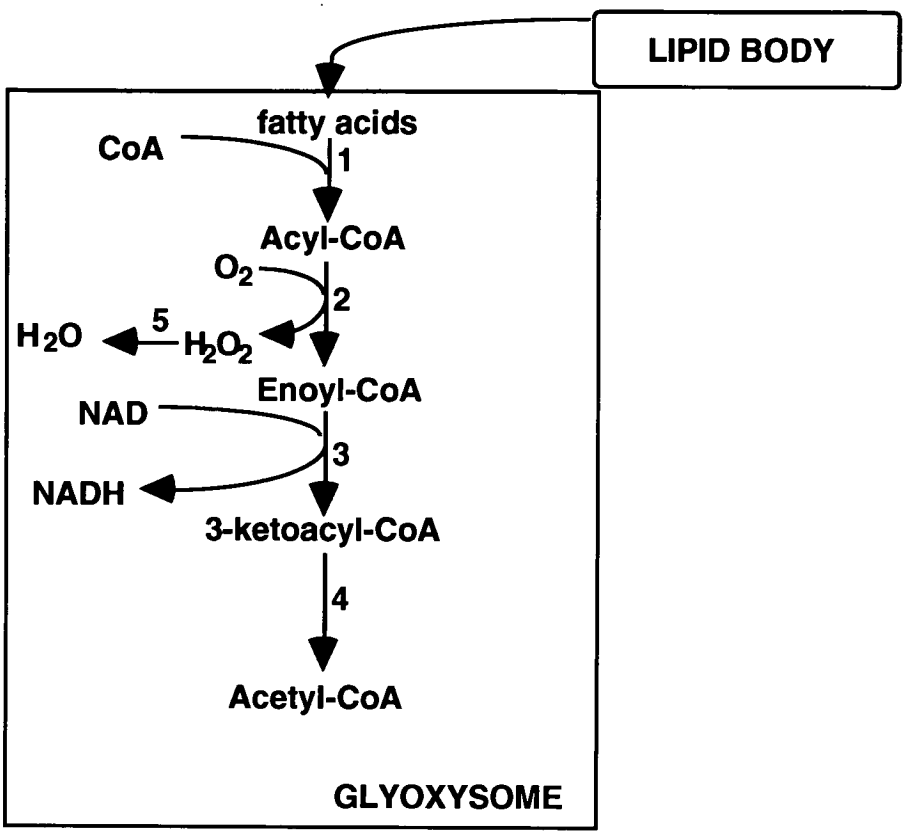


Figure 1. 2. The β–oxidation sequence of fatty acids in the glyoxysome. Free fatty acids are transported from the lipid body then converted into CoA derivatives first by acyl-CoA synthetase (1). The fatty acyl-CoAs are then degraded by the enzymes of the β–oxidation spiral into acetyl-CoA. All the β–oxidation spiral enzymes are found in the glyoxysome. 1, Acyl-CoA synthetase; 2, enoyl-CoA hydratase; 3, β-hydroxyacyl-CoA dehydrogenase; 4, acetyl-CoA acetyltransferase (thiolase); 5, catalase.

1. 1. 3. The glyoxylate cycle; the second step

Acetyl-CoA, the β-oxidation product is metabolised through the glyoxylate cycle (Figure 1. 1). Four of the five glyoxylate cycle enzymes are restricted to the glyoxysomes but aconitase is cytosolic (Courtois-Verniquet and Douce, 1993; De Bellis *et al.,* 1994). Two enzymes unique to the glyoxylate cycle are isocitrate lyase (ICL) (*threo*-D-isocitrate glyoxylate-lyase, EC 4. 1. 3. 1), which converts isocitrate to glyoxylate and succinate, and malate synthase (MS) (L-malate glyoxylate-lyase[CoA-acetylating], EC 4. 1. 3. 2) which catalyses the condensation of glyoxylate and acetyl-CoA to form malate. The glyoxylate cycle brings about the net conversion of two moles of acetyl-CoA to one mole of succinate, the end product of the cycle (Beevers, 1980). Succinate, the four carbon metabolic intermediate leaves the glyoxysomes and is converted to malate by TCA cycle enzymes in the mitochondrion, then oxaloacetate. Potentially the oxaloacetate used by PEPCK can come either from the mitochondrion (Figure 1. 1) or the glyoxysome (Figure 1. 5). The oxidation of NADH and the function of malate dehydrogenase (MDH) is discussed in more detail in section 1. 4. 3. The sequence of reactions for the conversion of oxaloacetate to sucrose occur in the cytosol (Nishimura and Beevers, 1979).

1. 1. 4. Reverse glycolysis; the last step

Oxaloacetate, originating either from the mitochondrion (Figure 1. 1) or glyoxysome (Figure 1. 5) is decarboxylated to PEP by PEPCK (see section 1. 6 and Chapter 5). This conversion is an ATP-dependent reaction just as are certain others required to convert PEP into sucrose by reverse glycolysis (Figure 1. 3). Sucrose is

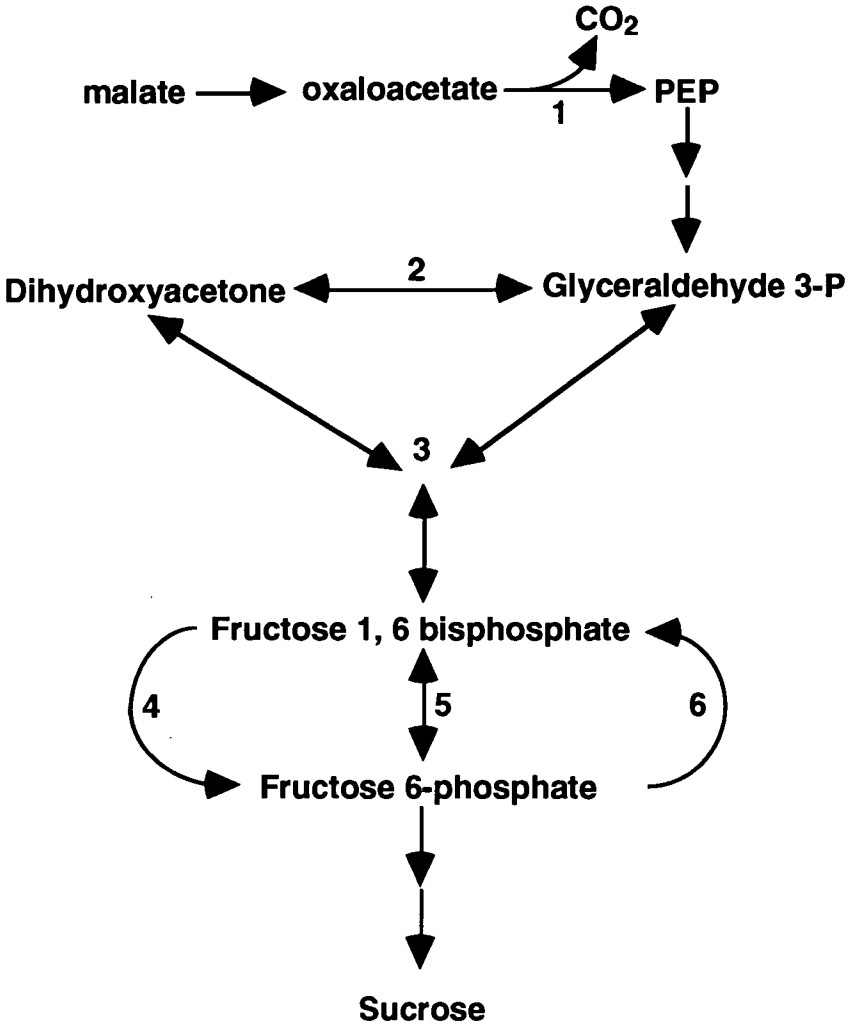


Figure 1. 3. The pathway of reverse glycolysis. The reactions involved in the conversion of C_4 acids to hexose phosphates during gluconeogenesis. 1, PEPCK; 2, triose phosphate isomerase; 3, aldolase; 4, fructose1,6-bisphosphatase; 5, pyrophosphate fructose 6-phosphate 1-phosphotransferase; 6, phosphofructokinase (Bryce and Hill, 1993).

the major product of the gluconeogenic pathway in germinating oilseed plants (Beevers, 1961; Nishimura and Beevers, 1979).

1. 2. Microbodies

Microbodies were first described by De Duve (1969), comprising a simple set of membrane-bounded organelles in the cells of eukaryotes. They have a simple structure; a single membrane, no internal membranes or ribosomes. They lack their own genome and possess characteristic enzymes including catalase and oxidases (Beevers, 1979; Huang *et al.*, 1983). Enzyme content and function are genetically and developmentally controlled by the nucleus because of the absence of a microbody genome.

1. 2. 1. Microbodies in plants

Microbodies are considered to have a ubiquitous distribution in most higher organisms. The term peroxisome is used to emphasize the role of microbodies in hydrogen peroxide (H_2O_2) metabolism. In plants, depending on their enzymatic complement and function, different forms of microbodies are distinguished; leaf-peroxisomes, glyoxysomes, unspecialised peroxisomes (Huang *et al.*, 1983) and gerontosomes (Vincentini and Matile, 1993). Therefore, the organelle carries out various roles in distinct cell types that appear to be determined by the environment or the differentiated state of the cell, and by the developmental stage of plant or plant organs.

1. 2. 2. Specialised peroxisomes in higher plants

Peroxisomes are commonly found in green tissues in higher plants. In particular, leaf-type peroxisomes are characterised by glycolate metabolism as part of the photorepiratory process (Tolbert, 1971). The activity of glycolate oxidase (GO) is much higher than in peroxisomes of other tissues. Leaf-type peroxisomes also contain other enzymes that are involved in photorespiration, including NADH-dependent hydroxypyruvate reductase (HPR) and serine:glyoxylate amino transferase (SGAT). Leaf-peroxisomes also contain enzymes for fatty acid β–oxidation (Gerbling and Gerhardt, 1987; Pistelli *et al.,* 1989).

Cotyledons become the first photosynthetic organs of the developing seedling in epigeous species, *eg*, cucumber, watermelon and pumpkin. Around 2 to 3 days following seed imbibition, mRNAs encoding peroxisomal enzymes appear and show light dependent accumulation (Feierabend and Beevers, 1972; Hondred *et al.,* 1987; Greenler and Becker, 1990). Gene expression studies for HPR have been extensively carried out recently (see section 1. 5).

Uninfected soybean root nodule cells show proliferation of peroxisomes which contain high uricase activity (Newcomb and Tandon, 1981; Van den Bosch and Newcomb, 1986). The enzyme uricase is involved in the pathway of ureide synthesis for *de novo* synthesis of purines from amino acids and the oxidation of purines to form ureides. This enzyme is responsible for the breakdown of uric acid to allantoin.

1. 2. 3. Glyoxysomes in oil-seed plant development

Since the discovery of the glyoxylate cycle (Kornberg and Beevers, 1957a, b), much work has been done to characterise the glyoxysome. The glyoxysome was named to emphasize its role in glyoxylate cycle metabolism in plants by Breidenbach and Beevers (1967). Morphologically, glyoxysomes are similar to other microbodies, and also contain catalase activity, characteristic of peroxisomes. Therefore the name glyoxysome recognises their distinct function in plant metabolism.

Glyoxysomes are observed primarily in developing seeds and in seedlings of fat-rich seed plants, for example watermelon, cucumber, pumpkin, castor bean, rape-seed *etc.*, and are unique to plant cells. They contain enzymes for β-oxidation of fatty acids, few peroxisomal enzymes and the glyoxylate cycle, a modification of the TCA cycle. They are found in storage tissues of oilseeds during germination where they are involved in the mobilisation of the lipid reserves to support heterotrophic growth of the seedling until it becomes autotrophic. Thus, glyoxysomes have an important function in germinating seeds and seedlings of oil-storing species.

Glyoxysomes contain the four sequential enzymes of the glyoxylate cycle, citrate synthase, ICL, MS, and gMDH, but do not contain aconitase, succinate dehydrogenase, fumarase, or an electron transport system linking NADH to O_2. Previously, aconitase has been classified as one of the glyoxysomal enzymes but it has recently been identified as a cytosolic enzyme (Courtois-Verniquet and Douce, 1993; De Bellis *et al.*, 1994). Therefore, the glyoxylate cycle operates in the glyoxysome and cytosol as shown in the modified glyoxylate cycle pathway (Figure

1. 1, Courtois-Verniquet and Douce, 1993).

1. 2. 4. Unspecialized microbodies

Other peroxisomes in some higher plant tissues, several algal, and fungal species metabolise certain compounds, but they have not been given specific names, so are called unspecialised peroxisomes. Unspecialised forms of peroxisomes have been reported from various plant tissues such as shoots, roots, tubers, petals and fruits (Huang and Beevers, 1971). They all contain catalase and often show low activities of glycolate oxidase and, in the majority of cases, uricase. Peroxisomes belonging to one of these classes probably occur in all plant cells. Biogenesis appears to be controlled partially by the regulated accumulation of constituents unique to a particular class of peroxisome (Comai *et al.*, 1989).

1. 2. 5. Gerontosome

An unusual type of microbody develops during senescence of green tissue. This process presumably occurs by microbody transition. A glyoxysome-like function has been reported during the breakdown of chloroplastic galactolipids in senescing barley leaves. The glycerol, phosphatidylcholine and acyl residues produced are eventually metabolised to respiratory substrates (Gut and Matile, 1989: Wanner *et al.*, 1991). This process is accompanied by the apparent induction of key enzymes of the glyoxylate cycle (Gut and Matile, 1988). Several research groups have demonstrated

the occurrence of glyoxysome-like organelles in senescent cotyledons, leaves and petals of a number of plant species (Landolt and Matile, 1990; De Bellis *et al.*, 1990, 1991; Pistelli *et al.*, 1991; De Bellis and Nishimura, 1991). However, senescing leaves show both photorespiration and uricase enzyme activities (Hong and Schopfer, 1981). Following these results, Vincentini and Matile (1993) have proposed a new term "gerontosome" to describe the multifunctional microbody in senescent organs.

1. 2. 6. Microbodies in other eukaryotes

The glycosome is a peroxisome-related organelle of unicellular flagellate protozoa (*Trypanosoma*) (Visser *et al.*, 1981). Trypanosomes are well adapted to life in the mammalian bloodstream. The energy for this organism is entirely dependent on glycolysis. A unique aspect of the glycolytic pathway in typanosomes is that it is located inside a microbody-like organelle called the glycosome (Opperdoes, 1987).

An association of glyoxylate cycle and β-oxidation enzymes within peroxisomes was observed in oleic acid induced yeast cell cultures but the specific function is unknown (McCammon *et al.*, 1990). In yeasts, the glyoxylate cycle supports the growth on acetate and shows glyoxysomal into peroxisomal transformation in response to changes in environmental conditions (Zwart *et al.*, 1983).

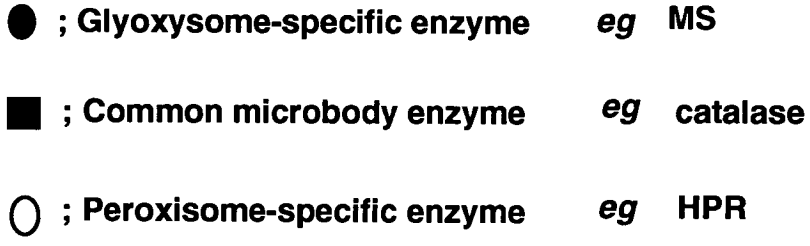
1. 3. Microbody transitions in epigeous plant development

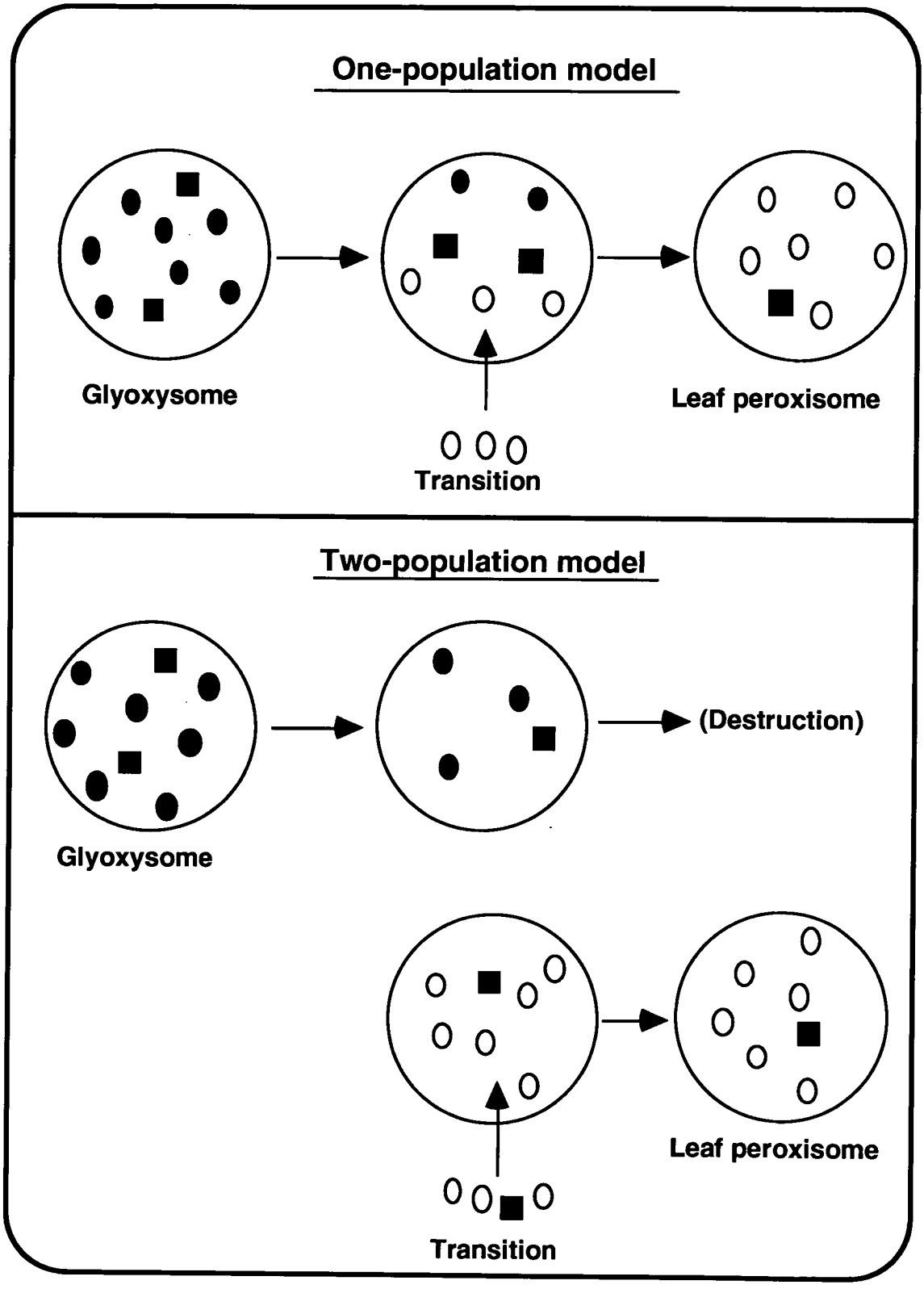
1. 3. 1. Transition of glyoxysome to peroxisome in epigeous plants

Biogenesis of the organelle is developmentally regulated; glyoxysomes are present primarily in developing seeds and in seedlings, whereas other peroxisomes are found in mature plant organs (Huang *et al.,* 1983; Trelease, 1984). Concerning the relationship between glyoxysomes and peroxisomes in the greening cotyledons of cucurbit seedlings, two hypotheses have been proposed. According to one, the peroxisomes are derived from pre-existing glyoxysomes by changes in the enzymatic components; the one-population model (Trelease *et al.*, 1971; Burke and Trelease, 1975; Sautter, 1986; Behrends *et al.,* 1990). According to the other, the old glyoxysomes are disintegrated and new peroxisomes emerge by *de novo* synthesis; the two-population model (Kagawa *et al.,* 1973; Kagawa and Beevers, 1975). These models are shown in Figure 1. 4.

The direct transformation of microbodies from glyoxysomes to leaf peroxisomes was observed in the cotyledons of some fat-storing seedlings using immunocytochemistry (Beevers, 1979; Titus and Becker, 1985; Nishimura *et al.*, 1986). During the microbody transition, glyoxysomal enzymes, such as MS and citrate synthase, are degraded in the microbodies, with coincident synthesis *de novo* of leaf peroxisomal enzymes, such as GO and HPR, which are transported into the peroxisomes during microbody transition. Glyoxylate cycle enzymes and peroxisomal enzymes were shown to be present in the same microbody during the transition stage, so demonstrating that the one-population model is correct.

Figure 1. 4. Simplified models for microbody transitions. The one-population model implies the simultaneous presence of glyoxysomal and peroxisomal enzymes in one and the same organelle during microbody transition. The two-population model suggests that glyoxysomal and peroxisomal enzymes are segregated into different microbodies during microbody transition.




One-population model
Glyoxysome
Leaf peroxisome
Transition
Two-population model
(Destruction)
Glyoxysome
Leaf peroxisome
Transition

1. 3. 2. Reverse transition from peroxisome to glyoxysome-like microbody in senescing tissues

A reverse transition of leaf peroxisomes to glyoxysome-like microbodies (or gerontosomes) could occur in the cotyledons during senescence and the glyoxylate cycle could function in the glyoxysome-like microbodies of senescing organs in connection with both the β-oxidation pathway and purine catabolism. Therefore a "double" transition could occur during the development of cucurbit cotyledons; the first from glyoxysomes to leaf peroxisomes during greening of the cotyledons; the second from leaf peroxisomes to glyoxysome-like microbodies (gerontosomes) during senescence. This hypothesis is also supported by the associated increase in the glyoxylate cycle enzymes and β-oxidation activities in peroxisomal fractions from senescing leaves and detached organs, and by the high specific activities of ICL and MS (Gut and Matile, 1988; Landolt and Matile, 1990; De Bellis *et al.*, 1990; De Bellis *et al.*, 1991; Pistelli *et al.*, 1992; Kim *et al.*, 1992; Graham *et al.*, 1992; McLaughlin, 1994). Immunocytochemical studies revealed such a microbody transition during senescence (Nishimura *et al.*, 1993). The enzyme content and function of microbodies can vary and appears to be determined by the control of gene expression and enzyme synthesis during plant development.

It is now generally accepted that newly synthesised proteins are translocated post-translationally into both types of peroxisomes (Trelease, 1984; Sautter, 1986; Behrends *et al.*, 1990). A consensus has emerged from numerous studies showing that the temporal expression of glyoxysomal enzymes (*eg*, catalase, ICL and MS) correlates with accumulation of their mRNAs, which is generally interpreted to reflect transcriptional regulation (Weir *et al.*, 1980; Smith and Leaver, 1986; Comai *et al.*,

1989). Similar conclusions were made for regulation of several leaf-type peroxisomal enzymes, for example, HPR and SGAT (Hondred, 1987).

1. 4. Glyoxylate cycle enzymes and gene expression

1. 4. 1. ICL and MS in oil-seed plants

ICL and MS enzymes are found within the glyoxysomes in oil-seed plants (Nishimura and Beevers, 1979; Huang *et al.*, 1983; Kindl, 1987). Therefore, they are useful markers for the presence of glyoxysomes and in the study of control of gene expression during a particular stage in development. The function of the glyoxylate cycle and characteristics of ICL and MS synthesis has been extensively studied in higher plants (Weir *et al.*, 1980; Smith and Leaver, 1986; Graham, 1989; Comai *et al.*, 1989; Turley and Trelease, 1990; Graham *et al.*, 1992;McLaughlin, 1994).

As described above, ICL and MS enzymes participate in reactions associated with lipid mobilisation in oilseed plant seedlings. In cucumber both enzymes appear in the cotyledons after 2 days of germination and reach a peak of activity on day 3 or 4 (Weir *et al.*, 1980; Smith and Leaver, 1986). During this time, the lipid reserves are mobilised. After this peak of enzyme activity, both ICL and MS decline rapidly in amount until they are undetectable by day 6 of germination in light-grown seedlings. These changes in the activities of ICL and MS are reflected by changes in the amounts of their transcripts (Smith and Leaver, 1986; S. Reynolds unpublished observations). RNA blot studies with ICL and MS cDNA clones from *Brassica napus*

showed that the genes in this plant exhibit similar expression patterns. The mRNAs begin to accumulate during late embryogeny, reach maximal levels in seedling cotyledons but transcripts were not detected in significant amounts in young leaves (Comai *et al.*, 1989). The patterns of expression of the two enzymes are found to be qualitatively similar but are quantitatively different. The studies indicate that expression of ICL and MS is controlled primarily at the transcriptional level (Comai *et al.*, 1989), but that translational or post-translational processes differentially affect accumulation of the two enzymes (Ettinger and Harada, 1990). The effect of hormones on the appearance of ICL and MS has been investigated in germinating castor bean seeds (Rodriguez *et al.*, 1987). Exogenously applied gibberellic acid appears to accelerate transcription and abscisic acid inhibits the accumulation of transcripts. However, these effects are not thought to be specific to genes involved in lipid mobilisation, but merely to affect the rate of transcription and hence protein synthesis in general.

Transgenic tobacco plants were produced (Graham *et al.*, 1990) containing 1.1 kbp of the 5' upstream region from the start of the coding region of cucumber MS, fused to the reporter gene β-glucuronidase (GUS). The transgenic plants showed synthesis of GUS in a spatial and temporal manner similar to that observed for MS in cucumber and indicates that transcription appears to be the primary controlling factor in the synthesis of MS.

1. 4. 2. ICL and MS in other plant species

ICL and MS are often used as a marker enzyme for the glyoxylate cycle and

glyoxysomes. Nevertheless, there are a few reports of MS activity without accompanying ICL in plants (Yamamoto and Beevers, 1960; Koller *et al.*, 1979; Miernyk *et al.*, 1979; Miernyk and Trelease, 1981). Also, ICL activity has been detected in pea leaves without MS (Hunt and Fletcher, 1977). Furthermore, ICL activity has been detected in mitochondria in pea leaves (Hunt and Fletcher, 1977), cultured rose cells (Hunt *et al.*, 1978), and wheat leaves (Singh and Naik, 1983) but not in microbodies. According to this, ICL and MS are found in various situations in higher plants and may show diversity in their roles. Recently, Kim *et al.*,(1988) reported operation of the glyoxylate cycle in mitochondria of cotyledon cells from starchy seeds of *Vigna cylindrica*. Therefore, they proposed a pathways of organic acid metabolism in mitochondria and peroxisomes of 2-day-old cotyledons of *V. cylindrica*. ICL activity was exclusively present in the peroxisomal fraction but MS was detected in both mitochondria and peroxisomes.

A recent report indicates homology between *DAL7*, a protein involved in allantoin degradation in *Saccharomyces cerevisiae*, and MS from castor bean (Rodriguez *et al.*, 1990). Allantoin is a toxic by-product of purine metabolism. The possibility that MS could have a second role in nitrogen recycling as well as carbon recycling is intriguing.

1. 4. 3. Glyoxysomal MDH

MDH ((S)-malate: NAD^+ oxidoreductase, EC 1. 1. 1. 37) catalyses the reversible interconversion of malate and oxaloacetate;

$$\text{Malate} + NAD^+ \xrightleftharpoons{\text{MDH}} \text{Oxaloacetate} + \text{NADH} + H^+$$

Several isoenzymes of NAD-dependent MDH are found in plants. The mitochondria contain an isozyme (mitochondrial MDH, mMDH) that is a component of the Krebs cycle where it oxidises malate to oxaloacetate (Leech and Ellis, 1961). The cytosol contains three isozymes in cucumber, which may participate in numerous pathways including the malate shuttle and gluconeogenesis (Liu and Huang, 1976). Another isozyme is present inside the glyoxysomes (gMDH), participating in the glyoxylate cycle (Huang *et al.*, 1974). The leaf-type peroxisomes also contain an isozyme in green leaves which functions in inter-organelle electron shuttling (Yamazaki and Tolbert, 1969; Rocha and Ting, 1970). Chloroplasts also contain isozymes but these are $NADP^+$-dependent (Metzler *et al.*, 1989).

The total activity of NAD-malate dehydrogenase in the cotyledons of cucumber seeds and seedlings increases 10 fold during the first 3 days of germination in the dark and then declines gradually to one third of the peak activity after 10 days (Liu and Huang, 1976). They also reported 5 isozymes of MDH activity from cucumber seedlings resolved by starch gel electrophoresis. One was identified as mMDH, one as gMDH and three cytosolic forms.

Upon greening of the cotyledons the glyoxysomes are converted into peroxisomes which co-operate with the chloroplasts and the mitochondria in photorespiration, permitting the recycling of phosphoglycolate into glycerate-3-phosphate and CO_2. The peroxisome imports glycolate produced by photorespiration in the chloroplast and converts it via glyoxylate into glycine. This is shuttled into the mitochondria, where two molecules of glycine condense to serine. Serine returns to the peroxisome and is converted into glycerate, which serves the carbon reduction cycle in chloroplasts. In the peroxisomes MDH provides NADH for

the reduction of β-hydroxypyruvate to glycerate. Malate is thought to enter from the cytosol in exchange for aspartate (Mettler and Beevers, 1980).

1. 4. 3. 1. Role of gMDH in the glyoxylate cycle during development

In glyoxysomes of germinating oilseeds, both MDH activity of the glyoxylate cycle and β-oxidation of fatty acids can produce NADH (Mettler and Beevers, 1980). For each sequence of β-oxidation, one NADH is generated, and for each turn of the glyoxylate cycle, another NADH can potentially be produced. In the simple representation of the glyoxylate cycle (Figure 1. 1), three moles of NAD^+ are reduced to NADH within the glyoxysome for every mole of succinate production from fatty acids (Beevers, 1980). Therefore, in this case the glyoxysome requires a continuous supply of NAD^+. However it is not clear if glyoxysomes can directly oxidise NADH, but it is readily reoxidised outside the glyoxysome, presumably by the mitochondrion (Huang *et al.*, 1983).

At least three different mechanisms could explain the oxidation of glyoxysomal NADH and the movement of electrons out of the glyoxysome;

(a) transport of NADH out of the organelle

(b) a malate/aspartate shuttle

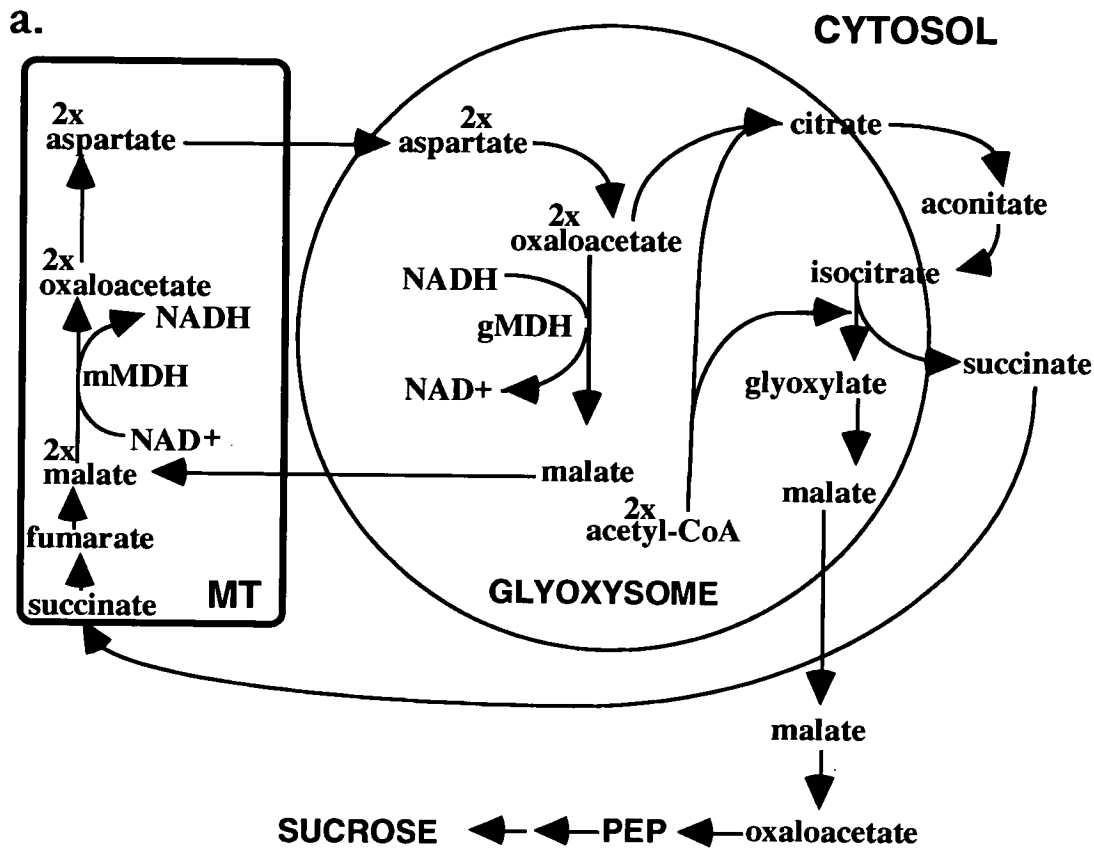
(c) a membrane redox system.

The first mechanism is thought to operate in liver peroxisomes (Ito *et al.*, 1981). The rapid transport of NADH through the glyoxysomal membrane could be a

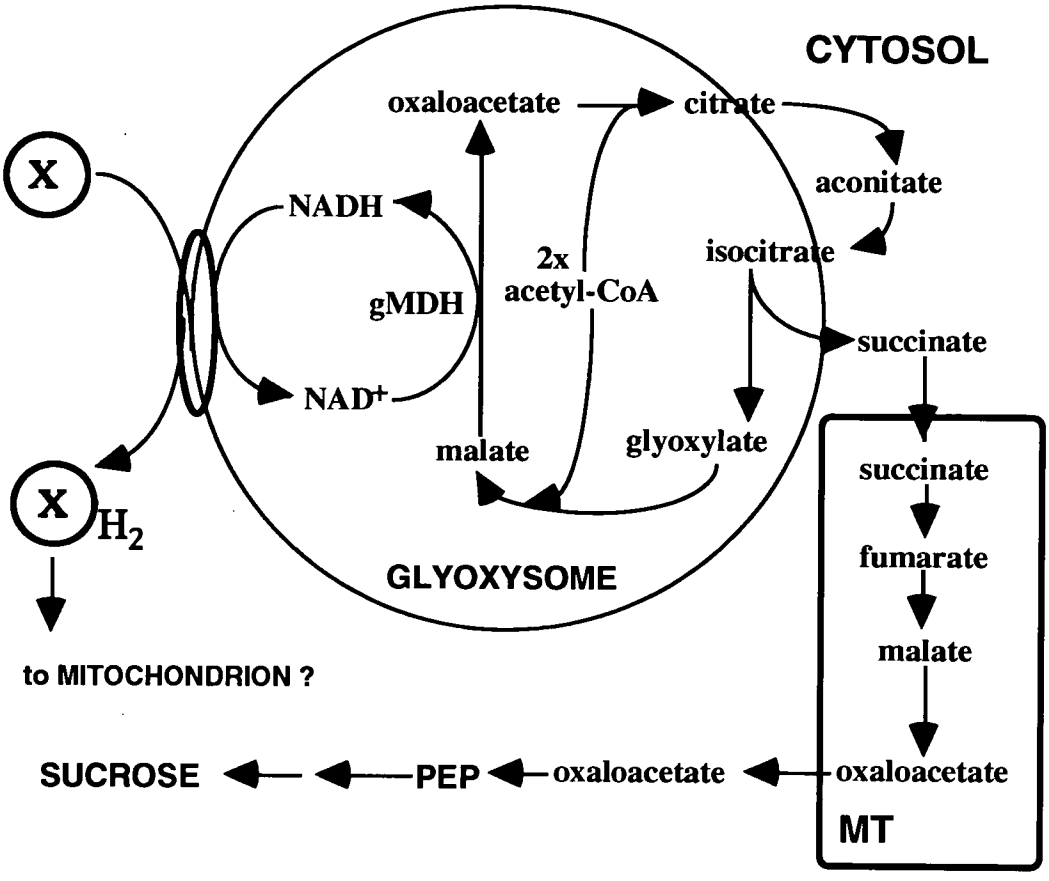
mechanism to allow NADH to be oxidized by mitochondria as has been observed for liver peroxisomes. But, the evidence does not support this possibility for glyoxysomes (Donaldson *et al.,* 1981). The latencies (i.e., increase in activity upon dissruption of the glyoxysomal membrane) of glyoxysomal dehydrogenases imply restricted membrane permeability to molecules the size of NADH. Also, isolated glyoxysomes retain NAD^+ and $NADP^+$ (Donaldson *et al.,* 1981; Donaldson, 1982).

The second mechanism has been proposed by Mettler and Beevers (1980) in which electrons are transferred from glyoxysomes to mitochondria as malate. Glyxoysomal MDH and aminotransferase are sufficiently active. The shuttle mechanism (Figure 1. 5a) needs the transport of malate and aspartate, through glyoxysomal and mitochondrial membranes. It also needs a glutamate-2-oxoglutarate shuttle to return amino groups to the shuttle in the glyoxysomal pathway. In as much as isolated intact mitochondria, but not glyoxysomes, are capable of reoxidising NADH, it was concluded that the NADH produced in the glyoxysomes is oxidised by the mitochondria. The presence of highly active glutamate oxaloacetate aminotransferase in glyoxysomes and mitochondria suggests that aspartate and malate may be utilised in a shuttle system transferring reducing equivalents generated from NADH between organelles. The shuttle theory accounts for a sustained regeneration of NAD^+ in the glyoxysomes without a direct transport of NADH to the mitochondria. According to this scheme, in the reactions of the glyoxysome, malate is not converted to oxaloacetate by gMDH, but gMDH instead reduces oxaloacetate to malate for subsequent export (Figure 1. 5a). Therefore, the shuttle mechanism requires regulated transport of aspartate, malate, glutamate and 2-oxoglutarate through mitochondrial and glyoxysomal membranes. Even though this proposed shuttle depends on the equilibrium of the MDH reaction in the glyoxysomes

Figure 1. 5. Simplified models for export of reducing equivalents from glyoxysomes. (a) Proposed malate-aspartate shuttle for the transfer of reducing equivalents between glyoxysomes and mitochondria providing for oxidation of NADH produced by glyoxysomal β-oxidation (Mettler and Beevers, 1980). (b) Donaldson's group proposed this membrane redox model (Alani *et al.,* 1990). Reduced NADH is reoxidized and recycled by glyoxysomal membrane electron transport proteins that transfer electrons to unknown acceptor molecules [X] outside the glyoxysomes. MT, Mitochondria.


a.
CYTOSOL
2x
aspartate
2x
oxaloacetate
NADH
mMDH
NAD+
2x
malate
fumarate
succinate
MT
2x
aspartate
2x
oxaloacetate
NADH
gMDH
NAD+
malate
2x
acetyl-CoA
GLYOXYSOME
citrate
aconitate
isocitrate
succinate
glyoxylate
malate
malate
SUCROSE
PEP
oxaloacetate

b.


CYTOSOL
oxaloacetate
citrate
aconitate
isocitrate
X
NADH
gMDH
NAD+
2x
acetyl-CoA
succinate
malate
glyoxylate
X
H2
GLYOXYSOME
succinate
fumarate
malate
oxaloacetate
MT
to MITOCHONDRION ?
SUCROSE
PEP
oxaloacetate

favouring malate formation, a potential weakness is the fact that MDH in the mitochondrion has a similar equilibrium to gMDH, but should favour oxaloacetate formation. Futhermore, such movement of metabolites has not been demonstrated yet.

According to the third explanation, reduced nucleotides are reoxidized and recycled by glyoxysomal membrane electron transport proteins that transfer electrons to accepter molecules outside the glyoxysomes (Figure 1. 5b). Such a mechanism is based on the observation of an electron transport chain within the glyoxysomal membrane (Fang *et al.*, 1987). The oxidation of NADH in a glyoxysome membrane redox system may allow β-oxidation and the glyoxylate cycle to be partially uncoupled from mitochondrial oxidative phosphorylation. NADH oxidation by the malate-aspartate shuttle would link β-oxidation to a higher level of ATP generation. Therefore, the balance of electron flow through the two routes would depend on the demand for ATP as fatty acid is oxidized and converted to sucrose. The redox components in the glyoxysomes become most active at the same developmental stages as glyoxylate cycle activity (Alani *et al.*, 1990). Thus, the glyoxysomal membrane redox system will develop in parallel with the process that generates NADH in the matrix. The extra-glyoxysomal electron acceptor is not known, but cytochrome *c*, ferricyanide and ascorbate can function *in vitro*. The increased metabolic flux through these pathways during germination requires a more active electron transport of reducing equivalents across the glyoxysomal membrane to external acceptors.

An important difference between the shuttle and the electron transport mechanisms is in the function of glyoxysomal NAD-malate dehydrogenase. In the shuttle mechanism gMDH functions to reduce oxaloacetate, whereas in the electron

transport mechanism it oxidises malate. Later in development when glyoxysomes have been converted into peroxisomes, the organelle must import reducing equivalents, and MDH then oxidises malate. The microbody changes from a net exporter of reducing equivalents into a net importer.

Recently, a peroxisomal short redox chain has been observed in potato tuber peroxisomes, which contains a *b*-type cytochrome (Struglics *et al.*, 1993). Nevertheless, the redox mechanism and its function is yet to be elucidated.

1. 4. 3. 2. Organelle targeting MDH isoenzymes; gMDH, mMDH and chloroplastic MDH (cMDH)

All the MDH isozymes are encoded in the nuclear genome and synthesized on cytoplasmic ribosomes, then imported into the microbodies, mitochondria and chloroplasts (Walk and Hock, 1977; 1978). A majority of enzymes for mitochondrial and chloroplast import are synthesised with a cleavable presequence at the amino terminus. In watermelon a 27 amino acid extra sequence is found in the mitochondrial precusor, and a 37 amino acid extra sequence in the glyoxysomal precusor (Gietl *et al.*, 1990; Gietl, 1992). Chloroplast targeted MDH contains a 57-residue chloroplastic transit peptide at the amino terminus (Metzler *et al.*, 1989). Although the three organelle-targeted MDHs are synthesised with an amino terminal extension, they show different peptide sequences. Therefore, it indicates that they are encoded in different nuclear genes.

Most microbody enzymes are synthesised at the mature size and targeted with

an internal or carboxy terminal recognition sequence (Keller *et al.,* 1991). However, the pre-gMDH molecule contains an amino terminal putative transit peptide of 37 amino acid residues which is clearly different from the presequence of the mitochondrial precusor (27 amino acids) and has no carboxy terminal extension (Gietl, 1990). This amino terminal extension is cleaved off upon association with or import into glyoxysomes. Another example of a microbody protein with such an amino terminal extension is peroxisomal thiolase (Preisig-Muller and Kindl, 1993). Subsequently the MDH holoenzyme comprising two identical subunits is assembled (Gietl and Hock, 1982; 1984). Mature polypeptides of gMDH and of mMDH are 319 and 320 amino acids residues long, respectively, thus, essentially the same length as MDHs from pig, rat, mouse, yeast and *E. coli.* Glyoxysomal MDH cDNA was isolated from watermelon RNA (Gietl, 1990). Subsequently, a watermelon mMDH cDNA was isolated and compared with other organisms by Gietl *et al.,* (1990). The homology between gMDH and mMDH has been shown to be 60% at the DNA level and 65% at the protein level.

It is thought that there are three classes of microbody targeting signals.

(a) the peroxisomal targeting signal (PTS); a tripeptide at the carboxy terminus

(b) the positive charge domains and carboxy terminal extentions found in most glycosomal proteins

(c) the amino terminal cleavable presequence or transit peptide found in peroxisomal thiolase and gMDH

The tripeptide sequence -[S/A/C]-[R/H/K]-L at the carboxy terminus is

thought to be a peroxisomal targeting signal in mammals, insects, plants and fungi (Gould *et al.,* 1989a; Keller *et al.,* 1991). SRL or SRM has been found at the carboxy termini of ICL, MS and catalase in several plants (Comai *et al.,* 1989; Graham *et al.,* 1989; Ni *et al.,* 1990a; Turley and Trelease, 1990). However, not all peroxisomal proteins contain such a targeting motif at the carboxy terminus. Peroxisomal thiolase and gMDH are exceptions which are synthesised as precursors (Gietl, 1990; Swinkels *et al.,* 1991; Gietl, 1992). They have amino terminal presequences which are cleaved upon import. The amino terminal presequences show limited similarity in cucumber with other organisms (Preisig-Muller *et al.,* 1993). Internal signals has been observed in catalase from *Saccharomyces cerevisiae* and acyl-CoA oxidase from *Candida tropicalis.* Catalase contains multiple topogenic sequences. The acyl-CoA oxidase contains two separated target signals, one at the amino terminus (amino acids 1-112) and an internal region (309-464) (Small *et al.,* 1988). According to these observations, the targeting of microbody proteins is rather complex.

1. 4. 4. Glyoxysomal citrate synthase

Citrate synthase (EC 4. 1. 3. 7) is located in glyoxysomes and mitochondria. As with malate dehydrogenase, citrate synthase is a component of both glyoxylate and citric acid cycles. Glyoxysomal citrate synthase activity considerably increases during germination in oil-seed plants together with ICL, MS and gMDH (Kindl, 1987). The glyoxysomal citrate synthase gene (or cDNA) has not been cloned yet. Therefore, we still do not have information about this enzyme in terms of protein targeting and gene expression.

1. 4. 5. Aconitase in higher plants

One of the TCA cycle enzymes aconitase (citrate[isocitrate] hydro-lyase, EC 4. 2. 1. 3) catalyses the dehydration of both isocitric and citric acids to form *cis*-aconitic acid. The reactions are reversible and so the enzyme brings about the interconversion of citric and isocitric acids. Two isoenzymes are found in sycamore cells, which are cytosolic and mitochondrial (Brouquisse *et al.*, 1987). Previously, one aconitase isoenzyme has been assumed to be present in glyoxysomes. However, Courtois-Verniquet and Douce (1993) reported the absence of aconitase from microbodies. Consequently, they suggested a modified glyoxylate cycle which requires a detour via the cytosol (Figure 1. 1). Similar results have been found for pumpkin aconitase (De Bellis *et al.*, 1994). However, there are no further molecular biological reports about aconitase isozymes or their genes.

1. 5. Peroxisomal enzymes and gene expression in green tissues

1. 5. 1. Catalase

Catalase (H_2O_2:H_2O_2 oxidoreductase; EC 1. 11. 1. 6) protects aerobic organisms from the toxic effects of H_2O_2 and other activated oxygen species by converting H_2O_2 to H_2O and O_2. The enzyme occupies as much as 10 to 25% of the total peroxisomal protein and possesses extremely high activity and catalytic turnover number (Huang *et al.*, 1983). It has a molecular weight ranging from 225,000 to 300,000 and consists of four identical subunits with heme as its prosthetic group. This is one of the marker enzymes for microbodies and is encoded by more than one

gene. Catalase is a tetrameric enzyme, and many isoenzymes can be constructed through the assembly of different subunits in various proportions. Therefore, biosynthesis of catalases is complicated during plant development. Two different subunits are assembled into five tetrameric isoforms of catalase in cotton (Ni *et al.*, 1990a; Ni *et al.*, 1990b). They reported on the molecular basis and regulatory mechanisms for the temporal expression of cottonseed catalase subunits by estimating steady-state levels of protein and mRNAs by measuring transcription rates in isolated nuclei (Ni and Trelease, 1991). They showed that the expression of glyoxysomal subunit 1 (SU 1) and leaf-peroxisomal subunit 2 (SU 2) are regulated primarily at the post-transcriptional level as opposed to the transcriptional level suggested for expression of other glyoxysomal and leaf-peroxisomal enzymes. Expression of the five tetrameric catalase isozymes during postgerminative growth of cotton seedlings were a consequence of interactions between two subunits (SU 1 and SU 2) synthesised from two distinct catalase genes. The time courses for steady-state levels of the mRNAs encoding these two subunits revealed two clearly separated peaks; the first at day 1 (SU 1) and the other at day 4 (SU 2). Accumulation of these mRNAs preceeded the accumulation of their corresponding proteins by at least 24 hours, suggesting temporal, pretranslational regulation of synthesis of both subunits. Results from run-on transcriptional assays showed that the transcripts encoding both subunits were synthesised together on days 1 through 5. Therefore, they have suggested that the accumulation of SU 1 and SU 2 mRNAs are controlled primarily at the post-transcriptional level, which has not previously been reported for catalase or any other peroxisomal enzymes.

Three catalase genes (*CAT*1, *CAT*2 and *CAT*3) were identified in maize and their developmental and tissue specific expression characterised (Skadsen *et al.*,

1990; Redinbaugh *et al.*, 1990). *CAT*1 is expressed when kernels mature while *CAT*2 encodes a protein fated for glyoxysomes in the embryo. *CAT*3 was activated in leaves and expressed in a circadian rhythm. Glyoxysomal catalase in sunflower also appears in several isoforms (Eising and Gerhardt, 1989; Eising *et al.*, 1990).

1. 5. 2. Glycolate oxidase

The enzyme from green leaves has been purified and extensively studied (Tolbert, 1981; Kindl, 1987; Tsugeki *et al.*, 1993). Glycolate oxidase (GO)(EC 1. 1. 3. 15) is a flavoprotein which catalyses the O_2 -dependent oxidation of α-hydroxy acids, in particular glycolate to glyoxylate and H_2O_2. It has a molecular weight of about 225,000 to 300,000 and consists of four identical subunits each having flavine mononucleotide (FMN) as the cofactor (Huang *et al.*, 1983). The gene is activated by illumination leading to a dramatic increase in enzyme activities responsible for the peroxisomal part of photorespiration (Tolbert, 1981; Kindl, 1987; Tsugeki *et al.*, 1993). However, the level of GO mRNA is low in cotyledons of germinating seeds (Ludt and Kindl, 1990).

The primary structure of GO has been elucidated from cDNAs encoding GO from spinach (Volokita and Somerville, 1987), lentil (Ludt and Kindl, 1990) and pumpkin (Tsugeki *et al.*, 1993). It has a PTS-like tripeptide (PRL) at the carboxy terminus but is slightly different from the proposed signal (SKL or ARL) for targeting to microbodies in lentil and pumpkin.

1. 5. 3. NADH-dependent hydroxypyruvate reductase

NADH-dependent hydroxypyruvate reductase (HPR) (EC 1. 1. 1. 29) has been studied to examine peroxisomal enzyme development in green tissues. HPR catalyses the conversion of hydroxypyruvate to glycerate with the oxidation of NADH. As with other peroxisomal enzymes in cotyledon development, HPR activity and transcript levels are strongly regulated during development and by light. Light-dependent accumulation of peroxisomal enzymes is observed in wheat leaves (Feierabend and Beevers, 1972), sunflower cotyledons (Schnarrenberger *et al.*, 1971), mustard cotyledons (Hong and Schopfer, 1981), watermelon seedlings (Kagawa and Beevers, 1975) and cucumber cotyledons (Trelease *et al.*, 1971).

In cucumber, HPR activity and transcripts are detected in cotyledons during the metabolic transition from heterotrophy to autotropy at about 2 to 3 days after imbibition (Greenler and Becker, 1990). Recent research about HPR shows transcriptional regulation of gene expression (Hondred *et al.*, 1987). cDNA and genomic clones (*hpr*-A and *hpr*-B) were isolated from cucumber recently (Greenler *et al*, 1989; Schwartz *et al.*, 1991) and promoter analysis also has been carried out using transgenic tobacco plants (Sloan *et al.*, 1993). The promoter analysis of the HPR gene showed *cis*-acting elements for light inducibility located between -299 and -218 from the transcription start. As in other light regulated genes, similar sequence motifs (I box and G box) have been found. Furthermore, Greenler and Becker (1990) reported light-regulated and organ-specific expression of the *hpr*-A gene in cucumber. Red-light induction of the HPR gene was observed and the reversion of the induction by far-red light also found in cucumber so indicating phytochrome involvement (Bertoni and Becker, 1993).

1. 6. PEPCK and gluconeogenesis in higher plants

1. 6. 1. PEPCK enzyme in eukaryotes; general features

The PEPCK (ATP: oxaloacetate carboxy-lyase (transphosphorylating) EC 4. 1. 1. 49; GTP: oxaloacetate carboxy-lyase (transphosphorylating) EC 4. 1. 1. 32) reaction was demonstrated first in rat liver (Utter *et al.*, 1954) and then in leaf extracts of several plants (Mazelis and Vennesland, 1957; Edwards *et al.*, 1971; Dittrich *et al.*, 1973). PEPCK catalyses the following reaction;

$$\textbf{oxaloacetate + ATP or GTP} \xleftrightarrow{\textbf{PEPCK}} \textbf{PEP + ADP or GDP + } CO_2$$

It is recognised as a key enzyme in gluconeogenesis in different organisms from bacteria to vertebrates. The enzyme has also been found in some invertebrates and eukaryotic microorganisms as a CO_2 -fixing enzyme (De Flombaum *et al.*, 1977; Rohrer *et al.*, 1986; Podkovyrov and Zeikus, 1993). The enzyme from animal tissues is only active with GTP or ITP whereas the plant enzyme is most active with ATP (Miller *et al.*, 1968; Edwards *et al.*, 1971). The enzyme has a metal requirement (Mg^{++} or Mn^{++}). The enzyme has been characterised earlier from microorganisms and animals (Cooper and Benedict, 1968; Ballard and Hanson, 1969). Gene structure and expression studies have been carried extensively with animal PEPCK (Beale *et al.*, 1985; Park *et al.*, 1990; Beale and Tishler, 1992; Park *et al.*, 1993). However, limited information is available on the characteristics of this enzyme in higher plants (Dittrich *et al.*, 1973; Laurence *et al.*, 1977). There is no molecular biological report yet from plants.

1. 6. 2. PEPCK (GTP) cDNAs (or genes) in mammals and microorganisms

Two PEPCK (GTP) isozymes are found in animal cells, which are mitochodrial and cytosolic (Ballard and Hanson, 1969). cDNAs and genes were isolated from several animals and insect; rat cytosolic (Beale *et al.*, 1985), chicken cytosolic and mitochondrial (Cook *et al.*, 1986; Weldon *et al.*, 1990), and fruit fly (Gundelfinger *et al.*, 1987). The lengths of the sequences are similar, varying from 607 to 640 amino acids. The peptide sequences also show about 50% homology with PEPCK of the anaerobic fungus *Neocallimastix frontalis* (Reymond *et al.*, 1992). Even though their biochemical function is similar, they do not show any comparable sequence homology with ATP-dependent PEPCK such as *Saccharomyces* (Stucka *et al.*, 1988), *Trypanosoma* (Parsons and Smith, 1989), *E. coli* (Medina *et al.*, 1990) and *Rhizobium* (Osteras *et al.*, 1991). However, interestingly, nucleotides binding regions (GXXXXGK) shows almost complete conservation between GTP- and ATP-dependent PEPCK.

1. 6. 3. PEPCK (ATP) in photosynthetic tissue and its roles; C_4, CAM and C_3 plants

High activity of this enzyme in leaves of some plants with the C_4 pathway of photosynthesis has been reported (Edwards *et al.*, 1971). PEPCK acts as a decarboxylase during photosynthesis in bundle sheath cells of specific plants ('PEPCK' type) which carry out C_4 photosynthesis. In these C_4 plants, CO_2 is released from oxaloacetate in the bundle sheath cells where the CO_2 is refixed by the C_3 cycle. The PEP produced by PEPCK is returned to the mesophyll cells for further

CO_2 fixation by PEP carboxylase. In some C_4 plants either a $NADP^+$- or NAD^+-dependent malic enzyme ('NAD-ME' or 'NADP-ME type') acts as the decarboxylase in bundle sheath cells (Ray and Black, 1976; Leegood, 1993). Nevertheless, in all C_4 plants the 4-carbon organic acids are formed in the mesophyll cells by the carboxylation of PEP by PEP carboxylase.

PEPCK activity has also been reported in some plants which show crassulacean acid metabolism (CAM) (Dittrich *et al.*, 1973). A diurnal acidification and deacidification is accompanied by a rhythm of CO_2 uptake, starch catabolism and malic acid synthesis in the dark, followed in the light by a reduction of CO_2 uptake with a coincident breakdown of malic acid and synthesis of starch. The PEPCK acts as a decarboxylase during the light period, providing CO_2 for the C_3 cycle and PEP for gluconeogenesis (Dittrich *et al.*, 1973).

PEPCK activity is hardly detectable in C_3 photosynthetic plant tissue but enzyme activity was observed in marrows cotyledons during the early germination stage (Leegood and ap Rees, 1978). The ATP-dependent enzymes of bacteria, yeast and plants are thought to be composed of several subunits, as dimers or tetramers. The only report of the enzyme molecular weight is from *Panicum maximum* , which is 360,000 (Urbina and Avilan, 1989).

1. 6. 4. Cellular localisation in plants

PEPCK is localised in the mitochondria and cytosol in some animal tissues (Ballard and Hanson, 1969). At an early stage in the study of PEPCK in plants, the

enzyme was first recognised as an organelle enzyme (chloroplasts or mitochondria) (Edwards *et al.*, 1971). However, the enzyme is found in the cytosolic fraction of germinating marrow cotyledons (Leegood and ap Rees, 1978). The enzyme is also localised in the cytosol in both C_4 and CAM plants (Ku *et al.*, 1980; Watanabe *et al.*, 1984; Urbina and Avilan, 1989).

1. 6. 5. Roles in lipid mobilising tissues in gluconeogenesis

Gluconeogenesis is the process by which plant cells synthesise sucrose and reducing sugars either from storage reserves in non-photosynthetic cells (in particular, in germinating seeds its provides energy and carbon skeletons), or directly from CO_2 in photosynthetic tissues. The synthesis of glucose (and hence other hexoses) can occur from non-carbohydrate substrates such as acetate, glycerol, pyruvate and succinate. In particular, this is an important pathway in non-photosynthetic tissues. As discussed earlier (see section 1. 1), a large number of seeds store lipid as their major reserve (*eg*, cucumber, watermelon, castor bean and rape) and this lipid provides energy in early germination. The mobilisation of this lipid on germination involves the oxidation of fatty acids to produce acetyl-CoA, the synthesis of malate from acetyl-CoA via the glyoxylate cycle and the subsequent synthesis of sucrose within the cytosol from this malate. The cytosolic enzymes PEPCK and MDH catalyse the conversion of malate into PEP via oxaloacetate (see Figure 1. 1). PEPCK is a key enzyme in this conversion and is found only in lipid-mobilising tissues in C_3 plants (Leegood and ap Rees, 1978).

1. 6. 6. The pathway of gluconeogenesis in lipid mobilising tissues

PEP from oxaloacetate can now be converted to fructose 1,6-bisphosphate (F1,6BP) via the triose phosphates glyceraldehyde 3-phosphate or dihydroxyacetone phosphate. There are three enzymes in plant cells involved in the interconversion of F1,6BP and the hexose phosphate, fructose 6-phosphate (F6P). These are ATP-F6P 1-phosphotransferase (phosphofructokinase, PFK), PP_i-F6P 1-phosphotransferase (PFP) and F1,6BP 1-phosphatase (F1,6BPase) (Figure 1. 3). The step from F1,6BP to F6P is mainly catalysed by F1,6BPase in an irreversible reaction. Hence the enzyme activity increases in parallel with the rate of lipid break down and sucrose synthesis (Thomas and ap Rees, 1972). Although the role of PFP is less clear, the enzyme also catalyses the production of F6P from F1,6BP with a freely reversible reaction. In castor bean endosperm the activity of PFP increases at the same time as F1,6Pase, and the enzymes are present at comparable levels. Therefore, it supports a gluconeogenic role for PFP rather than a glycolytic one. However, the control of the flux between triose and hexose phosphates is complicated. The flux may be controlled by the relative levels of these sugars, but the precise mechanism of this regulation in unknown (Bryce and Hill, 1993).

1. 7. Genetic and biochemical regulation of senescence

1. 7. 1. Genetic regulation of senescence in higher plants

The maturity-to-senescence transition is considered to be under the control of gene expression modulated by internal, positional signals and environmental factors.

Therefore, senescence may be a genetically controlled sequence of development during which there is synthesis of new proteins and increased synthesis of preexisting proteins (Skadsen and Cherry 1983; Kar and Feierabend, 1984; Woodson, 1987; Kawakami and Watanabe, 1988a, b, c; Thomas *et al.,* 1992). A number of enzymes, including proteases, ribonuclease, acid phosphatase, cellulase, and polygalacturonase, show increased activity during the early stages of senescence and this in part reflects new or increased synthesis of proteins. Thomas (1990) reported evidence of genes being turned off during senescence, but also clear instances of novel translatable RNAs appearing during the process. Strong evidence for genetic control of senescence comes from mutants which fail to show normal leaf senescence (Thomas *et al.,* 1992). Also, inhibitors of protein synthesis can delay flower senescence, showing a requirement for active protein synthesis in the senescence process (Wulster *et al.,* 1982).

In addition, decreased levels of nucleic acids have been recognised in a number of senescing tissues, including leaves, cotyledons and flowers (Chang *et al.,* 1985). They have also reported that in soybean cotyledons nuclear DNA declines by 25% by the final stages of senescence. There are also qualitative and quantitative changes in RNA, particulary rRNA, during senescence. For example, chloroplast rRNA is selectively degraded during senescence of cucumber, and tobacco leaves (Brady, 1988). A number of studies with several tissues, including senescing flowers (Woodson, 1987; Woodson and Lawton, 1988) and ripening fruit (Tucker and Laties, 1984; Lincoln *et al.,* 1987) have demonstrated that there is synthesis of proteins during this terminal phase of development. There are also large changes in the synthesis of RNA. The newly synthesised RNA includes rRNA, soluble RNA and poly $(A)^+$ RNA. Furthermore, changes have been observed in specific enzymes such

as glutamate dehydrogenase, glutamate oxaloacetate amino transferase, glutamate-pyruvate aminotransferase and $NADP^+$-dependent isocitrate dehydrogenase, which are involved in amino acid metabolism during senescence (Kar and Feierabend, 1984), and glutamine synthetase which plays a central role in nitrogen metabolism (Kawakami and Watanabe, 1988a; Kamachi, *et al.*, 1992).

1. 7. 2. Biochemical and physiological changes in membrane senescence

Senescence is the terminal stage of development in the life of a plant organ or a plant that leads ultimately to death. Senescence is a gradual metabolic process in higher plants. Membrane deterioration is an early and characteristic feature of senescence that results in increased permeability, loss of ionic gradients, and decreased activities of key membrane-associated enzymes including ion pumps. There are also major alterations in the molecular organization of the lipid bilayer in senescing membranes particularly the plasmalemma and microsomal membranes (Brown *et al.*, 1991). Decreases in bulk lipid fluidity have been reported for microsomal membranes from senescing cotyledons, flowers, leaves and fruit (Leshem *et al.*, 1984; Thompson *et al.*, 1987; Fobel *et al.*, 1987). There is also a strong temporal correlation between changes in lipid fluidity of senescing microsomal membranes and production of the superoxide anion by the membranes (Lynch and Thompson, 1984). This decrease in membrane lipid fluidity was attributible to an increase in the sterol:phospholipid ratio (or sterol: fatty acid ratio). It reflects a selective depletion of unsaturated fatty acids from the membranes by lipoxygenase and resulting increase in the saturated:unsaturated fatty acid ratio (Thompson, 1988).

Loss of chloroplast structural and functional integrity is a dominant feature of leaf senescence. There are also ultrastructural changes in senescing thylakoids. Even though granal and stromal thylakoids are progressively dismantled, the chloroplast envelope retains its integrity until the late stages of senescence (Woolhouse, 1984). Of particular interest is the finding that there were no changes in the fluidity of thylakoid membranes over a period during which they lose 90% of their noncyclic photosynthetic electron transport capability. It suggests that thylakoid membranes may senesce by a different mechanism than plasma or microsomal membranes (McRae *et al.*, 1985).

1. 7. 3. Glyoxysomal enzymes gene expression during senescence

The glyoxylate cycle was previously considered to operate primarily during the period after germination until the establishment of photosynthetic competence of the seedling. Recently, it has been demonstrated that detached leaves of barley show glyoxysomal enzyme activity when incubated in darkness for several days (Gut and Matile, 1988). The GUS reporter gene fused with the MS promoter has been transferred into tobacco (*Nicotiana plumbaginifolia*) plants. GUS activity has been detected in senescent leaves (Graham *et al.*, 1992). MS transcripts have also been detected in senescing cucumber cotyledons, leaves, petals and radicles (Graham *et al.*, 1990, 1992). Furthermore, the occurrence of ICL and MS has been reported in peroxisomes of dark-treated detached rice leaves, leaf-beet leaves and cotyledons of pumpkin (De Bellis *et. al.*, 1990) and spinach leaves (Landolt and Matile, 1990). During leaf senescence, chloroplasts become degraded while peroxisomes maintain their integrity and undergo changes in their enzyme complements (Kura-Hotta *et al.*,

1990). Furthermore, glyoxysomal enzymes have been detected in leaves of rice (*Oryza sativa* L.) and wheat (*Triticum durum* L.) from either naturally senescing or dark-treated plants (Pistelli *et al.*, 1991), cotyledons (De Bellis and Nishimura, 1991) and petals (De Bellis *et al.*, 1991). Isolated peroxisomes of detached rice and wheat leaves show ICL, MS and β-oxidation activities.

Leaf peroxisomes from dark-induced senescing leaves show glyoxylate cycle enzyme activities two to four times higher than naturally senescing leaves. It indicates that the final stage of leaf development could be associated with a reversed transition of peroxisomes into glyoxysomes (see section 1. 3). In addition, it indicates that biogenesis of glyoxysomes is regulated in part through the expression of key glyoxysomal enzyme genes. Furthermore, results support the hypothesis of Gut and Matile (1988) that, during foliar senescence, a leaf peroxisome to glyoxysome (gerontosome) transition occurs in a genetically regulated senescence process. Thus two peroxisomal transitions may take place within the same organ: the first during greening and the second during senescence.

According to these observations, it may be assumed that senescence includes a utilization and metabolism of fatty acids associated with gluconeogenesis. The possible source of acyl residues are the chloroplastic glycolipids which represent the most abundant group of foliar lipids. Apart from photorespiratory and glyoxysomal functions which appear to determine the enzyme composition of microbodies in senescence, the catabolism of purines produced upon the degradation of nucleic acids may represent a further function (Rodriguez *et al.*, 1990). Thus, the occurrence and possible localisation in microbodies of enzymes of purine metabolism should be investigated in senescent leaves. The breakdown of chloroplasts in senescent leaves is

associated with the metabolism of large quantities of acyl residues produced upon the hydrolysis of thylakoidal glycolipids. In senescent primary leaves of barley, galactolipids have been demonstrated to disappear without simultaneous accumulation of free fatty acids (Gut and Matile, 1989). According to these observations, galactolipids could be mobilised via β-oxidation and the glyoxylate-cycle and eventually used for gluconeogenesis. Some support for this hypothesis has been provided by Wanner *et al.,* (1991). If this is so, an increased activity of PEPCK might be expected, but this has not been investigated.

1. 8. Research aims and objectives

Very little is known about the enzymes of microbodies and the metabolic pathways which function during germination and senescence in higher plants. Furthermore, the factors which regulate gene expression and microbody development during senescence are not understood. First of all, characterisation of glyoxysomal MDH gene expression and enzyme synthesis was studied to compare with that of ICL and MS during cotyledon development. Even though a gMDH cDNA has been previously cloned from watermelon (Gietl, 1990), gene expression during plant development has not been investigated, and its role in senescence is not known. gMDH gene expression and possible metabolic roles during cotyledon development were therefore investigated.

The main part of the project aimed to isolate and characterise cDNAs encoding enzymes and other proteins synthesised in cotyledons during germination and senescence but absent from the intermediate photosynthetic stage of growth. In

this way it was anticipated isolating clones encoding enzymes of lipid hydrolysis, glycerol utilisation, fatty acid β-oxidation, the glyoxylate cycle and PEPCK, or possibly proteins involved in the regulation of gene expression, and the synthesis, transport and assembly of these enzymes.

Therefore a cDNA library was prepared from RNA of senescing cotyledons and screened with cDNA probes from day 3, day 14, detached and senescent cotyledons. The aim then was to try to identify any cDNAs derived from mRNAs coordinately synthesised with those of ICL and MS, and study gene expression and function during cotyledon development.

Chapter Two: Materials and Methods

2. 1. Biological materials

2. 1. 1. Plant material

Seeds of cucumber (*Cucumis sativus* L. cv Masterpiece) were purchased from W. K. McNair (Portobello, Edinburgh) and germinated as described previously (Becker *et al.*, 1978). Seeds were imbibed overnight at 4 °C in sterile-distilled water, then sown in a tray of wet vermiculite. The seedlings were maintained in a growth room with 12 h per day lighting and a constant temperature of 25 °C. Excised cucumber cotyledons, leaves and roots were quickly frozen in liquid nitrogen and kept at -70 °C until they were needed. For detached experiments, cotyledons were excised after 2 weeks of sowing and placed on wet filter paper in petri dishes and sealed with Parafilm (American Can Co.). These were incubated for up to 8 days under the same conditions used for seedling growth. For dark-treated cotyledons, the petri dishes were wrapped in aluminium foil and incubated under the same conditions.

2. 1. 2. *Escherichia coli* strains and their genotypes

Table 2. 1. *Escherichia coli* strains used in this study and their genotypes.

Strain	Genotype
HB101	*supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1* (Hanahan, 1983)
XL-1 Blue	*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1* *ac*F'[*proAB*$^+$ *lacI*^{q} *lacZ M15* Tn *10(tet*^{r}*)*] (Bullock *et al.*, 1987)
SURE	*recB recJ sbcC201 uvrC umuC*::Tn*5(kan*^{r}*) lac (hsdRMS) endA1 gyrA96 thi relA1 supE44* *ac*F'[*proAB*$^+$ *lacI*^{q} *lacZ M15* Tn *10(tet*^{r}*)*] (Stratagene)

2. 1. 3. Bacterial plasmids and bacteriophages

2. 1. 3. 1. Plasmids and λ vectors for cloning

Table 2. 2. *Escherichia coli* plasmid vectors and lambda DNA.

Plasmid/ λ Vector	Source
pBLUESCRIPT SK II$^+$	Stratagene
pBLUSECRIPT SK II$^-$	Stratagene
pBS	Stratagene
pGEX-3X	Pharmacia
λgt 11	Amersham
λZAP II	Stratagene

2. 1. 3. 2. Other recombinant plasmids

Table 2. 3. Other plasmids containing cDNAs from cucumber.

Plasmid	Source
pBSMS1.9	A full length cDNA clone encoding cucumber MS (Graham, 1989).
pBSICL1.4	A partial cDNA clone encoding cucumber ICL from Wayne Becker (unpublished).
pBSH18	A full length clone encoding cucumber HPR (Greenler, *et al.*, 1989).
pBRGS	A partial cDNA clone (0.4 kb) encoding cucumber cytosolic glutamine synthetase (This thesis).
pBR-UNKNOWN	Unknown cDNA clones from differential screening (This thesis)

2. 1. 4. gMDH cDNA clones

A cDNA encoding gMDH from watermelon has been isolated by Gietl (1990). The cDNA was generously given to this laboratory and used for isolation of cucumber gMDH cDNA clones (Table 2. 4).

Table 2. 4. Plasmids containing gMDH cDNA.

Plasmid	Source
pGEMEX gMDH	A full length cDNA clone encoding watermelon gMDH (Gietl, 1990)
pBgMDHc-3	A partial cDNA clone (248bp) encoding cucumber gMDH (This thesis)
pBgMDHc-7	A partial cDNA clone (484 bp) encoding cucumber gMDH (This thesis)
pBgMDHc-12	A partial cDNA clone (567 bp) encodeing cucumber gMDH (This thesis)
pBRgMDHc-1	A full length cDNA clone (1278 bp) encoding cucumber gMDH (This thesis)
pBRgMDHc-B16	A full length cDNA clone (1.25 kb) encoding cucumber gMDH (This thesis)

2. 1. 5. PEPCK cDNA clones

cDNA clones encoding PEPCK have been isolated from senescing cucumber cDNA library by differential screening in this study (Table 2. 5).

Table 2. 5. Plasmids containing cucumber PEPCK cDNA.

Plasmid	Source
pBR U1-10	A partial cDNA clone (411 bp) encoding cucumber PEPCK (This thesis).
pBRPCK-1A	A partial cDNA clone (0.4 kb) encoding cucumber PEPCK (This thesis).
pBRPCK-4A	A full length cDNA clone (2.4 kb) encoding cucumber PEPCK (This thesis)
pBRPCK-5A	A partial cDNA clone (1.1 kb) encoding cucumber PEPCK (This thesis)
pBRPCK-6A	A partial cDNA clone (1.1 kb) encoding cucumber PEPCK (This thesis)
pBRPCK-7A	A full length cDNA clone (2,408 bp) encoding cucumber PEPCK (This thesis)
pBRPCK-9A	A partial cDNA clone (1.9 kb) encoding cucumber PEPCK (This thesis)
pBRPCK-11B	A partial cDNA clone (1.7kb) encoding cucumber PEPCK (This thesis)
pGEX-PCK9A	A PEPCK cDNA fragment subcloned into bacterial over-expression vector pGEX-3X (This Thesis).

2. 1. 6. Antiserum

Table 2. 6. Sources of antiserum are outlined.

Antisera	Source
Anti-gMDH	Polyclonal antisera raised against watermelon glyoxysomal malate dehydrogenase, given by Christine Gietl. Antibody recognises two (or three) polypeptides in westerns with apparent molecular masses of 41 and 38 kDa.
Anti-PEPCK	Polyclonal antisera raised against purified preparations of a fusion between cucumber PEPCK and bacterial glutathione transferase expressed in *E. coli.* The antibody recognises a cucumber polypeptide in westerns with an apparent molecular mass of 74 kDa. (This Thesis)

Freeze-dried anti-gMDH antibody was reconstituted in sterile double distilled water and stored at -20 °C. Anti-PEPCK was stored at 4 °C after collecting the sera. Samples were dliuted 1:400 for anti-gMDH and 1:2,000 for anti-PEPCK in TBST (10 mM Tris-Cl pH 8.0, 0.9% (w/v) NaCl, 0.5% (v/v) Tween-20).

2. 2. Molecular biological techniques

2. 2. 1. General conditions

All solutions, pipette tips and microcentrifuge tubes used in the isolation and manipulation of nucleic acids were autoclaved at 15 p.s.i. for 20 min before use. Double distilled water was used at all times in the preparation of solutions. All glassware coming into contact with nucleic acids was baked at 120 °C overnight. All manipulations were carried out on ice unless otherwise stated. The concentration of nucleic acids in aqueous solution was determined by measuring the absorbance (A) at 260 nm and 280 nm. For RNA, 1 A_{260} unit is equivalent to 40 μg ml^{-1} and for DNA, 1 A_{260} unit is equivalent to 50 μg ml^{-1}.

2. 2. 2. Isolation of nucleic acids

2. 2. 2. 1. Isolation of bacterial plasmid DNA

Plasmid DNA was isolated from *E. coli* strains by the alkaline lysis method (Sambrook *et al.*, 1989). First, a single colony was inoculated in 5 ml Luria broth (LB) containing appropriate antibiotics and incubated for 12 to 16 h at 37 °C with continuous shaking. Isolation of plasmid DNA was carried out in 1.5 ml microfuge tubes. 1.5 ml of the culture was harvested by centrifugation in a microfuge tube for 30 sec at room temperature and the supernatant was discarded and the pellet air dried briefly. The pellet was resuspended in 100 μl GTE solution (50 mM glucose, 25 mM Tris-Cl pH 8.0, 10 mM EDTA (disodium ethylenediaminetetraacetate)) and incubated

at room temperature for 5 min. Then 200 µl of freshly prepared alkaline SDS (sodium dodecyl sulphate) solution (0.2M NaOH and 1% (w/v) SDS) was added and mixed, and placed on ice for 5 min. Next, 150 µl of ice cold potassium acetate solution (3 M potassium, 5 M acetate, pH 4.8) was added and mixed briefly, then incubated on ice for 5 min. Samples were centrifuged at 4 °C for 15 min and the supernatant was transferred to a fresh microfuge tube. An equal volume of phenol/chloroform (1:1) was added to the supernatant and vortexed vigorously for a few seconds, then centrifuged for 10 min at 4 °C. The supernatant was transferred to a fresh microfuge tube and precipitated with 2.5 volumes of ice-cold ethanol at -20 °C for 10 min. Nucleic acids were pelleted by centrifugation at 4 °C for 30 min. The pellet was washed in 70% (v/v) ethanol, dried in a Howe Gyro-Vap (V. A. Howe and Co Ltd, Banbury, U. K.) and resuspended in 20 to 30 µl of TE buffer (10 mM Tris-Cl pH 7.5, 1 mM EDTA). RNA was eliminated by the addition of 1 µl of RNaseA (1 mg ml^{-1}) and subsequent incubation at 37 °C for 10 min prior to restriction enzyme digestion and electrophoresis. The plasmid DNA samples were stored at -20 °C for further use.

2. 2. 2. 2. Isolation of cloned DNA fragments

First, DNA was digested with the appropriate restriction enzyme following the suppliers protocols. Then, DNA was electrophoresed on a 1.0% (w/v) agarose gel containing 1X TAE (40 mM Tris-acetate, 1 mM EDTA pH 8.0) and 0.2 µg ml^{-1} ethidium bromide. DNA fragments were visualised by long-wave UV (320 nm) illumination and were subsequently excised from the gel using a sterile scalpel. DNA fragments were purified using a Geneclean Kit (USB, Ohio, USA).

2. 2. 2. 3. Total RNA isolation from cucumber cotyledons

Plant materials were ground to a fine powder using liquid nitrogen with a baked pestle and mortar. The fine powder was transferred to a pre-chilled 50 ml COREX tube and 4 ml grinding buffer (100 mM Tris-Cl pH 8.5, 6% (w/v) *para*-aminosalicylate, 1% (w/v) tris-*iso*propylnaphthalene sulphonate) added per gram fresh weight of tissue. Then, immediately the same volume of phenol/chloroform/isoamyl alcohol (50:48:2 v/v/v) was added, the phenol having been saturated with 100 mM Tris-Cl pH 8.0 and containing 0.1% (w/v) 8-hydroxyquinoline. Each tube was vortexed for a few seconds. Centrifugation was carried out to separate cell debris and protein at 3,000 x *g* for 10-20 min at 4 °C. The supernatant was carefully transferred to a 30 ml COREX tube and extracted twice using phenol/chloroform/isoamyl alcohol (50:48:2 v/v/v) and separated at 10,000 x *g* for 30 min. After final extraction, the upper aqueous phase was removed to a fresh tube and nucleic acids were precipitated with 2.5 volumes of ethanol at -20 °C for between 1 h and overnight. Then, the pellet of nucleic acids was centrifuged as above and washed in 70% (v/v) ethanol, and all the liquid removed. The pellet was dissolved in 1 ml of DEPC (diethyl pyrocarbonate)-treated double distilled H_2O, and total RNA was selectively precipitated by adding an equal volume of 5 M lithium chloride to a final concentration of 2.5 M and incubated on ice overnight. After centrifugation at 10,000 x *g* for 30 min, the resulting pellet was washed in 2 M lithium chloride and the ethanol precipitation step was repeated to remove salt. Finally, the total RNA pellet was dissolved in DEPC-treated double distilled H_2O and the concentration was estimated in a Beckman spectrophotometer at 260 nm and 280 nm.

2. 2. 2. 4. Selective isolation of Poly (A)$^+$ RNA from total RNA

Isolation of poly(A)$^+$ RNA was carried out using Hybond^{TM}-mAP (messenger affinity paper) following the manufacturers protocol (Amersham, U. K.).

2. 2. 2. 5. Isolation of DNA from plant leaves

Approximately 2 g of leaf tissue was ground to a fine powder using a pestle, mortar and liquid nitrogen. 4.5 ml of extraction buffer (0.1 M Tris-Cl pH 8.0, 50 mM EDTA, 500 mM NaCl, 10 mM 2-mercaptoethanol) was added and the homogenate was incubated for a few minutes at 65 °C to thaw all tissue. Then, 100 µl of 10% (w/v) SDS was added, mixed well and incubated at 65 °C for 20 min. The sample was then centrifuged at 13,000 x *g* for 10 min and the supernatant was removed to a fresh 15 ml COREX tube. To precipitate the nucleic acids, 1.5 ml 5 M potassium acetate was added and placed on ice for 30 min. The resulting potassium dodecyl sulphate precipitate was sedimented by centrifugation at 13,000 x *g* at 4 °C for 30 min. The supernatant was removed to a 15 ml tube and 3.6 ml of isopropanol added, then incubated at -70 °C for at least 10 min. Centrifugation at 10,000 x *g* for 10 min sedimented the nucleic acids. The supernatant was removed and the pellet was dissolved in 4.2 ml of TE (10 mM Tris-Cl pH 7.5, 1 mM EDTA). To further purify, nucleic acids were reprecipitated by adding 480 µl of 3 M sodium acetate and 3 ml of isopropanol and incubated at room temperature for 10 min. After the final centrifugation at 10,000 x *g* for 30 min, the nucleic acid pellet was washed with 70% (v/v) of ethanol. The DNA was finally dissolved in 100 µl of TE and the DNA concentration measured at 260 nm.

2. 2. 3. A cDNA library construction from mRNAs of senescing cucumber cotyledons

2. 2. 3. 1. cDNA library system and general procedure

Firstly, yellow cotyledons (see Figure 3. 1) were collected from cucumber plants 5-6 weeks after sowing and quickly frozen in liquid nitrogen, then stored at -70 °C until needed. Secondly, total RNA and poly $(A)^+$ RNA were isolated successively as above (sections 2. 2. 2. 3 and 2. 2. 2. 4). RNA was dissolved in DEPC-treated double distilled water and stored -20 °C. The final concentration of the poly $(A)^+$ RNA was measured by absorption at 260 nm and 280 nm, and examined by TBE (45 mM Tris-borate, 1 mM EDTA pH 8.0) agarose gel electrophoresis.

Initially, 5 μg of poly $(A)^+$ RNA was used for first-strand cDNA synthesis. Then, all the other steps followed the manufacturer's standard protocol (Stratagene, La Jolla, USA). The first-strand cDNA synthesis was achieved by M-MuLV (Moloney murine leukemia virus) reverse transcriptase and primer. The primer is a fifty base oligo-nucleotide;

(5' GAGAGAGAGAGAGAGAGAGAACTAGTCTCGAGTTTTTTTTTTTTTTTT TT 3') and, designed with a "GAGA" sequence to protect the *Xho*I restriction enzyme (underlined) recognition site and 18 base poly dT sequence. The restriction site allows the finished cDNA to be inserted into the Lambda ZAP vector in a sense orientation (*Eco*RI to *Xho*I) after double digestion with *Eco*RI and *Xho*I with respect to the *LacZ* promoter. The Uni-ZAP vector allows *in vivo* excision of the pBLUESCRIPT phagemid, allowing the insert to be characterised in a plasmid system including restriction enzyme analysis and DNA sequencing.

At the end of this stage, the cDNA was purified through a Sephacryl S-400 column and monitored with a portable Geiger counter. cDNA was precipitated with ethanol and washed with 70% (v/v) ethanol. Finally, cDNA was dissolved in 20 µl of TE buffer and stored at -20 °C. The cDNA was ligated into the *Eco*RI and *Xho*I sites of λZAPII. After a 48 h ligation reaction at 4 °C, the whole reaction was used for a *in vitro* packaging. A Gigapack Gold II packaging extract (Stratagene, La Jolla, USA) was used for *in vitro* packaging. After the packaging reaction, the initial cDNA library was examined to estimate the number of clones and recombination ratio.

2. 2. 3. 2. Amplification of initial cDNA library

After *in vitro* packaging, the initial library was immediately amplified. Host cells (SURE) were grown at 30 °C overnight, then cooled on ice for 20 min and centrifuged for 15 min at 4 °C at 3,000 rpm to collect the cells. The cells were resuspended in ice cold 10 mM $MgCl_2$ and cell number adjusted to $A_{600} = 0.5$ and 1.0 respectively. 200 µl of cells ($A_{600} = 0.5$) were mixed with 10^4 pfu of the initial library in 10 ml test tubes, then incubated in a 37 °C waterbath for 15 min. 4 ml of pre-warmed top-agar (48 °C) was added to each tube and poured immediately to a prewarmed (37 °C) NZY-bottom agar plate. The plates were placed on a flat table for 10 min to solidify the top-agar mixture. The petri dishes were incubated at 37 °C for 5 to 6 h until clear plaques developed. 10 ml of phage buffer (50 mM Tris-Cl pH 7.5, 0.01% (w/v) gelatine, 1 mM NaCl, 7 mM $MgCl_2$) was added to each plate and incubated at 4 °C overnight with gentle shaking. The bacteriophage suspension was recovered from each plate and pooled into 50 ml sterile polypropylene tubes. After

centrifugation for 5 min at 4,000 x *g*, cell debris was removed and chloroform added to 0.5% (v/v) and stored at 4 °C. Finally, the library was titred and used for screening.

2. 2. 4. cDNA library screening for isolation of cucumber gMDH and PEPCK cDNAs using cloned DNA probes

2. 2. 4. 1. Preparation of plaque-lifts

Bacteriophage plaques were prepared as follows. For first-round screening, plaque numbers were adjusted to 1.0 x 10^5 for a 20 x 20 cm plate and for the second-round screening, plaque numbers were adjusted to between 100 and 200 for a 11 x 11 cm plate. Bacteriophage were incubated for 5 to 8 h at 37 °C, then placed in the cold room for 2 h to chill. Bacteriophage plaques were transferred for 2 min to Hybond-N membrane (Amersham, U. K.). The transferred phage DNA was denatured after lifting, by submerging the membranes in denaturation solution (1.5 M NaCl, 0.5 M NaOH, 1 mM EDTA pH 8.0) for 2 min. The membranes were then neutralised for 5 min by submerging in neutralization solution (1.5 M NaCl, 0.5 M Tris-Cl pH 8.0). Subsequently, membranes were rinsed for 30 sec in 0.2 M Tris-Cl pH 7.5, 2X SSC (1X SSC; 150 mM NaCl, 15 mM Na_3-citrate pH 7.0). The membranes were air dried on Whatman 3MM filter paper and UV-crosslinked on an autocrosslinker (Hybaid, Middlesex, U. K.) at 0.4 J cm^{-2} for 40 sec. Master agar plates and blotted membranes were stored at 4 °C to use for subsequent hybridisation and phage isolation.

2. 2. 4. 2. Plaque hybridisation

Firstly, plaque lift membranes were prehybridised in prehybridisation solution (6X SSC, 5X Denhardt's solution, 0.5% (w/v) SDS, 100 μg ml^{-1} denatured herring sperm DNA) for 1 to 4 h at 65 °C using a Hybaid incubator (Hybaid, Middlesex, U. K). Next, hybridisation was carried out for 16 to 20 h as for prehydridisation conditions. Then the membrane was washed according to the manufacturers protocol (Amersham, U. K.).

2. 2. 4. 3. Plaque isolation from masterplates after hybridisation

Plaques of interest were removed from the master agar plates using sterile Pasteur pipettes and transferred to sterile microfuge tubes containing 1 ml of phage buffer and 20 μl of chloroform. The tubes were vortexed and incubated overnight at 4 °C to release the Uni-ZAP™ XR phage particles into phage buffer Then, the tubes were centrifuged to eliminate debris and the supernatant was transferred to fresh tubes and stored at 4 °C.

2. 2. 4. 4. *in vivo* excision of pBLUESCRIPT from Uni-ZAP™ XR

Firstly, 200 μl of XL-1 Blue cells (A_{600}=1.0), 200 μl of Uni-ZAP™ XR phage stock (section 2. 2. 4. 3) and 1 μl of R408 helper phage (Stratagene, La Jolla, USA) were mixed in a 50 ml conical tube. The tube was incubated at 37 °C for 15 min. Then 5 ml of Superbroth medium was added and incubated 3 to 4 h at 37 °C in

a shaker. After incubation, the mixture was heated at 65 °C for 30 min, then centrifuged for 10 min at 3,000 x *g* using a Mistral centrifuge at 4 °C. The supernatant containing pBLUESCRIPT phagemid was transferred to a fresh tube and used for transformation. To plate the rescued phagemid, 10 µl of a 10^{-2} dilution of pBLUESCRIPT phagemid and 200 µl XL-1 Blue cells (A_{600} = 1.0) were mixed in a 5 ml sterile test tube and incubated in a 37 °C waterbath for 15 min. Subsequently, 10 to 50 µl was spread on fresh LB/ampicillin (50 µg ml^{-1}) plates and incubated overnight at 42 °C. Colonies which appeared were randomly selected and cultured in 5 ml LB/ampicillin broth, then a mini-prep plasmid DNA isolation carried out. Afterwards, mini-prep DNA was further analysed by restriction endonuclease digestion and agarose gel electrophoresis.

2. 2. 5. Nucleic acid hybridisation

2. 2. 5. 1. Preparation of radiolabelled DNA probe

Probe DNAs were labelled with α-[^{32}P]dCTP by the random primer method (Feinberg and Vogelstein, 1984). Firstly, 20 to 50 ng of the DNA fragment was denatured by boiling in a Dry Block for 4 to 5 min at 100 °C and then cooled on ice for 5 min. A reaction contained 5 µl of OLB (oligolabelling buffer) mix (250 mM Tris-Cl pH 8.0, 25 mM $MgCl_2$, 5 mM β-mercaptoethanol, 2 mM dATP, 2 mM dGTP, 2 mM dTTP, 1M HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid) pH 6.6, 1 mg ml^{-1} oligonucleotides), 1 µl 10 mg ml^{-1} BSA (bovine serum albumin), 1-2 units of DNA polymerase I (Klenow) (Gibco BRL, U. K.), 2 µl (20 µCi) α-[^{32}P] dCTP and sterile double distilled H_2O up to 25 µl. The reactions were incubated at

room temperature for 5 to12 h and stopped by addition of 200 µl of STOP solution (20 mM Tris-Cl pH 7.4, 20 mM NaCl, 2 mM EDTA, 0.25% (w/v) SDS). Unincorporated nucleotides were separated from radiolabelled DNA by passing the reaction mix through a 1 ml syringe packed with Sephadex G-50 (Pharmacia LKB, Sweden) and equilibrated with STE (TE pH 8.0, 100 mM NaCl). The syringe was centrifuged at 750 x *g* for 2 min and the elute collected. Labelled DNA probes were denatured by boiling for 4 to 5 min and cooled on ice before adding to hybridisation reaction.

2. 2. 5. 2. Northern blot hybridisation

10 µg total RNA samples were diluted in DEPC-treated double distilled H_2O to 6 µl and mixed with 19 µl denaturing solution (50% (v/v) formamide, 8% (v/v) formaldehyde, 1X MOPS (3-[*N*-morpholino]propanesulfonic acid) (20 mM MOPS, 5 mM sodium acetate pH 7.0, 1 mM EDTA, 100 ng ml^{-1} ethidium bromide). Samples were incubated at 65 °C for 5 min and chilled on ice. Then, 2.5 µl 10X sample buffer (50% (v/v) glycerol, 0.1 mg ml^{-1} bromophenol blue, 0.1 mg ml^{-1} xylene cyanol) was added and mixed. Total RNA was electrophoretically fractionated through an agarose/formaldehyde denaturing gel (16% (v/v) formaldehyde, 1X MOPS buffer, 1.5% (w/v) agarose) system using 1X MOPS buffer as electrode buffer. Then, RNA was blotted onto Hybond-N membranes (Amersham, U. K.) for 12 to 18 h using 20X SSPE (3.6 M NaCl, 0.2 M sodium phosphate pH 7.7, 20 mM EDTA) as the blotting buffer. Next, the membranes were air dried and UV-crosslinked by irradiation at 0.4 J cm^{-2} using a Hybaid crosslinker (Hybaid, Middlesex, U. K.).

Membranes were prehybridised (for 2 to 4 h) and hybridised at 42 °C in 50% (v/v) formamide, 5X SSPE, 5X Denhardt's solution (0.1% (w/v) BSA, 0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrrolidon)), 0.5% (w/v) SDS and 100 μg ml^{-1} denatured herring sperm DNA. cDNA fragments were radiolabelled for probes using the oligo labelling procedure and labelled probes were seperated through Sephadex G-50 column as described in section 2. 2. 5. 1. Labelled DNA probes were boiled for 5 min and chilled on ice, then added directly to the hybridisation reaction and incubated for 16 to 20 h. Membranes were washed twice in 2X SSPE/0.1% (w/v) SDS for 15 min, once in 1X SSPE/0.1% (w/v) SDS for 30 min 42 °C and once in 0.1X SSPE/0.1% SDS for 10 min at room temperature. The membranes were air dried and exposed to pre-flashed X-ray film for 12 to 96 h at -70 °C.

To reprobe the membrane, the probe was removed by incubating the membrane in 5 mM Tris-Cl pH 8.0, 2 mM EDTA, 0.1X Denhardt's solution for 1 h at 65 °C.

2. 2. 5. 3. Southern blot hybridisation

10 μg of genomic DNA was digested by restriction endonucleases and electrophoretically fractionated in a 0.8% (w/v) agarose (Sigma Chemical Co.) gel. After electrophoresis in agarose, the gel was placed in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 30 min and then for a further 30 min using fresh denaturing solution. Next, the gel was transferred to neutralizing solution (1.5 M NaCl, 0.5 M Tris-Cl pH 7.5, 1 mM EDTA) and left for 30 min then transferred to fresh solution for a further 30 min. Then, denatured DNA was transferred to Hybond-N membrane by

capillary blotting using 20X SSC (3.0 M NaCl, 0.3 M Na_3 citrate). After blotting, the Hybond-N membrane was air dried and UV-crosslinked at 0.4 J cm^{-2} using a Hybaid UV-Crosslinker (Hybaid, Middlesex, U. K). Prehybridisations and hybridisations were carried out at 65 °C in 6X SSC, 5X Denhardt's solution, 0.5% (w/v) SDS and 100 µg ml^{-1} denatured herring sperm DNA for 2 to 4 h and 16 to 20 h respectively. The hybridised membrane was washed exactly as described in the manufacturer's protocol (Amersham, U. K.).

2. 2. 6. Differential cDNA library screening

2. 2. 6. 1 General procedure

Bacteriophage plaque-lifts were prepared as above (section 2. 2. 4. 1) but the number of plaques was adjusted to 1 x 10^4 (for a 11 x 11 cm plate) for the first round screening. After the second round screening, pure plaques were isolated and stored at 4 °C (section 2. 2. 4. 3) for *in vivo* excision. All the other steps of phagemid preparation followed the manufacturer's standard protocol (Stratagene, La Jolla, USA) (see sections 2. 2. 4. 3 and 2. 2. 4. 4).

2. 2. 6. 2. Radiolabelled single stranded-cDNA probe preparation

Poly(A)$^+$ RNA was used as a template for radiolabelled cDNA probes. 5µg of poly(A)$^+$ RNA was used for each reaction. Firstly, RNA was heated at 65 °C for 3 min and cooled on ice. A reverse transcription reaction contained 1X reverse transcription buffer (50 mM Tris-Cl pH 8.5, 75 mM KCl, 8 mM $MgCl_2$, 4 mM DTT), 1.25 µg oligo $(dT)_{12-18}$ (Pharmacia LKB, Sweden), 10 mM dATP, 10 mM dGTP, 10

mM dTTP (Pharmacia LKB, Sweden), 5 µl (50 µCi) α-[^{32}P]dCTP (Amersham, U. K.), 0.5 µg BSA, 2.5 units RNasin (Pharmacia LKB, Sweden), 1,000 units M-MuLV reverse transcriptase (Promega) and DEPC-treated double distilled water up to 50 µl. The reaction was thoroughly mixed and briefly spun then incubated in a 37 °C water bath for 1 h. After that, template RNA was hydrolysed by adding 50 µl hydrolysis mixture (1% (w/v) SDS, 50 mM EDTA pH 8.0, 400 mM NaOH) and further incubated for 2 h at 37 °C. Subsequently, the reaction was neutralised by adding 2 µl glacial acetic acid, then extracted by phenol/chloroform. The aqueous supernatant was extracted once with chloroform. Radiolabelled DNA was separated on a Sephadex G-50 column as above (section 2. 2. 5. 1). 1 to 2 µl of the radiolabelled single stranded-cDNA probe was electrophoresed on an alkaline denaturing gel (section 2. 2. 6. 3) then subjected to autoradiography, to examine incorporation and size of products.

2. 2. 6. 3. Alkaline agarose gel electrophoresis

Agarose (1 g) was dissolved in 85 ml water by heating in a microwave oven. Then the gel was cooled to 60 °C, NaOH added to 50 mM and EDTA (pH 8.0) to 1 mM, and poured. After the gel was completely set (30 to 40 min), it was soaked in 1X alkaline electrophoresis buffer (50 mM NaOH, 1 mM EDTA). The sample was prepared by addition of 1 volume of 2X loading buffer (100 mM NaOH, 2 mM EDTA, 6% (w/v) Ficoll, 0.4% (w/v) bromocresol green, 0.1% (w/v) xylene cyanol FF) to radiolabelled cDNA probes. Then, samples were loaded into wells and electrophoresis carried out at 50 V until the dye has migrated two thirds of the length of the gel. At the end of the run, the gel was removed from the tank and soaked in 7%

(w/v) trichloroacetic acid for 30 min at room temperature with two changes. The gel was dried and products detected by autoradiography.

2. 2. 6. 4. Plaque DNA blot hybridisation

Prehybridisations and hybridisations were carried out as described in the section 2. 2. 4. 2. However, to block poly $(A)^+$ tails, polyuridylic acid poly U (Pharmacia LKB, Sweden) (250 $\mu g\ ml^{-1}$) was added to prehybridisation and hybridisation solutions.

2. 2. 6. 5. Plaque isolation and *in vivo* excision

Followed as above in sections 2. 2. 4. 3 and 2. 2. 4. 4.

2. 2. 7. DNA sequencing

2. 2. 7. 1. Template DNA (Double stranded) isolation

Template DNA (plasmids or phagemids) was isolated and purified as described in manufacturers suggested protocol (Pharmacia LKB Biotechnology, Uppsala, Sweden). This involved mini-prep plasmid DNA isolation (see section 2. 2. 2. 1), nucleic acid precipitation with isopropanol and phenol/chloroform extraction. Finally, the nucleic acids were precipitated with 0.3 M sodium acetate and 2.5

volumes of absolute ethanol and the pellet washed with 1 ml of 70% (v/v) ethanol. Then, the washed nucleic acids pellet was dried under vacuum and dissolved in 20 to 30 µl of TE buffer, and immediately proceeded with denaturing (with 0.5 M NaOH at room temperature for 10 min) and the sequencing reaction.

2. 2. 7. 2. Sequencing reaction and the sequencing gel

DNA sequencing of clones or subclones in pBLUESCRIPT SKII was carried out according to the dideoxy chain termination method of Sanger *et al.,* (1977). For the 5' to 3' direction, reverse primer (5' AACAGCTATGACCATG 3') was used and for the 3' to 5' direction, M13-40 primer (3' TGACCGGCAGCAAAATG 5') was used.

The sequencing reaction was carried out using a T7 DNA sequencing kit ™ (Pharmacia LKB Biotechnology, Uppsala, Sweden). Labelling of the DNA was by incorporation of α-[^{35}S]dATP during the second strand synthesis. Labelled products were fractionated by electrophoresis using a Sequi-Gen Nucleic Acid Sequencing Cell (Bio-Rad Laboratories, CA, USA) in a 6% (w/v) polyacrylamide gel as described in Sambrook *et al.,* (1989). Subsequently, the gels were soaked in 10% (v/v) acetic acid and 10% (v/v) methanol in distilled water for 40 to 60 min, then transferred to a sheet of filter paper (Whatman 3MM) and covered with Saran Wrap (Dow Chemical Company). The gel was dried using a Bio-Rad (Bio-Rad Laboratories, CA, USA) vacuum gel drier for 1 to 2 h at 80 °C. Finally, after removing the covered wrap, the gel was exposed to a CRONEX-4 X-ray film at room temperature for 18 to 96 h.

2. 2. 8. Anti-PEPCK antisera production

2. 2. 8. 1. Subcloning of a PEPCK cDNA fragment into an *E. coli* over-expression vector

Antisera against cucumber PEPCK were generated using as antigen, a fusion protein synthesised in *E. coli* . The strategy employed the over-expression plasmid vector pGEX-3X (Pharmacia LKB Biotechnology,Uppsala, Sweden)which had been modified (by Takeshi Takaha) to include extra *Nco*I, *Xho*I and *Hind*III sites (pGEX-301). This plasmid directs synthesis of an *E. coli* glutathione transferase fusion protein, from an IPTG (Isopropyl-β–D-thiogalactopyranoside)-inducible*tac* promoter. A 1.9 kb cDNA clone (pBRPCK-9A) was chosen and restriction digested with *Eco*RI (see section 5. 4. 1). The 1.5 kb *Eco*RI fragment was subcloned into the pGEX-301 *Eco*RI site. Orientation of the cDNA insert was established by restriction endonuclease digestion and agarose gel electrophoresis. The recombinant plasmid was named pGEX3-PCK. XL-1 Blue cells were transformed with pGEX3-PCK for protein synthesis.

2. 2. 8. 2. Induction of PEPCK fusion protein synthesis in *E. coli* cells and isolation of inclusion bodies

XL-1 Blue cells containing pGEX3-PCK plasmid were cultured at 37 °C overnight in 200 ml LB/ampicillin medium. The total volume of this culture was transferred and mixed with 600 ml fresh LB/ampcillin medium. After 1 h incubation at 37 °C, IPTG (final concentration 3 mM) was added then further incubated for 3 h.

To isolate inclusion bodies, firstly the culture was cooled on ice for 20 min, then centrifuged at 6,000 x *g* at 4 °C for 5 min. The cell pellets were resuspended in 10 volumes of 100 mM NaCl, 1 mM EDTA and 50 mM Tris-Cl (pH 8.0), then lysozyme was added to 1 mg ml^{-1} and incubated at room temperature for 30 min. Secondly, the mixture was centrifuged at 3,000 x *g* for 10 min at 4 °C and the pellet was resuspended in an equal volume of ice cold 100 mM NaCl, 1mM EDTA, 0.1% (w/v) sodium deoxycholate and 50 mM Tris-Cl (pH 8.0). Then the suspension was further incubated on ice with occasional mixing for 10 min. Next, $MgCl_2$ (final concentration of 8 mM) was added to the mixture and DNaseI added to a final concentration of 10 μg ml^{-1}. After further incubation on ice until the viscosity disappeared (about 30 min), the mixture was centrifuged at 3,000 x *g* for 10 min and the pellet was resuspended in 10 ml of 1 X SDS gel running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS). The crude inclusion body mixture was stored at -20 °C before futhrer purification.

2. 2. 8. 3. Purification of PEPCK fusion protein from *E. coli* cells

After *E. coli* inclusion body isolation, SDS-PAGE (polyacrylamide gel electrophoresis) was carried out to examine purity, then the fusion protein was further purified by electro-elution. Firstly, after SDS-PAGE, the gel was stained in 0.01% (w/v) Coommasie Brilliant blue in water, then the band of polyacrylamide containing the fusion protein was excised and the fusion protein was electro-eluted using prepared dialysis tubing (Sambrook *et al.*, 1989). Purified fusion protein was diluted in TBS buffer (10 mM Tris-Cl pH 8.0, 0.9% (w/v) NaCl) and stored at -20 °C.

2. 2. 8. 4. Sample preparation for injection of PEPCK antigen into rabbits

Pre-immune serum was taken from two rabbits one day before the first injection. For the first injection, an equal volume of fusion protein suspension (equivalent of 1,000 μg protein) and Complete Adjuvant (Sigma) were mixed vigorously using a vortex mixer until a thick emulsion developed. For the second and third injections, an equal volume of Incomplete Adjuvant (Sigma) and protein suspension (equivalent of 1,000 μg protein) were mixed as in the first injection. 500 μg protein was injected subcutaneously into each of two rabbits by staff of Edinburgh University Medical Faculty Animal Area. Rabbits were given second and third injections at 10 day intervals and bled 12 days after the third injection. Antisera were collected and sodium azide added to 0.01% (w/v) and, stored at 4 °C. Some serum samples were freeze-dried and stored at 4 °C.

2. 3. Western blot analysis

2. 3. 1. Plant protein sample preparation

Cotyledon samples were collected and stored as described above (see section 2. 1. 1). Frozen cotyledons were homogenized in PTES buffer (100 mM Tris-Cl pH 8.0, 2 mM EDTA, 1.0% (w/v) SDS, 18% (w/v) sucrose, 10 mM DTT (dithiothreitol), 1 mM PMSF (phenylmethyl sulfonyl fluoride), 5 mM aminocaproic acid). The homogenates were prepared by the addition of 2 volumes of the extraction buffer relative to weight of tissue. All extractions were carried out at 4 °C using a pre-cooled pestle and mortar. Then the homogenate was transferred to microcentrifuge

tubes and centrifuged at 10,000 x *g* at 4 °C for 20 min. Supernatant was stored as 50 µl aliquots at -20 °C. To determine the amount of protein, firstly SDS was removed by passing a 100 µl aliquot down a 1 ml syringe packed with Sephadex G-50, by centrifugation at 750 x *g* for 2 min. The amount of protein was determined using the Bio-Rad protein determination kit that is based on the Bradford assay protocol (Bradford, 1976).

2. 3. 2. SDS-PAGE

SDS-PAGE was carried out as described in Laemmli (1970). Typically 10% (w/v) acrylamide, 375 mM Tris-Cl pH 8.8, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulphate, 0.008% (v/v) TEMED was prepared for the resolving gel and poured into a mini-gel apparatus (Bio-Rad Laboratories, CA, USA) up to 2 cm from the top of the plates. Resolving gel overlay solution (0.375 M Tris-Cl pH 8.8, 0.1% (w/v) SDS, 79% (v/v) iso-propanol) was overlayed on top of the polymerising acrylamide. The gel was left for 30 to 60 min until polymerised. After washing out of the overlay solution using double distilled H_2O, the stacking gel (3.3% (w/v) acrylamide, 125 mM Tris-Cl pH 6.8, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulphate, 0.1% (v/v) TEMED) was poured over the resolving gel and a comb was inserted into the stacking gel. After polymerisation of the stacking gel, the comb was removed and wells were washed out using double distilled H_2O. Prior to electrophoresis, protein samples were heated in boiling water for 5 to 10 min. Electrophoresis was carried out using 1X Laemmli electrophoresis buffer (25 mM Tris-Cl pH 8.5, 192 mM glycine, 0.1% (w/v) SDS) at 20 mA until the marker dye had migrated to the interface between the

stacking gel and resolving. The current was then kept at 15 mA until the dye reached the bottom of the gel (approximately 2 h).

2. 3. 3. Protein transfer onto Hybond-ECL membrane

After eletrophoresis, the gel was removed from the apparatus and soaked in transfer buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, 10% (v/v) methanol) for 5 min. Hybond-ECL membrane (Amersham, U. K.) and 4 sheets of Whatman 3 MM paper were cut to the same size as the region of gel to be transferred. Hybond-ECL membrane was immersed in double distilled H_2O for 5 min and transferred to the transfer buffer for 10 min. After assembling the transfer apparatus, fractionated polypeptides were transferred at 350 mA for 2 h. Electro-blotted membrane was rinsed twice with double distilled H_2O and washed twice with TBST (10 mM Tris-Cl pH 8.0, 0.9% (w/v) NaCl, 0.5% (v/v) Tween-20). Occasionally, the membrane was stained with Ponceau-S solution (2% (w/v) Ponceau-S, 20% (w/v) trichloroacetic acid) for 5 min at room temperature. The membrane was washed with double distilled H_2O to visualise the transferred proteins. Then the stain was removed by several washes at room temperature with TBST .

2. 3. 4. Immunodetection of specific proteins

Firstly, non-specific binding was blocked by incubating the membrane overnight in TBST containing 5% (w/v) non-fat dried skimmed milk (Safeway Co.) at 4 °C and washed three times for 30 min with TBST at room temperature. Then,

incubation was carried out with primary antibody (Table 2. 6) diluted in TBST, for 1 to 2 h. Subsequently, the membrane was washed with TBST 6 times for 15 min. Second antibody (goat anti-rabbit IgG conjugated with horseradish peroxidase, Sigma) was diluted in TBST (normally 4 x 10^{-4}) and the membrane was incubated in diluted second antibody for 20 min. Unbound secondary antibody was removed by washing with TBST for 1 h with 6 changes at room temperature.

ECL immunodetection reagents (Amersham, U. K.) were used for the detection of antigens. Equal volumes of detection solutions 1 and 2 were mixed and the membrane was incubated in the detection mixture for 1 min. After blotting off the liquid, the signal was detected on CRONEX-4 X-ray film (Dupont, Frankfurt, Germany) for 1 to 10 min.

2. 4. Miscellaneous

2. 4. 1. Chemicals

All chemicals were purchased from Sigma Chemical Co. Ltd., and BDH Chemicals Ltd., unless otherwise stated.

2. 4. 2. Radiochemicals

α-[^{32}P]dCTP (3,000 Ci mmol^{-1})) and α-[^{35}S]dATP (500 Ci mmol^{-1}) were purchased from Amersham International plc.

2. 4. 3 Autoradiography Film

All X-ray films were CRONEX-4 (Dupont, Frankfurt, Germany) and all films were developed in an Agfa-Gaevert Gevamatic 60 automatic developer.

2. 4. 4 Bacteriological media

All strains of *E. coli* were grown in LB (Luria and Bertani) broth or LB agar at 37 °C, except XL-1 Blue cells for *in vivo* excision, which was grown at 30 °C. For phage library screening and *in vivo* excision, NZY top-agar and superbroth were used.

LB broth: 10g l^{-1} Bacto tryptone (Difco Laboratories, Detroit, USA), 5g l^{-1} Bacto yeast extract (Difco), NaCl 10g l^{-1} , pH 7.2. For LB agar, 1.5% (w/v) of Bacto agar (Difco) was added to LB broth.

NZY plates: 5g l^{-1} NaCl, 2g l^{-1} $MgSO_4$, 5g l^{-1} yeast extract, 10g l^{-1} casein hydrolysate, pH 7.5, 1.5% (w/v) Difco Agar.

Top agar: same as NZY plates but 0.7% (w/v) agarose replacing Difco Agar.

Superbroth: 5g l^{-1} NaCl, 20g l^{-1} Bacto-yeast extract, 35g l^{-1} Tryptone (Difco Laboratories, Detroit, USA), 0.005 M NaOH.

2. 4. 5. Computer analysis

Assembly and analysis of nucleotide and derived amino acid sequence data was carried out using the programs (Devereux *et al.*, 1984) of the University of Wisconsin Genetics Computer Group (UWGCG) through the VAX 8000 system at the University of Edinburgh.

Chapter Three:
Glyoxysomal NAD-malate dehydrogenase gene expression

3. 1. Rationale

The role of the glyoxylate cycle in the conversion of storage lipids to sucrose during post-germinative growth of oilseed plants is well characterised. The glyoxylate cycle enzymes ICL and MS are synthesised again during senescence of cotyledon, leaf and petal. Such glyoxylate cycle enzyme genes are also activated in dark-treated detached organs (leaf and cotyledon). This provides some evidence that the glyoxylate cycle may be reactivated during senescence. Furthermore, such glyoxylate cycle reactivation may be associated with a reverse transition of leaf peroxisome to glyoxysome during senescence. A full-length cDNA of cucumber gMDH was isolated and sequenced. The predicted amino acid sequence was compared with watermelon. The cDNA was used for examining synthesis of gMDH during cotyledon development (Figure 3. 1), to provide information on the role of gMDH during cotyledon development, especially senescence related to the glyoxylate cycle.

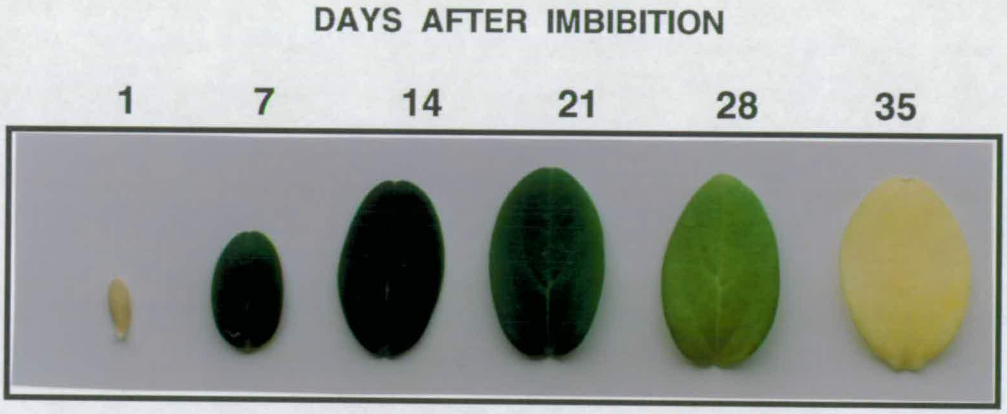


Figure 3. 1. Development of cucumber cotyledons at 1, 7, 14, 21, 28 and 35 days post-imbibition. Cucumber seeds were imbibed in sterile double distilled water at 4 °C for 16 h and sown in wet vermiculite and grown under a 12 h light/ 12 h dark photoperiod. Cotyledons were removed for photography (except day 1, where the whole seed is shown).

3. 2. Cloning of gMDH cDNA clones and sequence analysis

The watermelon gMDH cDNA clone (Gietl, 1990) was used to screen a cDNA library prepared from cotyledon mRNA after 3.5 days of germination (the library donated by W. Becker, USA). This produced incomplete cucumber gMDH cDNAs. At the first round screening, 10 positives were selected from about 300,000 plaques. Subsequently, they were used for second-round screening and 7 out of 10 plates gave positive signals. Single plaques were isolated from these plates and used for phage DNA isolation. As a result, seven λgt11 recombinants were selected from the cDNA library. After isolation of phage DNA with the plate lysate prodedure (Sambrook *et al.*, 1989), the DNA was restriction enzyme digested and the cDNA inserts examined by agarose gel electrophoresis. The cDNA insert sizes ranged from about 250 to 600 bp. The cDNA inserts were subcloned into the pBS *Eco*RI site to carry out DNA sequencing. The three longest clones were selected for sequencing (Figure 3. 2). Sequencing of these subclones was carried out with the dideoxy chain termination method (Sanger *et al.*, 1977), using vector primers. Nucleotide sequencing of the longest cDNA (567 bp) (pBgMDHc-12) revealed that the insert contained a 3' untranslated sequence, a poly $(A)^+$ segment and some of the protein coding sequence (corresponding to the carboxy terminal 117 amino acid residues), but lacked the 5' end of the coding sequence. The homology with gMDH of watermelon was 96% at a DNA level and 97% at a protein level. In view of this outcome, an alternative approach to isolate full length cDNA clones was the construction of a new library from senescing cucumber cotyledons. The advantage of preparing a library from senescing cotyledons was that senescence-specific sequences could also be isolated subsequently. A northern blot hybridisation using the

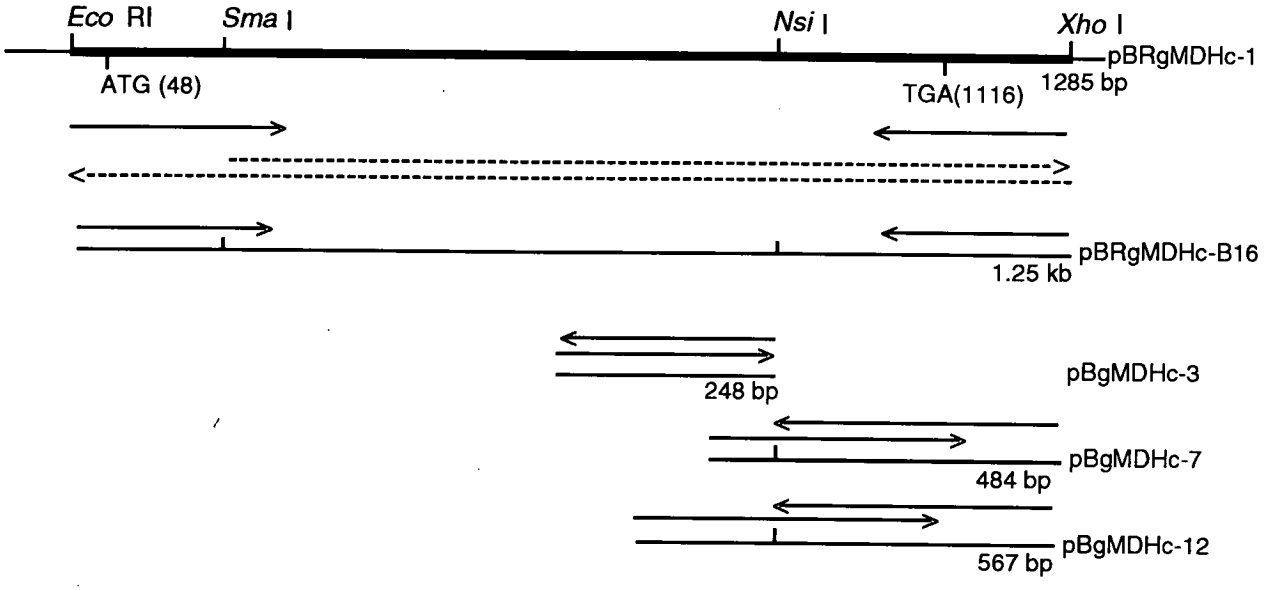


Figure 3. 2. Map of gMDH cDNAs from cucumber. Partial cDNA clones (pBgMDHc clones) were isolated from the day 3 mRNA derived cDNA library (courtesy of W. Becker). The two full-length cDNA clones (pBRgMDHc clones) were isolated from the senescent cotyledon cDNA library.

watermelon gMDH cDNA as a probe, showed that gMDH mRNA is present in senescent cotyledons (see Figures 3. 6 and 3. 7).

Then, a cDNA library (see section 2. 2. 3) was constructed from mRNAs isolated from yellow cucumber cotyledons (Figure 3. 1). The yellow cotyledons were carefully collected and were mostly from plants 5 weeks after seed imbibition. These cotyledons were almost completely yellow (over 99% yellow) and wilted or spotted cotyledons were eliminated.

The cDNA library was screened for cucumber gMDH cDNAs using the previously isolated partial gMDH cDNA fragment as the probe, and a full-length clone isolated. At the first-round screening, 22 positives were isolated from about 200,000 plaques and 12 out of the 22 positives were used for second-round screening. At the second-round screening, 11 out of the 12 plates showed a positive signal (not shown). After two-rounds of screening, the selected clones were analysed first by restriction enzyme digestion. The cDNA insert sizes ranged from 0.5 to 1.3 kbp (see Table 2. 4). The longest cDNA clone (pBRgMDHc-1) was sequenced on both strands, and revealed a full-length gMDH cDNA (Figures 3. 2 and 3. 3). The cDNA sequence matched completely with the previously isolated incomplete cDNA sequences except a difference at the site of the 3' end poly $(A)^+$ tail addition (Figure 3. 3). Another gMDH cDNA (pBRgMDHc-16B) isolated from the senescent library was also partially sequenced and shown to be only 13 bp shorter than pBRgMDHc-1 but otherwise showed no differences in nucleotide sequence.

Figure 3. 3. Full-length cDNA and predicted amino acid sequence of gMDH from cucumber. The underlined amino acid sequence indicates putative microbody targeting amino-terminal presequence. The predicted polypeptide sequence has 356 amino acids, the same number as watermelon. Arrow heads on the 3' end non-translated region indicate poly $(A)^+$ addition sites. a, from two partial cDNA clones (pBgMDHc-7 and 12); b, from two full-length cDNA clones (pBRgMDHc-1 and 11B). Putative poly $(A)^+$ addition signal is typed bold.

```
   1  TTCTGATCTTGGAGCGGTGGGTTTCTTTTCTCTTTCTGCAACTAACTATGCAGCCGATTC
                                                  M  Q  P  I  P    5

  61  CTGATGTTAATCAACGCATTGCTCGAATCTCTGCTCATCTTCATCCTCCCAAGTATCAGA
        D  V  N  Q  R  I  A  R  I  S  A  H  L  H  P  P  K  Y  Q  M   25

 121  TGGAGGAGAGTTCAGTTTTGAGGAGGGCGAATTGCCGGGCCAAAGGCGGAGCTCCCGGGT
        E  E  S  S  V  L  R  R  A  N  C  R  A  K  G  G  A  P  G  F   45

 181  TCAAAGTCGCAATACTTGGGGCTGCCGGTGGCATTGGCCAGCCACTTGCGATGTTAATGA
        K  V  A  I  L  G  A  A  G  G  I  G  Q  P  L  A  M  L  M  K   65

 241  AGATGAATCCTCTGGTTTCTGTTCTGCATCTATATGATGTAGTCAATGCCCCTGGCGTCA
        M  N  P  L  V  S  V  L  H  L  Y  D  V  V  N  A  P  G  V  T   85

 301  CTGCTGATATTAGCCACATGGACACGGGTGCTGTGGTGCGTGGATTCTTGGGGCAGCAGC
        A  D  I  S  H  M  D  T  G  A  V  V  R  G  F  L  G  Q  Q  Q  105

 361  AGCTAGAGCGTGCGCTTACTGGCATGGATCTTGTTGTAATCCCTGCGGGCGTTCCTCGGA
        L  E  R  A  L  T  G  M  D  L  V  V  I  P  A  G  V  P  R  K  125

 421  AACCAGGAATGACAAGGGATGATCTATTCAAAATAAACGCAGGAATTGTCAAGACTCTTT
        P  G  M  T  R  D  D  L  F  K  I  N  A  G  I  V  K  T  L  C  145

 481  GCGAAGGGATTGCAAAGTGTTGTCCTACAGCCATTGTCAACCTGATCAGTAATCCTGTGA
        E  G  I  A  K  C  C  P  T  A  I  V  N  L  I  S  N  P  V  N  165

 541  ACTCCACCGTGCCCATTGCAGCTGAAGTTTTCAAGAAAGCTGGAACTTATGATCCAAAGC
        S  T  V  P  I  A  A  E  V  F  K  K  A  G  T  Y  D  P  K  R  185

 601  GACTTCTAGGAGTTACAATGCTTGACGTCGTCAGAGCCAATACCTTTGTGGCAGAAGTAT
        L  L  G  V  T  M  L  D  V  V  R  A  N  T  F  V  A  E  V  L  205

 661  TGGGTCTTGATCCTCGGGATGTTAATGTTCCAGTTGTTGGCGGTCATGCTGGTGTAACCA
        G  L  D  P  R  D  V  N  V  P  V  V  G  G  H  A  G  V  T  I  225

 721  TTTTACCCCTTCTATCTCAGGTCAAGCCTCCAAGTTCTTTCACACAAGAAGAGATTAATT
        L  P  L  L  S  Q  V  K  P  P  S  S  F  T  Q  E  E  I  N  Y  245

 781  ACCTGACTGATAGGATTCAAAATGGTGGAACAGAAGTTGTTGAGGCCAAAGCAGGAGCTG
        L  T  D  R  I  Q  N  G  G  T  E  V  V  E  A  K  A  G  A  G  265

 841  GTTCAGCAACTCTCTCAATGGCATATGCTGCCGTTAAGTTTGCAGATGCATGCCTCAGGG
        S  A  T  L  S  M  A  Y  A  A  V  K  F  A  D  A  C  L  R  G  285

 901  GCTTAAGAGGAGATGCTGGTGTTGTTGAATGCGCGTTCGTGTCTTCTCAGGTGACCGAAC
        L  R  G  D  A  G  V  V  E  C  A  F  V  S  S  Q  V  T  E  L  305

 961  TTCCATTCTTTGCAACAAAAGTACGACTTGGCCGCAATGGTATAGATGAAGTATACTCCC
        P  F  F  A  T  K  V  R  L  G  R  N  G  I  D  E  V  Y  S  L  325

1021  TTGGCCCGCTAAATGAGTACGAGAGGATTGGATTGGAGAAAGCAAAGAAAGAGTTGGCAG
        G  P  L  N  E  Y  E  R  I  G  L  E  K  A  K  K  E  L  A  G  345
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1081  GAAGCATTGAGAAAGGAGTTTCCTTCATCAGAGGCTGAAGAGATGCCAATTACAATTAGT
        S   I   E   K   G   V   S   F   I   R   G   *                  356

1141  TTTAATAGAAACATTCTGTCTCTTATAGATTACTTGTCCCATATGTTCTCCTAGAGATTG

1201  AAGTTGAAACCACACTTCTTTTTATACTAATAAAACTATATCGCCATCATGTCGATATTT

1261  AATGCACAACCAAAATGGTTGGATTAAAAAAAAAAAAAAAAAAAA
           ^                    ^
           a                    b
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3. 2. 1. Predicted cucumber gMDH amino acid sequence

The predicted cucumber amino acid sequence was compared with its watermelon counterpart. As can be seen in Figure 3. 4, the two polypeptide sequences show extremely high homology (97%). There is also a highly conserved putative microbody targeting amino terminal transit peptide of 37 amino acids. This very high conservation is perhaps not surprising since cucumber and watermelon are closely related.

3. 3. Genomic DNA southern blot analysis

Genomic DNA was isolated to examine the gMDH gene copy number in cucumber. Purified leaf DNA was digested with 4 different restriction enzymes (*Bam* HI, *Eco*RI, *Hind*III and *Xba*I) for 2 h, then more restriction enzymes was added to each digestion reaction and incubated at the appropriate temperature for a further 6 h. None of these restriction enzymes has a site in the cucumber gMDH cDNA. After digestion, DNA was electrophoresed and blotted on to Hybond-N membrane. Finally, DNA was detected using the full-length gMDH cDNA as a probe (Figure 3. 5). According to this result, cucumber has one copy of the gene encoding gMDH, because single fragments are detected with three of these enzymes. *Hind* III gives two strong, and one faint band of hybridisation. This result could be due to a *Hind* III site (or two) in intron(s). The lack of sequence differences between five cDNAs is also consistent with the presence of a single gene. This is further discussed in the next section (section 3. 4). The gene will subsequently be referred to as *mdh*G, and products of this gene referred to as gMDH mRNA or protein.

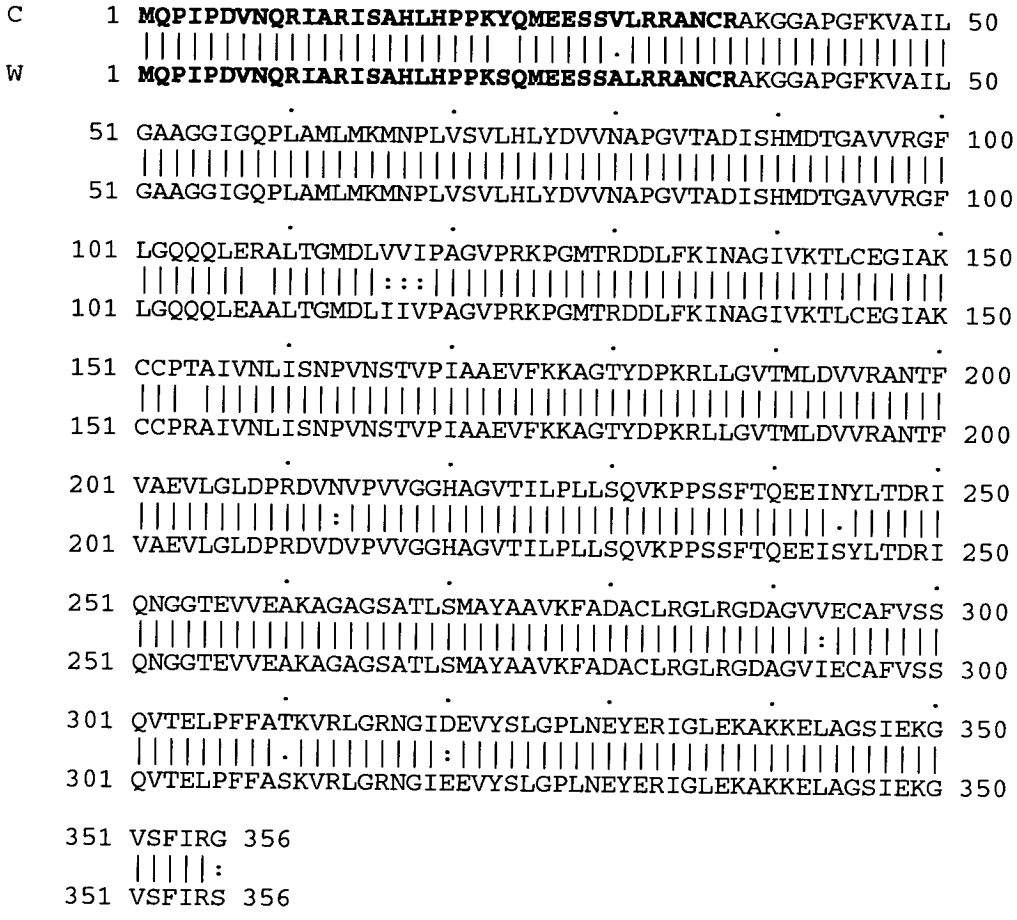


Figure 3. 4. Peptide sequence alignment of cucumber gMDH (C) with watermelon (W). Both peptide sequences contain 356 amino acids together with 37 amino acid amino terminal putative microbody targeting presequence (bold type). The two peptide sequences show 97% identity.

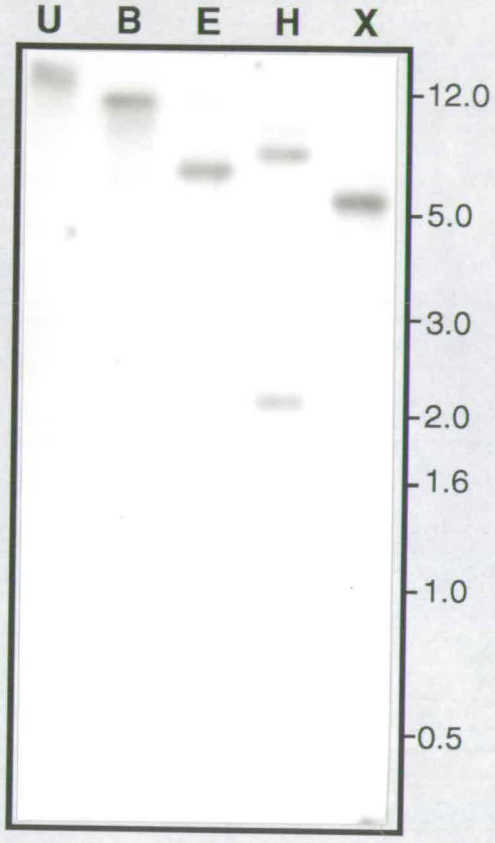


Figure 3. 5. Southern blot analysis for cucumber *mdh*G gene copy number. DNA was isolated from 3 week old first leaves and digested with *Bam*HI, *Eco*RI, *Hind*III and *Xba*I. The DNA was fractionated on a 0.8% (w/v) agarose gel and probed with the full-length cucumber gMDH cDNA (pBRgMDHc-1). The cDNA has no sites for the enzymes used in this analysis. Markers (kbp) are from a 1 kb DNA ladder (Gibco BRL, U. K.). (U, undigested; B, *Bam*HI; E, *Eco*RI; H, *Hind*III; X, *Xba*I).

3. 4. Detection of glyoxysomal and peroxisomal enzyme transcripts during cotyledon development

3. 4. 1. Changes in transcripts of gMDH, HPR, ICL and MS during cotyledon development

Cucumber seeds were imbibed first in sterile distilled water at 4 °C for 16 h, and sown in wet vermiculite and grown under a photoperiod of 12 h light, 12 h dark. Cotyledons were harvested at day 1 (after 24 h of sowing) and thereafter every 24 h to day 7, and after that at weekly intervals until completly senescing (Figure 3. 1). Total RNA samples were isolated and purified from each cotyledon sample, and used in northern blot analysis to determine the pattern of amounts of mRNAs for gMDH, HPR, ICL and MS, during development. In cucumber, growth is characterised by the transition of cotyledons from a storage to a photosynthetic organ and subsequent senescence (Figure 3. 1). This change of function to a photosynthetic organ was reflected in the appearance and then gradual decline of ICL and MS transcripts in the first 4 days of growth followed by the appearance of transcripts encoding the photorespiratory enzyme HPR (Figure 3. 6).

The development of glyoxysomal MDH activity in watermelon cotyledons during the first few days following seed imbibition was reported earlier (Walk and Hock, 1977), and showed a similar pattern to ICL and MS. The simultaneous occurrence of gMDH, ICL and MS transcripts are observed during the first 2 to 3 days of germination (Figure 3. 6). Transcripts for all three enzymes are clearly

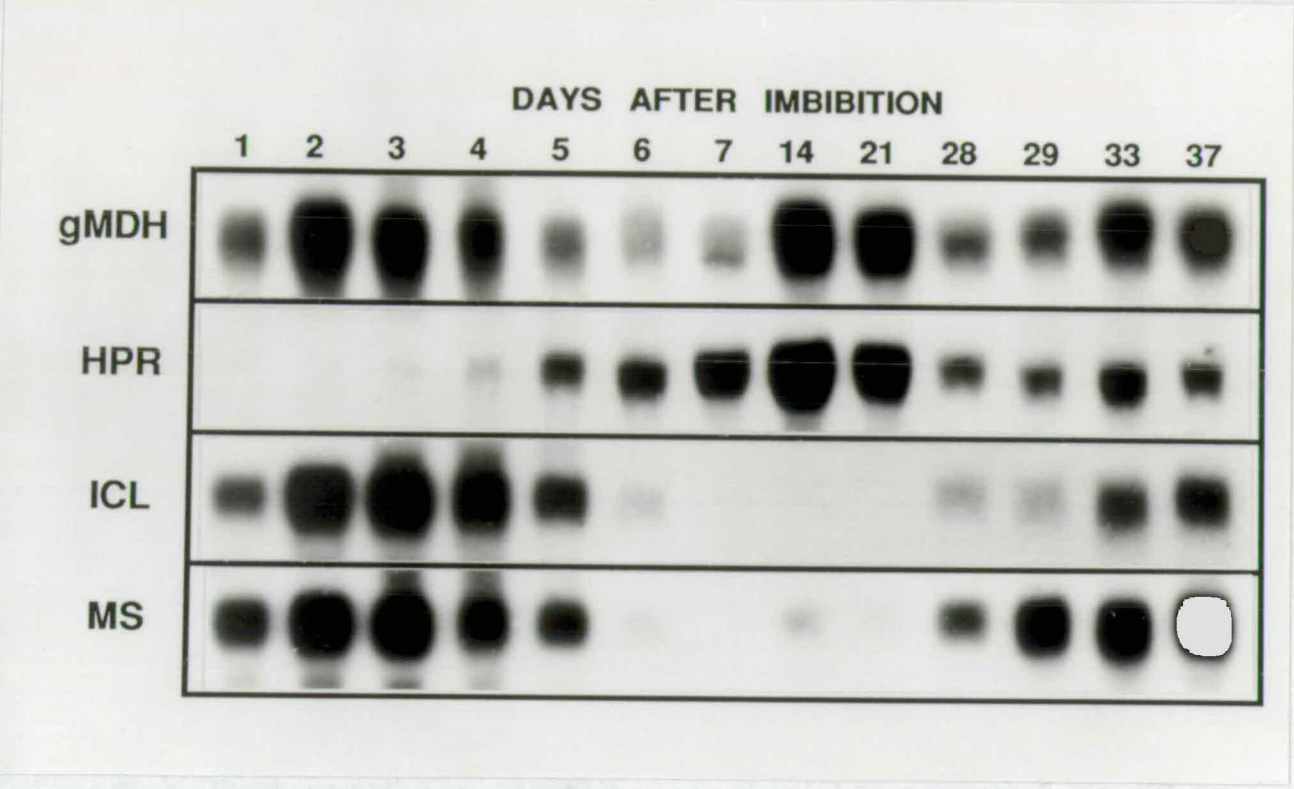


Figure 3. 6. Developmental changes in glyoxysomal and peroxisomal enzyme transcript abundance in cotyledons during growth of cucumber. Total RNA samples were isolated and purified from each cotyledon sample. Equal amounts (10 μg) of total RNA were electrophoretically fractionated on a denaturing horizontal gel. RNA within the gel was transferred onto Hybond-N membrane following the manufacturers protocol (Amersham, U. K.). The membrane was probed and exposed to X-ray film successively with the gMDH, HPR, ICL and MS cDNA fragments, each labelled with α-[^{32}P]dCTP by oligo-labelling procedure.

detected during early germination then decline markedly after 4 to 5 days. ICL and MS transcripts are not detected after 1 week but gMDH transcripts are detected at a low level. Then, a second peak of gMDH mRNA appears in green cotyledons.

This second peak corresponds exactly with the appearance of HPR transcripts, consistent with the role of MDH in photorespiration. Subsequently both gMDH and HPR transcripts decrease in amount, presumably as photosynthetic capacity is reduced. Finally, in senescing cotyledons, the mRNAs for ICL, MS, gMDH and HPR all increase in amount (Figure 3. 7). The reappearance of ICL and MS in senescing cotyledons has been reported previously (De Bellis and Nishimura, 1991; Graham *et al.,* 1992). The increase in amount of gMDH mRNA might indicate that gMDH is synthesised for glyoxylate cycle activity in senescing cotyledons. An increase in amount of HPR mRNA was also observed (Figure 3. 7). A similar increase in the amount of GO mRNA was also seen (not shown).

Careful inspection of the autoradiographs shown in Figure 3. 6 suggested that the peak of gMDH and HPR mRNAs might precede that of ICL and MS in senescence. Therefore, similar experiments were carried specifically to examine the level of expression of the glyoxysomal and peroxisomal enzyme genes in different senescing stages. Senescing cotyledons were collected carefully according to the extent of chlorophyll loss i.e., 70%, 80%, 90% and completely yellow (>99%) after 4 or 5 weeks of germination. The cotyledon samples were classified into the four senescing stages, I, II, III and IV. Total RNAs were isolated and northern blot analyses carried using cDNA fragments from gMDH, HPR, ICL and MS as probes after radiolabelling. Firstly, the northern filter was hybridised with gMDH, then the first probe was removed and the filter reprobed successively with ICL, MS and HPR

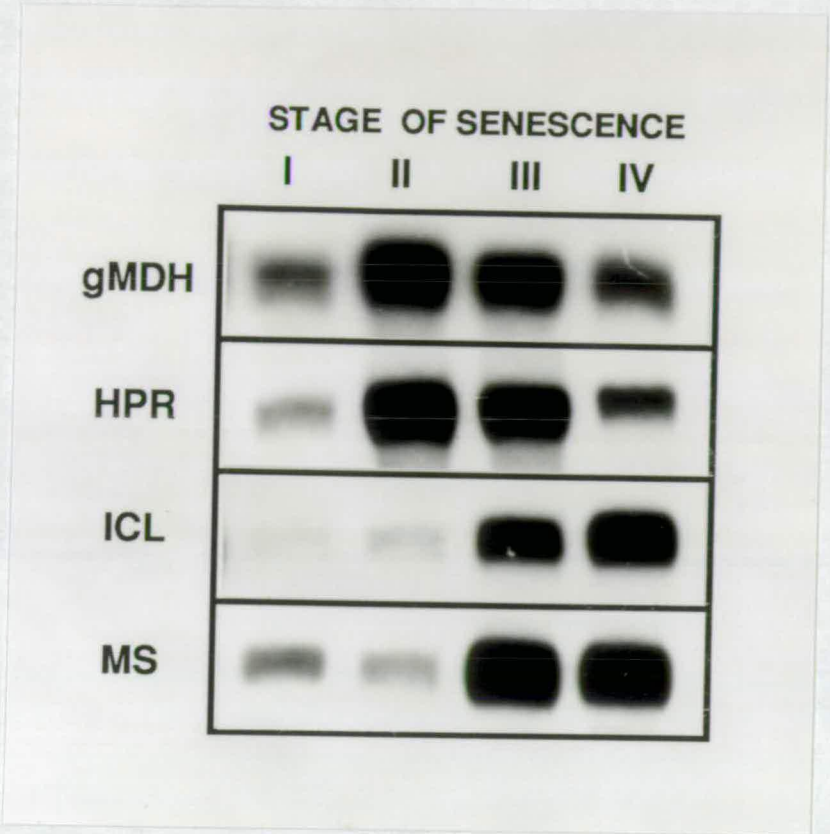


Figure 3. 7. Changes of transcripts of glyoxysomal and peroxisomal enzymes in senescing cotyledons. Cotyledons were collected according to the yellow area 4 to 5 weeks after germination. 10 μg of total RNA was loaded in each sample then northern blot analyses carried out using cDNA fragments as probes. I, 70% yellow; II, 80% yellow; III, 90% yellow; IV >99% yellow.

(Figure 3. 7). The filter was also reprobed with a PEPCK cDNA fragment (see Chapter 5). The mRNAs for ICL and MS show similar accumulation mainly at stages III and IV but transcripts were detected already at stages I and II at a low level. In the case of gMDH and HPR, the accumulation of the transcripts appear one step earlier at stages II and III, then decline at stage IV. GO showed a similar pattern (not shown). The results demonstrate again that the expression of these genes is activated during senescence, but the non-coordinate pattern of expression is not understood. Assays of enzyme amounts and activities in senescence, would be important to help understand the function of these enzymes.

Detection of ICL and MS proteins has been confirmed in senescing cucumber cotyledons by immunoblot analyses (Mclaughlin, 1994). This result agrees with previous observations from several plant species (De Bellis and Nishimura, 1991; De Bellis *et al.,* 1991; Graham *et al.,* 1992). Nevertheless, although reappearance of the glyoxylate cycle enzymes during senescence is generally accepted, the function of the glyoxylate cycle enzymes is not clear. It might indicate the presence of multi-functional microbodies (gerontosomes) (Vincentini and Matile, 1993), although the function of enzymes of the photorespiratory pathway in such microbodies is unknown.

The two periods of relatively low levels of gMDH transcripts during cotyledon development may correspond to those times when there is no net import or export of reducing equivalents across the glyoxysomal/peroxisomal membrane (see Chapter 7).

The gMDH cDNA detects only a single band in southern blot hybridisation indicating a single gene. The results shown here indicate that this single gene is expressed throughout cotyledon growth. This implies that in cucumber a single gene encodes glyoxysomal and peroxisomal MDH.

3. 5. Immunodetection of gMDH (or perosixomal MDH) protein during cotyledon development

Antiserum raised against the gMDH enzyme of watermelon (Courtesy of C. Gietl) was used to provide information concerning changes in the levels of protein during cotyledon growth. Additionally, data regarding the postulated glyoxysome to leaf-type peroxisome transition and reverse transition associated with greening and senescence might be obtained. The antiserum recognises numerous bands, among which are two or three of approximately 40 kDa, the size expected for gMDH. The two detected in cotyledons at day 3, are detected through cotyledon development (Figure 3. 8). The identity of these bands is unknown. One possibility is that they represent pre-gMDH and mature gMDH but it seems unlikely that the presursor accumulates *in vivo*. More likely, the antibody recognises mMDH or cytosolic MDH in addition to gMDH. The relative amounts of the two bands detected, change during cotyledon growth and senescence.

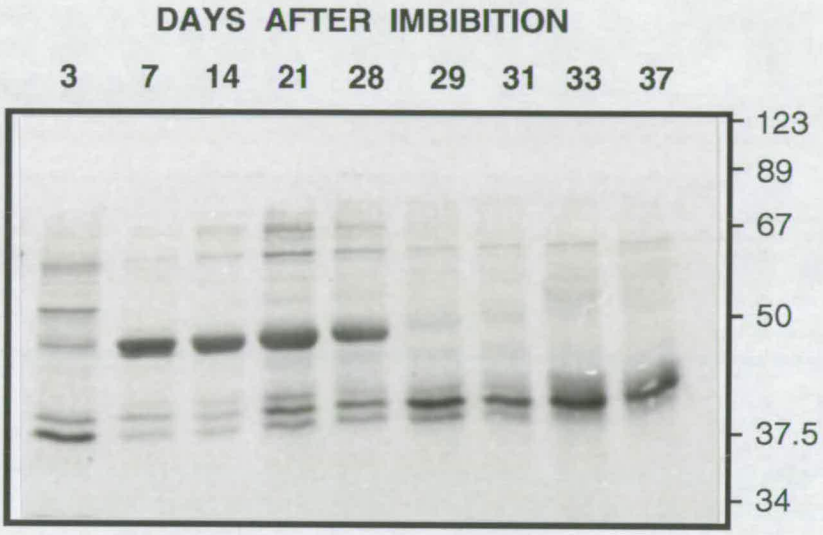


Figure 3. 8. Developmental changes in MDH protein during cotyledon growth. Immunoblot analysis of total protein isolated from cotyledons at intervals until 37 days. Total protein was extracted and 10 μg of each was separated on a 10% (w/v) SDS-polyacrylamide gel, transferred to Hybond-ECL membrane and the presence of MDH was detected with watermelon anti-gMDH antibody. Molecular weight (kDa) markers are β−Galactosidase (123), Fructose-6-Phosphate Kinase (89), Pyruvate Kinase (67), Ovalbumin (50), Lactic Dehydrogenase (37.5) and Triosephosphate Isomerase (34) (Prestained markers, Sigma).

3. 5. Conclusion

From the data presented in this Chapter it is clear that the longest cDNA clone (1,285 bp) has an insert containing a 5' non-coding region and 3' end poly(A)+ segment, so was full-length. The cDNA sequence showed 94% homology at the DNA level and 97% at the amino acid sequence level with watermelon gMDH. No inconsistencies were encountered between previously isolated incomplete cDNAs and the newly isolated cDNA clones from the senescing cDNA library. It shows not only that the cDNA library construction was effective but is also consistent with the proposal that a single gene encodes gMDH in cucumber. Furthermore, it is also consistent with the operation of the glyoxylate cycle in senescing cotyledons.

From the data presented in this Chapter it is clear again that gMDH shows a similar expression pattern during cotyledonary development (especially in germination) with ICL and MS except the second peak in the green cotyledons. Furthermore, there is synthesis of the glyoxysomal ICL, MS and gMDH during the latter stage of cotyledonary senescence. Although the role of glyoxylate cycle enzymes is still unclear, it clearly supports the proposal that the glyoxylate cycle is regenerated in senescing tissues, and the development of multifunctional microbodies (gerontosomes) in senescent cotyledons is suggested by the presence of HPR and GO mRNAs.

Chapter Four:
Differential screening for cDNA clone(s) of mRNAs coordinately expressed during germination and senescence in cotyledons

4. 1. Rationale

Several research groups have been investigating developmental and metabolic regulation of glyoxysomal enzyme gene expression in plants. In particular, *icl* and *ms* genes have been studied (Comai *et al.*, 1989; Graham *et al.*, 1990; Graham *et al.*, 1992). However, we do not yet have much information about other glyoxylate cycle enzyme gene(s), for instance citrate synthase and aconitase. Nor do we have much information about the synthesis of enzymes of lipid mobilisation, β-oxidation, and gluconeogenesis.

Therefore a differential screen was carried out to isolate molecular clones encoding proteins synthesised at both germination and senescence stages of cotyledon development, but absent at the intermediate photosynthetic stage of growth. In other words, to isolate cDNA clones from mRNAs which are expressed together with ICL and MS during germination and senescence (see Figure 3. 6). In this way, isolating clones encoding enzymes of lipid hydrolysis, glycerol utilisation, β-oxidation, the glyoxylate cycle and PEPCK, or possibly proteins involved in the regulation of ICL and MS synthesis, was anticipated. Furthermore it was thought possible that clones encoding other enzymes or proteins might be isolated, so providing evidence for the operation of other metabolic pathways or developmental processes at both stages of

cotyledon development. The isolation of such clones might provide a starting point for elucidating new aspects of ICL and MS function and synthesis.

4. 2. Preparation of a cDNA library from RNA of senescing cotyledons and differential screening

A cDNA library was made from polyadenylated RNA of senescing cucumber cotyledons (see Figure 3. 1). The polyadenylated RNA was used for a northern blot hybridisation with ICL and MS probes to ensure the abundance of the glyoxylate cycle enzyme transcripts. The northern blot hybridisation showed clear abundance of these two key glyoxysomal enzyme transcripts (not shown). The initial library was amplified and examined to check the number of plaques and frequency of recombinants. The initial library produced 1.7 x 10^6 pfu from a packaging reaction (500 µl) and about 86% recombinants. Therefore, approximately 6.8 x 10^7 independent recombinant phages were produced from 5 µg RNA. The frequency of recombinants dropped to 60% three days after *in vitro* packaging. Therefore, the next library was amplified immediately on the same day of packaging to retain a high level of recombinants. The amplified library gave 2.6 x 10^9 pfu ml^{-1} and 85% recombinants. The library was stored at 4 °C after addition of chloroform (final concentration 0.5% (v/v)) and used for screening.

From the library, approximately 300,000 independent clones were screened with radiolabelled single stranded-cDNA probes derived from RNA of cotyledons 3 days and 14 days (green) post imbibition, at the first attempt of library screening. At the second attempt of library screening a third probe (RNA from cotyledons 30 to 35 days (yellow) post-germination) and a fourth probe (from green cotyledons detached

and incubated in the dark for 8 days) were prepared. The fourth probe also contained ICL and MS sequences (data not shown, but see chapter 6). The use of these probes is further explained in Figure 4. 1.

4. 2. 1. Radiolabelled single stranded-cDNA probe preparation.

Single stranded-cDNA probes were prepared as described in section 2. 2. 6. 2. An aliquot of each cDNA probe was sampled and analysed by alkaline agarose gel electrophoresis after phenol/chloroform extraction and Sephadex G-50 chromatography. Depending on different mRNA sources, incorporation ratios showed differences and so filters were subsequently exposed for different lengths of time (Figure 4. 2). The whole cDNA probe was added to each hybridisation reaction.

4. 2. 2. The first experiment; first-round screening

Firstly, phage plaques were formed in two large square petri dishes (20 x 20 cm) that contained about 100,000 plaques each. Then, duplicate plaque lifts were prepared and hybridised with day 3 and day 14 probes, then phages were taken from day 3 positive but day 14 negative plaque areas (not shown). In this way, 32 positives were selected, then 16 out of the 32 best candidates were further selected. The plaques were used for second-round screening the same as the first round screening procedure.

Figure 4. 1. Experimental procedure for differential screening. A library screening plan was designed as shown in this figure. The library was first screened with 2 probes (day 3 and day 14 cotyledon mRNA derived cDNA probes) using duplicate filters. Then in the case of the second experiment, the probes were removed and filters reprobed with two other probes (detached and senescing cotyledon mRNA derived cDNA probes). Positive signals common to day 3, detached and senescence probes were selected. At the second-round screening, a small number of chosen plaques were rescreened with day 3 and day 14 mRNA derived cDNA probes. Finally, day 3 positive but day 14 negative pure single plaques were further analysed.

TOTAL RNA; From day 3, day 14, senescing and dark-incubated detached cotyledons

↓

POLY(A)+ RNA; Selective isolation from total RNAs

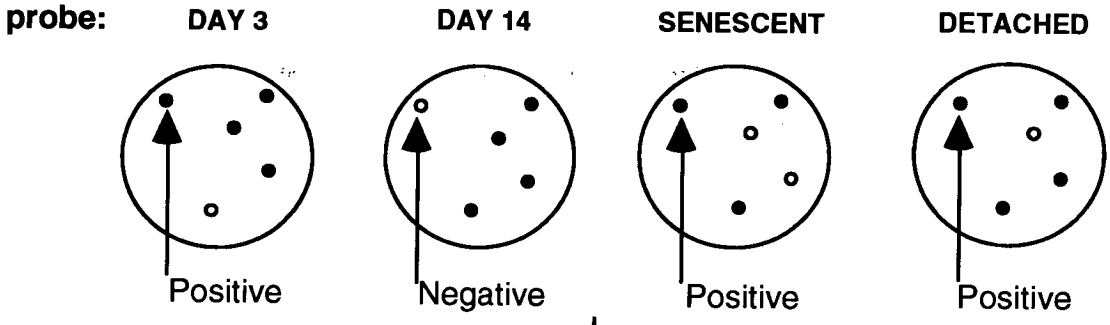
↓

Radiolabelled single stranded cDNA probes preparation

↓

Plaque hybridisation

↓



↓

Plaque isolation

↓

***in vivo* excision of inserts**

↓

Phagemid DNA isolation

↓

DNA sequencing and identification

↓

Northern blot and expression pattern analysis

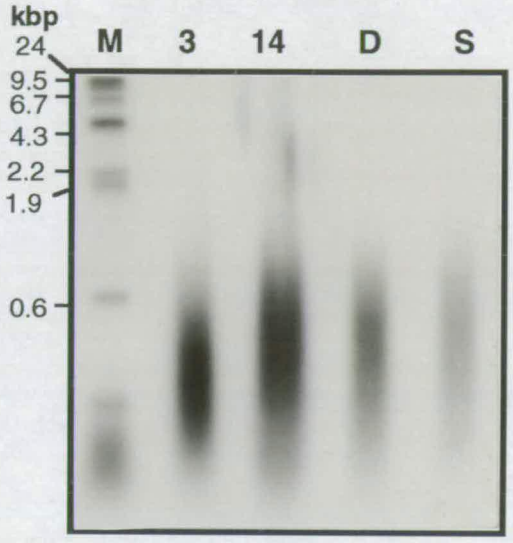


Figure 4. 2. An autoradiograph of radiolabelled single stranded-cDNA probes from an alkaline agarose gel. Purified mRNAs were radiolabelled with α-[^{32}P]dCTP by reverse transcription using M-MuLV reverse transcriptase (see section 2. 2. 6. 2). M, labelled DNA (lambda DNA *Hind*III digested) size markers (kb); 3, RNA from day 3 cotyledons; 14, RNA from day 14 cotyledons; D, RNA from detached and dark-incubated cotyledons; S, RNA from senescing cotyledons.

4. 2. 3. The second experiment; first-round screening

Duplicate plaque lifts were prepared as in the first experiment. But approximately 100,000 plaques were formed in 10 separate small square petri dishes (12 x 12 cm) (i.e., 10,000 plaques in one petri dish). In the second attempt of library screening the first probes were removed and rehybridised with the third and fourth probes after marking the positives on the X-ray films of filters hybridised with the day 3 probe. In this way, the positive signals from the day 3, senescing and detached cotyledon probes, and negative signals from the day 14 probe (Figure 4. 3) were found. As many as possible candidate clones (positives from day 3, senescent and detached but negatives from day 14) were selected, and characterised into three groups according to the signal strength with the day 3 probe, for example strong, medium and weak. In this way, 24 out of 96 candidate clones were further selected and titred for the second-round screening.

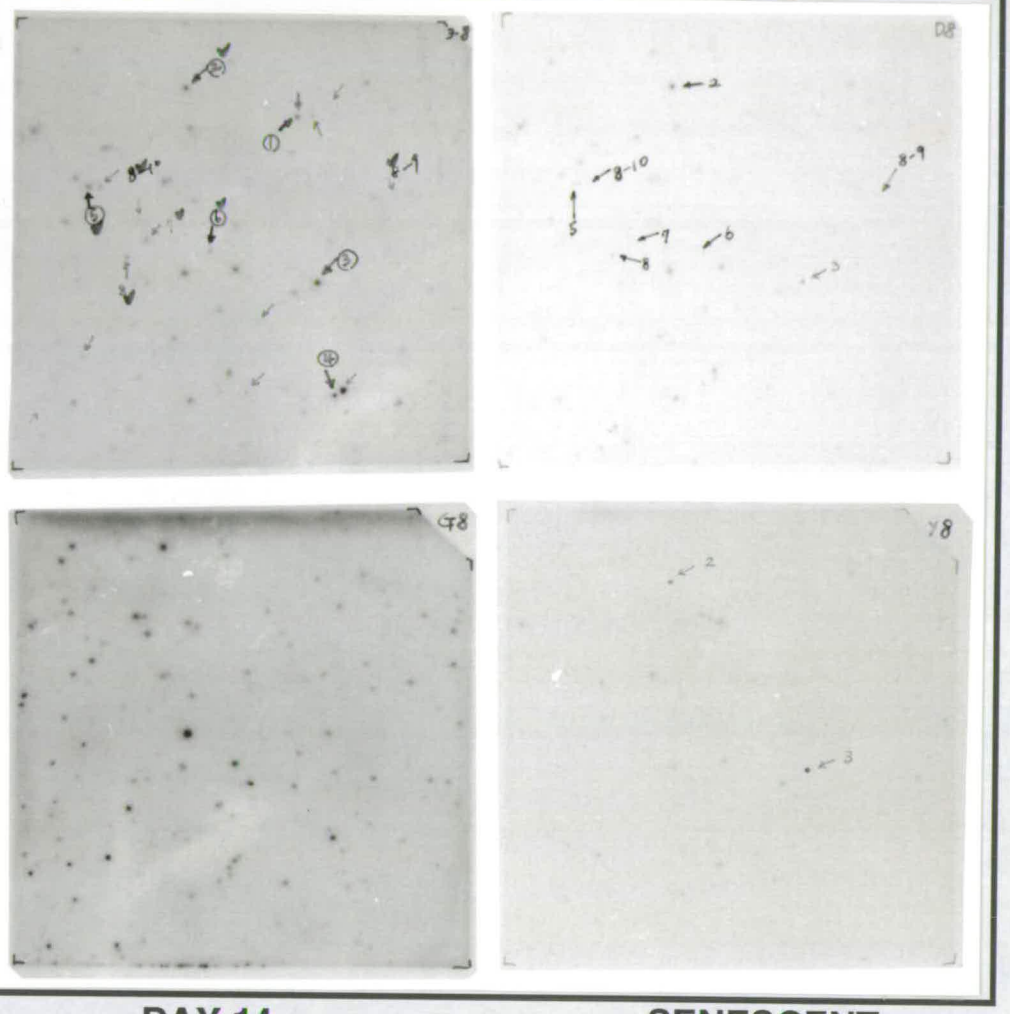
4. 2. 4. The second-round screening

For each selected agar plug from the first-round screening (both first and second experiments), around 100 to 200 plaques were used for the second-round screening (Figure 4. 4). Duplicate plaque lifts were hybridised with day 3 and day 14 mRNA derived cDNA probes. In this way single plaques were isolated, which showed positive hybridisation to the probe from day 3 but negative with the day 14 probe. After the second-round screening, single plaques were available for subcloning and further analysis. A total 40 clones were isolated from the two experiments of differential library screening and 26 out of the 40 clones were examined further with DNA sequence analysis and northern hybridisation.

Figure 4. 3. Selected autoradiographs (from plate No. 8) from the first-round screening in the second experiment. Duplicate filters were hybridised with 4 different cDNA probes. Arrows indicate selected signals chosen for the second-round screening.

DAY 3

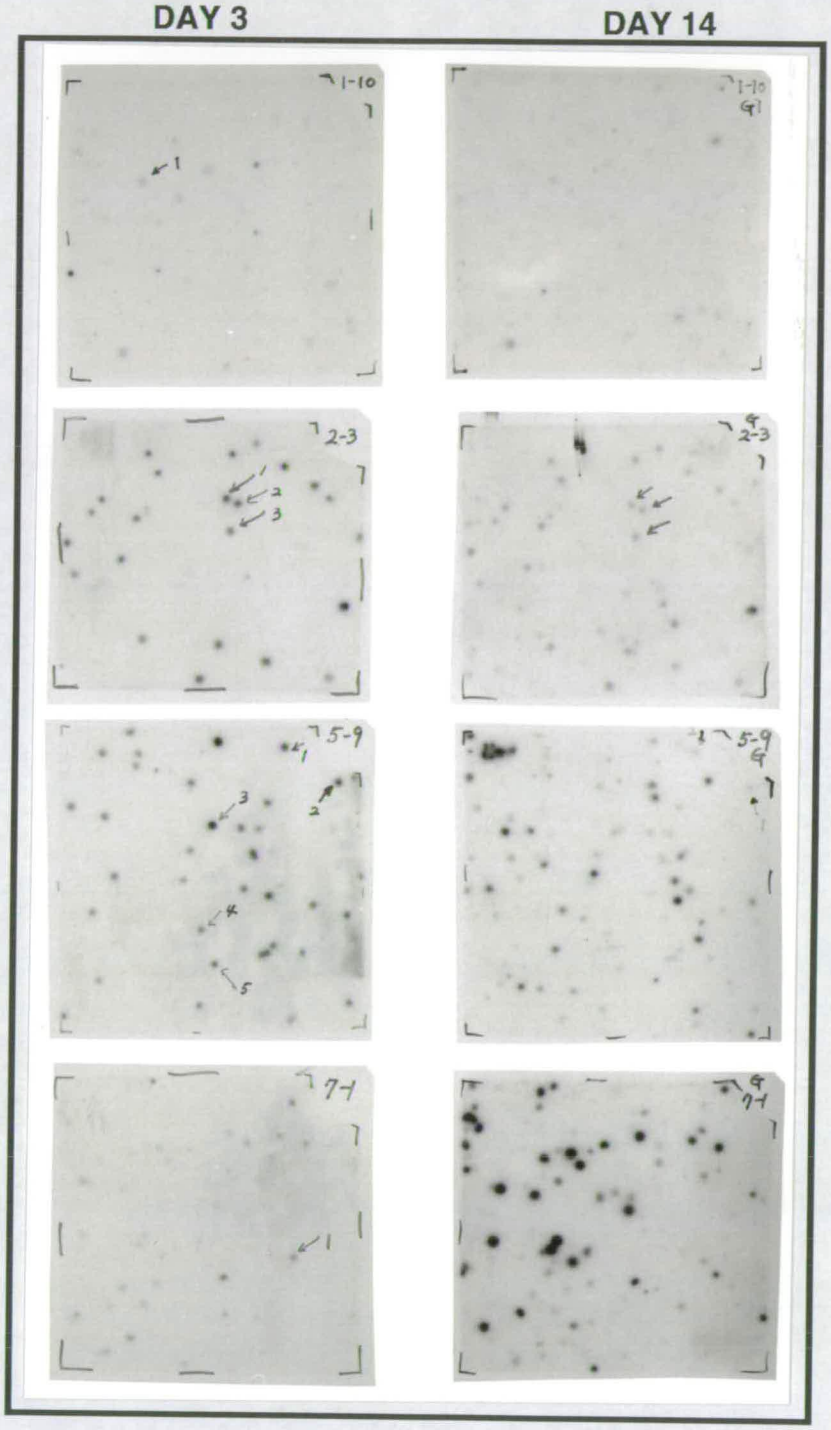
DETACHED



DAY 14

SENESCENT

Figure 4. 4. Autoradiographs from the second-round screening. Selected positives from the second experiment of the library screening were probed with day 3 and day 14 cDNA probes using duplicate filters. Arrows indicate selected individual plaques for further analysis.


DAY 3
DAY 14
1-10
1-10
2-3
2-3
5-9
5-9
7-1
7-1

4. 3. Analysis of selected cDNA clones

4. 3. 1. DNA sequence and northern blot analysis

After the two-rounds of screening, single plaques were isolated and subcloned in pBLUESCRIPT SKII$^-$ phagemid vector by the *in vivo* excision procedure. Firstly, the clones were sequenced from the 5' end and the Gene Bank/EMBL Databases searched for related sequences. In the case of unknown clones (i.e., no similarity shown from the available Databases), northern blot analysis was carried out using the cDNA fragments as probes to examine (and also compare with ICL) the developmental expression pattern in cotyledons (Figure 4. 5). A summary of the results is given in Tables 4. 1 and 4. 2.

Most of the selected cDNA clones showed a relatively strong signal with the day 3 probe but they were mostly negative with the day 14 probe. However, over half the clones originated from chloroplast DNA at the first attempt of screening. One of the chloroplast originated cDNA clones (U3-2, Table 4. 1) was used for northern blot analysis to analyse the expression pattern during development (Figure 4. 5). The clone shows constitutive expression during all developmental stages (Figure 4. 5). Chloroplast related cDNA clones were also isolated in the second attempt, at a similar level. Northern blot analysis with unidentified clones as probes showed that most demonstrate constitutive expression, except U1-10, U2-1, U7-1, U7-4 and U8-2-3 (encoding glutamine synthetase) clones (Figure 4. 5, Tables 4. 1 and 4. 2). Even though the U8-2-3 cDNA clone shows a similar expression pattern to ICL and U1-10, the clone has not been further investigated. This sequence shows homology with cytosolic glutamine synthetase, rather than chloroplastic glutamine synthetase.

Figure 4. 5. Northern blot analyses with isolated clones. Total RNA was isolated from cotyledons at days 3, 7, 14, 21 and 28 after imbibition, and from senescent cotyledons. 10 μg of total RNA was electrophoretically fractionated on a formaldehyde denaturing gel, then transferred on to a Hybond-N membrane. The membranes were hybridised with isolated cDNA fragments. ICL was used for comparison. GS encoded glutamine synthetase.

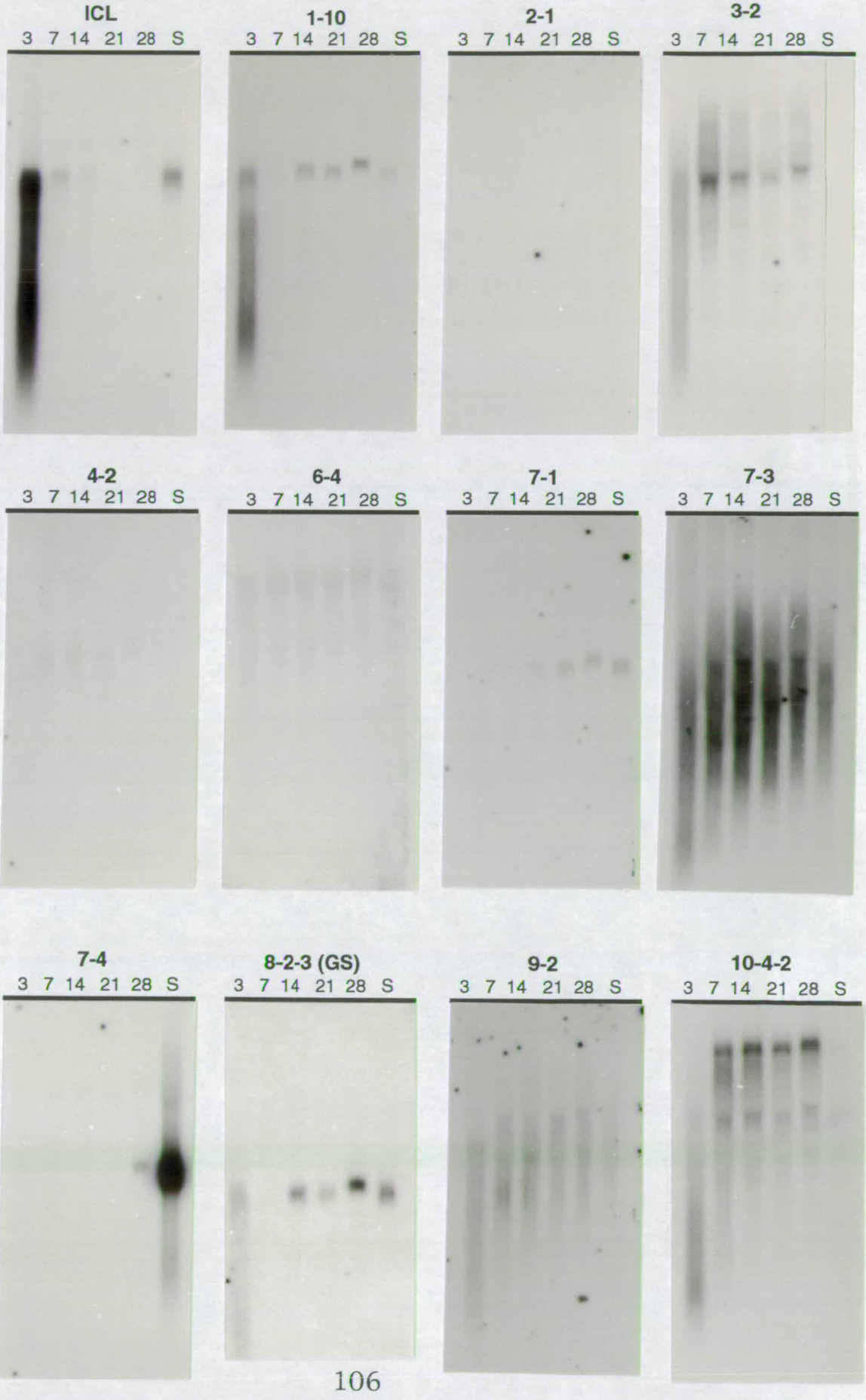

ICL
3 7 14 21 28 S
1-10
3 7 14 21 28 S
2-1
3 7 14 21 28 S
3-2
3 7 14 21 28 S
4-2
3 7 14 21 28 S
6-4
3 7 14 21 28 S
7-1
3 7 14 21 28 S
7-3
3 7 14 21 28 S
7-4
3 7 14 21 28 S
8-2-3 (GS)
3 7 14 21 28 S
9-2
3 7 14 21 28 S
10-4-2
3 7 14 21 28 S

Table 4. 1. Clone characterisation by DNA sequencing, Gene Bank/EMBL Database searching and northern blot analysis, from the first-attempt of library screening.

Clone	cDNA size(kb)	Data Base Information	Northern
2-1	1.0	Unknown	ND (?)
2-2	0.7	Chl	ND
3-2	0.4	Chl	Constitutive
7-1	0.7	Unknown	Senescence
7-3	1.1	Unknown	Constitutive
7-4	0.4	Unknown	Senescence
8-1	0.4	Chl	ND
9-1	1.4	Chl	ND
9-2	0.7	Unknown	Constitutive
17-1	0.2	Chl	ND
17-2	0.3	Unknown	Constitutive
18-4	1.2	Chl	ND
20-1	0.4	Chl	ND
23-1	1.4	ICL	germination/senescence

Note: Chl, chloroplast DNA originated; ND, No Data; Senescence, senescence-specific expression; Constitutive, constitutive expression pattern (see Figure 4. 5.)

Table 4. 2. Clone characterisation by DNA sequencing, Gene Bank/EMBL Database searching and northern blot analysis from the second-attempt of library screening.

Clone	cDNA size(kb)	Data Base Information	Northern
1-10	0.4	(PEPCK)	similar to ICL (?)
2-3	2.1	Chl (*rpl* 16)	ND
3-5-1	0.2	Chl	ND
3-5-2	3.0	Chl	ND
4-2	1.0	Unknown	Constitutive (?)
5-9	0.5	Chl (*rpl* 16)	ND
6-4	0.7	Unknown	Constitutive (?)
8-2-2	0.4	MS	ND
8-2-3	0.4	Glutamine synthetase	similar to U1-10
9-10	3.1	Chl	ND
10-4-2	0.7	Unknown	Constitutive
10-10	1.4	25S rRNA	ND

Note: Chl, chloroplast DNA originated; ND, No Data; Constitutive, constitutive expression pattern (see Figure 4. 5.) Note that the PEPCK cDNA was only identified as such after isolating longer cDNA clones (see Chapter 5).

Interestingly, one of the unknown clones (U7-4) shows strong expression in yellow cotyledons (Figure 4. 5) which was first detected 4 weeks after germination. It seems to be a senescence related gene product but has not been further investigated. Another clone (U7-1) also shows a senescence related pattern but the transcripts are detected at an earlier stage (around 2 weeks post-germination) of development (Figure 4. 5). This clone has not been further investigated either.

Most of the remaining research was focused on the U1-10 clone, since, additional northern blot analysis (see Figure 5. 7) using RNA gel blots which had been previously hybridised with ICL and MS probes, showed similarity of expression pattern during cotyledon development (Figure 5. 7). The U1-10 clone was identified as encoding PEPCK only after rescreening the library using U1-10 cDNA insert (see Chapter 5).

4. 3. 2. Characterisation of isolated cDNA clones

cDNA inserts are variable in size from 0.3 kb to 3.0 kb. At the later stage of screening, remarkably long chloroplast DNA derived clones (U2-3, U3-5-2 and U9-10) were identified. As can be seen from Tables 4. 1 and 4. 2, a large number of chloroplast DNA derived clones were isolated from the senescing cotyledon cDNA library. Several are ribosomal RNA derived sequences. This can in part be explained by contamintion of poly $(A)^+$ RNA with chloroplast RNA during purification at the earlier stage of cDNA library construction. However, it is not clear why such chloroplast RNA should have served as a template for cDNA synthesis, nor why such clones should have been isolated during the screening procedure. However for this reason, a subtracted probe library screening should be considered before another

attempt. Nevertheless, chloroplast DNA related clones actually gave a positive signal with the day 3 probe but negative with the day 14 probe, which could be explained as a technical problem during the probe preparation or hybridisation reaction.

Nevertheless, the isolation of cDNAs encoding ICL and MS showed that the screening procedure was effective, as originally intended. Isolation of a cytosolic glutamine synthetase cDNA and two senescence related clones will be useful at the next stage of the project. Most importantly however, isolation of a cDNA encoding PEPCK, which had not previously been cloned, is an important step forward. The characterisation of the PEPCK cDNA and features of PEPCK gene expression are discussed in Chapters 5 and 6.

4. 4. Conclusion

Much research has been carried out on the glyoxylate cycle enzymes and their genes, and their metabolic and genetic regulation during plant development. Several research groups have isolated cDNAs and genes encoding ICL and MS. Furthermore, the reappearance of these enzymes also has been demonstrated in senescing plant tissues. These observations raised the possibility of the isolation of other glyoxylate cycle enzyme cDNA clone(s) using differential screening. Therefore, a cDNA library was obtained from RNA of day 3 dark-incubated cucumber cotyledons (courtesy of W. Becker, USA). It was used first for gMDH cDNA clone isolation. However, the library showed problems in quality. Most of the cDNAs were incomplete, covering only the middle part or 3' end. Hence, a cDNA library was constructed from mRNAs isolated from senescing cucumber cotyledons. Such a library was considered potentially very useful for isolating other senescence related sequences. This library

was screened first for gMDH cDNAs and a full-length clone isolated successfully as described in Chapter 3. Therefore the library was used to isolate other clones potentially related in lipid mobilisation and other metabolic pathways. Although, minor technical problems were encountered in the differential screening, the library was screened successfully since, the screening produced cDNAs encoding PEPCK, ICL, MS, two senescence related sequences, one cytosolic glutamine synthetase and several unknown clones.

Chapter Five:
Identification and characterisation of the phospho*enol*pyruvate carboxykinase (PEPCK) cDNA clone and gene expression in cucumber during development

5. 1. Rationale

As discussed in previous chapters (Chapters 1, 3 and 4) postgerminative growth of embryos and senescence of photosynthetic organs are characterised by the appearance of the glyoxylate cycle and β-oxidation enzymes which are involved in the utilisation of products of lipid breakdown (Beevers, 1961; Gut and Matile, 1988). During postgerminative growth, the four-carbon acids produced by the glyoxylate cycle and mitochondrial activity are used as substrates for gluconeogenesis. The net conversion of lipid into sucrose can be demonstrated (Beevers, 1961; 1980). In senescence the role of the glyoxylate cycle is less clear although it has been suggested that gluconeogenesis also occurs (Wanner *et al.*, 1991).

The isolation of cDNA clone U1-10 (see Chapter 4) derived from a mRNA expressed with ICL and MS in germination and senescence, suggested that this sequence may encode a protein involved in an aspect of glyoxylate cycle function. In order to investigate this possibility further, it was considered necessary to try to isolate a full-length cDNA for this protein.

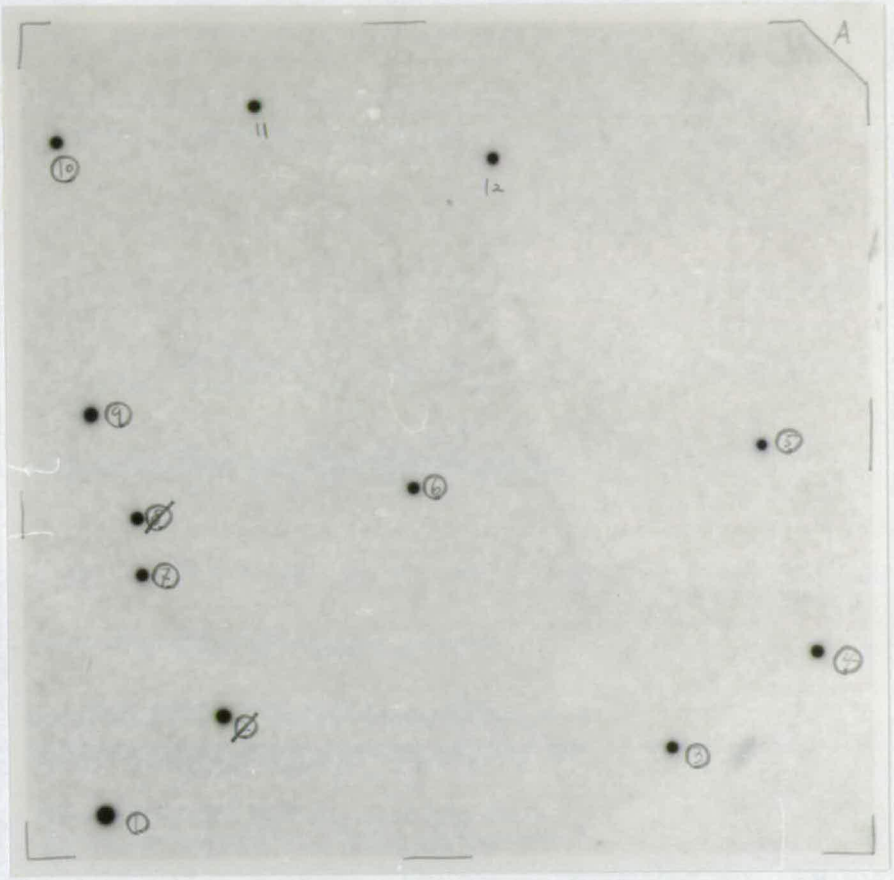
5. 2. cDNA library screening and DNA sequence analysis

5. 2. 1. Full-length cDNA clone isolation

The U1-10 clone was used as a probe to isolate further homologous cDNAs. At the first-round screening, 24 positive plaques were selected from about 200,000 phage plaques (Figure 5. 1). Then, 12 out of the 24 positive plaques were further selected and used for second-round screening.

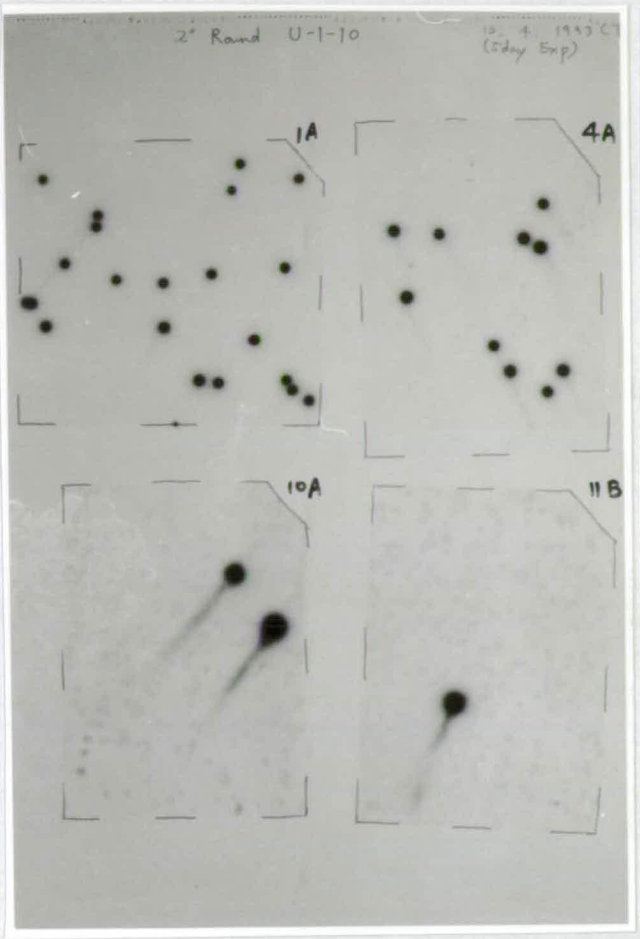
At the second-round screening, 9 out of the 12 plates gave a positive signal (Figure 5. 2). These clones were sub-cloned into pBLUESCRIPT by *in vivo* excision and restriction enzyme analysed to examine the cDNA inserts. The cDNA inserts were mostly longer than the U1-10 clone with sizes from 0.4 kb to 2.4 kb (Table 5. 1). The ends of four new cDNAs and the complete pBRPCK-7A cDNA were sequenced (Figure 5. 3). The DNA sequence analysis of the 3' ends revealed them to be identical with the U1-10 clone. Even though some clones showed longer 3' end extensions, no sequence heterogeneity was observed when all six cDNA sequences were compared. Hence, it indicates that they were probably derived from a single gene.

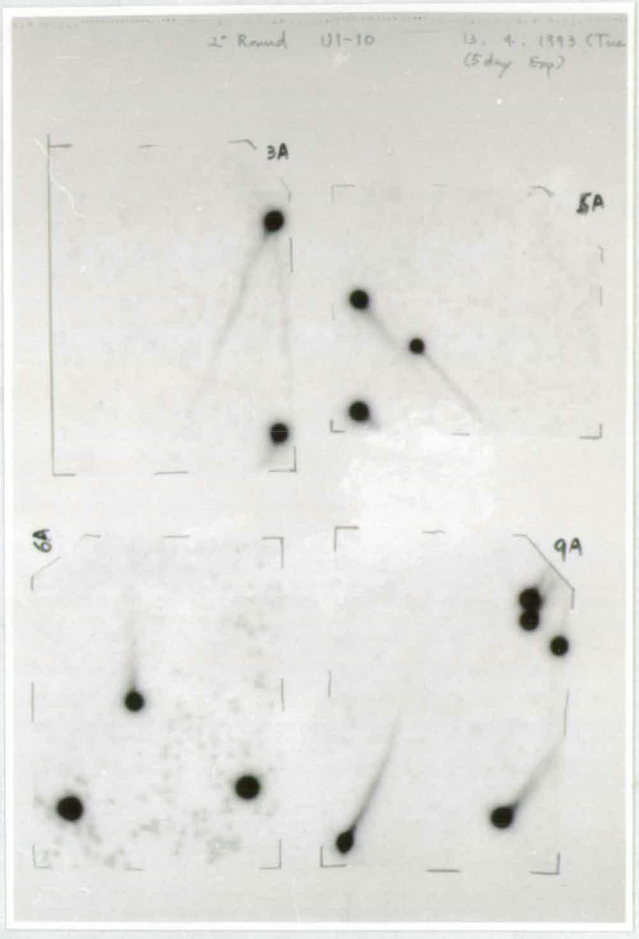
Figure 5. 1. First-round screening for homologous U1-10 cDNA clones. About 200,000 plaques were used for the first-round screening. Two square petri dishes (20 x 20 cm) were used for forming plaques and DNA hybridisation to the two plaque lifts was carried out using radiolabelled U1-10 cDNA fragment. The positive areas were removed from the master plates then used for the second-round screening.


A
11
10
12
9
5
6
8
7
3
2
1


B
7
8
6
5
1
2
4
3
9

Figure 5. 2. Second-round screening using the positive plaques from the first-round screening. The positive plaques were titred and about 100 to 200 plaques formed on square petri dishes (12 x 12 cm). Then plaque lifts were hybridised as in the first-round screening. The single positive plaques were used for further analyses.


2° Round U-1-10
13. 4. 1993 CT
(5day Exp)
1A
4A
10A
11B


2° Round U1-10
13. 4. 1993 CTua
(5day Exp)
3A
5A
6A
9A

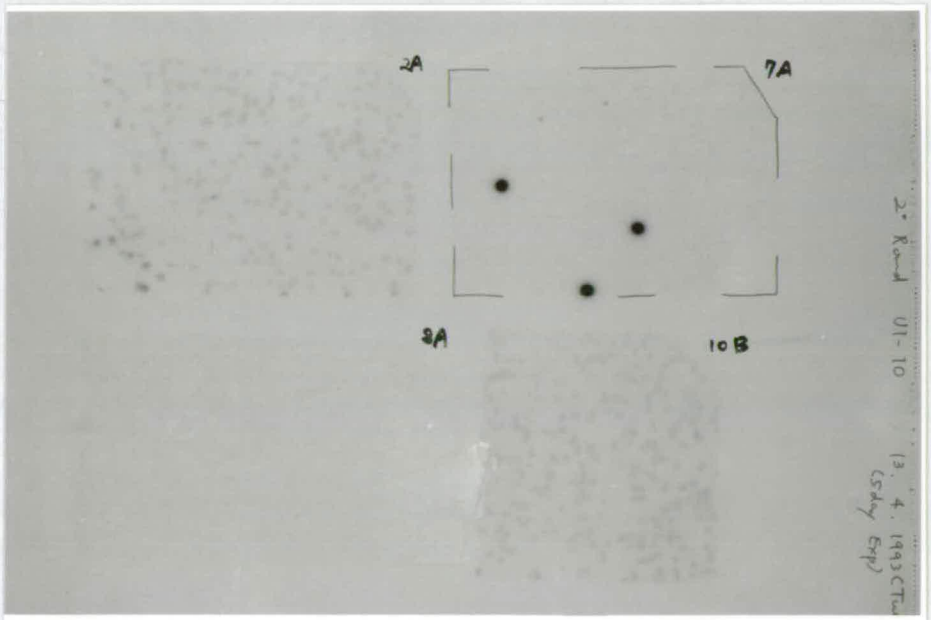

2A
7A
8A
10B
2° Round U1-10
13. 4. 1993 CTua
(5day Exp)

Table 5. 1. U1-10 homologous cDNA clones

Clone	cDNA insert size	Sequence
U1-10	0.4 kb	Complete
pBRPCK-1A	0.4 kb	NO
pBRPCK-4A	2.4 kb	Both ends
pBRPCK-5A	1.1 kb	Both ends
pBRPCK-6A	1.1 kb	NO
pBRPCK-7A	2.4 kb	Complete
pBRPCK-9A	1.9 kb	Both ends
pBRPCK-11B	1.7 kb	Both ends

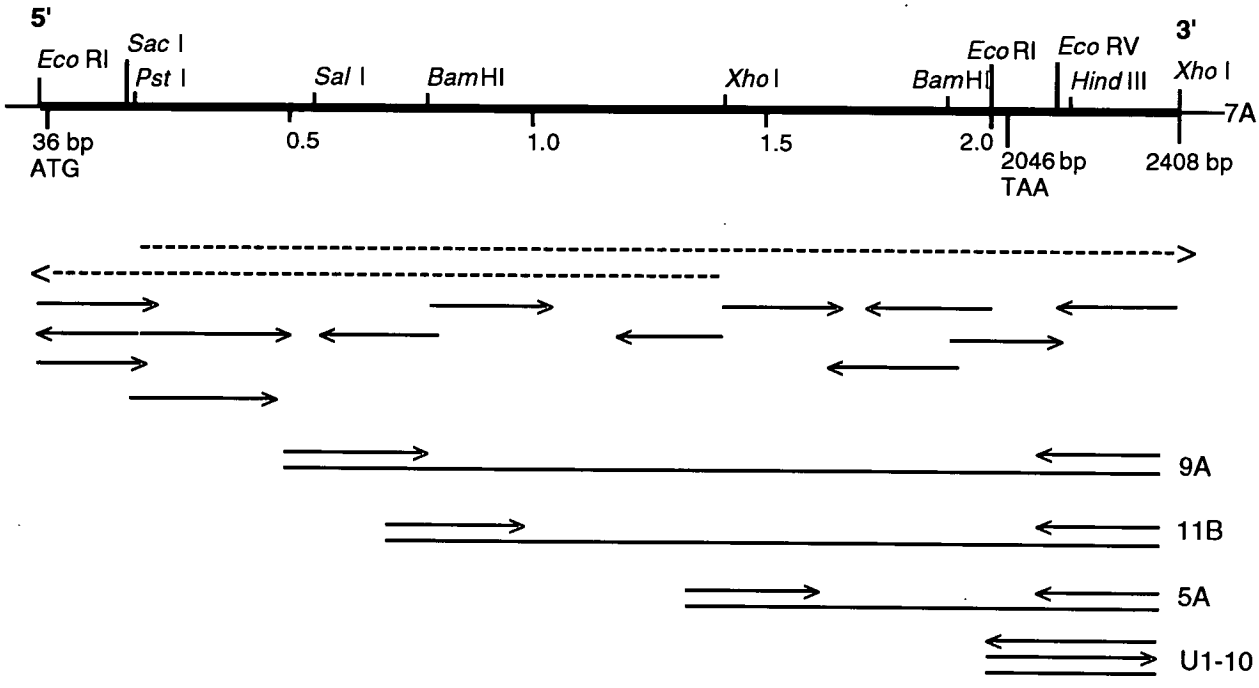


Figure 5. 3. U1-10-related cDNA clones restriction enzyme map and DNA sequencing strategy. The longest clone 7A was chosen for determining the full sequence. For sequencing both strands, *Exo*III -deletion was applied to the appropriate restriction enzyme sites (*Sac*I for sense and *Xho*I for antisense direction). Dotted lines indicate *Exo*III deletion and sequencing. Arrows indicate sequencing directions from specific restriction sites. Numbers (0.5, 1.0. 1.5 and 2.0) indicate kbp.

5. 2. 2. Identification of cDNA encoding PEPCK and sequence comparison with other organisms

Comparison of the U1-10 cDNA sequence with other sequences in the Gene Bank and EMBL Data Bases had revealed no similarities (see Chapter 4). This was not surprising in view of the fact that it covers only 0.4 kb at the 3' end. The sequences at the 5' ends of the longest cDNAs (pBRPCK-4A and 7A) also failed to reveal any homology, but cDNAs pBRPCK-9A and 11B showed homology with cDNAs or genes encoding PEPCK from *Saccharomyces, Trypanosoma, Rhizobium* and *E. coli.* This homology was approximately 50 to 60% at the amino acid sequence level and indicated very strongly that the pBRPCK cDNA clones encode cucumber PEPCK. cDNAs pBRPCK-7A and 4A were identical at both 5' and 3' ends and so are thought to be sibling clones.

5. 2. 3. Full-length PEPCK cDNA sequence analysis

The longest cDNA (pBRPCK-7A) was completely sequenced by employing exonuclease III to create a series of deletions in both directions. In addition restriction enzyme sites were employed for sub-cloning and sequencing other fragments. The complete sequence is shown in Figure 5. 4. Sequence analysis was also carried out on fragments of cDNAs pBRPCK-7A, 9A and 11B for comparison with pBRPCK-7A. No sequence differences were seen.

Figure 5. 4. Full-length cDNA encoding PEPCK from cucumber. The 5' end contains two possible translation start codons (at 36 and 186 bp). The underlined amino acid sequence shows limited sequence homology with the putative microbody targeting amino terminal presequences of peroxisomal thiolase and gMDH (Preisig-Muller and Kindl, 1993). The predicted polypeptide sequence produces 670 amino acid and a molecular weight of 74,397 Da. The 3' untranslated region does not contain putative polyadenylation signals (AATAAA) 10 to 30 nucleotides upstream of the identified polyadenylation sites. However, related motifs (underlined) are found at positions 2332 (AATAAT) and 2384 (AATATT). The 3' end contains three different poly (A)$^+$ addition sites indicated by arrow heads. a, U1-10 and pBRPCK- 5A ; b, pBRPCK-9A and 11B; c, pBRPCK-7A and 4A

```
   1  CTCTTATCCATCACAAATCTCCGAGGATACAAAAAATGGAGAATGAGGGGAAAGATAACG
                                        M  E  N  E  G  K  D  N  G    9

  61  GCGAATTTAGCTTTGTGAGTGATGGAGGAGCGGAGACAGGACGGAGAGGACTGCCGAAGA
      E  F  S  F  V  S  D  G  G  A  E  T  G  R  R  G  L  P  K  I   29

 121  TTCACACGGAGAAAAACGCGCCGACGACGGAGAGAGATATATGTCATGATGATAGTACGA
      H  T  E  K  N  A  P  T  T  E  R  D  I  C  H  D  D  S  T  T   49

 181  CACCGATGAGAGCTCGGACGTTGGAGCATCTTCATTCACTGCAGAAAAAGCGATCGACGC
      P  M  R  A  R  T  L  E  H  L  H  S  L  Q  K  K  R  S  T  P   69

 241  CGACCACTCCATTGACGGACAGTCAGGGAGTTTTTTCCCCTGTTTCTGAAGCCGAACGTC
      T  T  P  L  T  D  S  Q  G  V  F  S  P  V  S  E  A  E  R  Q   89

 301  AAAAGCAGCAGCTTATCTCAATCAGTGCTTCATTGGCGTCGCTGACAAGAGAAACTGGGC
      K  Q  Q  L  I  S  I  S  A  S  L  A  S  L  T  R  E  T  G  P  109

 361  CGAAGTTAGTGAAAGGCGATCCAGAGAAAAAGAAGGAGGCCCACAAAGCATCAGTATTGG
      K  L  V  K  G  D  P  E  K  K  K  E  A  H  K  A  S  V  L  D  129

 421  ACCATCTTCATTTTGGAGAGCCCATATTGAACTTGAGTGACAGCGCCCTCAAGTCCACCC
      H  L  H  F  G  E  P  I  L  N  L  S  D  S  A  L  K  S  T  H  149

 481  ACATCCTCTACAATCCCTCTCCCGCCGAGCTTTACGAGCAAGCGATCAAGTACGAGAAAG
      I  L  Y  N  P  S  P  A  E  L  Y  E  Q  A  I  K  Y  E  K  G  169

 541  GGTCATTCATAACGTCGACAGGGGCTTTGGCCACTCTTTCAGGAGCCAAAACGGGAAGAT
      S  F  I  T  S  T  G  A  L  A  T  L  S  G  A  K  T  G  R  S  189

 601  CGCCTATAGACAAAAGAGTTGTTAAAGATGACACCACTGAAAAGGAGCTTTGGTGGGGCA
      P  I  D  K  R  V  V  K  D  D  T  T  E  K  E  L  W  W  G  K  209

 661  AGGGATCACCTAATATTGAGATGGATGAACATACTTTCTTAATCAATAGAGAGAGAGCTG
      G  S  P  N  I  E  M  D  E  H  T  F  L  I  N  R  E  R  A  V  229

 721  TCGATTACCTGAACTCCCTTGATAAGGTATTTGTGAATGATCAGTTCTTGAACTGGGATC
      D  Y  L  N  S  L  D  K  V  F  V  N  D  Q  F  L  N  W  D  P  249

 781  CCGAAAACCGAATCAAAGTCCGAATCGTTTCAGCCCGAGCCTATCATTCCTTGTTCATGC
      E  N  R  I  K  V  R  I  V  S  A  R  A  Y  H  S  L  F  M  H  269

 841  ACAACATGTGCATTCGACCAACTGCTGGAGAGCTGGAGGACTTTGGGACTCCGGATTTCA
      N  M  C  I  R  P  T  A  G  E  L  E  D  F  G  T  P  D  F  T  289

 901  CAATATACCATGCTGGGCAGTTTCCTTGTAATCGTTACACTCACTATATGACTTCTTCCA
      I  Y  H  A  G  Q  F  P  C  N  R  Y  T  H  Y  M  T  S  S  T  309

 961  CCAGTATAGATATGAATCTTGATAGGAAGGAAATGGTCATTCTTGGTACTCAATATGCTG
      S  I  D  M  N  L  D  R  K  E  M  V  I  L  G  T  Q  Y  A  G  329

1021  GCGAAATGAAGAAAGGCCTCTTTAGTTTAATGCATTATCTCATGCCGATGCGCCAGATTT
      E  M  K  K  G  L  F  S  L  M  H  Y  L  M  P  M  R  Q  I  L  349
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1081  TGTCTCTTCATTCTGGTTGCAACATGGGCAAAAATGGAGACGTCGCCCTTTTCTTTGGAT
        S  L  H  S  G  C  N  M  G  K  N  G  D  V  A  L  F  F  G  L  369

1141  TATCAGGTACTGGGAAGACCACGTTGTCTACAGATCATAATAGGTACTTAATAGGGGATG
        S  G  T  G  K  T  T  L  S  T  D  H  N  R  Y  L  I  G  D  D  389

1201  ATGAACACTGCTGGAGCGATAATGGTGTATCGAACATTGAAGGCGGTTGCTATGCCAAAT
        E  H  C  W  S  D  N  G  V  S  N  I  E  G  G  C  Y  A  K  C  409

1261  GCATCGACTTGTCGAGGGAAAAGGAGCCTGACATTTGGAATGCTATCAAGTTCGGGACTG
        I  D  L  S  R  E  K  E  P  D  I  W  N  A  I  K  F  G  T  V  429

1321  TTCTTGAGAATGTGGTGTTTGATGAGCACACTAGAGAAGTTGATTACTCTGAAAAATCCG
        L  E  N  V  V  F  D  E  H  T  R  E  V  D  Y  S  E  K  S  V  449

1381  TTACAGAGAACACTCGAGCGGCGTATCCCATTGAATACATTCCTAATGCTAAAATCCCCT
        T  E  N  T  R  A  A  Y  P  I  E  Y  I  P  N  A  K  I  P  C  469

1441  GCGTTGGCCCTCATCCAAAGAATGTAATTCTTCTTGCTTGTGATGCATTTGGAGTTCTCC
        V  G  P  H  P  K  N  V  I  L  L  A  C  D  A  F  G  V  L  P  489

1501  CACCAGTGAGCAAGCTGAGCTTGCCTCAGACTATGTACCATTTTATCAGTGGCTACACTG
        P  V  S  K  L  S  L  P  Q  T  M  Y  H  F  I  S  G  Y  T  A  509

1561  CTTTGGTGGCTGGAACTGAGGAGGGTGTGAAAGAGCCACAGGCAACATTCTCTGCTTGTT
        L  V  A  G  T  E  E  G  V  K  E  P  Q  A  T  F  S  A  C  F  529

1621  TTGGAGCAGCATTCATAATGTTGCATCCATCCAGATATGCAGCAATGCTAGCTGAGAAGA
        G  A  A  F  I  M  L  H  P  S  R  Y  A  A  M  L  A  E  K  M  549

1681  TGAAAAAACACGGTGCCACGGGATGGCTCGTAAACACCGGTTGGTCAGGAGGAAGCTATG
        K  K  H  G  A  T  G  W  L  V  N  T  G  W  S  G  G  S  Y  G  569

1741  GAAGTGGTAACAGGATCAAGTTAGCCTACACAAGGAAGATTATCGATGCAATCCACTCAG
        S  G  N  R  I  K  L  A  Y  T  R  K  I  I  D  A  I  H  S  G  589

1801  GAGCGCTTTTGGAAGCAAACTACAGCAAGACTCGGGTGTTTGGCCTTGAGATTCCTGATG
        A  L  L  E  A  N  Y  S  K  T  R  V  F  G  L  E  I  P  D  A  609

1861  CTATTGAGGGAGTTCCTTCACATATCTTGGATCCAATAAACACGTGGTCAGACAAAGATG
        I  E  G  V  P  S  H  I  L  D  P  I  N  T  W  S  D  K  D  G  629

1921  GCTATCATGAGACATTGCTGAAGTTGGGTGGTCTGTTTAAGAAGAACTATGAAGGAATTC
        Y  H  E  T  L  L  K  L  G  G  L  F  K  K  N  Y  E  G  I  H  649

1981  ATACTTACCAAGTGGAGAGGGACAGTGAATTGGCTGAGGAGATTCTTGCAGCTGGGCCTA
        T  Y  Q  V  E  R  D  S  E  L  A  E  E  I  L  A  A  G  P  T  669

2041  CCTTG**TAA**TAAAATGGTTAATGGAGGAGCATTTCATGAATTTGGATGAAGAATGATATCA
        L  *                                                          670
2101  TGAAATGGCTGGCGTTGGTTATATTTTACAGATGAATGTGTGAAAGCTTCGAAGTTGAGA

2161  GTTTTTGCGGATAAACAAGATATTGCATAGTCCATAGTTTTTTTCTCCTGTGATTTATGT

2221  TGTAAAATCATAACGTTTATATTTGTGATTTGGGCTTTCAAGGCCTGAACAAAGTATGGT
```

2281 CTTTTAAATTGTAATTTTATAGCAGAATCTGCTATTCACAATCTATACATGAATAATATG

2341 TCAAGTTGACCTTTAAGAGCATTATGTTTCTTCCAATCAATCAAATATTACTTCTTATTT

^ ^

a b

2401 TAGATTCTAAAAAAAAAAAAAAAAAA

^

c

The sequence of pBRPCK-7A is 2,408 bp long, excluding the poly(A)$^+$ tail. It contains two potential translation start codons at the 5' end of the cDNA (at 36 and 186bp) but the first one is presumably used (Kozak, 1984; Joshi, 1987). When all five different cDNAs are compared, there are three different sites for the attachment of the poly(A)$^+$ tail at positions 2355, 2361 and 2408 (marked with arrow heads in Figure 5. 4). Sequences which might function as polyadenylation signals are found at positions 2332 (AATAAT) and 2384 (AATATT) (Figure 5. 4). All five cDNAs contained poly(A)$^+$ sequences at the 3' ends, ranging in size from 16 to 24 bases.

5. 2. 4. Predicted peptide sequence analysis and sequence comparison with other organisms

The cDNA and deduced polypeptide sequence are shown in Figure 5. 4. There are two methionine residues at the amino terminal end (amino acids 1 and 51). They produce polypeptides of 670 and 620 amino acids with molecular weights of 74,397 and 68,966, respectively. It is assumed that the first methionine codon represents the amino terminal end (Kozak, 1984; Joshi, 1987). This conclusion is supported by immunoblotting and size estimation of the polypeptide from cotyledons (see sections 5. 4 and 5. 5). Comparison of the cucumber PEPCK with the *Saccharomyces, Trypanosoma* , *Rhizobium* and *E.coli* PEPCK gives 43-57% overall identity of residues (Figure 5. 5). The *Saccharomyces* PEPCK (Stucka *et al.*, 1988) shares 57% identical residues with cucumber PEPCK. The overall identity drops to 49% when one compares the sequence of PEPCK from the African unicellular

Figure 5. 5. Alignment of cucumber PEPCK with four other ATP-dependent PEPCK peptide sequences. The conserved ATP-binding site (GXXGXGKT, amino acids 368 to 375) is shown in bold-type. Underlined amino terminal region shows limited sequence homology with the putative transit peptide for peroxisome targeting.

```
Cucumber              MENEGKDNG EFSFVSDGGA ETGRRGLPKI HTEKNAPTTE   39
Yeast     .......... .......... .......... .......... ..........
Rhizobium .......... .......... .......... .......... ..........
E. coil   .......... .......... .......... .......... ..........
Trypanosoma .......... .......... .......... .......... ..........
Consensus ---------- ---------- ---------- ---------- ----------

Cucumber  RDICHDDSTT PMRARTLEHL HSLQKKRSTP TTPLTDSQGV FSPVSEAERQ   89
Yeast     .......... .......... .......... .......... ..........
Rhizobium .......... .......... .......... .......... ..........
E. coli   .......... .......... .......... .......... ..........
Trypanosoma .......... .......... .......... .......... ..........
Consensus ---------- ---------- ---------- ---------- ----------

Cucumber  KQQLISISAS LASLTRETGP KLVKGDPEKK KEAHKASVLD HLHFGEPILN  139
Yeast     .......... .......... .....MSPSK MNATVGSTSE VEQKIRQELA
Rhizobium .......... .......... .......... ........ME QLGTRNPRTD
E.coli    .......... .......... .......... ....MRVNNG LTPQELEAYG
Trypanosoma .......... .......... .......... .......... ..........
Consensus ---------- ---------- ---------- ---------E -L----P-L-

Cucumber  LSDSALKSTH ILYNPSPAEL YEQAIKYEKG SFITSTGALA TLSGAKTGRS  189
Yeast     LSDEV...TT IRRNAPAAVL YEDGLK.ENK TVISSSGALI AYSGVKTGRS
Rhizobium WKRSVFRPLG RSLQLRAAEL YEEAF..ARR GALTAHGALC ARTGQHTGRS
E. coli   ISDVHDIVYN PSYDLLYQEE LDPSLTGYER GVLTNLGAVA VDTGIFTGRS
Trypanosoma ......MPPT IHRNLLSPEL VQWALKIEKD SRLTARGALA VMSYAKTGRS
Consensus LSDSV--P-- I--NL--AEL YE-ALK-E-- --LT--GALA --SG-KTGRS

Cucumber  PIDKRVVKDD TTEKELWWGK GSP....NIE MDEHTFLINR ERAVDYLNSL  235
Yeast     PKDKRIVEEP TSKDEIWWGP VNK....HV. LKEHGL..ST VKAADYLRTR
Rhizobium PKDKYVVRDA ATGDQIWWDN .......NSA ISPENFERLR QDMLAHAKGM
E. coli   QKISISVRDD TTRDTFWWAD KGKGKNDNKP LSPETWQHLK GLVTRQLSG.
Trypanosoma PLDKRIVDTD DVRENVDWGK V......NMK LSEESFARVR KIAKEFLDTR
Consensus PKDKR-V-DD TT-D--WWG- V-K----N-- LSEE-F-R-R --A---L--R

Cucumber  DKVFVNDQF. LNWDPENRIK VRIVSARAYH SLFMHNMCIR PTAGELEDFG  284
Yeast     DHIYIVDRF. AGWDPKYRIK VRVVCARAYH ALFMTNMLIR PTEEELAHFG
Rhizobium S.LYVQDLVG AGQSGKCVAD ARRHRIR.WH SLFIRNLLIR PPREGLASF.
E. coli   KRLFVVDAFC GA.NPDTRLS VRFITEVAWQ AHFVKNMFIR PSDEELAGF.
Trypanosoma EHLFVVDCF. AGHDERYRLK VRVFTTRPYH ALFMRDMLIV PTPEELATFG
Consensus -HLFVVD-F- AGWDP--R-K VR----RAYH ALFM-NMLIR PT-EELA-FG

Cucumber  TPDFTIYHAG QF.PCNRYTH YMTSSTSIDM NLDRKEMVIL GTQYAGEMKK  333
Yeast     EPDFTVWNAG QF.PANLHTQ DMSSKSTIEI NFKAMEMIIL GTEYAGEMKK
Rhizobium LPKLTIIDLP SF.KANPERH GCRGETIIAC DLTKGLVLIG GTSYAGEMKK
E. coli   KPDFIVMNGA KCTNPQWKEQ GLNSENFVRL T.DRAHAADC GTWYGGEMKK
Trypanosoma EPDYVIYNAG EC.KADPSIP GLTSTTCVAL NFKTREQVIL GTEYAGEMKK
Consensus -PDFTI-NAG -F--AN---- G--S-T-I-- NL---E-VIL GTEYAGEMKK
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```
Cucumber   GLFSLMHYLM PMRQ.ILSLH SGCNMG.KNG DVALFFGLSG TGKTTLSTDH  381
   Yeast   GIFTVMFYLM PVHHNVLTLH SSANQGIQNG DVTLFFGLSG TGKTTLSADP
Rhizobium  SVFTVLNYLL P.NKAVMPMH CSANVG.PAG DTAIFFGLSG TGKTTLSADP
 E. coli   GMFSMMNYLL PLK.GIASMH SSANVGEK.G DVAVFFGLSG NGKTAF.PRP
Trypanosoma GILTVMFELM P.QMNHLCMH ASANVG.KQG DVTVFFGLSG TGKTTLSADP
Consensus  G-FTVM-YLM P---N-L-MH SSANVG-KNG DVA-FFGLSG TGKTTLSADP

Cucumber   NRYLIGDDEH CWSDNGVSNI EGGCYAKCID LSREKEPDIW NAI.KFGTVL  430
   Yeast   HRLLIGDDEH CWSDHGVFNI EGGCYAKCIN LSAEKEPEIF DAI.KFGSVL
Rhizobium  NRTLIGDDEH GWSDKGVFNF EGGCYAKAIR LSEAAEPEIF ATTRRFGTVM
 E. coli   KRRLIGDDEH GWDDDGVFNL KAAGYAKTIK LSKEAEPEIY K.LSVVMALL
Trypanosoma HRNLIGDDEH VWTDRGVFNI EGGCYAKAIG LNPKTEKDIY DAV.RFGAVA
Consensus  -R-LIGDDEH -WSD-GVFNI EGGCYAK-I- LS-E-EPEI- -A---FG-VL

Cucumber   ENVVFDEHTR EVDYSEKSVT ENTRAAYPIE YIPNAKIPCV .GPHPKNVIL  479
   Yeast   ENVIYDEKSQ VVDYDDSSIT ENTRCAYPID YIPSAKIPCL ADSHPKNIIL
Rhizobium  ENVVLDER.R APDFDNGSLT ENTRIAYPLD FIPNA.SETG TAPQPRTIIM
 E. coli   ENVTVREDG. TIDFDDGSKT ENTRVSYPIY HIDNIVRPVS KAGHGRRLSL
Trypanosoma ENCVLDKRTG EIDFYDESIC KNTRVAYPLS HIEGALSKAI AG.HPKNVIF
Consensus  ENVV-DE-TR --DFDD-S-T ENTR-AYPI- -IPNAKIP-- A-PHPKNVIL

cucumber   LACDAFGVLP PVSKLSLPQT MYHFISGYTA LVAGTEEGVK .EPQATFSAC  528
   Yeast   LTCDASGVLP PVSKLTPEQV MYHFISGYTS KMAGTEQGVT .EPEPTFSSC
Rhizobium  LTADAFAVLP PIAKLTPEQA MYHFLSGYTA KVAGTEKGVT .EPEATFSTC
 E. coli   LTAHAFGVLP PVSRLTADQT QYHFLSGFTA KLAGTERGIT .EPTPTFSAC
Trypanosoma LTNDAFGVMP PVARLTSAQA MFWFVMGYTA NVPGVEAGGT RTARPIFSSC
Consensus  LT-DAFGVLP PVSKLT--Q- MYHFLSGYTA KVAGTE-GVT -EPEPTFS-C

Cucumber   FGAAFIMLHP SRYAAMLAEK MKKHGATGWL VNTGWSGGSY GS.GNRIKLA  577
   Yeast   FGQPFLALHP IRYATMLATK MSQHKANAYL INTGWTGSSY VSGGKRCPLK
Rhizobium  FGAPFMPRHP SEYGNLLKDL MPRNGVTCWL VNTGWTGGAY GTGS.RMPIK
 E. coli   FGAAFLSLHP TQYAEVLVKR MQAAGAQAYL VNTGWNGS.. ...GKSISIK
Trypanosoma FGGPFLVRHA TFYGEQLAEK MQKHNSRVWL LNTGYAGGRA DRGAKRMPLR
Consensus  FGAPFL-LHP --YA--LA-K M--HGA--WL VNTGW-GGSY GSGGKR-PLK

cucumber   YTRKIIDAIH SGALLEANYS KTRVFGLEIP DAIEGVPSHI LDPINTWSDK  627
   Yeast   YTRAILDSIH DGSLANETYE TLPIFNLQVP TKVNGVPAEL LNPAKNWSQG
Rhizobium  VTRALLSAAL DGSLNRPAFR TDANFGFACR SRCRRRRRPY SRPALDLVGQ
 E. coli   DT..... .....AHYR RISRFAG*.. ..... .....
Trypanosoma VTRAIIDAIH DGTLDRTEYE EYPGWACTSR STSPKCRSIC *.....
Consensus  -TRAIIDAIH DGSL-R--Y- --P-F----P S---GVP--- L-PA--WS--

Cucumber   DG.YHETLLK LGGLFKKNYE GIHTYQVERD SELAEEILAA GPTL*.....  670
   Yeast   ESKYRGAVTN LANLFVQNFK IY.....Q DRATPDVLAD WSSIRVNETC*
Rhizobium  AKPTMPRPGG WSTCSSPTSP SSRAMSTAAC AIRPPGTKLA AE*.....
 E. coli   ..... ..... ..... ..... .....
Trypanosoma ..... ..... ..... ..... .....
Consensus  ---Y------ L--LF--N-- ---------- ----P--LAA ----------
```

flagellate protozoa *Trypanosoma* (Parsons and Smith, 1989) and further drops to 43% from *Rhizobium* (Osteras *et al.,* 1991) and *E.coli* (Medina *et al.,* 1990) PEPCK. No PEPCK sequence from a plant has been reported.

The coding sequence is significantly longer at the amino terminal end than other PEPCK sequences. This is the most distinctive feature in cucumber PEPCK. This observation raises the possibility that the amino terminal sequence in the plant enzyme may have a particular function *eg*, protein transport into an organelle. Interestingly, this region of the protein shows some similarity with the putative transit peptides of gMDH and other peroxisomal enzymes (not shown). The region surrounding the possible ATP-binding site is completely conserved suggesting that this sequence is important for function (Figure 5. 5).

According to the polypeptide sequence comparison, the PEPCK enzyme can be divided into three regions. Region I is the amino terminal domain which shows variability in peptide sequence and length. This region is much longer in cucumber than any other PEPCK proteins. Region II (amino acids 260 to 590) is a highly conserved (60-70%) central domain. A consensus sequence of an ATP-binding site (GXXGXGKT) is found in this region (amino acid 368 to 375) that is almost completely conserved in all five proteins. Region III is a carboxy terminal domain which also shows variability in both sequence and size (Figure 5. 5).

5. 2. 5. Genomic DNA southern blot analysis

Southern blot hybridisation experiments with cucumber leaf DNA digested with a range of restriction endonucleases and the full-length cDNA as a probe reveals a simple pattern of hybridising bands consistent with the presence of only one PEPCK gene (Figure 5. 6). The cDNA contains no *Xba*I site, one *Hind* III site, one *Eco*RI site and two *Bam*HI sites (Figure 5. 3). In each case the number of hybridising bands is exactly as predicted by a single gene containing those sites. The lack of sequence heterogeneity among five independent cDNAs also supports the conclusion that there is only one PEPCK gene (hereafter designated *pck*).

5. 3. Developmental changes of PEPCK transcript in cucumber cotyledons

Northern hybridisation with RNA isolated from cotyledons at daily intervals for the first week and weekly intervals thereafter (Figure 5. 7) shows that PEPCK mRNA has a size of approximately 2.4 kb, and that this RNA increases dramatically in amount in cotyledons during the first two days immediately post imbibition, then declines significantly, and increases again to a low level in yellow cotyledons. This pattern of expression is very similar to that of ICL and MS (see Figure 3. 6), except that PEPCK mRNA is detected at a low level in green cotyledons (see also Figure 4. 5). The pattern of mRNA accumulation during the first few days of growth corresponds very well with the accumulation of protein detected with the PEPCK antibody (see section 5. 5).

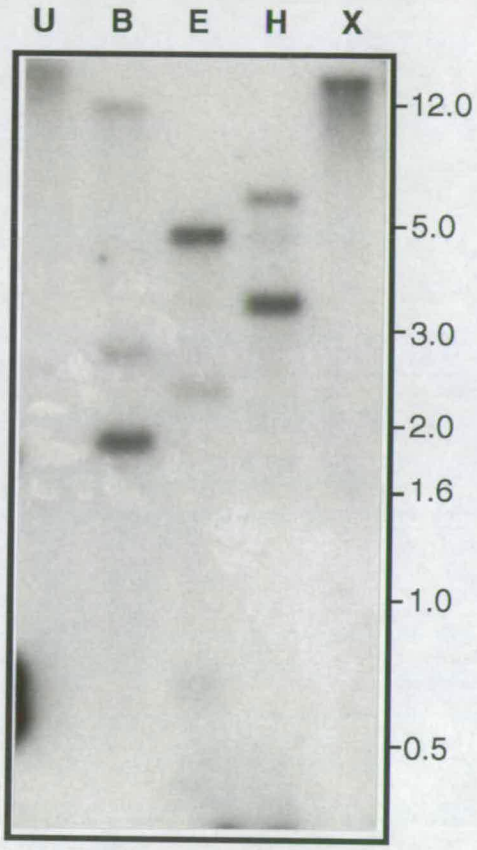
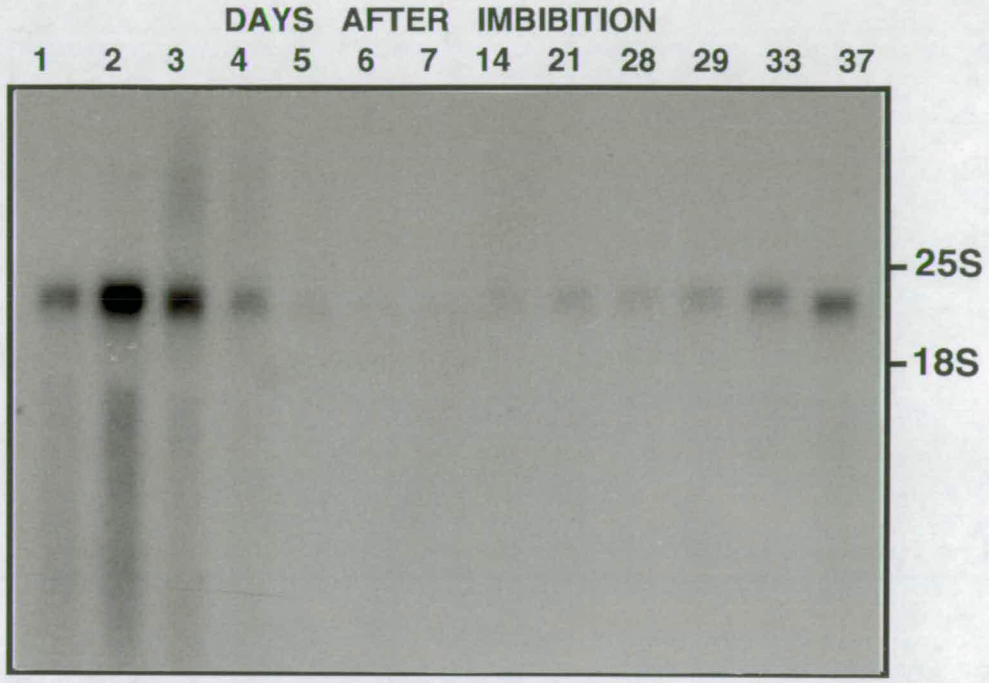


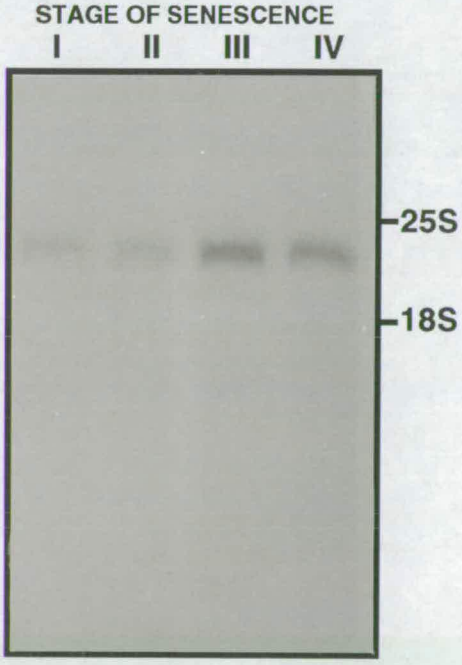
Figure 5. 6. Southern blot analysis for cucumber *pck* gene copy number. DNA was isolated from 3 week old first leaves and digested with *Bam*HI, *Eco*RI, *Hind*III and *Xba*I. The DNA was fractionated on a 0.8% (w/v) agarose gel and probed with the full-length cucumber PEPCK cDNA (pBRPCK-7A). The cDNA has no *Xba*I site, one *Hind*III site, one *Eco*RI site and two *Bam*HI sites. Markers (kbp) are from a 1 kb DNA ladder (Gibco BRL, U. K.). (U, undigested; B, *Bam*HI; E, *Eco*RI; H, *Hind*III; X, *Xba*I).

Figure 5. 7. Northern blot analyses using PEPCK cDNA fragment. The blots had been previously hybridised with ICL, MS, gMDH and HPR probes as described in Chapter 3. a, Developmental changes of PEPCK mRNA in cucumber cotyledons; b, Changes of PEPCK mRNA during cotyledon senescence. Cotyledon samples and RNA preparation was described earlier (see Figures 3. 6 and 3. 7).

a.


DAYS AFTER IMBIBITION
1 2 3 4 5 6 7 14 21 28 29 33 37
25S
18S

b.


STAGE OF SENESCENCE
I II III IV
25S
18S

The pattern of expression in senescence was further investigated by hybridising the northern filter show in Figure 3. 7 with the PEPCK probe. The result (Figure 5. 7b) shows a very similar pattern of mRNA accumulation to that of ICL and MS.

5. 4. Cucumber PEPCK antibody preparation using an *E. coli* over-expression system

5. 4. 1. Subcloning of pBRPCK-9A *Eco*RI fragment into *E. coli* over-expression vector pGEX-301

After DNA sequence scrutiny, pBRPCK-9A cDNA was chosen as suitable and convenient for using an *Eco*RI fragment to create a fusion construct in pGEX-301 (Figure 5. 8). The vector pGEX-301 can create a translational fusion with bacterial glutathione S- transferase (GST) (Smith and Johnson, 1988). Therefore, both plasmid DNAs (pBRPCK-9A and pGEX-301) were first digested with *Eco*RI. Then, the digested DNA was electrophoresed on an agarose gel and the required bands were removed under long wave UV illumination using sterile scalpels. The DNA bands were purified by Geneclean kit (UBS, Ohio, USA). Insert (1.5 kb pBRPCK-9A *Eco* RI fragment) and vector (*Eco*RI digested pGEX-301) were ligated and XL-1 Blue cells transformed. Even though few colonies contained the insert, one of them was inserted in the right direction. Orientation of the insert was confirmed by restriction enzyme analysis and the clone named pGEXPCK-9A.

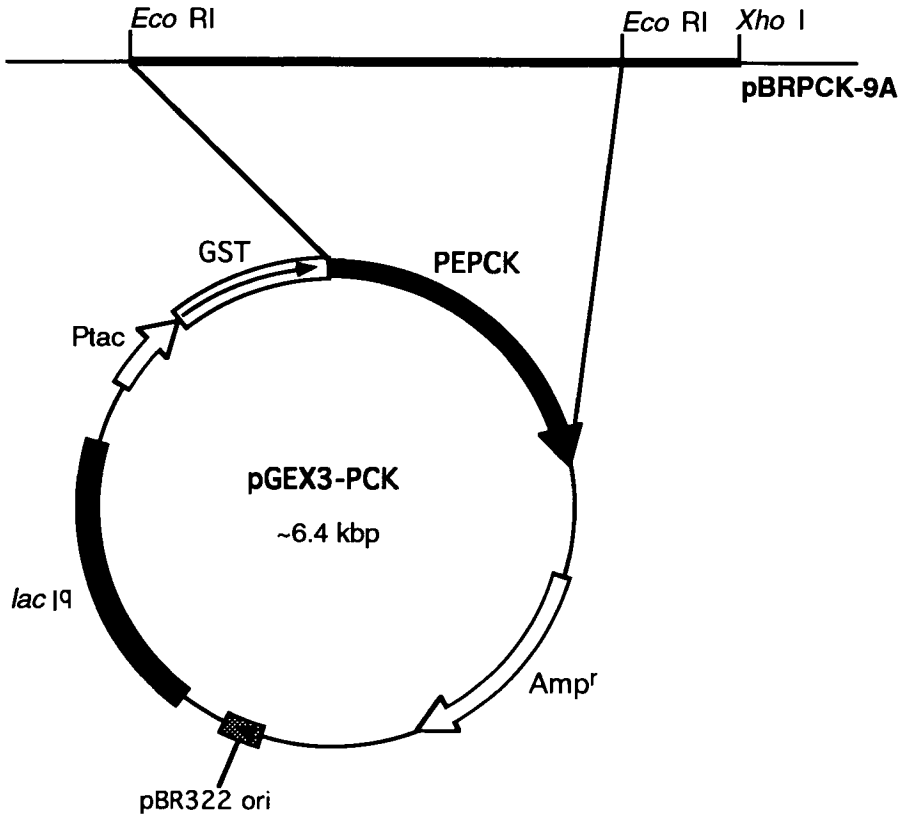


Figure 5. 8. Subcloning of the pBRPCK-9A *Eco*RI fragment into pGEX-301 *E. coli* over-expression vector. A 1. 5 kb *Eco*RI frament was prepared from the pBRPCK-9A plasmid then ligated into *Eco*RI digested pGEX-301. The recombinant plasmid was used for XL-1 Blue cells transformation to achieve over-expression in the *E. coli* cells.

Over-expression was achieved successfully by incubation with IPTG and produced an 88,000 molecular weight fusion protein as expected (Figure 5. 9). This protein was purified from inclusion bodies by SDS-PAGE and used to raise antibodies in rabbits.

5. 4. 2. Antigen preparation for injection preliminary test of antisera

The fusion protein was injected three times (10 to 12 day intervals) into two rabbits separately. Before the first injection, pre-immune antisera were sampled. One day before the third injection, a small amount of serum was also collected and the antisera tested against purified bacterial fusion protein (antigen) using a dot blot procedure. As a result of this, antigen recognizing antibody was identified (not shown). Ten days after the final boost, antisera were collected and incubated at room temperature to form blood clots. The antisera were further incubated at 4°C overnight and finally antisera were collected by centrifugation. The antisera were stored at 4°C in aliquots after addition of sodium azide (0.02%, w/v) and some was freeze dried at -54 °C then stored at 4 °C.

5. 4. 3. Test of antisera against plant protein extracts

Antisera were tested against native antigen (bacterial protein) and extracts of cucumber cotyledons (not shown). Initially, protein samples were prepared in PTES-buffer (100 mM Tris-Cl pH 8.0, 2 mM EDTA, 1.0% (w/v) SDS, 18% (w/v) sucrose, 10 mM DTT, 1 mM PMSF, 5 mM aminocaproic acid) and SDS-PAGE performed,

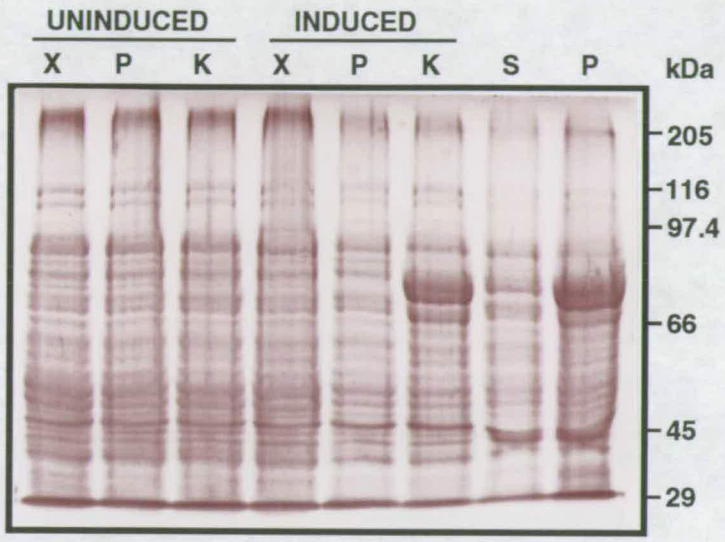
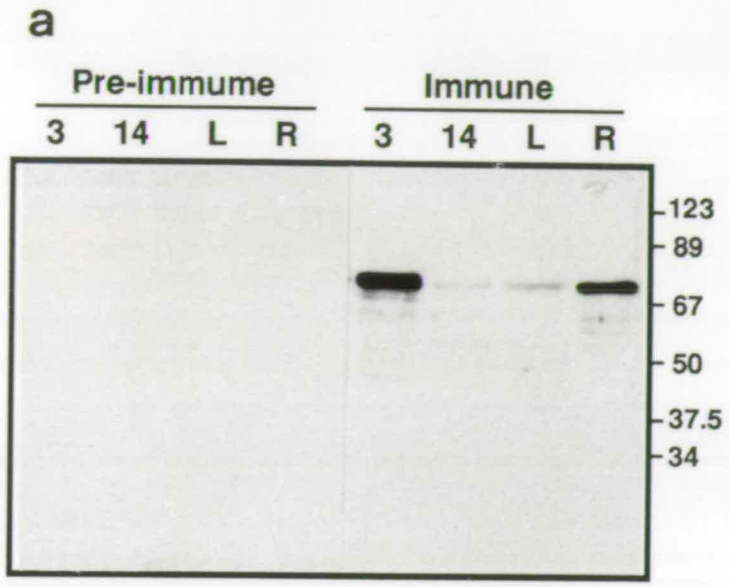


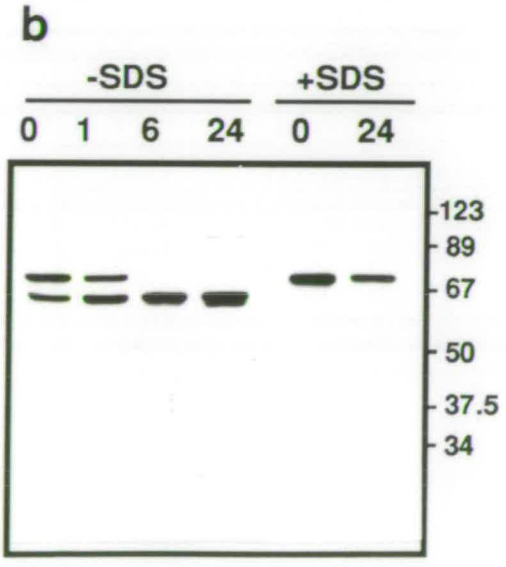
Figure 5. 9. Induction of fusion protein synthesis in *E. coli.* XL-1 Blue cells were transformed with the over-expression plasmids. LB broth was inoculated with a sample of overnight culture and grown at 37 °C. After 1h, 3 mM IPTG was added and further cultured for 3 h. The culture was centrifuged and the cells collected. The cells were lysed with SDS sample buffer and SDS-PAGE carried out then stained with Coomassie Brillitant Blue R. X, XL-1 Blue cells only; P, pGEX-301 plasmid transformed XL-1 Blue cells; K, pGEX-PCK plasmid transformed XL-1 Blue cells; S, supernatent from the induced pGEX-PCK transformed XL-1 Blue cells after ultra sonification and centrifugation of the culture; P, pellet from the induced pGEX-PCK transformed XL-1 Blue cells. Molecular weight markers (Sigma) are Myosin (205), β-Galactosidase (116), Phosphorylase b (97.4), Bovine Albumin (66), Egg Albumin (45) and Carbonic Anhydrase (29).

then the fractionated proteins were transferred onto Hybond-ECL membrane using wet electro-blotting method (Sambrook *et al.,* 1989). The protein transfer efficiency was slightly affected by the concentration of the SDS in the transfer buffer. Routinely, lower concentrations (less than 0.1% (w/v)) of SDS was quicker and efficient. Occasionally, the membrane was stained with Ponceau-S to examine transfer efficiency and known molecular weight masses marked using soft pencil. Subsequently, the membrane was destained in TBST with several changes. Then, the membrane was blocked with TBST buffer containing 5% (w/v) skimmed milk at 4 °C overnight. Then the membrane was incubated with anti-PEPCK antibody, then with secondary antibody (horseraddish peroxidase conjugated goat anti-rabbit IgG, Sigma) at 40,000 fold dilutions for 20 min. After washing the membrane with the TBST buffer, the membrane was visualised with ECL immunodetection reagent (Amersham, U. K.). A 2,000 fold dilution of PEPCK serum gives sufficient signal to detect PEPCK protein (Figure 5. 10). Pre-immune serum did not detect any bands (Figure 5. 10a). A 74 kDa polypeptide was detected in cotyledon extracts, the size corresponding with that predicted from the PEPCK cDNA sequence. The 74 kDa PEPCK polypeptide was also detected in leaves at a low level and roots at a significant level (Figure 5. 10a) indicating PEPCK gene expression in a range of plant tissues. However, the function is unknown in cucumber leaves and roots.

A different protein extraction buffer, PTE (100 mM Tris-Cl pH 8.0, 2 mM EDTA) showed the presence of two clear bands (74 kDa and 62 kDa) in a day 3 cotyledon sample (Figure 5. 10b). The 62 kDa polypeptide is thought to be a proteolytic cleavage product from the intact polypeptide (74 kDa) during the sample preparation. The 62 kDa polypeptide is seen when proteins are extracted in PTE-

Figure 5. 10. Test of anti-PEPCK antibody. a, Protein samples were extracted with PTES-buffer (100 mM Tris-Cl pH 8.0, 2 mM EDTA, 1% (w/v) SDS, 18% (w/v) sucrose, 10 mM DTT, 1 mM PMSF, 5 mM aminocaproic acid) from day 3 cotyledons (3), day 14 cotyledons (14), first leaves (L) and roots (R). Then, equal amounts (10 μg) of protein samples were electrophoresed on a polyacrylamide gel containing SDS. The proteins were transferred on to a Hybond-ECL membrane, then incubated with pre-immune and immune serum. b, Effect of SDS on PEPCK protein stability. Extracted protein samples were incubated at room temperature for 0, 1, 6 and 24 h then stored at -20 °C. One extract was prepared with PTE-buffer (-SDS) and the other with PTES-buffer (+SDS). The samples were electrophoresed and PEPCK detected after transfer to Hybond-ECL membrane. Molecular weight (kDa) markers (Prestained markers, Sigma) are β–Galactosidase (123), Fructose-6-Phosphate Kinase (89), Pyruvate Kinase (67), Ovalbumin (50), Lactic Dehydrogenase (37.5) and Triosephosphate Isomerase (34).


a
Pre-immume
Immune
3
14
L
R
3
14
L
R
123
89
67
50
37.5
34


b
-SDS
+SDS
0
1
6
24
0
24
123
89
67
50
37.5
34

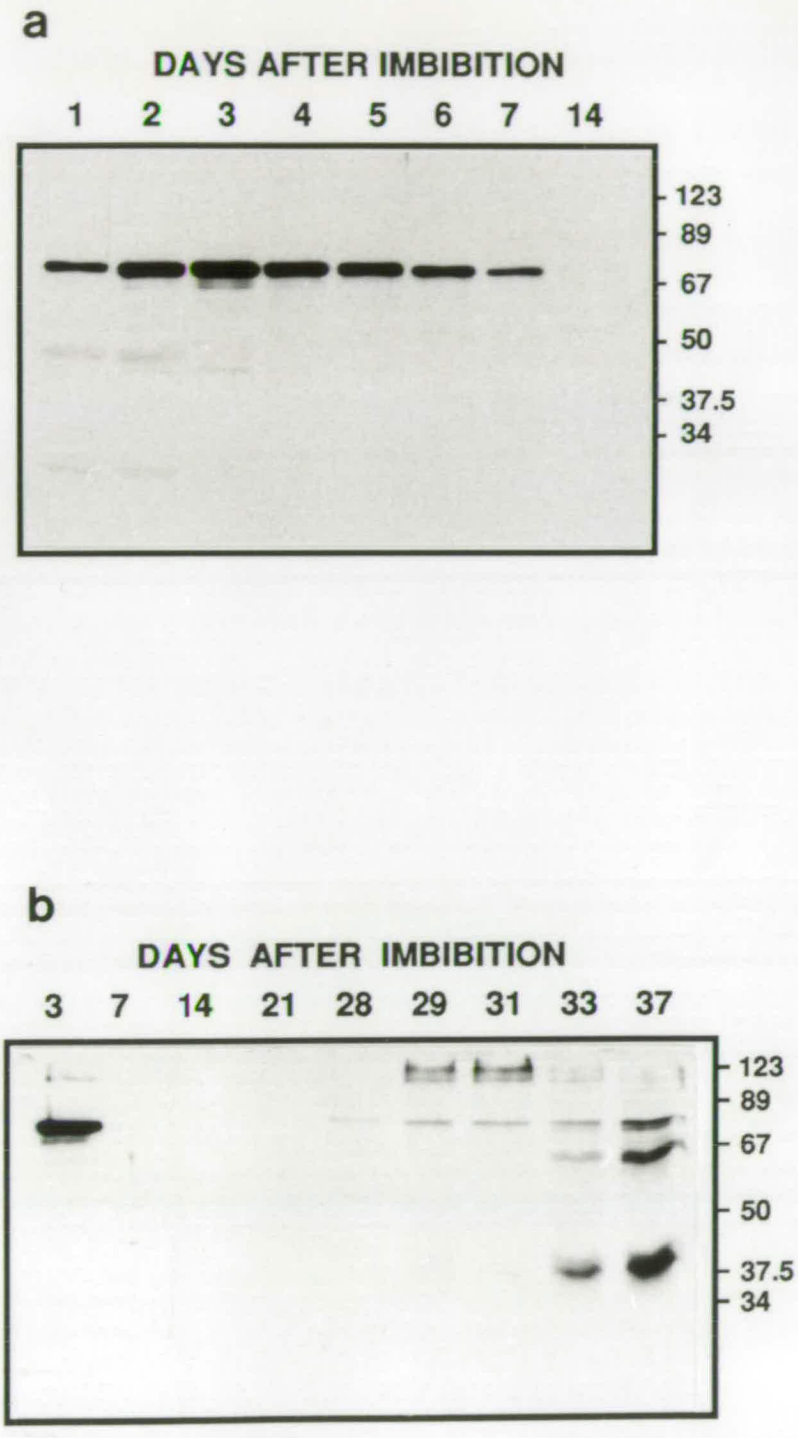
buffer, but not when the extraction buffer contains SDS and the other protease inhibitors PMSF and aminocaproic acid (Figure 5. 10b). When an extract is prepared with PTE-buffer and the sample incubated at room temperature, there appears to be a conversion of the 74 kDa polypeptide into the 62 kDa polypeptide. This does not occur in the buffer with SDS (Figure 5. 10b).

5. 5. Immunodetection of PEPCK protein during cotyledon development

5. 5. 1. Developmental changes of PEPCK protein in cotyledon growth

Protein was isolated from cotyledons from a similar experiment to that for northern blot analysis, and analysed by western blotting using the anti-PEPCK antibody. The amount of 74 kDa PEPCK polypeptide changes in amount (Figure 5. 11) in parallel with changes in PEPCK mRNA level (Figure 5. 7). These results agree with the earlier observation of the changes in PEPCK enzyme activity in germinating marrow cotyledons (Leegood and ap Rees, 1978) but thereafter, there is no further report of PEPCK synthesis in plant development especially in oil-seed plants. In extracts of senescent cotyledons there is evidence of polypeptide breakdown, including the 62 kDa polypeptide, even though extracts were prepared with PTES-buffer. The 62 kDa polypeptide might therefore exist *in vivo* in senescent tissue.

Figure 5. 11. Western blot analyses of PEPCK protein during cotyledon growth. a, Day 1 to 7 and 14; b, day 3, 7, 14, 21, 28, 29, 31, 33 and 37 after imbibition. Cotyledons were collected as described in the northern blot analysis. Cotyledons were ground in pre-chilled pestle and mortar using liquid nitrogen. The gound powder was homogenated in PTES-buffer. Equal amounts (10 μg) of protein samples were electrophoresed on a 10% (w/v) SDS polyacrylamide gel, then western blotting carried out using ECL immunodetection system. Molecular weight (kDa) markers (Prestained markers, Sigma) are β–Galactosidase (123), Fructose-6-Phosphate Kinase (89), Pyruvate Kinase (67), Ovalbumin (50), Lactic Dehydrogenase (37.5) and Triosephosphate Isomerase (34).


a
DAYS AFTER IMBIBITION
1 2 3 4 5 6 7 14
123
89
67
50
37.5
34
b
DAYS AFTER IMBIBITION
3 7 14 21 28 29 31 33 37
123
89
67
50
37.5
34

5. 6. Conclusion

The U1-10 related clones showed DNA sequence identity with PEPCK encoding cDNA (gene) sequences from *Saccharomyces* (59%), *Rhizobium* (57%) and *Trypanosoma* (54%). The predicted amino acid sequence exhibited less identity : *Saccharomyces* (54%), *rhizobium* (42%) and *trypanosoma* (47%) respectively. However, *E. coli* PEPCK which does not appear on a nucleotide basis, gives 42% identity at the amino acid level. However, the identity increases by about 10% in the middle of the molecule for *Saccharomyces* (70%), *Rhizobium* (60%),*Trypanosoma* (64%) and *E. coli* (60%). According to these observations, cloning of a cDNA clone encoding PEPCK is established. Subsequently it was learnt that the PEPCK enzyme purified from cucumber contains the peptide -KKHGATGWLVNT- (R. Walker and R. Leegood, personal communication) which is seen in the sequence deduced from pBRPCK-7A at position 550 to 561 (Figure 5. 4). The GTP-dependent PEPCK protein consists of 607 to 647 amino acid (molecular weight range about 69,000 to 71,000) and contains highly conserved GTP-binding GXXXXGK sequence which is similar to that of ATP-binding domain (GXXGXGKT) in the ATP-dependent PEPCK polypeptide.

As discussed earlier (Chapter 4 and Figure 5. 7), northern blot hybridisation shows remarkable similarity with that of ICL and MS during development. Western blot analysis also shows a similar pattern of PEPCK protein synthesis. Therefore gluconeogenesis may be operating at two developmental stages, germination and senescence. However, further research is required for a firm conclusion to be reached.

The predicted molecular weight of the subunit of cucumber PEPCK is 74 kDa (in the case of the first methionine) or 69 kDa (for the second methionine). However, the antibody recognizes two polypeptides (74 kDa and 62 kDa) in some cases, in particular when PTE-buffer (-SDS) is used for extraction of plant proteins (Figure 5. 10). However, the smaller molecular weight polypeptide did not appear when proteins were extracted with PTES-buffer (+SDS > 0.5% w/v). It could be explained by proteolytic cleavage of the intact polypeptide without protease inhibitors such as SDS and PMSF in the extraction buffer. Therefore, it is likely that the higher molecular polypeptide is the intact subunit of the PEPCK enzyme.

Chapter Six:
Potential metabolic regulation of gMDH and PEPCK synthesis in cucumber cotyledons

6. 1. Rationale

Inhibition of photosynthetic gene expression has been observed in response to sucrose and glucose, which are photosynthetic end products, using a protoplast transient gene expression method (Sheen, 1990). It is proposed that genes involved in metabolic pathways are regulated by fluctuation of internal and external metabolites in muticellular higher plants as in unicelluar bacteria and yeast (Miller and Reznikoff, 1978; Struhl, 1985). Previously, external feeding of sucrose or glucose to leaves has been shown to cause a significant decrease in photosynthesis (Foyer, 1988; Huber, 1989). Based on these observations, it is likely that sugars can play a crucial role in the biochemical feedback regulation of carbon assimilation. Moreover, sugars can act as the modulators of source and sink interactions according to the capacity of photosynthate utilisation (Sheen, 1990; Sonnewald and Willmitzer, 1992).

In bacteria and fungi, genes involved in lipid mobilisation, including ICL and MS are seen to be repressed by glucose and derepressed by acetate in the absence of any other carbon source. In *E. coli* , genes encoding ICL and MS are both located on the *ace* operon. These enzymes in *E. coli* are not directly induced by acetate or acetyl-CoA (Maloy & Nunn, 1982). This operon appears to be under the control of two other genes (*Icl*R and *Fad*R), one of which is also involved in the control of fatty acid degradation (Cortay *et al.*, 1991). They also found formation of the complex

between IclR protein and the *ace* operator/promoter region to be impaired by PEP but insensitive to acetate, acetyl-CoA, pyruvate and oxaloacetate.

In addition, transcriptional repression of photosynthetic genes with acetate was found in green algae (Steinbiss and Zetsche, 1986; Kindle, 1987). The acetate repression was also found in the mesophyll cells of maize seedlings (Sheen, 1990). However, the effective concentration is 100 times lower than that of sugars for transcriptional repression (10 mM to 50 mM for sucrose and 0.25 mM to 1 mM for acetate) because of the intrinsic concentration ranges (Gehard *et al.*, 1987). In plant cells, acetyl-CoA which is the direct cellular derivative of acetate, has a central role in gluconeogenesis and respiration. In oilseed plants, glyoxysomes are exceptionally abundant in germinating seedlings for metabolising fat. The seedling cells generate energy using acetyl-CoA that is the initial input for the glyoxylate cycle and TCA cycle. Therefore, these observations in the plant kingdom from green algae to higher plants, suggest an important role of acetyl-CoA (or acetate) in cellular metabolism.

In plants, there are reports of metabolic regulation of gene expression in photosynthetic genes (Sheen, 1990; Krapp *et al.*, 1993), genes encoding enzymes of starch metabolism (Rocha-Sosa *et al.*, 1989; Muller-Rober *et al.*, 1990) and ICL and MS (Kudielka and Theimer, 1983a,b; Graham *et al.*, 1992). This chapter describes the effect the addition of sucrose has on the synthesis of gMDH and PEPCK under conditions where glyoxylate cycle enzyme synthesis occurs.

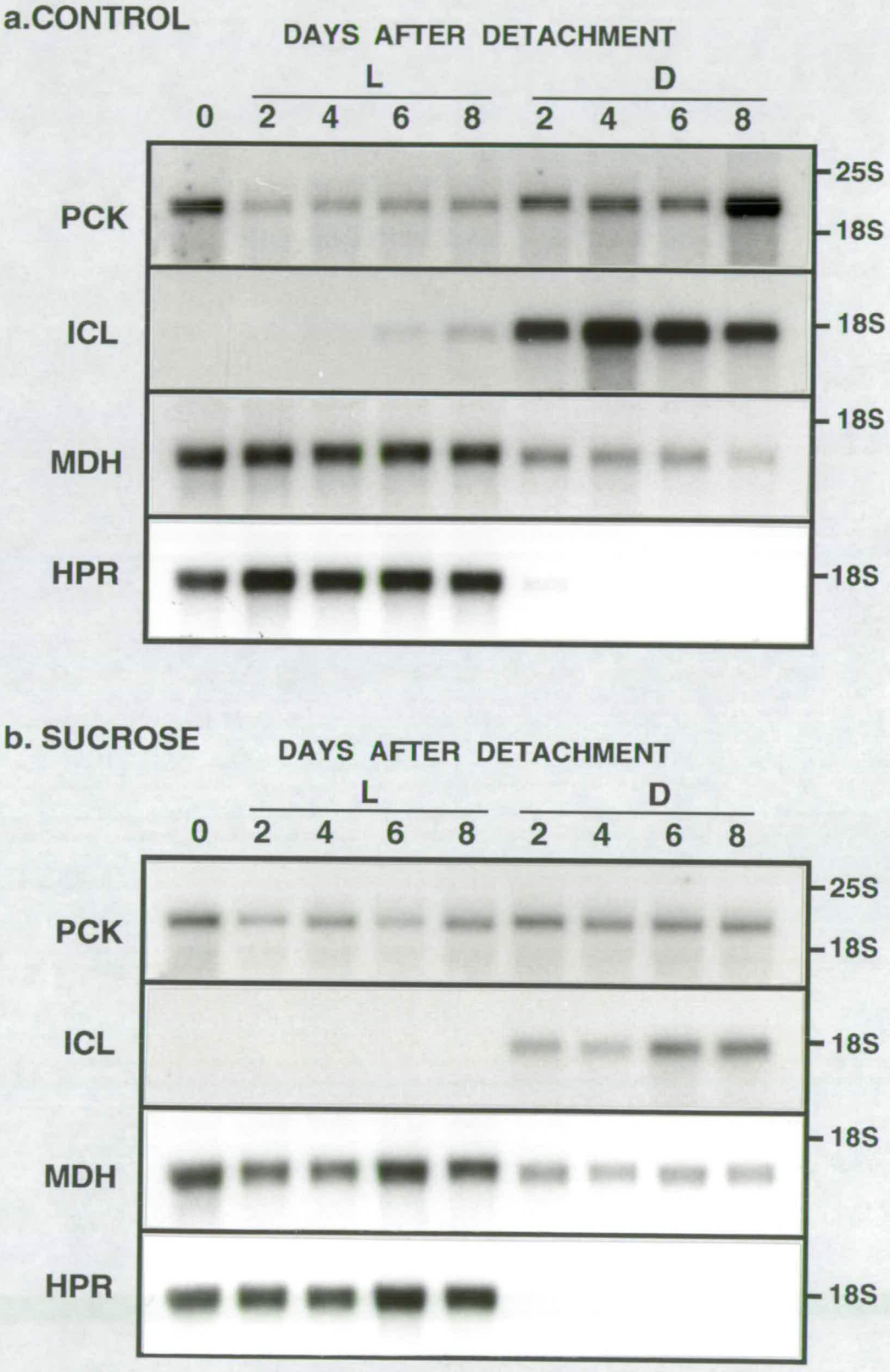
6. 1. 1. Effect of light and exogenous sucrose on *mdh*G and *pck* gene expression in detached cotyledons

Previous studies have shown that green cotyledons detached and incubated in darkness, accumulate ICL and MS mRNAs and proteins (McLaughlin, 1994). This accumulation is inhibited by light and by exogenously supplied sucrose, indicating a metabolic control of gene expression. To determine if *pck* and *mdh*G gene expression is similarly activated and controlled, detached cotyledons were incubated in darkness and in light, in the absence and presence of 25 mM sucrose, for up to 8 days.

Northern hybridisation of RNA isolated from these cotyledons is shown in Figure 6. 1. The same filters were hybridised successively with probes for PEPCK, ICL, gMDH and HPR. The results indicate that gMDH mRNA amount is decreased by dark incubation but the level of the gMDH mRNA is sustained in the light. The PEPCK mRNA is also not increased by dark incubation until 8 days, in contrast to that of ICL (and MS) which increases dramatically after only two days. The PEPCK transcripts were constant until 6 days incubation then, increased slightly in amount at day 8 but the amount of PEPCK mRNA in light-incubated cotyledons decreases slightly. Expression of the *hpr* gene is dramatically affected by light which shows strong expression in the light but almost undetectable in the dark incubated cotyledons.

In the presence of 25 mM exogenous sucrose, *icl* gene expression is strongly repressed in the dark and further decreased in the light. The *mdh*G gene is unaffected by sucrose so that the transcript was detected at a similar level to that of the minus sucrose treatment (Figure 6. 1).

Figure 6. 1. Northern blot analyses of RNA from detached cotyledons. Cotyledons were detached at day 14 after sowing. The cotyledons were incubated in the light or in the dark as described in Chapter 2, and the absence or presence of sucrose (25 mM) for up to 8 days. Total RNA (10 μg per lane) was electrophoretically factionated on a formaldehyde denaturing agarose gel. Then the RNA was blotted onto Hybond-N membrane and hybridised with PEPCK, ICL, gMDH and HPR probes. Autoradiographs were exposed for lengths of time between 20 to 96 h to give suitable exposures. (a) Control; (b) Plus sucrose. L, light; D, dark.


a.CONTROL
DAYS AFTER DETACHMENT
L
D
0 2 4 6 8 2 4 6 8
PCK
ICL
MDH
HPR
25S
18S
18S
18S
18S
b. SUCROSE
DAYS AFTER DETACHMENT
L
D
0 2 4 6 8 2 4 6 8
PCK
ICL
MDH
HPR
25S
18S
18S
18S
18S

However, *pck* gene expression is slightly affected by 25 mM exogenous sucrose in the dark incubated cotyledons. In particular, cotyledons incubated 8 days (in the dark) show repression of *pck* gene expression. These results indicate that the *pck* gene may be subject to metabolic control, but the nature of this control is different to that of *icl* and *ms*.

6. 1. 2. Immunodetection of MDH in detached cotyledons

Detached cotyledons were prepared as described for the northern blot analysis. Protein sample preparation and western blotting were carried out as described earlier (see Chapter 2). The watermelon anti-gMDH antibody again recognises two major polypeptides as in developing cotyledons (see Figures 3. 8 and 6. 2). The apparent molecular weights are 42 kDa and 38 kDa. The identity of these two bands is again unknown. One possibility is that they represent pre-gMDH and mature gMDH but it seems unlikely that the presursor accumulates *in vivo*. More likely, the antibody recognises mMDH or cytosolic MDH in addition to gMDH. The amount of both bands increase following detachment, and there appears to be a further increase after 8 days in the dark. In the presence of sucrose the lower band decreases in amount. The significance of these changes is not clear since we do not know which band is gMDH (Figure 6. 2).

6. 1. 3. Immunodetection of PEPCK in detached cotyledons

Anti-PEPCK antibodies were employed to visualise PEPCK polypeptide

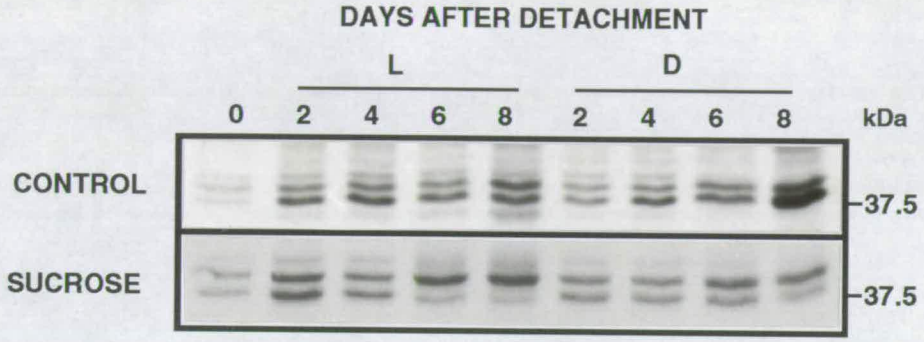


Figure 6. 2. Immuno detection of gMDH protein in detached cotyledons. Cotyledons were treated as described in Figure 6. 1. Proteins were extracted with PTE (100 mM Tris-Cl pH 8.0, 2 mM EDTA) buffer. Proteins were electrophoretically fractionated on a 10 % (w/v) polyacrylamide gel containing SDS. The proteins were transferred onto a Hybond-ECL membrane and watermelon anti-gMDH antibody added first, and then secondary antibody (horseraddish peroxidase linked goat anti-rabbit IgG, Sigma). Reactants were detected using the ECL immunodetection reagent (Amersham, U. K.), and exposed to X-ray film. Molecular weight marker (Prestained markers, Sigma) is Lactic Dehydrogenase (37.5). L, light; D, dark

levels in the same experiment as the northern blot analyses (Figure 6. 1). In the case of PEPCK, this analysis was complicated by the fact that low levels of PEPCK exist in this tissue and that both 74 and 62 kDa polypeptides were detected in varying proportions (Figure 6. 3), together with some non-specific reaction with the large subunit of ribulose*bis*phosphate carboxylase (not shown). This made it difficult to establish if the amount of PEPCK changed significantly. The use of extraction buffer containing SDS (PTES) did not eliminate the 62 kDa polypeptide, suggesting that it could exist *in vivo* in detached cotyledons. In cotyledons extracted in buffer without SDS, PEPCK is observed mainly as the 62 kDa polypeptide. In cotyledons incubated without sucrose, the amount of this polypeptide is similar in all samples except 8 days incubated in the dark which shows a slight increase similar to that of the transcript (see Figures 6. 1a and 6. 3a). In cotyledons extracted in buffer with SDS, both 62 and 74 kDa polypeptides are seen, and a small band (Figure 6. 3). It is difficult to see how the total amount of PEPCK changes. In cotyledons incubated with sucrose (Figure 6. 3b) the amount of 62 kDa protein is very high after 6 and 8 days in the light. This does not correspond to a change in transcript level (Figure 6. 1) so either a translational or post-translational control operates leading to accumulation of PEPCK or the antibody is reaching with another protein of similar size.

6. 2. Discussion

6. 2. 1. Roles of gMDH in detached cotyledons

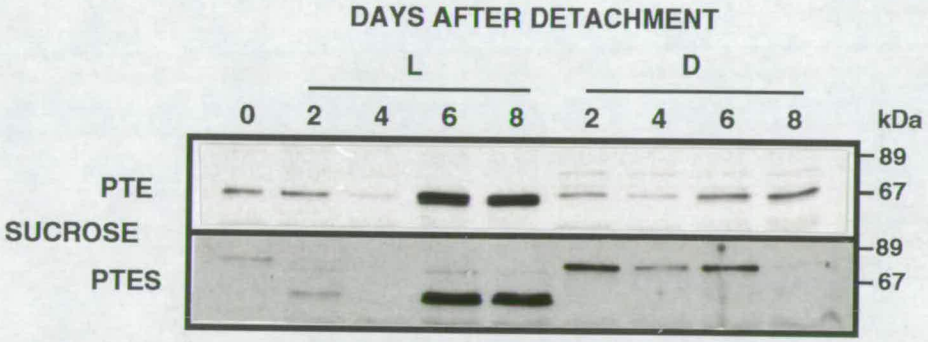
As mentioned earlier, *icl* and *ms* gene activation was observed in dark incubated detached cotyledons (McLaughlin, 1994). The genes are dramatically activated within 2 days of detachment in the dark (Figure 6. 1a). Such activation does

Figure 6. 3. Immuno detection of PEPCK protein in detached cotyledons. Cotyledons were detached as described in northern blot analyses for detached experiments. Proteins were extracted with PTE- and PTES-buffers. The proteins were transferred onto a Hybond-ECL membrane and PEPCK antibody added first, and then secondary antibody (horseradish peroxidase linked goat anti-rabbit IgG, Sigma), Reactants were detected using the ECL immunodetection reagent (Amersham, U. K.), and exposed to X-ray film. (a) Detached cotyledons were incubated in sterile distilled water as a control; (b) Detached cotyledons were incubated in 25 mM sucrose. L, light; D, dark. Molecular weight markers (Prestained markers, Sigma) are Fructose-6-Phosphate Kinase (89) and Pyruvate Kinase (67).

a.


DAYS AFTER DETACHMENT
L
D
0
2
4
6
8
2
4
6
8
kDa
89
67
PTE
CONTROL
89
PTES
67

b.


DAYS AFTER DETACHMENT
L
D
0
2
4
6
8
2
4
6
8
kDa
89
67
PTE
SUCROSE
89
PTES
67

not take place when the cotyledons are incubated in the light. The synthesis of ICL and MS in such detached photosynthetic organs has been explained as a response to senescence (Gut and Matile, 1988) and as a metabolic control (Graham *et al.*, 1992). In the case of gMDH, expression of the gene is affected by light i.e., the transcript level is decreased in the dark. The situation does not change in sucrose treated cotyledons. In contrast to ICL and MS, sucrose had no apparent effect on gMDH mRNA and protein levels in the light or dark. The gMDH role may be different in the light and in the dark. In the light, gMDH may function together with HPR (and SGAT) in photorespiration (McLaughlin, 1994), but in the dark, gMDH may be involved in the glyoxylate cycle together with ICL and MS (see Figures 6. 1 and 6. 2).

A difference between the detached and naturally senescing cotyledons is apparent. While *icl*, *ms* and *mdh*G genes are coordinately expressed in cotyledons during germination and senescence, they are not in detached cotyledons. These observations imply that the function of the glyoxylate cycle enzymes could be different in detached cotyledons to that in germination and senescence, but accurate assays of enzyme activities are required before further conclusions can be made.

6. 2. 2. Roles of PEPCK in detached cotyledons

The *pck* gene is coordinately expressed with the *icl* and *ms* genes in germination and in senescing cotyledons. These observations suggest that gluconeogenesis is occuring in both situations, producing sucrose for export from the cotyledon. In contrast, results here cast doubt on the proposal that the same is happening in detached cotyledons, since *pck* gene expression is not activated, when that of ICL and MS is dramatically activated. This does not exclude the possibility

that some gluconeogenesis occurs in detached cotyledons. However, an alternative role for the glyoxylate cycle in such tissues is an anapleurotic one. In this case 4-carbon acids may be provided to maintain mitochondrial citric acid cycle activity, possibly as a source of carbon skeletons for aminotransferase activity and salvage of nitrogen. This would be consistent with the observed accumulation of asparagine in starved cultures (Douce *et al.,* 1991). There might therefore be a distinct difference in gene expression and metabolic activity in naturally senescing cotyledons as compared to dark-incubated detached cotyledons. These results add further caution to the use of detached organs as a model for studying senescence (Graham *et al.,* 1992; Becker and Apel, 1993).

6. 2. 3. Metabolic regulation

Detached cotyledons, while not necessarily providing a good model for studying some aspects of senescence, can be used to investigate factors which control gene expression. Detached cotyledons incubated in the dark rapidly accumulate ICL and MS mRNAs but not gMDH and PEPCK. Thus the signal which activates ICL and MS is not effective with gMDH and PEPCK. In contrast sucrose can repress synthesis of ICL, MS and PEPCK, though PEPCK seems less sensitive. This lower sensitivity may reflect the fact that ICL and MS mRNAs are apparently very much more abundant than that of PEPCK in the dark, in the absence of sucrose, and therefore show a greater relative repression by sucrose. In detached cotyledons in the light, endogenous sucrose accumulates five-fold (McLaughlin, 1994) which may explain the small decrease in PEPCK mRNA level (Figure 6. 1). However, it can not be supposed that sucrose is directly responsible for regulating gene expression (Graham *et al.,* 1992) but rather a product of sugar metabolism. It would not be surprising to

find that the *pck* gene is subject to metabolic control since an increasing number of plant genes encoding enzymes of primary metabolism have been found to be responsive. Furthermore, the vertebrate PEPCK gene, although encoding a different type of PEPCK enzyme (requiring GTP), has been extensively investigated and shown to be subject to glucose repression, acting through a signal transduction pathway employing cyclic AMP (McGrain *et al.,* 1992). Further work is required on the cucumber *pck* gene to determine if it responds to metabolites. It may possibly require much higher concentrations of sugar to achieve effective repression. For example, photosynthetic genes require between 30 and 100 mM sucrose for effective repression while *icl* and *ms* genes require only 5 mM (I. Graham personal communication).

Chapter Seven: General Discussion

7. 1. Synthesis of MDH

Southern blot analysis indicated the existence of only one gene encoding microbody targeted MDH in cucumber. The isolation of cDNA clones from two different cDNA libraries (one from germinating cotyledon RNA and the other one from senescent cotyledon RNA) with no sequence differences, is consistent with one gene expressed at two (or three) developmental stages. Earlier studies of the synthesis of MDH provided evidence for only one MDH enzyme in glyoxysomes and peroxisomes during cucumber cotyledon development (Liu and Huang, 1976). Therefore one gene probably encodes a single microbody MDH for function in both glyoxysomes and peroxisomes. In considering terminology, it may be more accurate to call it peroxisomal MDH rather than gMDH. Furthermore, recent studies of the import of glyoxysomal proteins (ICL and MS) revealed that these proteins were imported into several classes of peroxisome (leaf-type and root) (Onyeocha *et al.*, 1993) with a carboxy terminal targeting determinant (Olsen *et al.*, 1993). These studies suggest that protein import does not play a regulatory role in determining peroxisome function and that similar import machinery is present for glyoxysomes and peroxisomes. Nevertheless, the putative microbody targeting amino terminal transit peptide of gMDH has not been studied, therefore it should be further investigated fuse to CAT (Chloramphenicol acetyltransferase) or GUS gene using mutations and fusions of the sequence.

The pattern of *mdh*G expression in cucumber cotyledons is similar to that of *icl* and *ms* in germination and senescence. Moreover, *mdh*G gene expression is similar to *hpr* in green cotyledons. These results are consistent with a role in the glyoxylate cycle in germination and senescence, and in photorespiration in green cotyledons. However, the HPR transcript is detected in senescing cotyledons as well. gMDH protein detection with the watermelon antibody showed a complex result because of the non-specific binding. Therefore, it is necessary to obtain a specific antibody (*eg,* monoclonal) to measure changes in amount of the protein during cotyledon development. Enzyme assay also will be useful to understand the synthesis and regulation of gMDH enzyme during plant development.

The gene may have *cis*-acting element(s) in common with *icl, ms* and *hpr.* According to the detached cotyledon experiments, the *mdh*G gene may exhibit light regulation similar to *hpr.* Isolation of the gene will allow promoter analysis and comparison with *icl, ms* and *hpr.* Furthermore, GUS gene fusions, promoter deletions and assay in transgenic plants can be applied to identify control sequences in the future.

7. 2. Function of MDH in plant development

According to northern hybridisation, genes encoding gMDH, ICL and MS are coordinately expressed during germination, presumably for the glyoxylate cycle. The second increase of the MDH transcript in green cotyledons may be required for photorespiration. In senescent tissue, the role of MDH enzyme is not certain. The detection of the transcript with ICL and MS implies involvement in the glyoxylate

cycle. But expression with HPR and GO may imply a photorespiratory role in senescent tissues. Therefore, these observations may indicate a multifunctional role for MDH in plant development especially in senescence. Furthermore, according to the suggestion that multifunctional microbodies exist in senescing tissues (Vincentini and Matile, 1993), there may be another metabolic role in senescing tissue such as amino acid or purine metabolism.

The two phases of lower levels of expression of the *mdh*G gene may correspond to the periods when there is no net import or export of reducing equivalents between glyoxysomes (peroxisomes) and the cytosol or mitochondria. However, immunodetection of the MDH protein did not show a similar pattern as the northern hybridisation, therefore again indicating the importance of a microbody specific MDH antibody to examine this apparent discrepancy. The exact function of MDH in the glyoxylate cycle is not certain, even though MDH is involved in the cycle (Mettler and Beevers, 1980; Donaldson, 1982).

7. 3. Molecular cloning of proteins synthesised in germination and senescence.

The method used here (differential screening) was successful, but there were some unexplained results (*eg,* cDNAs derived from chloroplast DNA and senescence specific sequences). In the future, for another cloning experiment, a subtractive library or subtractive probes might be better (Sive and John, 1988). However, this screening procedure will only detect clones from abundant RNAs. It may not isolate clones of regulatory proteins which are expressed at a low level, such as signal

transducton proteins or transcription factors. Furthermore, regulatory molecules might be constitutive, but their activity change in development, so they can never be cloned using differential screening. Other approaches may therefore need to be adopted, such as screening for mutants which show aberrant patterns of gene expression.

7. 4. Characteristics of cucumber PEPCK protein

The sequence of a full length cDNA shows that cucumber PEPCK is similar to *E. coli, Rhizobium, Trypanosoma* and *Saccharomyces*. However, the sequences do not show comparable sequence similarity with GTP-dependent PEPCK DNAs and peptides. Therefore, they are different enzymes. The enzyme is thought to be cytosolic in plants but mammals contain isoenzymes of GTP-dependent PEPCK in the cytosol and the mitochondria. However, the predicted cucumber polypeptide shows an unusual feature at the amino terminus which does not appear in other ATP-dependent PEPCK polypeptide sequences. The cucumber PEPCK has about 110 more amino acids at the amino terminus. In immunodetection experiments, the PEPCK antibody recognises two polypeptides, 74 and 62 kDa, in PTE-buffer (-SDS) extracted protein samples. Apparently the intact molecule (74 kDa) is particularly susceptible to a specific proteolytic degradation in crude extracts, which removes about 12 kDa, but thereafter the molecule is stable. It is interesting that the PEPCK polypeptide is about 12 kDa longer than the enzyme of other organisms, and raises the possibility that it is this amino terminal sequence which is removed. Furthermore, the 62 kDa PEPCK has no loss of activity and is thought to be an artefact of isolation (R. Leegood, personal communication). Even though the cucumber PEPCK has been observed to be a cytosolic enzyme, the 12 kDa piece may have some function in

regulation. The ATP-dependent PEPCK enzyme is thought to be a tetrameric structure in plants with a molecular weight of about 300,000, but it is not conclusive. The purification and characterisation of PEPCK of C_3 plants has not been reported, nor has any molecular clone encoding a plant PEPCK been described. As described earlier (Chapters 1 and 5), PEPCK plays an important role in both C_4 and CAM plants for the synthesis of carbohydrates, and in gluconeogenesis in C_3 plants. Therefore, it will be particulary interesting to compare the enzyme of C_3 plants with that found in PEPCK-type C_4 plants, since they might be subject to different types of regulation. In view of these considerations, there are many questions to be answered about PEPCK in plants.

7. 5. Synthesis of PEPCK in plants

According to Southern hybridisation analysis, a single gene apparently encodes PEPCK in cucumber. Northern blot analyses show that the *pck* gene expression pattern is similar to that of the *icl* and *ms* during germination and senescence. The amount of these RNAs increase dramatically during the first 2 days of germination then decline significantly, and increase again to a low level in senescing cotyledons. They show very similar developmental regulation of gene expression at two different developmental stages. PEPCK is synthesised and accumulates rapidly in cotyledons during the few days immediately after seed imbibition, together with ICL and MS. This result is expected, based on several observations of the enzymes involved in the gluconeogenic pathway by which storage lipid is converted into sugars to support growth until photosynthesis begins. The accumulation of these enzymes in senescing tissues, suggests that gluconeogenesis is

again taking place, presumably using membrane lipids as the carbon source (Gut and Matile, 1988; Pistelli *et al.,* 1991). All these observations suggest the regeneration of the glyoxylate cycle, gluconeogenesis and a microbody transition in senescing tissues.

Green tissues (cotyledons and leaves) contain the PEPCK protein at a low level. Furthermore, roots contain a significant amount of the polypeptide, indicating *pck* gene expression in a range of plant tissues. However, it is unknown if this PEPCK is enzymatically active, or what function it might have in photosynthetic tissues. It seems unlikely that it has a gluconeogenic role in mesophyll and palisade cells since sugars will be derived from photosynthesis. It will be important in future research to determine which leaf cells contain PEPCK. The function and tissue distribution of the PEPCK enzyme in roots is again unknown. Therefore, gene expression and enzyme synthesis studies have to be applied more extensively in the future to determine where PEPCK is synthesised and what function it has. Genetic manipulation could be applied to leaves and roots using antisense RNA to investigate the effect on growth and metabolism. PEPCK antibodies and cDNAs are useful to find out the cellular localisation in plant cells using immunocytochemistry and *in situ* hybridisation. Promoter analysis will be available when the gene is cloned, then the promoter could be fused with the GUS gene. The gene fusion can be transferred to some convenient plant species (*eg,* tobacco, potato or *Arabidopsis*) using well establised tissue culture and transformation techniques to produce transgenic plants. Then, the transformants can be analysed with blue staining to detect GUS activity, and so deduce where the *pck* promoter is active. It will also be interesting to investigate metabolic regulation of enzyme synthesis in growth and development.

7. 6. Metabolic and genetic regulation of the synthesis of gMDH and PEPCK

Metabolic regulation of the *lac*-operon is a well-known model in bacteria. Regulation of gene expression by metabolite levels is widespread in microorganisms. One example is carbon catabolite repression. In plants, the metabolic regulation of photosynthetic genes (Sheen, 1990; Krapp *et al.*, 1993), *icl* and *ms* genes (Kudielka and Theimer, 1983a,b; Graham *et al.*, 1992) were observed. As described in Chapter 6, sucrose or glucose are major metabolic regulators for plant genes which are involved in the main metabolic pathways, such as photosynthesis, TCA cycle and lipid metabolism. Acetate is also thought to be a important plant gene regulator in major metabolic pathways. For the two key enzyme genes *icl* and *ms,* sucrose (or glucose) repression was proposed in the1970s (Longo and Longo, 1970; Slack *et al.*, 1977) in post-germination and in cultured cells. In the present work sucrose was applied to detached cotyledons to examine sucrose repression, and RNA probed with three glyoxysomal enzyme cDNA probes and PEPCK. The results were similar to those of the previous cotyledon detachment and protoplast experiments (Mclaughlin, 1994) in that *icl* and *ms* genes are repressed, but the glyoxysomal *mdh* gene is affected by light rather than by exogenous sucrose. The *pck* gene is slightly affected by the 25 mM exogenous sucrose but it is not a conclusive result. As a result of these observations, glyoxysomal *mdh* gene expression may not be controlled by metabolites, but seems to be controlled more by developmental and environmental factors. According to these results, it would appear to be that the glyoxylate cycle may not be important for gluconeogenesis in starvation. Thus, there might be another function(s) for the glyoxylate cycle in plant development *eg,* anapleurotic, providing oxaloacetate for the TCA cycle or for amino acid synthesis (Douce *et al.*, 1991).

Therefore, accurate enzyme assays and further metabolic studies will be necessary in the future. One of the surprising observation is the lack of metabolic response of the *pck* gene in view of results with the animal GTP-dependent PEPCK. Higher concentrations of sucrose (>50 mM) may be required to reach effective repression of the gene as is the case for photosynthetic genes (Sheen, 1990) or other metabolite(s) *eg,* glucose or acetate also need to be examined.

7. 7. Expression of genes encoding GTP-dependent PEPCK in animals

A large amount of research on GTP-dependent PEPCK has been carried out in recent years because of the importance in animal metabolism. The studies include nucleotide sequences analyses (Beale *et al.,* 1985; Cook *et al.,* 1986; Gundelfinger *et al.,* 1987; Weldon *et al.,* 1990), promoter analyses (Bokar *et al,* 1988; Hall *et al.,* 1992; Cheyette *et al.,* 1992) and regulation of gene expression (Park *et al.,* 1990; Park *et al.,* 1993) in several animal tissues. The PEPCK protein occurs in two isozyme forms in vertebrates: 1) a cytosolic form (PEPCK-C) whose levels are modulated by the effect of glucan (mediated by cAMP), and hormones such as insulin, and glucocorticoids on the rate of transcription of its gene and, 2) a form present in the matrix of the mitochondria (PEPCK-M) whose activity appears to be constitutive (Garber *et al.,* 1972). However the enzyme activity is variable in different species and tissues. Although the isoenzymes of PEPCK have similar characteristics including identical maximum velocities and cofactor requirements, the isozymes show different molecular weights, differences in isoelectric point and lack of cross-reactivity with polyclonal antibodies (Weldon *et al.,* 1990). Furthermore, deduced amino acid

sequences of PEPCK-C from rat and chicken show a clear distinction from the mitochondrial protein (Hod *et al.,* 1982). They also found important properties of PEPCK-M. First, PEPCK-M is initially synthesised as a precursor polypeptide which is processed to the mature form that has a similar molecular weight to that of PEPCK-C. Therefore, the isozymes are encoded by separate nuclear genes indicating that they are synthesised independently of each other. But both PEPCK enzymes hydrolyse GTP during PEP formation, and the protein sequence and predicted secondary structure show well conserved GTP-binding sequences. The GTP-dependent PEPCKs also contain a well conserved central region including the three consensus sequence elements (GXXXXGK, DXXG and NKXD) associated with other GTP-binding domains such as found in human c-H-*ras* (Dever *et al.*, 1987). The first conserved sequence, GXXXXGK, is also found in ATP-dependent PEPCK.

Transcription of the gene for GTP-dependent PEPCK shows a complex pattern of regulation. The expression of this gene is limited mainly to liver, kidney and adipose tissues, and its transcription in liver is acutely stimulated by glucan (via cAMP), glucocorticoids, and thyroid hormone but inhibited by insulin (Park *et al.,* 1990). Transcription of the gene is also developmentally regulated, with little expression in the liver until birth. *Cis*-acting elements are commonly thought to regulate transcription by binding to specialised nuclear proteins. Therefore, many *cis*-acting elements may be present in the promoter-regulatory region of this gene to confer these multiple modes of regulation. The first, cyclic AMP responsive elements (CRE) were identified in several genes including PEPCK-C from the rat (Short *et al.,* 1986) and chicken (Hod *et al.,* 1984). A common element is a core 8-bp motif, T(G/T)ACGTCA, which is required for the induction of gene transcription by cAMP. Furthermore, a single protein from rat liver was found to bind to multiple elements in

the promoter including CRE (Quinn *et al.*, 1988). The transcription factor CCAAT/enhancer-binding proteins-α and β (C/EBP-α and β) in liver and adipose tissue also bind to the promoter (Park *et al.*, 1990; Park *et al.*, 1993). However, there must be other DNA-binding proteins which confer hormonal responsiveness, tissue specificity, and developmentally regulated expression to the PEPCK gene.

7. 8. Further research on the plant *pck* gene

By comparison with the GTP-dependent PEPCK study, information about the ATP-dependent PEPCK gene, and its expression and regulation is lacking especially in higher plants. Therefore, one of the first objectives in future will be the cloning of the plant gene(s). Then, the project can proceed to identify the regulatory sequence elements and factors which participate in the control of the PEPCK gene(s). Identification of *cis*-acting sequences will involve applying mutagenesis to the promoter fragment of the PEPCK gene *in vitro* and examining the effects of the alterations on expression in transgenic plants and protoplasts via the GUS gene. Site directed mutagenesis of those sequences identified as putative *cis*-acting elements will be particulary informative to compare with the GTP-dependent PEPCK counterparts. Furthermore, it would allow a comparison of the *ms* (Graham *et al.*, 1990) and *icl* (S. Reynolds personal communication) gene regulatory sequences and may identify elements involved in apparent coordinate regulation of these genes. Secondly, identification of DNA binding proteins which react with the genetically defined regulatory elements would initially involve gel retardation and DNA footprinting studies. Isolation of the gene(s) encoding such *trans*- acting factors may be possible using screening techniques that enable cDNA clones from an expression

library to be isolated due to the ability of the expressed protein to bind specifically to a *cis*-acting element. Recent advances in molecular biology including the development of the PCR (polymerase chain reaction) can be used to isolate cDNA fragments from amino acid sequence data (Gould *et al.*, 1989b). Potentially, one of these techniques could be successful in the isolation of proteins regulating transcription of the PEPCK gene(s).

Finally, opportunities for the genetic manipulation of PEPCK synthesis, and hence modifications to plant metabolism and growth can be considered. Until the function of this enzyme in roots and leaves is known, it is not easy to predict what changes might be made. However, one proposal has been made to use PEPCK to create a CO_2 concentrating mechanism in chloroplasts of C_3 plants. This involves transferring PEPCK into the chloroplasts, where it may decarboxylate oxaloacetate and so increase CO_2 concentration for fixation by ribulose*bis*phosphate carboxylase (R. Leegood, personal communication). Although not possible to predict the outcome, this proposal shows that there are potentially exciting possibilities for plant genetic manipulation. Another example might be found in fruit ripening where conversion of organic acids to sugar may involve PEPCK (Blanke *et al.*, 1988).

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