The regulation and synthesis of malate synthase and isocitrate lyase in senescent and detached cotyledons of cucumber (*C. sativus*).

I hereby declare that the data presented in this thesis for examination for the degree of PhD at the University of Edinburgh is entirely the results of my own efforts.

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Acknowledgements.

Firstly, I would like to thank Dr. Steve Smith for giving me the opportunity to "do" a PhD and for his quiet patience throughout my time in Edinburgh. My thanks go out to everyone past and present who have made my days in Edinburgh enjoyable, Stefan, Ian Oliver, Anne, Dae-Jae, Takashi, Susan, Caroline, Matt, Andrew Hudson, Richard, Andrew Allen, Marc, Heather, Ian Graham and Shirley. Finally, I would like to thank Steve Rolf and Paul Quick for allowing me time off while I put the finishing touches to my thesis. This work was supported by SERC and the Buroo.

Abbreviations

A	absorbance
ADP	adenosine 5'-diphosphate
ATP	adenosine 5' -triphosphate
ß-ME	ß-mercaptoethanol
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
Ci	Curie
CoA	coenzyme A
dATP	2' deoxyadenosine 5' triphosphate
dCTP	2' deoxycytidine 5' triphosphate
dGTP	2' deoxygaunosine 5' triphosphate
DNA	deoxyribonucleic acid
dTTP	2' deoxythymidine 5' triphosphate
DTT	dithiothreitol
FADH	flavine adenine dinucleotide (oxidised)
$FADH_2$	flavine adenine dinucleotide (reduced)
g	relative centrifugal force
kbp	kilo base pair
kDa	kiloDalton
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger RNA
Na_2EDTA	ethlenediaminetetraacetic acid (sodium salt)
NAD^+	nicotinamide adenine dinucleotide (oxidised)
NADH	nicotinamide adenine dinucleotide (reduced)
NADP	nicotinamide adenine dinucleotide phosphate (oxisdised)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NH_3	ammonium
nm	nanometre, 10^{-9} metre
Pi	inorganic phosphate
p.s.i.	Ibs inch^{-2}
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
SDS	sodium lauryl sulphate
TBS	Tris buffered saline
TEMED	N, N, N', N', -tetramethylethylendiamine
Tris	tris- (hydroxymethyl)- methylamine
Tween-20	polyoxyethylene (20)-sorbitanmonolaurate
PVP	polyvinylpyrrolidone
(v/v)	volume:volume ratio
(w/v)	weight:volume ratio

Abstract

The expression and role of malate synthase (MS) and isocitrate lyase (ICL) during senescence and following detachment is investigated. After 7 weeks plant growth immunoblot analysis indicate the coordinate accumulation of MS and ICL. However, this increase in MS and ICL does not correlate with the decline in the thylakoid lipids monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG). A detectable decline in these lipid classes is observed 2 weeks after plant growth. Further analysis of changes in chlorophylls, carotenoids, protein and total RNA content confirm that senescence of cucumber cotyledons very soon after full greening. These data indicate that the glyoxylate cycle does not appear to play amy significant role in the disassembly of chloroplast membranes during the earlier stages of senescence. In detached cucumber cotyledons the synthesis of MS and ICL is detectable in dark incubated cotyledons within 48 hours. This appears to be primarily due to an increase in the transcripts that encode these proteins. However, the demonstrable increase in MS and ICL levels occurs prior to any detectable decline in chlorophyll, carotenoids and galactolipids. This indicates that the glyoxylate cycle may play an additional role to the metabolism of products of chlroplast membrane degradation. The role sucrose may play in controlling the synthesis of MS and ICL was also investigated. The presence of 25 mM sucrose was sufficient to greatly reduce the synthesis of MS and ICL uder conditions were the synthesis of these proteins would normally occur in detached cucmber cotyledons and protoplasts.

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CHAPTER 1

INTRODUCTION

1.1. Microbodies of higher plants.

It is now generally accepted that microbodies are present in virtually all cells of higher plants (Huang *et al.*, 1983). Electron microscopical studies of peroxisomes reveal them to be small organelles (0.5-1.5 μm in diameter), bounded by a single membrane and occasionally contain a semicrystalline matrix known as a nucleoid (Frederick *et al.*, 1975, Huang *et al.*, 1983). In addition, most microbodies contain a primitive respiratory chain which leads to the production of H_2O_2, which is converted to H_2O and O_2 via the action of catalase (Huang *et al.*, 1983).

1.1.1. Biogenesis of peroxisomes.

1.1.1.1. Peroxisome ontogeny.

Most of the biochemical data concerning the biogenesis of microbodies in higher plants has come from the analysis of fatty seedling tissue. Within a few days of germination there is usually a rapid increase in the number of a specialised type of microbody, glyoxysomes and synchronous development of glyoxysomal enzymes (for review see Beevers, 1979). As the amount of storage lipid reserves declines, there is a concomitant decrease in the activity of glyoxysome specific enzymes. The rapid accumulation of glyoxysomes during this period offers a good experimental system in which to study the biogenesis of microbodies in general.

Over the past 15-20 years considerable controversy has been generated between investigators proposing different models to explain the biogenesis of microbodies and much conflicting data have been presented. The earliest model used to describe peroxisome biogenesis was one that implicated the E.R. via vesiculation (Goldman and Blobel, 1978). Ultrastructural studies provided some evidence to support this model; peroxisomes are often found in close proximity to the E.R. (Rhodin, 1963; Novikoff and Shin, 1964). However, the proposal that this observation demonstrates continuity between the organelles has been challenged (Lazarow *et al.*, 1980). E.R. synthesised proteins are typically glycoslyated (Hirschberg and Snider, 1987). However, biochemical evidence demonstrating the presence of peroxisome-localised glycoproteins has been contradictory (compare Frevert and Kindl, 1978 and Riezman *et al.*, 1980). Improved biochemical techniques and the application of molecular biological

techniques have allowed more rigorous analysis of the targeting of peroxisomal proteins. All the recent evidence supports a simple post-transitional mechanism for the targeting of microbody localised proteins.

A significant body of research indicates that many peroxisomal membrane and matrix proteins are synthesised on free polyribosomes and subsequently imported post-translationally (Borst, 1986; Borst, 1989; Fujiki *et al.*, 1984; Lazarow and Fujiki, 1985; Maeshima *et al.*, 1987). Consequently, such observations have seriously weakened the E.R. vesiculation model and earlier evidence supporting this model has been reconsidered. It has been proposed that peroxisomes are formed from the vesiculation of the E.R. and that proteins are subsequently imported post-translationally (Trelease, 1984). Other workers reject any involvement of the E. R. in the formation of peroxisomes and postulate that these organelles arise in a manner similar to that considered for mitochondria and chloroplasts; by growth and division of pre-existing organelles with matrix and membrane proteins being post-translationally added (Lazarow and Fujiki, 1985).

Recently Chapman and Trelease (1991) have demonstrated that during the period of storage lipid mobilisation, non polar and phospholipids are transferred from lipid body membranes to glyoxysomal membranes. Additionally, this report did not provide any indication for the direct involvement of the E.R. in the biogenesis of glyoxysomes (Chapman and Trelease, 1991). Consequently, it would appear that it is unlikley that the E.R. plays any significant role in the biogenesis of glyoxysomes. Most of the data concerning microbody biogenesis in higher plants come from the analysis of glyoxysomes and care must be exercised in extrapolating such data to describe formation of peroxisomes in non fatty tissue. For example, it is clear that lipid bodies are unlikely to play any significant role in the biogenesis of leaf-type peroxisomes in leaves. However, the cotyledons of plants that exhibit epigeal germination do provide a useful opportunity to study the relationship between glyoxysomes and leaf-type peroxisomes.

1.1.1.2. Glyoxysome-peroxisome transition.

Following germination, peroxisomes in the cotyledonary cells of cucumber and other oilseed species perform two successive and distinct metabolic roles (Becker *et al.*, 1978; Huang *et al.*, 1983). Firstly, peroxisomes (glyoxysomes) are involved

in the mobilisation of storage lipid and, following greening, are involved in photorespiration. During the transition the activities of enzymes specific to glyoxysomes and leaf-type peroxisomes are both present (Becker *et al.*, 1978; Hondred *et al.*, 1985, 1987; Weir *et al.*, 1980). The mechanism underlying this transition has been investigated by a number of researchers over the past 15-20 years and a number of models have been proposed to explain it (Figure 1.1.). Beevers (1979) suggested a two population model proposing that during the transition, two biochemically distinct microbodies are present; glyoxysomes and leaf-type peroxisomes and that a selective degradation of glyoxysomes occurs during greening. Alternatively, it was postulated that a single population of microbodies existed and the change in peroxisome morphology and biochemistry associated with plant development was due entirely to differences in enzyme composition (Tolbert, 1971). A variation of the one population model proposes that a transition occurs by the continual synthesis and degradation of microbodies which as development proceeds will contain different complements of proteins (Schopfer *et al.*, 1976).

The most convincing evidence for the one population model comes from double-label immunocytochemistry of cotyledon cells during greening. Using two sizes of protein A-gold linked to antibodies raised against cucumber ICL and SGAT it was unequivocally demonstrated that both these marker proteins were localised in the same microbody, indicating that only one population exists which contain both sets of enzymes (Titus and Becker, 1985). Similar observations have been made during the greening of cotyledons in other Cucurbits (Sautter, 1987; Nishmura *et al.*, 1986).

Evidence in support of the two population model is based largely on the work of Kagawa and Beevers (1975), who describe a major loss of protein from a purified glyoxysomal fraction in dark-grown watermelon, which is accelerated in light. Additionally, there is evidence of membrane synthesis in peroxisomes during greening (Kagawa *et al.*, 1975). The validity of these observations is questionable because of the contamination of these peroxisomal fractions, firstly with protein bodies and then chloroplasts. The period of apparent glyoxysome degradation is in fact due to the decline in storage protein bodies that contaminate the peroxisome fraction (Becker *et al.*, 1978). Similarly, an increase in the amount of membrane lipids in peroxisome fraction is due primarily to the presence

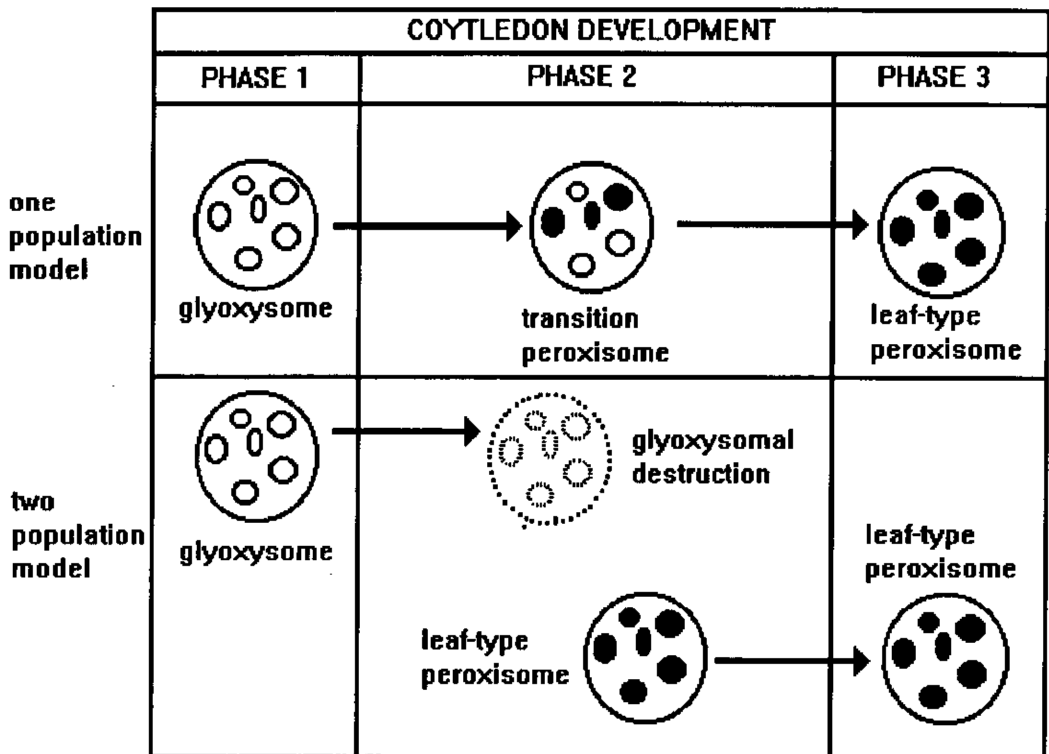


Figure 1.1. Proposed models that have been developed to explain the transition from glyoxysome to leaf-type peroxisome in the cotyledons of plant species that exhibit epigeal germination. At the onset of germination glyoxysomes are the predominant microbody (phase 1). Green cotyledons are characterised by the presence of leaf-type peroxisomes (phase 3). During greening (phase 2) glyoxysomes and leaf-type peroxisomes are both present. The one population model proposes that during development glyoxysomes are directly converted into leaf type peroxisomes. During this transition cotyledon cells would be characterised by the presence of transition peroxisomes that contain glyoxysome and leaf-type peroxisomal enzymes. The two population model proposes that glyoxysomes are specifically degraded during greening and leaf-type peroxisomes arise *de novo.*

of contaminant chloroplast that will increase during greening (Huang *et al.*, 1983). Currently, there appears to be an overwhelming body of evidence that supports the one population model (Figure 1.1.) first proposed by Trelease *et al.* (1971).

1.1.1.3. Protein targeting: the PTS motif.

Generally, most microbody proteins are synthesised at their mature molecular weight; there does not appear to be any change in molecular weight following import (Roberts and Lord, 1981; Kruse *et al.*, 1981; Gerdes *et al.*, 1981; Zimmermann and Neupert, 1980). This is in contrast to the established mechanism that leads to the targeting of nuclear-encoded proteins to chloroplasts and mitochondria (for review see Von Heijne *et al.*, 1989). As there does not appear to be any detectable modification of the imported protein, such as the proteolytic cleavage of a presequence, this implies that the signal responsible for targeting resides on the mature protein. Until recently the signal responsible for targeting was not known. Recently, the work of Gould and co-workers lead to the elucidation of a peroxisomal targeting signal (PTS) that appears to be conserved in animals, plants, protists and fungi.

Firefly luciferase is a peroxisomal localised protein. Fusion of the region of cDNA encoding the carboxy portion of luciferase to the gene encoding the cytosolic reporter protein, chloramphenicol acetyl transferase (CAT) lead to the targeting of the protein to peroxisomes (Gould *et al.*, 1987). It was subsequently reported that similar putative PTS motifs are localised at the carboxy terminus of several other peroxisomal proteins (Gould *et al.*, 1988; Miyazawa *et al.*, 1989; Small and Lazarow, 1987; Small *et al.*, 1988). Additionally, Gould *et al.* (1988) and Miyazawa *et al.* (1989) observed that the PTS sequence; serine, alanine or cysteine as the first amino acid, lysine, histidine or arginine as the second and leucine as the third position is common to the C-terminus of several peroxisomal proteins of mammals, insects, plants and fungi. Furthermore, it was demonstrated that antibodies that specifically recognise the PTS motif, serine-lysine-leucine, could detect proteins in the peroxisomes of fungi, plants and animals (Keller *et al.*, 1990).

From the analysis of the carboxy-terminal regions of several higher plant peroxisomal proteins, it would appear that putative PTS sequences are present in

Table1.1 C-terminal sequences of higher plant peroxisomal proteins. Putative targeting motifs are indicated in bold type. Asterisks represent introduced spaces to better align sequences.

Protein / Source	Sequence
Malate synthase	
Brassica napus(Comai et al., 1989a)	L T L A V Y D H I V A H Y P I N * A **S R L**
Castor Bean (Rodriquez et al., 1990)	L T L D V Y N N I V I H Y P K N * S **S R L**
Cucumber (Graham et al., 1989)	L T L D V Y N Y I V I H H P R N * L **S R L**
Cottonseed (Turley et al., 1990a)	L T L D V Y N Y I V I H H P K N V L **S R L**
Isocitrate lyase	
Castor bean (Beeching anf Northcote)	R P G A M E M G S A G S E V V A K **A R M**
Cottonseed (Allen et al., 1990)	R P G A G N I G S E G N L V V A K **A R M**
Brassica napus (Comai et al., 1989b)	R P G A A G M G E G T S L V V A K **S R M**
Cucumber (Reynolds pers comm.)	R P G A V N L G E C G N V V V A K **S R M**
Catalase	
Castor bean (Gonzalez, 1991)	Q C D K S L G Q K L A **T R L** N V K P S I
Cootonseed (Ni et a l., 1990)	Q A D K S V G Q K L A **S L L** N V R P S I
Sweet potato (Sakajo et al., 1987)	Q A D R S L G Q K V A **S R L** N I R P S I
Maize (Redinbaugh et al., 1987)	Q C D A A L G Q K L P **S R L** N L K P S I
Pea (Isin and Allen, 1991).	Q C D R S L G Q K L A **S H L** N M R P S I
Glycolate oxidase	
Spinach (Volokita and Somerville 1987)	S H I A A D W D G P * * S **S R A** V **A R L**
Lentil (Ludt and Kindl, 1990)	E H I V A D W D T P R I Q **P R A** L **P R L**
Uricase II	
Soybean (Nguyen et al., 1985)	P T D E P H G S I Q A S L **S R L** W **S K L**
Hydroxypyruvate reductase	
Cucumber (Greenler et al., 1989)	N V S P P A A S P S I V N A **K A L** G N A

the majority of cases (Table 1.1.). One enzyme that appears to contain a PTS is glycolate oxidase from spinach (Volokita and Somerville, 1987). Recently, transgenic tobacco plants containing a chimeric gene encoding a fusion protein composed of the reporter gene ß-glucuronidase (GUS) fused to the last-six C-terminal amino acids of glycolate oxidase were produced. It was subsequently demonstrated that this C-terminal region of this gene was sufficient to direct GUS protein to peroxisomes (Volokita, 1991). Consequently, these data provide additional evidence that support the proposal that the mechanism of targeting of peroxisomal proteins is conserved between evolutionary distinct organisms.

1.1.1.4. Targeting associated with proteolytic cleavage.

Although for most peroxisomal proteins there appears to be no post-translational proteolysis associated with import, exceptions are known. In higher plants, the most notable example is NAD-dependant malate dehydrogenase (MDH). In watermelon and cucumber there are 5 isozymes of MDH (Walk and Hock, 1976; Liu and Huang, 1976). In watermelon cotyledons, both mitochondrial (mMDH) and glyoxysomal (gMDH) isozymes are synthesised *in vitro* as higher molecular weight precursors (Gietl and Hock, 1982). In particular, gMDH is synthesised *in vitro* with a molecular weight of 41-38 kDa, that is larger than the 33 kDa of the mature protein (Gietl and Hock, 1984). Similar data have been reported for gMDH in cucumber cotyledons (Riezman *et al.*, 1980). Subsequent analysis of cDNA clones encoding gMDH demonstrate that there appeared to be no sequence similar to the PTS implicated in targeting of other peroxisomal proteins (Gietl, 1990). Additionally, comparison of mMDH and gMDH indicates that there is a region at the amino-terminal of gMDH that shows no significant homology with the mitochondrial enzyme (Gietl, 1990). By analogy with the established import mechanism of nuclear-encoded chloroplast and mitochondrial proteins it is believed that these additional sequences may be involved in targeting of these proteins into peroxisomes. Until recently there was no good evidence to support this assumption.

A second protein that is synthesised as a larger precursor is 3-ketoacyl-CoA thiolase from rat liver (Bodnar and Rachubinski, 1990; Hijikita *et al.*, 1990). In rat two forms of thiolase (thiolase A and B) exist that are localised in peroxisomes and following targeting amino portions of both proteins are removed (Hijikita *et al.*, 1987; 1990). Expression of a fusion protein composed of the putative

targeting sequence linked to the cytoslic protein CAT leads to the localistion of this fusion protein to peroxiosmes (Swinkels *et al.*, 1991). In addition, sequence comparison of cucumber thiolase with other thiolases and gMDH reveals conserved elements in the amino terminal that may be involved in the targeting of peroxisomal proteins (Preisig-Muller and Kindl, 1993 Table 1.2). From these data it would appear that a second mechansim for the targeting of peroxisomal proteins exists.

1.1.2. Peroxisome nomenclature and biochemistry.

Despite the ubiquitous distribution of peroxisomes in higher plants the enzyme composition and metabolic function of these organelles remains to be determined for most tissues. Currently, three types of microbodies have been identified that have an established metabolic function. Leaf-type peroxisomes found in photosynthetic tissue, peroxisomes localised in uninfected cells of the root nodules of some legumes and glyoxysomes in fat storing cells of oilseed species

1.1.2.1 Leaf-type peroxisomes: the C2 pathway.

Leaf-type peroxisomes are microbodies that are involved in photorespiration and contain most of the enzymes of the glycolate pathway (for review see Leegood, 1993). The photorespiratory pathway is presented in Figure 1.2. RuBisCO (ribulose 1,5-bisphosphate carboxylase oxygenase) can both oxygenate and carboxylate RuBP (ribulose 1,5-bisphosphate). Oxygenation leads to the formation of glycerate 3-phosphate and glycolate 2-phosphate. Glycolate 2-phosphate cannot be utilised by the Calvin cycle and consequently this represents a loss of assimilated carbon. The glycolate cycle is thought to be an attempt to recover this lost carbon. Glycolate produced in the chloroplast, is oxidised in the peroxisome to form glyoxylate and H_2O_2 that is subsequently converted into water and oxygen by the action of catalase (Huang *et al.*, 1983). Glycolate is transaminated to form glycine which is transported into the mitochondrion where it is utilised in the production of CO_2 and serine. Serine can be utilised in peroxisomes in the formation of glyceric acid. It is generally considered that photorespiration is not an efficient process as 25% fixed carbon is lost as CO_2.

As cotyledon greening occurs in cucumber, the enzymes serine:glyoxylate aminotransferase (SGAT) and NADH dependant hydroxypyruvate reductase (HPR)

Table 1.2. Sequence comparison of the amino terminal regions of peroxisomal proteins that are proteolytically processed following import. Asterisks represent introduced gaps to allow better alignment of sequences, regions of homology are indicated in bold. Taken from Preisig-Muller and Kindl, 1993. Rat thiolase A (Hijikita *et al.*, 1987), rat thiolase b (Bodnar and Rachubinski, 1991), cucumber thiolase (Preisig-Muller and Kindl, 1993), yeast thiolase (Einerhand *et al.*, 1991), human thiolase (Bout *et al.*, 1988) and watermelon gMDH (Gietl, 1990).

Protein			
Rat thiolase B	M M S E S V G E T S A N	H R L Q V V L G * H L	A G E S E S S S A * * * * *
Rat thiolase A	M	H R L Q V V L G * H L	A G E S E S S S A * * * * *
Cucumber thiolase	M E K A I	N R * Q S I L L H H L	H P * * * S S S A Y T N E S
Yeast thiolase	M S	Q R L Q S I K D * H L	V L E A M * G K G E S K R *
Human thiolase	M	Q R L Q V V L G * H L	R S P A D S C W M P * * * *
Watermelon gMDH	M Q P I P D V * * * * N	Q R I A R I S A * H L	H P P * * * * K S Q M E E S

Protein		
Rat thiolase B	* L Q A A P C	* * * * S A T F
Rat thiolase A	* L Q A A P C	* * * * S A G F
Cucumber thiolase	S L S A S V C	A A G D S A S Y
Yeast thiolase	* * * * * * *	* * * * * * * L
Human thiolase	* * Q A A P C	L * * * S G * P
Watermelon gMDH	A L R R A N C	R A * * K G G A

10

which are marker enzymes of the glycolate cycle accumulate in leaf-type peroxisomes in a light dependant manner (Becker *et al.*, 1978; Trelease *et al.*, 1971). Subsequently it was demonstrated that the light-stimulated accumulation of the photorespiratory enzymes SGAT and HPR was brought about primarily by an increase in the amount of mRNA encoding these proteins (Hondred *et al.*, 1987; Greenler *et al.*, 1989). Compatible data have been presented concerning the germination of mustard (Hong and Schopfer, 1981; Schopfer *et al.*, 1976), sunflower (Schnarrenberger *et al.*, 1971), watermelon (Kagawa *et al*, 1973; Kagawa *et al*, 1975) and lentil (Ludt and Kindl, 1990).

Surprisingly, leaf-type peroxisomes have been found in leaves of plant species that do not appear to have photorespiration (Frederick and Newcomb, 1971; Hilliard *et al.*, 1971), including plants that exhibit crassulacean acid metabolism (Kapil *et al.*, 1975; Herbert *et al.*, 1978) and even plants with achlorophyllous leaves (Gruber *et al.*, 1972). Additionally, in the C4 species *Atriplex rosea*, it was shown that the enzyme compliment of leaf-type peroxisomes was similar in bundle sheath cells and in mesophyll cells (Huang and Beevers, 1972). The significance of these observations is not clear.

1.1.2.2. Peroxisomes in uninfected cells of nodules of nitrogen fixing legumes.

A second specialised, though poorly characterised, type of peroxisome is found in the uninfected cells of root nodules of some legumes. In legumes such as soybean and cowpea, symbiosis with the nitrogen fixing bacterium *Rhizobium* occurs in root nodules leading to the formation of the ureides allantoin and allantoic acid. Nodule development is coupled with an increase in the size and number of peroxisomes in the uninfected cells. In soybean the proliferation of peroxisomes is associated with the synthesis of at least one peroxisomal enzyme, uricase (Nguyen *et al.*, 1985). Allantoin and allantoic acid are formed the oxidation of purines. It has been suggested that purines are synthesised in plastids and these are then subsequently oxidised in the peroxisomes. In this scheme purine xanthine is first converted to uric acid which is then converted to allantoin by the uricase. The action of uricase liberates H_2O_2 which is converted to H_2O via the action of catalase. The localisation of uricase provides good evidence that peroxisomes are involved in metabolism of fixed nitrogen in some legumes.

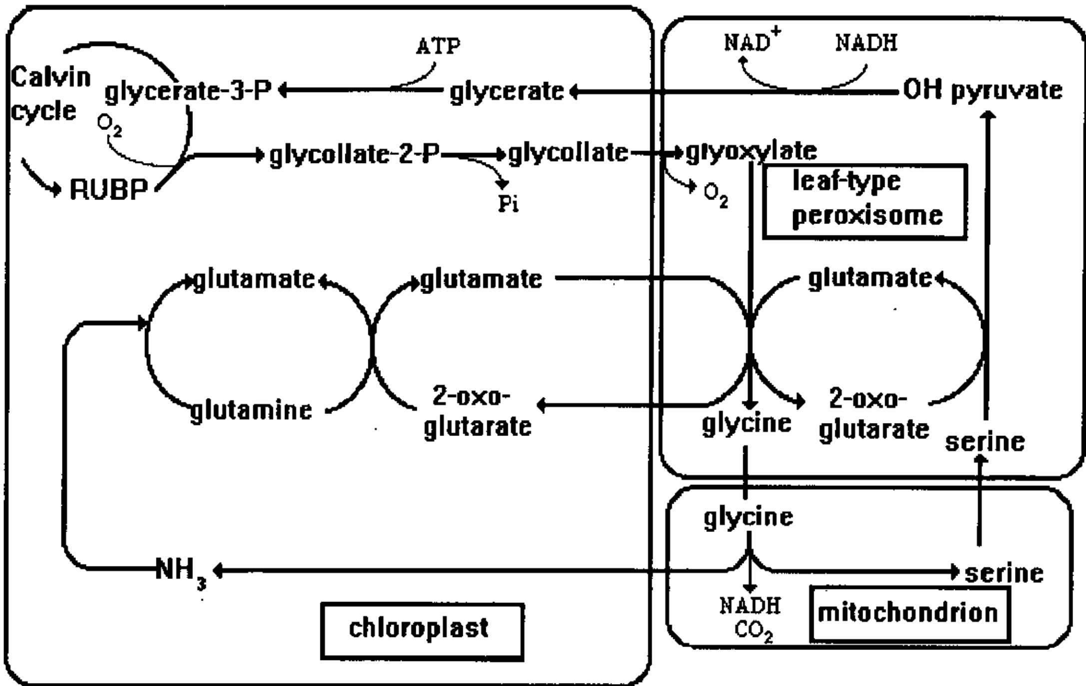


Figure 1.2. The photorespiratory pathway and its subcellular location. RuBisCO in the chloroplast can oxygenate or carboxylate RuBP. Oxygenation causes the formation of glycolate 2-P that cannot be utilised by the Calvin cycle. The photorespiratory pathway leads to the production of glycerate 3-phosphate a substrate of the Calvin cycle. During this process, 25% of the carbon is lost.

1.1.2.3. Glyoxysomes.

The third type of peroxisome is that found in the fatty tissue of higher plants, these contain the enzymes of the glyoxylate cycyle and are consequently termed glyoxysomes. The function of glyoxysomes in higher plants has been studied extensively and in particular their role in the conversion of storage triacylglycerols to sugars is well established (for review see Huang *et al.*, 1983).

1.2. The function and regulation of the glyoxylate cycle.

1.2.1. Conversion of fats to sugars: ß-oxidation and the glyoxylate cycle.

Triacylglycerides, located in membrane bound organelles termed lipid bodies, are the major site of storage lipids in seeds and are converted in four stages into sucrose that is subsequently translocated to the axis.

1. Hydrolysis of storage triacylglycerols to form fatty acids and glycerol.
2. ß-oxidation of fatty acids to give acetyl-CoA.
3. Conversion of acetyl-CoA to 4-carbon acids (malate or succinate) by the enzymes of the glyoxylate cycle.
4. Conversion of 4-carbon acids to sucrose via the enzyme phospho*enol*pyruvate carboxykinase (PEPCK) and reverse glycolysis.

During the early stages of seedling growth the activity of lipases increases and leads to the production of fatty acids and glycerol (For review on plant lipases see Huang, 1987). There appears to be some uncertainty as to the subcellular localisation of these enzymes. In castor bean there is an acid lipase associated with the lipid body membrane (Ory *et al.*, 1968) which has a high activity prior to germination, declining to a lower level as storage lipid is mobilised. Additionally, the glyoxysomes contain an alkaline lipase (Muto and Beevers, 1974) which shows a much greater activity with monoglycerides as substrate than with di- of tri-glycerides (Huang *et al.*, 1987). The significance of the specificity of this enzyme remains unclear (Huang *et al.*, 1987). The lipid body acid lipase activity demonstrated in castor bean endosperm appears to be absent in many other oilseed species (Huang, 1987). Two classes of oilseed species exist in terms of lipase activity (Huang *et al.*, 1983). The first group possess an acid or neutral lipase in the lipid body and the activity of these enzymes increases upon the onset of germination. *Brassica*, mustard and cottonseed appear to be part of this group.

Other plants including cucumber, soybean and peanut do not have any lipid body associated lipase activity. These plant species appear to mobilise storage lipid via the action of a glyoxysomal alkaline lipase (Huang *et al.*, 1983). It has been observed that lipid bodies are closely appressed to glyoxysomes, however, the precise mechanism that brings about the transfer of fatty acids from lipid body to glyoxysome remains to be established.

In higher plants the enzymes involved in the ß-oxidation of fatty acids to acetyl-CoA in fatty tissue are localised exclusively in glyoxysomes (Cooper and Beevers, 1969). There are four steps in the ß-oxidation cycle (Figure 1. 3.). Firstly, the fatty acid is activated to its acyl-CoA derivative by the action of fatty acyl-CoA synthases. This is subsequently oxidised by fatty acyl-CoA oxidase producing the enoyl-CoA derivative and H_2O_2. This is then converted to the 3-ketoacyl-CoA derivative, both reactions are catalysed by the so-called multifunctional protein. The removal of acetyl-CoA from the 3-keto ester by reacting with CoA is catalysed by the enzyme thiolase. This leads to the formation of a fatty acyl-CoA molecule having a carbon chain that is two carbons shorter that the starting material. In animal cells, ß-oxidation occurs in the mitochondrion and the peroxisome. There is some evidence that demonstrates that ß-oxidation may also be located in plant mitochondria of non-fatty tissue (Burgess and Thomas, 1986; Masterton *et al.*, 1990; Thomas and Wood, 1986; Wood *et al.*, 1984, 1986). This is in contrast to other observations that confirm the peroxisome as the sole site of fatty acid ß-oxidation in non-fatty tissue (Gerhardt, 1983; Macey, 1983; Macey and Stumpf, 1983). It is clear therefore, that further analysis of the function and site of enzymes involved in fatty acid ß-oxidation in non-fatty tissue is required.

Acetyl-CoA is converted to 4-carbon acids via the action of the glyoxylate cycle (Figure 1.4.). In higher plants, this cycle was first described in castor bean endosperm during the period of lipid utilisation (Kornberg and Beevers, 1957). Unique to the cycle are the enzymes malate synthase (MS) and isocitrate lyase (ICL). Combined with the TCA cycle enzymes aconitase, citrate synthase and malate dehydrogenase, this pathway brings about the conversion of two molecules of acetyl-CoA into 4-carbon acids such as succinate and malate (Figure 1.4.). The characteristic enzymes of the glyoxylate cycle, namely MS and ICL, are present in the fat storing tissue of several plant species (for review see Huang *et al.*, 1983).

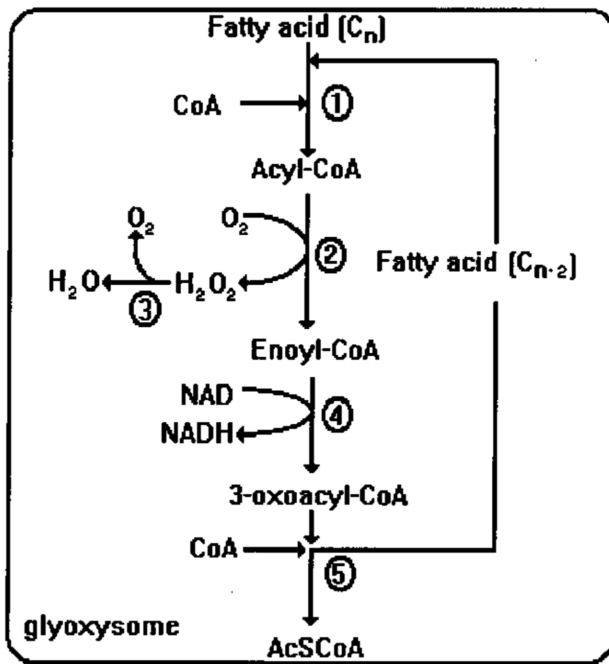


Figure 1.3. The β-oxidation of fatty acids. Fatty acid produced from the action of lipases on storage lipid is coverted to acetyl CoA and NADH by the enzymes of β-oxidation that are located in glyoxysomes. One cycle of the pathway produces acetyl CoA and a fatty acid chain 2 carbons less, this is also a substrate of β-oxidation enzymes. Consequently, 2 carbons are removed from a fatty acids with each turn of the cycle. Reactions are catalysed by; 1 fatty acyl-CoA synthase, 2 and 4 multifunctional enzyme, 3 catalase, and 5 thiolase.

Succinate dehydrogenase and fumarase are additional enzymes required for the operation of the glyoxylate cycle. However, these enzymes are located exclusively in the mitochondria and consequently, it is necessary that transport of metabolites between glyoxysomes and mitochondria must occur. Additionally, there must be a mechanism for the re-oxidation of NADH produced in the glyoxysome during fatty acid ß-oxidation. It has been reported that glyoxysomal membrane contains an electron transfer chain, which can oxidise NADH *in vitro*, however the significance of this observation remains to be fully established (Fang *et al.*, 1987). Currently, there appears to be more support for the mitochondrial oxidation of NADH generated in the glyoxysome (Cooper and Beevers, 1969; Lord and Beevers, 1972; Beevers, 1978). This is supported by the evidence demonstrating a malate-aspartate shuttle for the transfer of carbon or reducing equivalents between glyoxysomes and mitochondria (Mettler and Beevers, 1980; Millhouse *et al.*, 1983). Consequently, the reaction scheme used to illustrate the glyoxylate cycle (Figure 1. 4.) is based on such data.

The final stage in the conversion of lipid to sucrose occurs in the cytosol and is essentially the reverse of glycolysis (Nishimura and Beevers, 1979). Unique to this pathway are the enzymes phosph*enol*pyruvate carboxykinase (PEPCK), which converts oxaloacetate to phosph*enol*pyruvate. A second enzyme, fructose-1,6-bisphosphate 1-phosphatase (FBPase) catalyses the subsequent conversion of phosph*enol*pyruvate to hexose phosphate. Sucrose is then formed from fructose-6-phosphate and UDP-glucose by sucrose phosphate synthase.

1.2.2. Synthesis of malate synthase and isocitrate lyase in higher plants.

MS and ICL are unique to the glyoxylate cycle and, as a consequence, have been used as marker enzymes in the analysis of the developmental regulation of the synthesis of this pathway. In higher plants, the synthesis of these enzymes has been demonstrated during embryogenesis, post-germinative growth, and senescence. Several aspects of the glyoxylate cycle have been described in cucumber (Becker *et al.*, 1978; Graham *et al.*, 1992; Lamb *et al.*, 1978; Riezman, *et al.*, 1980; Smith and Leaver, 1986; Weir *et al.*, 1980).

In higher plants, MS is a octamer made from identical subunits (Koller and Kindl, 1977). MS has been purified to homogeneity from cucumber cotyledons

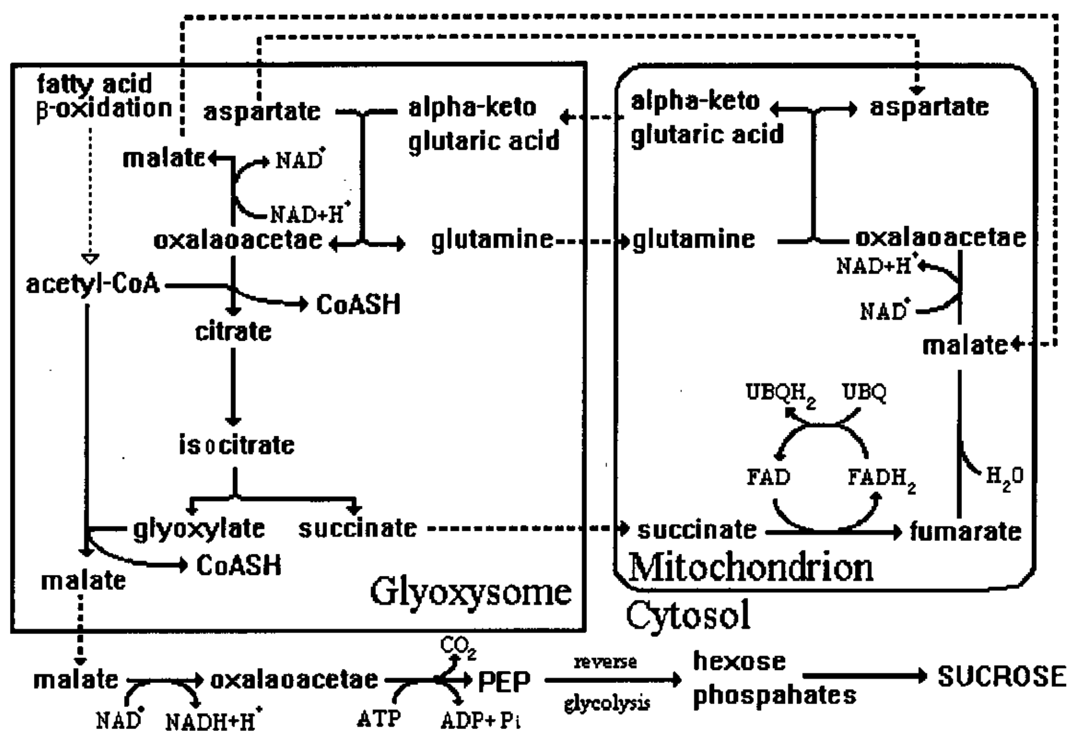


Figure 1.4. The conversion of acetyl CoA to sucrose.Acetyl- CoA is produced from the β-oxidation of fatty acids. This is subsequently converted to succinate by enzymes of the glyoxylate cycle. Cleavage of isocitrate and the formation of malate are catalysed by the enzymes ICL and MS respectively. Both enzymes are specific to the glyoxylate cycle. The malate produced by the glyoxylate cycle is transported to the cytosol were it is converted to sucrose. Succinate is transported to mitochondria were it is involved in the production of oxaloacetate that is required for the conversion of acetyl CoA to citrate, the initial reaction of the glyoxylate cycle, and the oxidation of NADH produced during fatty acid β-oxidation.

(Koller and Kindl, 1977; Lamb *et al.*, 1978; Riezman *et al.*, 1980). There appeared to be conflicting data concerning the size of the isolated subunit of MS; Riezman and co-workers reported the subunit of MS with an apparent molecular weight of 57 kDa, this is 6-7 kDa smaller than that reported earlier (Koller and Kindl, 1977). The apparent molecular weight of the subunit, isolated by Koller and Kindl, is in good agreement with that determined from the primary sequence of isolated cDNA and genomic clones encoding cucumber MS (Graham *et al.*, 1989). Given that the gene encoding MS is present as a single-copy sequence, this would indicate that there is only one gene that encodes this protein in cucumber (Graham *et al.*, 1989). Originally, this apparent discrepancy was considered to be due to post-translational glycosylation of the 57 kDa MS species (Riezman *et al.*, 1980). However, the possibility that the E.R. is involved in the synthesis of MS now appears to be unlikely and consequently the data reported concerning the isolation of a 57 kDa glycosylated form of MS has to be reconsidered. In other plant species the isolation of clones encoding this protein indicate that MS has an apparent molecular weight of 63-64 kDa (Comai *et al.*, 1989a; Mori *et al.*, 1991; Rodriquez *et al.*, 1990).

In higher plants ICL appears to be an tetramer. Two ICL subunits with apparent molecular weights of 63 and 61.5 kDa have been isolated from cucumber (Lamb *et al.*, 1978; Riezman *et al.*, 1980; Weir *et al.*, 1980). The analysis of the primary sequence of a full-length cDNA clone encoding cucumber ICL predicts a molecular weight of 64 kDa which is in good agreement with the larger isolated form (S. J. Reynolds, personal communication). Additionally, Southern blot analysis indicate that ICL exists in the genome of cucumber as a single copy sequence (S. J. Reynolds, personal communication). Consequently, it would appear that both forms of ICL in cucumber may be the product of a single gene and that post-translational modification may lead to the formation of both isoforms. The possibility that cucumber ICL is glycosylated has been excluded (Riezman *et al.*, 1980). Similarly, two immunologically identical isoforms of ICL have been purified from soybean and there is no evidence for glycosylation (Polanowski and Obendorf, 1991). From the analysis of cloned sequences and purified preparations it would appear that ICL in higher plants has an apparent molecular weight of 63-64 kDa (Allen *et al.*, 1988; Beeching and Northcote, 1987; Comai *et al.*, 1989b; Polanowski and Obendorf, 1991; S. J. Reynolds, personal communication).

1.2.2.1 Post germinative growth.

The major body of research concerning the synthesis and physiology of MS and ICL in cucumber, describes the activity of these enzymes during post germinative growth. Similar results have been demonstrated in other plant species (Allen *et al.*, 1988; Beevers, 1980; Comai *et al.*, 1989b; Dietrich, *et al.*, 1989; Rodriguez *et al.*, 1987; Turley and Trelease, 1990).

During germinative and post-germinative growth of cucumber the activity of MS and ICL increased in cotyledons from undetectable levels to reach a peak 3-4 days after seed imbibition (Becker *et al.*, 1978). Thereafter a decline in enzyme activities of MS and ICL was observed. However, in light grown seedlings this decline occurred far quicker than for that observed in dark-grown plants (Becker *et al.*, 1978). The isolation of MS and ICL from cucumber cotyledons allowed the preparation of polyclonal antisera that specifically recognise these proteins (Lamb *et al.*, 1978; Riezman *et al.*, 1980). The use of such antisera demonstrated that changes in the activities of MS and ICL are paralleled by the changes in the amounts of the corresponding mRNAs that are translated *in vitro* (Weir *et al.*, 1980). Western blot analysis of total protein isolated from cucumber cotyledons also indicated that the pattern of MS accumulation is similar to the demonstrable enzyme activity (Smith and Leaver, 1986). Additionally, the use of cDNA clones encoding cucumber MS and ICL indicated that the developmental synthesis of MS and ICL is due primarily to changes in the steady-state levels of the transcripts encoding these proteins (Smith and Leaver, 1986; S. J. Reynolds, personal communication). Transgenic plants were produced containing 1.1 kbp of the 5' region immediately upstream of the start of the coding region of cucumber MS fused to the reporter gene ß-glucuronidase (GUS). Subsequent analysis of the synthesis of GUS indicated that expression of the reporter gene occurs in a spatial and temporal manner similar to observed for MS in cucumber and indicates that transcription appears to be the primary controlling factor in the synthesis of MS (Graham *et al.*, 1989).

Collectively, these data indicate that an increase in transcription of the genes encoding MS and ICL may bring about the synthesis of MS and ICL. This has been demonstrated during post-germinative growth of *Brassica napus* seedlings (Comai *et al.*, 1989b; Ettinger and Harada, 1990). However, there have been reports that MS and ICL are phosphorylated in castor bean endosperm and *E. coli*

respectively (Yang *et al.*, 1988; Robertson *et al.*, 1988). Consequently, it is important to be aware that post-translational phosphorylation of MS and ICL may play an important role in the regulation of these enzymes.

1.2.2.2. Synthesis of MS and ICL during embryogenesis.

During maturation of cotton and cucumber seeds, glyoxysomal enzyme activities develop in gloxysomes in a non coordinate manner (Choinski and Trelease, 1978; Choinski *et al.*, 1981; Frevert *et al.*, 1980; Kindl *et al.*, 1980; Mierynk and Trelease, 1981b). In addition, malate synthase activity has been detected in dry seed from 17 other oilseed species (Miernyk *et al.*, 1979). The full significance of the glyoxylate cycle remains to be fully established and it appears that the pattern of synthesis of MS and ICL is species specific.

In cottonseed the activity of MS and ICL is observed at different stages of seed development (Choinski and Trelease, 1981). MS enzyme activity begins to increase at 45 days after anthesis, whereas ICL does not appear until 12 hours post imbibition (Choinski and Trelease, 1981, Miernyk and Trelease, 1981). Activities of other glyoxylate cycle, ß-oxidation enzymes and catalase appear to exhibit a coordinate increase in enzyme activity from 22 days after anthesis (Bortman *et al.*, 1981; Choinski and Trelease, 1981, Miernyk and Trelease, 1981). *In vitro* translation of RNA isolated from developing cotton embryos (22 days post anthesis) indicated the presence of mRNA encoding MS nearly 2 weeks prior to any demonstrable increase in enzyme activity and it was consequently proposed that the MS was transcribed at the same time as other genes encoding glyoxylate and ß-oxidation cycle and that some post-transcriptional mechanism prevented the accumulation of MS (Turley and Trelease, 1987). Similarly, RNA slot blots demonstrated the presence of transcripts encoding ICL in developing watermelon seeds, but western blot analysis failed to detect protein (Allen *et al.*, 1988). This is in contrast to the observed increase in MS and ICL activity in developing *Brassica napus* embryos were a coordinate increase in MS and ICL is observed. This is due primarily to increase in transcription of the genes encoding these proteins (Ettinger and Harada, 1990; Comai *et al.*, 1989b). The physiological significance of the presence of MS and ICL in developing seeds is not known. However it has been proposed that during this period MS may be involved in the synthesis of citrate (Miernyk and Trelease, 1981).

1.2.2.3. Synthesis of MS and ICL during senescence.

Following detachment and incubation of barley leaves in darkness it was observed that the activity of MS and ICL increased from undetectable levels (Gut and Matile 1988). Furthermore, this increase in glyoxylate cycle enzyme activity was coupled with a decline in the amount of the thylakoid lipid monogalactosyl diacylglycerol (MGDG). It was concluded from these data that products of thylakoid lipid degradation were further metabolised by the glyoxylate cycle (Gut and Matile, 1988). The detachment of green tissue and incubation in darkness is commonly used to induce senescence (Thimann, 1980). Therefore, it was postulated that during senescence, MS and ICL are involved in the metabolism of components of the thylakoid membrane (Gut and Matile, 1988). Subsequent analysis confirmed the observation that glyoxylate cycle enzyme synthesis increased in dark incubated detached organs and these enzymes were localised in peroxisomes (De Bellis *et al.,* 1990; De Bellis and Nishimura, 1991; Graham *et al.,* 1992). Additionally the induction of MS and ICL has been observed in naturally senescing leaves, petals and cotyledons from several different plant species (De Bellis *et al.,* 1991; Graham *et al.,* 1992; Pistelli, *et al.,* 1991). In addition to the activity of glyoxylate cycle enzymes there was also an increase in enzymes of ß-oxidation and given that there is a decline in the amount of MGDG it would seem acceptable to propose that the induction of these enzymes is associated with the breakdown of lipids during senescence.

Incubation of germinating seed in the light leads to a rapid decline in MS and ICL as photosynthesis is activated, but this decline is less pronounced in the dark (Allen *et al.,* 1988; Smith and Leaver, 1986; Weir *et al.,* 1980). Transfer of plants from light to darkness also leads to an induction of MS and ICL (Birkhan and Kindl, 1990; Graham *et al.,* 1992; Pistelli *et al.,* 1991). It has been proposed that the synthesis of glyoxylate cycle enzymes is repressed in tissue where sucrose is present and that reduction in sucrose content during senescence or following dark incubation of plants and excised organs may lead to an induction of the synthesis of these enzymes (Graham *et al.,* 1992). This model is based partly on data that indicate that the synthesis of glyoxylate cycle enzymes in bacteria, fungi and algae is substrate inducible and repressed by sugars.

1.3. Metabolic control of synthesis of the glyoxylate cycle.

1.3.1. Bacteria and fungi.

The glyoxylate cycle was first described in *Psuedomonas* sp. where it provides a means of growth on acetate (Kornberg and Marsden, 1957). This pathway was subsequently shown to support growth on acetate or other 2-carbon molecules in other bacteria (Hillier and Charnetsky, 1981; Kornberg, 1966), yeasts (Zwart *et al.*, 1983) and filamentous fungi (Armitt *et al.*, 1976; King and Casselton, 1977; Sjogren and Romano, 1967). In some of these cases it has been shown that MS and ICL synthesis is induced by growth on acetate and repressed by glucose (Kornberg, 1966; Sjogren and Romano, 1967).

Acetate non-utilising mutants of the ascomycete fungi *Neurospora crassa* and *Aspergillus nidulans* have been isolated (Armitt *et al.*, 1976; Flavell and Fincham, 1968). A number of genes involved in acetate utilisation in these fungal species have subsequently been cloned and have been found to encode MS (Connerton, 1990; Sandeman and Hynes, 1989; Thomas *et al.*, 1988) and ICL (Ballance and Turner, 1986). Despite the established relationship between glyoxylate cycle synthesis and the presence of acetate it is very unlikely that this metabolite directly leads to the expression of the genes encoding MS and ICL. This is observed from the analysis of the synthesis of glyoxylate cycle enzymes in *Escherichia coli.*

In *E. coli,* the structural genes that encode MS (*aceB*) and ICL (*aceD*) are clustered on the *ace* operon (Brice and Kornberg, 1968). The expression of these genes is negatively controlled by two gene products IclR and FadR (Maloy and Nunn, 1982). It has recently been demonstrated that phosph*enol*pyruvate, not acetate, acetyl CoA or acetyl-phosphate, specifically interacts with IclR and prevents binding of this factor with the promoter/operator of the *ace* operon *in vitro* (Cortay *et al.*, 1991). Consistent with this is the observation that in the fungus *Coprinus cinereus acu-1* mutants that lack acetyl-CoA syntheses activity are unable to synthesise ICL in the presence of acetate (Casselton and Casselton, 1974; King and Casselton, 1977) indicating that acetate *per se* is insufficient to activate the synthesis of ICL. In addition, growth of *E. coli* on fatty acids which does not lead to the synthesis of acetate also leads to the induction of synthesis of MS and

ICL (Maloy and Nunn, 1982). In plants, it might be more reasonable to anticipate that a similarly complex mechanism controls the expression of MS and ICL.

In *Saccharomyces cerevisiae*, mutants exist that are unable to degrade allantoin (Yoo and Copper, 1988). One gene, DAL7, is highly inducible when grown on allantoin as the sole source of carbon. However, the identity of the protein encoded by this gene was not known. Following sequence analysis of a cDNA clone encoding MS from castor bean it was demonstrated that DAL7 is likely to encode the gene for MS in *Saccharomyces cerevisiae* (Rodriquez *et al.*, 1990). Consequently this provides evidence that MS may play a role in the degradation of purines. It is not clear if ICL and other enzymes of the glyoxylate cycle would be similarly involved; this is because glyoxylate is produced from the breakdown of urideoglycolic acid and ICL action is obviated.

1.3.2. Metabolic control of glyoxylate cycle synthesis in algae and higher plants.

The incubation of *Euglena* cells in ethanol and acetate leads to an increase in the activity of MS and this occurs primarily through changes in the transcription of the gene encoding this proteins (Horrum and Schwartzbach, 1981; Woodcock and Merret, 1980). Similarly in *Chlorella* cells there appears to be an increase in the amount of ICL synthesised following the incubation of cells in acetate (McCullough and John, 1972). However, in both cases the presence of sugars or light in these cells leads to a decline in the activity of glyoxylate cycle enzymes.

The most convincing evidence that metabolites may play some part in the control of glyoxylate cycle enzyme synthesis in higher plants comes from studies using anise (*Anisum pimpinella* L.) suspension cultures. Removal of sucrose from the growth medium was associated with the induction of MS and ICL enzyme activity, that was subsequently enhanced by the addition of acetate to the medium. Repression of glyoxylate cycle activity occurred upon the addition of sucrose to the medium (Kudielka *et al.*, 1981; Kudielka and Theimer, 1983a, b). Sucrose and glucose have also been reported to inhibit the development of MS and ICL activity in fat storage tissue during periods of rapid lipid mobilisation (Hock and Beevers, 1966; Lado *et al.*, 1968, Longo and Longo, 1970; Slack *et al.*, 1977).

A second example of the induction of the glyoxylate cycle has been observed during sucrose starvation of maize root tips (Dieuaide *et al.*,1992). After the removal of sucrose from the growth medium there was a rise in the activity of fatty acid ß-oxidation and MS. Surprisingly, attempts to detect the activity of ICL were unsuccesful. In some cases attempts to assay ICL have proven difficult (Fusseder and Theimer, 1976). Consequently, it is not clear if the observation that ICL was not detected in maize root tips during sucrose starvation is not due to problems with the assay. However, there is evidence that at least part of the glyoxylate cycle is synthesised during sucrose starvation.

1.4. Senescence.

Senescence may be defined as those changes which lead to the death of an organism or some part of it. This is in contrast to ageing which is defined as all changes in time without reference to death as a consequence (Medawar, 1957). Fundamentally, senescence involves the systematic dismantling of biological organisation at both the tissue and cellular levels. For example, flower petals senesce following fertilisation and whole plant populations after seed set. This implies, that to a certain extent, senescence is genetically predetermined, but there are other environmental factors that can induce premature senescence such as ozone, drought and sulphur dioxide (Chia *et al.*, 1984; Nooden, 1988; Pauls and Thompson, 1980).

Senescence is a genetically programmed sequence of events during which there is a synthesis of new proteins and an increased synthesis of pre-existing proteins. A number of enzymes, including proteases, ribonuclease, invertase, acid phopshatase, cellulase and polygalacturonase, show increased activity and this in due primarily to an increased synthesis of protein (Mattoo and Aharoni, 1988). That senescence is genetically controlled is further supported by the isolation and characterisation of "non-yellowing" mutants of *Festuca pratensis* (Thomas, 1977) and non ripening varieties of tomato such rin, nor and Nr (Grierson *et al.*, 1985). It is apparent that the shift from predominantly synthetic to predominately degradative process that characterises senescence is subject to genetic regulation.

Senescence can occur at various levels of biological organisation from individual cells to entire plant populations. Localised senescence of a group of

cells within developing leaves of *Monstera* plants contributes to the morphology of the leaves (Nooden, 1988). The development of oil cavities in citrus peel is brought about by the differentiation of cells below the epidermis that divide, enlarge and senescence. This leads to the generation of a cavity containing oil droplets (Nooden, 1988). Abscission of flowers leaves and fruit can also be attributed to the senescence to a specialised layer of cells in the abscission zone (Sexton and Woolhouse, 1984). Whole plant senescence is characteristic of monocarpic plants and usually is associated with the transition from vegetative growth to the reproductive phase of development. It appears that certain parts of the plant can influence the timing and pattern of senescence in other parts of the plant. For example, in soybean photosynthates are mobilised for the growth of the developing fruit at the expense of the other parts of the plant. Polycarpic plants have longer life spans that can extend for up to thousands of years. Generally, in polycarpic plant species, only a proportion of total assimilate is directed to the formation of fruit and consequently senescence of the whole plant is more protracted that in monocarpic species.

1.4.1. Hormonal regulation of senescence.

It is of interest to note that all major plant growth regulators have been implicated in the control of senescence, but the mechanisms underlying the hormonal regulation of senescence remain to be elucidated. It is now proposed that the hormonal regulation of senescence is brought about by an interaction of several plant growth regulators.

1.4.1.1. Ethylene.

The dominant effect of ethylene is in the promotion of senescence and the action of ethylene has been studied extensively, particularly with respect to the processes of fruit ripening and flower senescence. The role of ethylene in leaf senescence is less well characterised.

Although leaves do produce ethylene it has been argued from studies of detached leaves that ethylene production has no effect (Thimann, 1980). This has been countered by the proposal that detachment may lead to an increase in ethylene to saturating levels and as a consequence render the application of this hormone ineffective in eliciting any responses associated with senescence (Mattoo and

Aharoni, 1988). There is some evidence that yellowing of leaves is attributed to the presence of ethylene and the application of exogenous ethylene leads to the degradation of protein (Steffens, 1983) and chlorophyll (Kao and Yang, 1983). Ethylene treatment also leads to the induction of chitinase and ß-1,3-glucanase in bean leaves (Mauch and Staehelin, 1989). However, decline in RNA levels is a prominent feature of senescence and the application of ethylene rarely appears to affect the rate of RNA degradation or activity of RNase (Abeles, 1973; Sacher and Salminen, 1969).

Endogenous levels of ethylene have been determined during the senescence of cotton leaves and it was reported that ethylene levels declined with age (Hall *et al.*, 1957). However, in other species senescence was coupled with a climacteric-like rise in ethylene similar to that observed during fruit and flower development. This climacteric rise occurred close to the time of chlorophyll degradation in some species (Gepstein and Thimann, 1981; Lau *et al.*, 1977; Kao and Yang, 1983).

Aminoethoxyvinylglycine (AVG), that can inhibit the synthesis of ethylene, and silver ions and CO_2, that can block ethylene action, have been used in the study of this plant growth regulator. In tobacco leaf discs AVG and Ag^+ ions retarded the appearance of the respiratory climacteric and loss of chlorophyll (Aharoni and Lieberman, 1979). Ag^+ also retards chlorophyll loss in senescing rice leaves (Kao and Yang, 1983) and in excised oat leaves (Gepstein and Thimann, 1981). In this latter study AVG did not delay the onset of senescence but did modify the rate of senescence. In addition, Ag^+ was ineffective in inhibition of chlorophyll loss in darkness. From these data it was concluded that ethylene did not initiate the onset of senescence, but could modify the rate at which the process progressed and that ethylene plays a role in chlorophyll degradation in the light only (Gepstein and Thimann, 1981).

Most studies concerned with the role of ethylene in leaf senescence are carried out on excised leaves and consequently the data obtained form such analysis may not reflect the changes occurring during natural senescence. However, it does appear that ethylene is likely to play some role in the regulation of leaf senescence.

1.4.1.2. Abscsisic acid.

The role of abscisic acid (ABA) during senescence is controversial and remains to be established. Treatment with exogenous ABA has been shown to promote various senescence-like process.

For example, it has been shown in a number of occasions that treatment of excised leaves with exogenous ABA leads to chlorophyll loss (for review see Nooden, 1988). In addition, chlorophyll synthesis, photosynthetic activity, protein and nucleic acid synthesis have all been reported to be inhibited following the application of exogenous ABA (Colquhoun and Hillman, 1972; Cornic and Miginiac, 1983; De Leo and Sacher, 1970; Manos and Goldthwaite, 1975; Wheda and Kuraishi, 1978). Exogenous ABA can also induce the degradation of protein and nucleic acids (Colquhoun and Hillman, 1972; De Leo and Sacher, 1970; Manos and Goldthwaite, 1975; Halvey *et al.*, 1974). Additionally, ABA can also accelerate the deterioration of chloroplast, plasmamembrane and tonoplast (Mittelheuser and van Speigelstein, 1969) and induce the degradation of acyl lipids (Hancock *et al.*, 1983).

However, in some instances ABA does not appear to cause the induction of senescence. For example, although exogenous application of ABA induces senescence of rose flowers, this only occurs if leaves are removed from the flowering stem (Halvey *et al.*, 1974). A possible explanation is that ABA causes stomatal closure in the leaves and this may slow down senescence (Halvey *et al.*, 1974). It has also been observed that although exogenous application of ABA accelerates senescence, it has no demonstrable effect on attached leaves (Smith *et al.*, 1968). This may reflect poor uptake or translocation of ABA or the compensatory affects of other plant growth regulators. In addition ABA induces the senescence of *Rhoeo* leaves only under low light conditions (De Leo and Sacher, 1970).

Similarly, determinations of endogenous ABA levels have not provided consistent information concerning the role of this hormone during senescence. Generally, ABA levels increase during senescence (Nooden, 1988). However, in young leaves of *Xanthium strumarium* levels of ABA are higher than for that observed in senescent leaves (Raschke and Zeevaart, 1976). Colquhoun and Hillman (1975) failed to find any correlation between ABA levels and stage of senescence of bean leaves. In soybean, ABA is translocated from leaves to fruit

and consequently will accumulate in leaves following removal of developing fruit (Nooden and Obermeyer, 1981; Setter *et al.*, 1980). As this treatment prevents senescence of the leaves this would appear inconsistent with the proposal that ABA can induce senescence.

It is clear that the role of ABA in senescence still remains to be determined unambiguously. For example, it has been proposed that ABA plays no role in senescence (Kelly and Davies, 1988), or that it is likely to be the second most important phytohormone involved in senescence after ethylene (Nooden, 1988). It has also been proposed that ABA may play a role in stress response of plants and that the synthesis of this hormone may occur as an indirect consequence of senescence, rather being a direct effector of the process (Leshem *et al.*, 1986).

1.4.1.3. Auxins.

Generally, auxins appear to delay senescence, although there are well documented instances in which the hormone has no demonstrable effect (for review see Nooden, 1988). In addition, auxins are involved in senescence during the differentiation of xylem vessels (Jacobs, 1952), senescence of petals (Nichols, 1984) and leaves (Mishra and Gaur, 1980).

In those cases were auxins delay senescence this hormone appears to alter the levels of several enzymes and to delay chlorophyll degradation, membrane deterioration, proteolysis and RNA degradation. In some of the instances were auxins appear to induce senescence it has been demonstrated that this may be due to an indirect effect auxin has on the synthesis of ethylene. In carnation flowers, treatment with exogenous auxin accelerates senescence by stimulating the synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase one of the enzymes involved ethylene biosynthesis (Wulster *et al.*, 1982). Studies of *Vanda* flowers have indicated that following pollination, there is a transfer of auxin from pollen to stigma and this leads to the synthesis of ethylene in the style (Burg and Dijkman, 1967). Similarly, it has been determined that the effect of auxins on fruit development occurs via the action of ethylene (McGlasson *et al.*, 1978).

Like other plant growth regulators the precise role of auxin during senescence has not been determined fully. Nooden (1988) has stated, from the combined

studies with endogenous and exogenous auxins, a role for auxin in the delaying of senescence is implied. In contrast, Thimann (1980) has suggested that auxins have not been found to be very potent in retarding leaf senescence. It would appear that a clear role for auxins in senescence remains to be established.

1.4.1.4. Cytokinins.

Since 1957, when Richmond and Lang reported that treatment of *Xanthium* leaves with kinetin inhibited the loss of protein, there has been considerable interest in the role of cytokinin during senescence (Letham and Palni, 1983; Van Staden *et al.*, 1988). Generally, cytokinins prevent or delay leaf senescence (for review see Van Staden *et al.*, 1988). It is important to be aware that the effects of cytokinins are more pronounced for detached leaves (Thimann, 1980) and for younger leaves, than older leaves (Naito *et al.*, 1978).

It has been proposed that a decline in endogenous cytokinin levels may play a part in leaf senescence (Van Staden *et al.*, 1988). There is clear evidence that application of endogenous cytokinins delays the breakdown of protein, chlorophyll and nucleic acids that normally occur during senescence and in some cases this may be due to a inhibition of the increase in RNase and protease activity (Legocka and Szweykowska, 1983; Sodek and Wright, 1969; Wyen *et al.*, 1972). Exogenous cytokinin also appears to stimulate protein synthesis and in one case this was attributed primarily to an increase in the synthesis of RuBisCO (Spencer and Wildman, 1964). This is consistent with additional reports of cytokinins stimulating the accumulation of key photosynthetic enzymes and chlorophyll, and inducing chloroplast replication and grana formation in partially senescent leaves (Boasson *et al.*, 1972; Caers *et al.*, 1985; DeBoer and Feiersbend, 1974; Fletcher *et al.*, 1973; Naito *et al.*, 1978; Purohit, 1982; Tsuji *et al.*, 1979). Cytokinins also appear to prevent membrane deterioration and this appears to be due, at least in part, by the inhibition of lipoxygenase (Grossman and Leshem, 1978) and lypolytic enzymes (Legocka and Szweykowska, 1983; Solomos, 1988; Wyen *et al.*, 1972). The removal of young leaves delays senescence and in some cases leads to regreening. Removal of young leaves also results in an increase in the cytokinin content of the remaining leaves (Colbert and Beevers, 1981; Van Staden and Carmi, 1982). The most convincing evidence that cytokinins retard senescence comes from the analysis of transgenic plants containing the *tmr* gene from *Agrobacterium tumefaciens* (Smart *et al., 1991*). This gene encodes a protein that

produces cytokinins and analysis of transgenic plants that contained the *tmr* gene fused to the 35S CaMV promoter demonstrated that a high amount of cytokinin is coupled with a delay in the progression of senescence (Smart *et al*, 1991).

1.4.1.5. Gibberellins.

The effect of exogenous gibberellins on senescence has been studied extensively. However, no precise role for gibberellins has been established during senescence.

In general, the application of gibberellins to leaves retards chlorophyll loss and inhibits protein degradation (for review see Nooden, 1988). There are also notable exceptions. Exogenous gibberellins promote chlorophyll loss in Brussels sprouts (Thomas, 1977), inhibit it in peanut and bean (Nooden, 1988) and has no effect on chlorophyll levels in rye grass (Thomas, 1975). Also, the response to gibberellins is dependant on whether leaves are excised or intact; detached rice leaves do not respond to gibberellins, whereas attached leaves do show altered pattern of senescence (Harada and Nakayama, 1982). The age of leaves also appears to be an important factor. Gibberellins retard chlorophyll loss in discs form mature leaves and induce the loss in discs from younger leaves (Thomas, 1975). This may reflect the fact the gibberellins levels may change during leaf growth.

Gibberellins and cytokinins can both inhibit senescence by preventing the production of ethylene (Mattoo and Aharoni, 1988). However, for some plant species the synthesis of ethylene can only be prevented by one and not the other hormone. For example, in *Taraxacum* leaves, the synthesis of ethylene is inhibited by gibberellin but the application of cytokinin has no effect (Fletcher and Osborne, 1966).

1.4.1.6. Other factors.

Free fatty acids are an additional factor that may play a role in regulating senescence. There is a large increase in free fatty acid, in particular linolenic acid during senescence of carnation flowers, due to the action of lypolytic acid hydrolase on phospholipids and galactolipids (Brown *et al.*, 1987). Linoleic acid has been shown to inhibit photosynthesis in isolated chloroplasts (Mve Akmba and Siegenthaler, 1979) and to cause chlorophyll loss (Ueda and Kato, 1982). It has

also been reported that polyunsaturated fatty acids may be converted to jasmonic acid (Lynch *et al.*, 1985). Methyl jasmonate and jasmonic acid both inhibit growth and promote senescence (Vick and Zimmerman, 1987). Chlorophyll loss in excised oat leaves and ethylene production during fruit ripening have been linked to methyl jasmonate (Saniewski and Czapski, 1985; Ueda *et al.*, 1981; Vick and Zimmerman, 1987). The precise roles of methyl jasmonate and jasmonic acid during senescence remain to be determined, but could be of potentially great interest.

1.4.2. The enigma of chlorophyll degradation.

The most obvious event associated with senescence of leaves and photosynthetic cotyledons is loss of chlorophyll (Thomas and Stoddart, 1980). It must be remembered that loss of chlorophyll does not necessarily correlate with changes in other parameters that occur during senescence (Hardwick *et al.*, 1968; Woolhouse, 1967). This is further illustrated by analysis of the "stay-green" mutants of soybean (Kahanak *et al.*, 1978) and the "non yellowing" mutants of *Festuca pratensis* (Thomas and Stoddart, 1975). In these mutants there appears to be no difference between timing of abscission and many of the characteristics associated with senescence are very similar when mutant and wild type are compared. However, it is usually a good indicator of the process of senescence. Knowledge of the pathway of chlorophyll degradation would aid our understanding of senescence.

Surprsingly, it is not clearly understood how chlorophyll degradation occurs (for reviews see Hendry *et al.*, 1987; Matile, *et al.*, 1987). One possible candidate is the enzyme chlorophyll oxidase, the action of this leads to the formation of chlorophyll *a-1*. There is some evidence from studies of detached *Phaseolus vulgaris* and *Hordeum vulgare* leaves that may indicate that chlorophyll degradation may involve the formation of chlorophyll *a-1*. In dark-incubated detached leaves the amount of chlorophyll *a-1* increases from undetectable levels to reach a maximum and then declines (Maunders *et al.*, 1983). This decline, 10-12 days after excision, indicates that chlorophyll *a-1* is not an end product of chlorophyll degradation, but is an intermediate. Chlorophyll *a-1* was not detected in attached naturally senescent leaves and it was proposed that chlorophyll degradation proceeds at a faster rate and does not accumulate to detectable amounts in attached tissue (Sexton and Woolhouse, 1984). In addition, the activity of

chlorophyll oxidase appears to be activated by fatty acids that could be from the breakdown of thylakoid lipids (Shimuzi and Tamaki, 1963; Thomas 1982). The premise that the formation of chlorophyll *a-1* is the first step of chlorophyll degradation is supported by the observation of a similar reaction that initiates the catabolism of other cyclic molecules such as heme (Brown and Troxler, 1982).

A second candidate enzyme that may be involved in chlorophyll degradation is chlorophyllase. This enzyme catalyses both the formation and hydrolysis of the phytol ester bond and has been implicated in the degradation of chlorophyll. (Kuroki *et al.*, 1981). The strongest evidence comes from the observation that the addition of chlorophyllase to isolated chloroplasts lead to the breakdown of chlorophyll (Amir-Shapira *et al.*, 1986, 1987). Additionally there is a positive correlation between the breakdown of chlorophyll and an increase in chlorophylase in ripening citrus fruit (Hirschfield and Goldsmith, 1983; Amir-Shapira *et al.*, 1987). However, a significant body of research indicates that *in vivo* the primary role of chlorophyllase is in chlorophyll biosynthesis (Jones, 1979). For instance, the amount of chlorophyllase declines during senescence of *Raphanus* leaves (Hendry *et al.*, 1987; Philips *et al.*, 1968). A third possible mechanism for chlorophyll degradation is via a non-enzymatic photochemical process (Maunders and Brown, 1983).

1.4.3. Changes in membrane composition and organisation during senescence.

Membrane deterioration is a characteristic feature of senescence which leads to increased permeability and decline in the activities of membrane bound enzymes. From biochemical and ultrastructural analysis of the changes in membrane structure a fairly well characterised series of events occurs. Ultrastructural analysis demonstrates that chloroplast disassembly occurs prior to that of other membranes.

1.4.3.1. Chloroplast membranes.

From ultrastructural analysis of the changes in senescing leaves and green cotyledons, deterioration of chloroplast membranes is one of the earliest observable changes (Barton, 1966; Butler and Simon, 1967; Dodge, 1967; Ikeda and Ueda, 1964). This is supported by biochemical analysis of the change in lipid content of

senescing green cotyledons and leaves: thylakoid localised lipids MGDG, DGDG (digalactosyl diacylglycerol) and SQ (sulphoquinovsyl diglyceride) decline earlier than other lipids localised in plasmamembrane and tonoplast (Ferguson and Simon, 1973; Fong and Heath, 1977; Harwood *et al.*, 1982; Huber *et al.*, 1976).

At the structural level, chloroplast membrane deterioration is characterised by the separation of the inner and outer membranes, and degeneration of the outer envelope (Butler and Simon, 1971; Batt and Woolhouse, 1975). This is then followed by the degradation of thylakoid located proteins (Thomas, 1977) and the appearance of numerous plastoglobuli that are rich in carotenoids. Associated with senescence of thylakoid membranes is increased crystallisation (McKersie and Thompson, 1978) and permeability (Dhindsa *et al.*, 1981; Pauls and Thompson, 1984). The latter change in membrane organisation appears to be linked to lipid peroxidation and a loss of fatty acids from the bilayer (Dhindsa *et al.*, 1981; Pauls and Thompson, 1984).

Knowledge of the metabolism associated with breakdown of chloroplast lipids is scarce in comparison with the detailed knowledge concerning the biosynthesis of galactolipids (for review see Douce and Joyard, 1987). Galactolipases have been detected and purified from several plant species. However, the localisation of these enzymes has not been unambiguously identified, nor has any positive correlation with an increase in the activity of these enzymes and senescence been established (Anderson *et al.*, 1974; Helmsing, 1989; Kaniuga and Gemel, 1984; O'Sullivan *et al.*, 1987). It was thought that the breakdown of galactolipids would lead to an increase in the free fatty acid content of senescent leaves and cotyledons. However, in barley, the very small pools of fatty acids present in leaves prior to senescence declined to below the levels of detection following the induction of senescence (Gut and Matile, 1989). In detached, dark incubated leaves of barley and tobacco, decline in galactolipids was accompanied with a temporary increase in PC with a similar acyl residue content to the thylakoid localised lipids (Gut and Matile, 1989; Koiwai *et al.*, 1981). This has lead to the proposal that galactolipid degradation is essentially the reverse of the established biosynthetic pathway: galactolipids are converted via diacylglycerol to PC which is exported from senescent chloroplasts and lead to the generation of PC which has a similar pattern of 18:3/18:3 acyl residues.

The observation that the dark-induced decline in MGDG in barley leaves is coupled with an increase in the activity of glyoxylate cycle enzymes MS and ICL (Gut and Matile, 1988) has lead to the proposal that acyl residues of PC may then be subsequently subjected to ß-oxidation and the products converted to sugars via the glyoxylate cycle and reverse glycolysis (Wanner *et al.*, 1991).

1.4.3.2. Microsomal membranes.

In addition to the changes in the membranes of chloroplasts, it is now becoming apparent that there are major alterations in the molecular organisation of the lipid bilayer of membranes of senescent organs, particularly, plasmamembrane and microsomal membranes. This is demonstrated by the loss of membrane phospholipid (Thompson, 1988).

Decrease in lipid fluidity has been reported for microsomal membranes from senescing cotyledons, flowers and leaves (Fobel *et al.*. 1987; Leshem *et al.*, 1984; Thompson *et al.*, 1987) and in the plasmamembrane from ripening fruit and senescing flowers (Thompson, 1988). During the senescence of carnation flowers this decrease in bulk-lipid fluidity coincides with the climacteric increase in ethylene synthesis (Thompson *et al.*, 1982). Moreover, addition of ethylene to presenescent flowers accelerates the onset of the decline in microsomal membrane lipid fluidity (Thompson *et al.*, 1982). This loss of fluidity is attributable to an increase in the sterol : phospholipid ratio and the saturated : unsaturated fatty acid ratio that is due to increased lipoxygenase activity (Fobel *et al.*, 1987). This is in contrast to the observation that associated with thylakoid deterioration there is no significant change in membrane bulk fluidity (McRae *et al.*, 1985). Therefore, it would appear that the mechanism that brings about the deterioration of thylakoid membranes differs from that of plasma or microsomal membranes. Freeze-fracture electron microscopy of senescent cow pea cotyledons demonstrates a second change in the properties of extraplastidial membranes (Platt-Aloia and Thomson, 1985). Membranes from young tissue posses smooth membranes with evenly distributed intramembranous particles. As senescence proceeds, these particles aggregate indicating the formation of gel-phase lipid domains. X-ray diffraction analysis has also demonstrated an increase in gel-lipid domains associated with senescence of microsomal membranes (for review see Thompson, 1988).

Change in lipid structure can influence the activity of membrane localised enzymes and receptors. For example, a decrease in membrane fluidity can lead to the displacement of proteins from the membrane bilayer (Borochov and Shiniyzky, 1976). Several membrane-associated enzymes, including Ca^{2+}-ATPase and K^{+}-ATPase show decreased activity during senescence (Lai and Thompson, 1972; Paliyath and Thompson, 1988). Additionally,a change in the affinity of membrane-associated binding sites for ethylene during the senescence of carnation flowers correlated with decreased membrane fluidity (Brown *et al.*, 1986). Four lipid-degrading enzymes, phospholipase D, phosphatidic acid phosphatase, lipoxygenase and lipolytic acyl hydrolase, are associated with microsomal membranes isolated from senescent *Phaseolus vulgaris* cotyledons (Paliyath and Thompson, 1988). Collectively, these enzymes are capable of degrading lipids and bringing about changes in the lipid bilayer, including loss of lipid phosphate, loss of fatty acids, an increase in the sterol: fatty acids ratio. However, there is no evidence that indicates that these changes in membrane organisation are due to the activity of these enzymes *in vivo*. This is supported by the observation that these enzymes are found in the membranes of non senescing carnation flowers (Brown *et al.*, 1991).

1.4.4. Changes in cellular homeostasis.

Loss of cellular homeostasis is an inevitable consequence of structural deterioration and is considered by some workers to be a pivotal feature of senescence (Brown *et al.*, 1991). For example, a decline in the integrity of membranes leads to a leakiness of cell and organelles. This leads to the removal of ionic and metabolite gradients and could lead to cascade of events that cause senescence. Moreover, this loss of membrane integrity is a very early feature of senescence; in carnation petals, there is a significant increase in membrane leakiness before the climacteric rise in ethylene synthesis and petal unrolling (Eze *et al.*, 1986). The formation of gel-phase lipid regions in the bilayer leads to the formation of leaky membranes (Barber and Thompson, 1980) that have been detected in very young petals of rose and carnation (Brown *et al.*, 1991; Farragher *et al.*, 1987).

Under normal conditions of homeostasis, the Ca^{2+} concentration in the apoplasmic space, the E. R. and vacuole is above millimolar levels. In the

cytoplasm Ca^{2+} concentration is maintained below 1 μM by Ca^{2+} ATPases (Hepler and Wayne, 1985). The enzymes phospholipase D and phosphatidate phosphatase are stimulated by micromolar levels of Ca^{2+} (Paliyath and Thompson, 1988). Additionally, the Ca^{2+} stimulated activity of phosphatidate phosphates appears to be mediated by calmodulin (Paliyath and Thompson, 1987). Consequently, it has been argued that disruption of Ca^{2+} homeostasis may be an inherent feature of senescence (Ferguson, 1984).

The identity of the factor that brings about the initial stimulus is unknown. Experiments with microsomal vesicles isolated from carnation petals and pre-loaded with Ca^{2+} have indicated that ethylene does not directly stimulate the release of Ca^{2+} (Paliyath *et al.*, 1987). Inositol triphosphate causes a release of Ca^{2+} only at high, non physiological concentrations (Paliyath and Thompson, 1987). However, membranes isolated from natural and ethylene-induced senescent carnation flowers exhibited a significantly reduced Ca^{2+}-ATPase activity (Paliyath and Thompson, 1988). It is presumed that during the initial stages of senescence there is an increase in cytosolic Ca^{2+} that in turn promotes other processes associated with senescence.

These senescence promoting effects of Ca^{2+} must be distinguished from the senescence-delaying affects that have been reported for the exogenous application of Ca^{2+}. In these instances the bulk of Ca^{2+} is introduced to the cell wall, were it serves as a cross-linking component between polygalacturonase chains (Knee, 1973; Poovaiah *et al.*, 1988). Additionally, Ca^{2+} is thought to become associated with the plasmamembrane and preserve membrane stability (Legge *et al.*, 1982; Lieberman and Wang, 1982). Thus the senescence delaying capability of exogenous Ca^{2+} is considered to be primarily due to extracellular effect of stabilising cell walls and the external surface of the plasmamembrane.

1.4.5. Changes in nucleic acids during senescence.

Decreases in the level of DNA have been reported in a number of senescing leaves (Harris *et al.*, 1982; Dyer and Osborne, 1971) In particular, the senescence of soybean cotyledons was accompanied by a 25% decline in nuclear DNA content (Chang *et al.*, 1985). However, this decline was due primarily to the loss of repeated DNA sequences within the genome (Chang *et al.*, 1985). This

provides evidence that senescence is under genetic control and this requires that coding regions of the genome remain intact until the latter stages. This is supported by the observation that the synthesis of the chloroplast encoded D-1 protein of photosystem II (PSII) remains unchanged in senescent bean leaves (Roberts *et al.*, 1987). Further information concerning the effects of senescence on nuclear and chloroplast DNA comes indirectly from regreening studies. For example, the observation that senescent bean leaves, devoid of chlorophyll and chlorophyll binding proteins can be induced to regreen (Venkatarayappa *et al.*, 1984) would imply, that, at least, the coding regions of nuclear and chloroplast DNA are maintained intact.

There are also qualitative and quantitative changes in RNA during senescence. In particular, levels of rRNA decline in senescent bean and wheat leaves (Brady and Scott, 1977; Makrides and Goldthwaite, 1981) and chloroplast rRNA is selectively degraded during senescence of *Perilla*, cucumber and tobacco leaves (see Brady, 1988). There is also a decline in rRNA during the early stages of fruit ripening (Rattanapanoone *et al.*, 1977). However despite the general decrease in RNA, *in vitro* translation of mRNA has shown that there is an increase in some transcripts (Kawakami and Watanabe, 1988a, b: Malik, 1987; Skasden and Cherry, 1983; Thomas, 1990; Watanabe and Imaseki, 1982). However, few of these transcripts have been identified: other than transcripts that encode MS (Graham *et al.*, 1992; De Bellis *et al.*, 1991) or are related to fruit ripening mRNAs (Davies and Grierson, 1989). Notable exceptions are transcripts that encode the cytosolic glutamine synthetase of radish and rice (Kamachi *et al.*, 1991; Kawakami and Watanabe, 1988c).

1.4.6. Change in protein content during leaf senescence.

During leaf senescence, protein levels normally decline in a temporal pattern that is approximate to the decline in chlorophyll. Given the role of leaves in photosynthesis most of the studies concerned with changes in protein during senescence demonstrate a decline in proteins involved in both dark and light reactions of carbon fixation.

Senescence of leaves is associated with a decline in photosynthetic capability, and this is due, at least in part, to decreased synthesis of photosynthetic enzymes.

For example, in wheat and soybean leaves, the amount of translatable mRNAs encoding the large and small subunits of RuBisCO appear to decline simultaneously with the progression of senescence (Jiang *et al.*, 1993; Spiers and Brady, 1981). Synthesis of thylakoid membranes proteins including the α and ß subunits of ATPase, the 68 kDa protein of photosystem I (PSI) and LHCPII also decrease during senescence (Roberts *et al.*, 1987). In barley, at least 85% of the decline in soluble protein is due to the degradation of RuBisCO (Friedrich and Huffaker, 1980). It is of interest that the synthesis of the D-1 protein of PSII (also known as the 32-kDa herbicide-binding protein) continues unchanged even in senescent bean leaves that demonstrate an 85% decline in non-cyclic photosynthetic electron transfer (Roberts *et al.*, 1987). From such data it could be argued that the decline in protein content is regulated and as a consequence there is a differential decline in the content of specific proteins.

Regulated decline in protein content is also observed from the study of changes in thylakoid membranes. Non-cylic photosynthetic electron transport activity declined by about 80% during senescence of bean leaves (Jenkins and Woolhouse, 1981). This decline is considerably greater than the decline in PSI or PSII activity. Consequently, it was proposed that the rate of electron transfer between PSI and PSII may become rate limiting in photosynthetic electron transport in senescent leaves (Jenkins and Woolhouse, 1981). Subsequent analysis has demonstrated that there is a decline in the activity of the cytochrome f/b6 complex (Holloway *et al.*, 1983). Immunological studies have demonstrated that in senescing bean and oat leaves cytochromes *f* and *b6* are depleted before other photosynthetic thylakoid proteins, although all the major thylakoid localised proteins eventually decline (Ben David *et al.*, 1983; Roberts *et al.*, 1987).

In general, most estimates indicate that the rate of protein synthesis declines during senescence (for review see Brady, 1988). In expanded leaves of *Perilla*, undetectable amounts of radiolabelled precursors became incorporated into RuBisCO and consequently indicate that the decline in protein amount is due primarily to cessation of synthesis. This has also been observed in cucumber cotyledons and *Capsicum* leaves (Brady, 1988). The relative rate of synthesis of other chloroplast proteins also decline during senescence of spinach leaves (Silverthorne and Ellis, 1980). However the synthesis of the 32 kDa herbicide-binding polypeptide of PSII remained unchanged. Subsequently it has been

observed that it is rapidly synthesised in senescent bean leaves (Roberts *et al.*, 1987). It has been proposed that the continued turnover of this polypeptide indicates a functional chloroplast system for protein synthesis still exists in senescent tissue.

Ubiquitin is a small abundant protein present in all cells including higher plants (Viestra, 1987). Ubiquitin binds to proteins and has been shown to degrade proteins. In higher plants, it has recently been demonstrated that ubiquitin is involved in the degradation of phytochrome (Shanklin *et al.*, 1987; Jabben *et al.*, 1998a, b). However, the role of ubiquitin during senescence has not been determined.

1.5. Project aims.

The work presented in this thesis is based on the observation that the activity of MS and ICL increased in detached, dark-incubated barley leaves (Gut and Matile, 1988). Additionally, it was subsequently reported that synthesis of MS occurs in detached and senescent organs of cucumber (Graham *et al.*, 1992). Additionally, a model was presented that proposes that the expression of the gene encoding MS is controlled by metabolites (Graham *et al.*, 1992). The role of the glyoxylate cycle during post germinative growth has been studied extensively. Consequently, cDNA clones and specific antibodies are available to allow an investigation the expression of MS and ICL genes during senescence and in detached cucumber cotyledons. It is anticipated that a role for glyoxylate cycle during senescence may be determined from such analysis. In addition, the use of protoplasts and detached cotyledons provides a useful experimental system in which factors that may regulate the synthesis of MS and ICL can be determined. In particular, the effect sucrose has on the expression of genes encoding MS and ICL.

CHAPTER 2

MATERIALS AND METHODS

2.1. Biological Material

2.1.1. Plant Material

Cucumber seed (*Cucumis sativus* L. cv. "Masterpiece") was obtained from W. K. McNair, Portobello, Edinburgh. Plants were grown as described previously (Becker *et al.*, 1978): Seed was imbibed overnight at 4 °C in distilled water, then planted in a tray of vermiculite. Seedlings were subsequently incubated in a 12 h photoperiod under a light intensity of 130 μEinsteins m^{-2} s^{-1}, at a constant temperature of 25 °C. For experiments involving detached cotyledons seedlings were grown under these conditions for 14 days. Cotyledons were detached and placed, adaxial side upwards, on moist filter paper in petri dishes and sealed with Parafilm (American Can Co.). Petri dishes of dark treated cotyledons were wrapped in aluminium foil and placed adjacent to light treated cotyledons that were incubated under the same conditions used to grow cucumber seedlings. In experiments concerned with feeding of metabolites to detached, dark-incubated cotyledons, media was made with 2.35 g l^{-1} MS basal medium and buffered to pH 5.8 with 0.1 M KOH. In experiments concerned with cotyledonary senescence seed were imbibed overnight and grown at high density (1-2 seedlings cm-2) and incubated under a 12 h photoperiod with a light intensity of 120 μ Einsteins m-2 s^{-1} at a constant temperature of 25 °C.

2.1.2. *Escherichia coli* strains and their genotypes.

Table. 2.1. Strains of bacteria used in this study and their genotypes.

Strain	Genotype
NM522	*F-, supE, thi, D(lac-proAB), Dhsd5 (r-m-),[F', proAB, laclqZDM15].* (Messing, 1979).
JM101	*supE, thi, D(lac-proAB),[F',traD36, pro AB+, laclqZDM15].* (Messing, 1979).

E. coli was grown at 37 °C on minimal medium (MM) solid medium (15 g l^{-1} Bacto agar, 2 g l^{-1} $(NH_4)_2SO_4$, 14 g l^{-1} K_2PO_4, 1 g l^{-1} Na citrate, 0.2 g l^{-1}

$MgSO_4$, 0.2 % (w/v) glucose, 1 mg ml^{-1} thiamine hydrochloride). Bacterial strains transformed with plasmid DNA were grown at 37 °C on solid Luria broth (Bacto tryptone 5 g l^{-1}, Bacto yeast 10 g l^{-1}, 1% (w/v) Bacto agar (Difco Labs, Detriot), 10g Sodium chloride, pH 7.2). Medium was sterilised by autoclaving as described in 2.4.6.

2.1.3. Bacterial Plasmids.

Table 2.2. Sources and descriptions of plasmids.

Plasmid	Source
pBS	Stratagene.
pBSMS1.9	A full length cDNA clone encoding cucumber malate synthase (Graham *et al.*, 1989).
pBSICL1.4	A partial cDNA clone encoding cucumber isocitrate lyase, Gift from Wayne Becker (unpublished).
pBSH18	A full length cDNA clone encoding a cucumber NADH-dependant hydroxypyruvate reductase (Greenler *et al.*, 1989).
pBS/1539	A partial cDNA clone encoding a small subunit of ribulose-1,5-bisphosphate carboxylase oxygenase from cucumber (Greenland, *et al.*, 1987).
pBS/989	A partial cDNA clone encoding the chlorophyll *a/b* binding protein from cucumber (Greenland, *et al.*, 1987).

2.1.4. Antiserum.

Table 2.3. Sources of all antisera are outlined |.

Antisera	Source
Anti-MS	Polyclonal antisera raised against purified preparations of cucumber MS (Riezman *et al.*, 1980). Antibody recognise single polypeptide in westerns with an apparent molecular mass of 63 kDa (Smith and Leaver, 1986).
Anti-ICL	Polyclonal antisera raised against purified preparations of cucumber ICL (Lamb *et al.*, 1980). Antibody recognise single polypeptide in westerns with an *Mr* 63,500.
Anti-SGAT	Polyclonal antisera raised against purified preparations of cucumber SGAT (Hondred *et al.*, 1985). Antibody recognise two polypeptides in westerns with apparent molecular mass of 47 and 45 kDa.

Antisera had been stored freeze-dried at -40 °C for 6-8 years. Samples were diluted 1:2000 for anti-MS and anti-SGAT and 1:20,000 for anti-ICL in TBS-Tween (20 mM Tris, 137 mM NaCl, 0.5% (v/v) Tween-20, pH 7.6). Following use diluted primary antibody was stored at -20 °C and used 3-4 times in subsequent experiments.

2.2. Plant growth analysis.

2.2.1. Cotyledon cell number determination.

Determination of cotyledon cell number was based on protocols reported by Rickless and Brown, (1949) and Becker *et al.* (1978). Tissue was incubated in 5 ml 5% (w/v) chromic acid at 4 °C for 48 h and broken up by gently passing through a Pasteur pipette. Cell number from aliquots of sample were counted using a haemocytometer slide.

2.2.2 Cotyledon dry and fresh weight determination.

Cotyledon fresh weight was determined to the nearest 10 mg and then dried in a forced draught oven until no change in dry weight to the nearest 1 mg occurred.

2.2.3. Carotenoid and chlorophyll determination.

Cotyledonary pigment was determined by the method described previously (Holm, 1954). Cotyledon tissue was frozen with liquid nitrogen, ground to powder using a mortar and pestle and 3 volumes of acetone was added. Extracts were incubated at 65 °C for 5 min, cooled and centrifuged at room temperature at 2,500 *g* for 2 min. The supernatant was removed to fresh tubes and chlorophyll extracted from the pellet a further two times, or until the pellet became colourless. Absorbance at 662, 644 and 441 nm was estimated. Concentrations of chlorophyll *a*, *b* and carotenoids were determined using the following equations.

Chlorophyll a (μg ml^{-1}) = 9.78(A662) + 0.99 (A644)

Chlorophyll b (μg ml^{-1}) = 21.4(A644) + 4.65 (A662)

Carotenoids (μg ml^{-1}) = 4.69(A441) - 0.267 (chlorophyll a + chlorophyll b)

2.2.4. Extraction and analysis of lipid.

To prevent degradation of lipid all manipulations were carried out on ice or at 4 °C. Cotyledon tissue was frozen in liquid nitrogen and ground to a powder using a mortar and pestle, transferred to cooled tubes containing 3 volumes of chloroform: methanol: acetic acid (100:50:1 v/v) and vortexed. Samples were centrifuged at 2,500 *g* for 5 min and the lower phase removed to fresh tubes. Protein was removed from the organic phase by the addition of 1/4 volume 100 mM KCl followed by centrifugation at 2,500 *g* for 10 min. The lower organic phase was removed to fresh tubes and dried using a vacuum centrifuge. The pellet was resuspended in chloroform: methanol: acetic acid (100:50:1 v/v) to a final concentration of 10μl per cotyledon and stored at -20 °C before application of samples to TLC plates.

TLC analysis was carried out on "Merck" silica gel TLC plates. Two solvent mixes were used for separation; acetone: benzene: water (91:30:8 (v/v), Phol, *et al.*, 1970) and chloroform: methanol: water: glacial acetic acid (170: 30: 20:7 (v/v), Nichols *et al.*, 1963). Total cotyledonary lipid equivalent to one cotyledon and 50 μg lipid standards (Lipid Products, Surrey) were spotted onto the plate and separation carried out in a TLC tank until the solvent front was approximately 1 cm from the top of the plate. The plate was removed from the tank and dried at room temperature for 5 min. Lipid was visualised by spraying the plate with

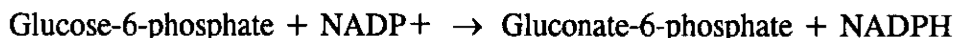
H_2SO_4: methanol (1:1, v/v) and baking the plate at 140 °C for 2-3 min. Lipid classes were identified by comparison with standard lipids and characteristic relative mobility.

2.2.5. Sucrose determination.

Determination of sucrose content was based on enzymatic conversion of sucrose to glucose and the subsequent inversion to glucose to form gluconate 6-phosphate. Firstly, endogenous glucose is determined and following inversion the amount of sucrose determined. At pH 7.6, hexokinase (HK) catalyses the phosporylation of of D-glucose.

D-Glucose + ATP $\rightarrow$ Glucose-6-phosphate + ADP

In the presence of glucose-6-phospahate dehydrogenase (G-6-P DH), glucose-6-phosphate is converted to gluconate-6-phosphate, this oxidisation is coupled with the reduction of NADP+ to NADPH



Glucose-6-phosphate + NADP+ $\rightarrow$ Gluconate-6-phosphate + NADPH

The amount of NADPH produced is stochiometric with the amount of glucose and is measured by absorbance at 340 nm.

Sucrose is hydrolysed to glucose and fructose by the enzyme ß-fructosidase (invertase).

Sucrose + H_2O $\rightarrow$ D-Glucose + D-Fructose

The determination of glucose (after inversion) is then carried out according to the principle outlined above. The sucrose content is calculated from the difference in the glucose concentration before and after enzymatic inversion.

Cotyledons were frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle and transferred to tubes containing 3 volumes of methanol: chloroform: water (12: 5: 3). Samples were votrexed and centrifuged at 12,000 *g* for 5 minutes. The pellet was discarded and the sucrose content of the supernatant

was determined using a sucrose determination kit (Boehringer Mannheim). Firstly, the glucose present in the sample is hydrolysed by the addition of 100 μl of Solution 1 (Tris-Cl pH 6.7, 2.5 mg ml^{-1} NADP, 6 mg ml^{-1}) and 500 μl solution 2 (290 U ml^{-1} HK, 145 U ml^{-1} G-6-P DH) to 50 μl of sample and incubation at 25 °C for 5 mins. The absorbance at 340 nm was determined in a Phillips PU8625 spectrophotometer. Following complete conversion of glucose to gluconate, determined by no change in A340, 10 μl of supension 3 (citrate buffer, pH 4.6; 7.2 U ml^{-1} ß-fructosidase). Following incubation at 25 °C the absorbance at 340 nm was determined.

The concentration of sucrose was determined from the following equation.

$$C = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A \ (mg\ ml^{-1})$$

V = final volume (ml)

v = sample volume (ml)

MW = molecular weight of the substance to be assayed (sucrose 342.30)

d = light path (cm)

ε = absorption coefficient of NADPH at 340 nm (6.3, l x $mmole^{-1}$ x cm^{-1})..

2.2.6. Protoplast isolation and culture.

Cucumber plants were grown as described in section 2.1.1. for 7 days. Detached cotyledons were placed in 70% (v/v) ethanol for 1 min and transferred to 0.5% (w/v) Na hypochlorite for 10 min. Na hypochlorite was removed by rinsing cotyledons 4-5 times with sterile dd H_2O Cotyledon tissue was transferred to petri dishes containing 475 mM mannitol, 25 mM sucrose, 0.7% (w/v) cellulase Onozuka R10 (Yakult Honsha Co. Ltd., Japan) and 1% (w/v) macrozyme R10 (Yakult Honsha Co. Ltd., Japan) and sliced using a sharp scalpel. Tissue was then incubated for 12-14 h at room temperature in the dark with continuous shaking.

Protoplasts were passed through 1 layer of muslin to remove large cell debris, transferred to sterile 50 ml Corning tubes and centrifuged at 50 *g* at room temperature for 5 min. The pellet was gently resuspended in 25 ml culture medium (500 mM mannitol, 0.235% (w/v) Murashige and Skoog medium, 0.89 μM 6-benzylaminopurine, 0.45 μM 2,4-dichlorophenoxyacetic acid, 5.37 μM α-

Naphthaleneacetic acid) and centrifuged at 50 *g* for 5 min. The pellet was subsequently washed a further two times. Protoplast yield and viability was determined using a haemocytometer slide. Protoplasts were incubated in culture medium supplemented with different carbon compounds in 15 ml Corning tubes and wrapped in aluminium foil for 48 h at 25 °C. Protoplasts were harvested by centrifugation at 50 *g* for 5 min and resuspended in 1x Laemmli buffer.

2.3. Molecular Biology Techniques.

2.3.1. General conditions.

All microfuge tubes, pipette tips and solutions containing heat stable compounds were sterilised by autoclaving at 15 p.s.i. at 120°C for 20 min Autoclaved double distilled water (ddH_2O) was used to make up all solutions that contained heat labile compounds and then subsequently filter sterilised by passing solution through a 0.2 μm filter (Gelman Science, USA). All glassware was baked at 120 °C for at least 12 h. For the extraction and analysis of RNA, diethyl pyrocarbonate was added to all solutions to a final concentration of 0.1% (v/v) and incubated at 37 °C for 12-16 h before autoclaving. All manipulations were carried out on ice unless otherwise stated and latex gloves were worn to prevent contamination of samples by nucleases.

2.3.2. Quantification of nucleic acid solutions.

The absorbance of samples at 260 nm and 280 nm in a Phillips spectrophotometer was used to estimate the purity and yield of nucleic acid solutions. A260 of 1.0 was taken as a concentration of 50 μg ml^{-1} for double stranded DNA and 40 μg ml^{-1} for RNA. An A260/A280 ratio of 2.0 for RNA and 1.8 DNA for was taken as an indication of the absence of contaminating protein or phenol.

2.3.3. Small-scale isolation of plasmid DNA.

Isolation of plasmid DNA from small culture volumes of *E. coli* was carried out in 1.5 ml microfuge tubes and is based on the procedure described by Holmes and Quigley (1981). A single colony was used to inoculate 5 ml LB containing appropriate antibiotic and grown for 6-12 h at 37 °C. 1.5 ml of the culture was harvested by centrifugation in a microfuge for 2 min at room temperature, the

supernatant was discarded and pellet resuspended in 250 μl STET solution (8% (w/v) sucrose, 5% (w/v) Triton X-100, 50 mM EDTA, 50 mM Tris-Cl pH 8.0). 20 μl freshly prepared 10 mg ml^{-1} lysozyme in STET was added, samples were heated in a dry block at 100 °C for 1 min and placed on ice. An equal volume of 5 M LiCl was added and the samples incubated on ice for 5-30 min. Samples were centrifuged at 4°C for 15 min and pellet removed using a sterile toothpick. The supernatant was precipitated, with sufficient ice-cold ethanol to fill the tube, at -80 °C for 30-60 min (or overnight at -20 °C). Nucleic acids were pelleted by centrifugation at 4 °C for 10 min. The pellet was washed in 70% (v/v) ethanol, dried in a Howe Gyro-Vap (V. A. Howe and Co Ltd, Banbury) and resuspended in 10-15 μl TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA). RNA was removed by the addition of 1 μl of 1 mg ml^{-1} RNase A and subsequent incubation at 37 °C for 1 h prior to electrophoresis.

2.3.4 Large-scale isolation of plasmid DNA.

Isolation of plasmid DNA was carried out according to standard methods (Sambrook *et al.*, 1989) A single *E. coli* colony was transferred, using a sterile loop, to a 5 ml culture of LB containing appropriate selective antibiotic. After overnight incubation at 37 °C, the culture was transferred to a flask containing 500 ml LB and selective antibiotic. The culture was then incubated at 37 °C until A_{650} reached approximately 1.0. Chloramphenicol was then added to a final concentration of 150 μg ml^{-1}. The culture was then incubated at 37°C for 12-16 h with continuous shaking.

Cells were harvested by centrifugation at 16,300 *g* at 4 °C and resuspended in 3 ml sucrose mix (25% (w/v) sucrose, 50 mM Tris-Cl pH 8.0, 40 mM EDTA). 500 μl freshly made 10 mg ml^{-1} lysozyme in sucrose mix and 500 μl 500 mM EDTA, pH 8.0 was added and the sample gently mixed on ice for 5 min. 6 ml of Triton mix (1% (v/v) Triton X-100, 50 mM Tris-Cl pH 8.0, 40 mM EDTA) was added, the sample mixed gently and left on ice for 10 min. After bacterial lysis (as judged by increased viscosity of the sample) the lysate was centrifuged at 39,000 *g* for 45 min at 4 °C. The supernatant was poured into a sterile measuring cylinder, ensuring that as much of the pellet was retained in the centrifuge tube as possible. The volume of the supernatant was then made up to 16 ml with TE buffer, 15.20 g CsCl was dissolved in this sample and 800 μl 5 mg ml^{-1} ethidium bromide was then added. The sample was placed in a Beckmann heat sealing tube and liquid

paraffin was added to fill the tube. Ultracentrifugation was carried out at 110,000 *g* at 18 °C for 48 h.

After establishment of the gradient, a 19 gauge syringe needle was inserted into the top of the tube. A second 19 gauge syringe needle was then inserted into the tube, below the band of plasmid DNA. Using a 1 ml syringe, plasmid DNA was removed to a sterile glass test tube. An equal volume of isopropanol saturated with NaCl solution was added to the sample, the two phases were mixed and the sample left on ice for 2-3 min. The upper phase was removed and fresh isopropanol was added, this was repeated until the lower aqueous phase became colourless. CsCl was diluted by the addition of 2 volumes of TE buffer and DNA precipitated by the addition of 6 volumes of ice-cold ethanol and placed at -20°C for 12-16 h. Plasmid DNA was pelleted by centrifugation at 16,300 *g* for 10 min at 4 °C. The resultant pellet was gently resuspended in 3 ml TE buffer and 300 μl 3 M Na acetate pH 5.5 was added. DNA was precipitated again by the addition of 2.5 volumes ice-cold ethanol and samples were left at -20 °C for 12-16 h, or at -80 °C for 30 min. After centrifugation at 16,000 *g* for 10 min at 4 °C, the pellet was resuspended in 100-200 μl TE buffer. Purity and yield of the preparation was determined by measuring the absorbance of the sample at 260 and 280 nm in a Phillips PU8625 spectrophotometer.

2.3.5. Isolation of cloned DNA fragments.

DNA was digested with the appropriate restriction enzyme according to suppliers instructions, size fractionated by electrophoresis through a 0.8% (w/v) agarose gel containing 1x TAE (40 mM Tris, 5.7ml/l glacial acetic acid, 1 mM EDTA) and 0.1 μg ml^{-1} ethidium bromide. Fragments were visualised by illumination at 312 nm and were subsequently isolated using a "Geneclean" kit (UBB, Ohio. no. BIO 101). The piece of agarose containing the restriction fragment to be isolated was cut from the gel and 2.5 - 3 volumes of NaI stock solution added (supplied with kit assuming 1 g agarose = 1 ml NaI). The agarose slice was then melted at 55 °C for 5 min. "Glassmilk" (supplied with kit) was added (5 μl for <5 μg DNA and an additional 1 μl for every 0.5 μg above 5 μg) and the contents gently mixed and incubated on ice for 5 min. The DNA-"Glassmilk" complex was pelleted by centrifugation for 5 sec in a microfuge and the supernatant discarded. The pellet was washed with 500 μl ice-cold "NEW" solution (supplied with kit) by vortexing and the complex pelleted by centrifugation

for 5 seconds. The pellet was washed with "NEW" solution an additional two times. DNA was eluted from the pellet by addition of 5-10 μl TE buffer or sterile double distilled water, incubation at 50°C for 2-3 min and centrifugation for 2 min. The DNA solution was removed to a fresh microfuge tube, the pellet was re-eluted a second time. Yield was determined by electrophoresis of an aliquot through a 0.8% (w/v) agarose gel containing 1x TAE and 0.1 μg ml^{-1} ethidium bromide.

2.3.6. Plasmid transformation of E. coli.

Transformation of *E. coli* cells (JM101 or NM522) with plasmid DNA was routinely carried out by a standard method described by Cohen *et al.* (1972) and Sambrook *et al.* (1989).

A single bacterial colony was picked from a petri dish containing solid minimal medium (see sections 2.1.2.) and was used to inoculate 5 ml sterile LB. The culture was grown overnight at 37 °C with continuous shaking. 1 ml of the culture was used to inoculate 100 ml LB, incubated at 37 °C with continuous shaking until A600 reached 0.2-0.4 and decanted to two sterile 50 ml corning tubes and incubated on ice for 10-15 min. The cells were centrifuged at 2,500 *g* for 10 min at 4 °C. The supernatant was carefully decanted and the tubes left to drain for 2-3 min. The bacterial pellet was then resuspended in 25 ml sterile 100 mM CaCl2 and left on ice for 10-15 min. Centrifugation of the samples was repeated and the pellets resupended in 1 ml ice-cold 100 mM CaCl2.

1-50 ng plasmid DNA, or ligation mix in a volume up to 10 μl was mixed with 200 μl competent cells and 100 μl transformation mix (10 mM Tris-Cl pH 7.2, 1 mM EDTA, 1 mM MgCl2) and incubated for 30 min on ice. Samples were incubated at 42 °C for 2 min, 1 ml of LB was added and cells incubated at 37 °C for 1 hour. Cells were then centrifuged in a microfuge at high speed for 2 min at room temperature, resuspended in 250 μl LB and an aliquot was plated on LB agar containing the appropriate antibiotic. Transformants were selected after overnight incubation at 37 °C.

2.3.7. Small-scale isolation of total DNA from higher plant tissue.

Isolation of genomic DNA from small amounts of tissue was based on the method described by Dellaporta *et al.* (1982). 1 g of tissue was frozen in liquid nitrogen and immediately ground to a fine powder using a mortar and pestle. 4.5 volumes extraction buffer (100 mM Tris-Cl pH 8.0, 500 mM NaCl, 50 mM EDTA, 10 mM ß-mercaptoethanol) was added and the extract divided equally between six microfuge tubes. 100 μl 10% (w/v) SDS was immediately added, samples incubated at 65 °C for 20 min, centrifuged at room temperature for 5 min and the supernatant removed to fresh tubes. Precipitation of protein from the supernatant was achieved by the addition of 250 μl of 5 M K acetate pH 5.5 and subsequent incubation on ice for 10-20 min. Protein was pelleted by centrifugation for 5 min and the supernatant removed to fresh tubes. 600 μl ice-cold isopropanol was added to the supernatant and samples incubated at -20 °C for 20-30 min. Samples were centrifuged at room temperature for 5 min and the pellet was resuspended in 700 μl TE buffer. 75 μl 3 M Na acetate pH 5.5 was added, nucleic acids precipitated a second time by the addition of 500 μl isopropanol and incubation at room temperature for 10 min. After centrifugation the pellet was washed with 70% (v/v) ethanol and the pellet partially dried. The pellet was resuspended in 20-30 μl TE buffer or sterile ddH_2O and incubated at 65 °C for 10 min. Samples were pooled and yield determined by electrophoresis of a 10 μl aliquot on a 0.8% (w/v) agarose gel containing 1x TAE and 0.1 mg ml^{-1} ethdium bromide. Samples were pre-treated by incubation with 1 μl 1 mg ml^{-1} RNase A for 5-10 min at 37 °C prior to electrophoresis.

2.3.8. Isolation of total RNA from higher plant tissue.

Isolation of higher plant total RNA was by a modification of Parish and Kirby (1966). 1-2 g plant material was frozen in liquid nitrogen, ground to a fine powder using a baked mortar and pestle and was immediately transferred to cooled tubes containing 5 volumes of extraction buffer (100 mM Tris-Cl pH 8.5, 6% (w/v) *para*-aminosalicylic acid, 1% (w/v) Tris-isopropylnapthalene). An equal volume of phenol : chloroform (1:1, v/v) was added and samples immediately vortexed for 10-15 secs. Samples were centrifuged a 4 °C for 10 min at 2,500 *g*. After centrifugation the upper aqueous phase was transferred to baked Corex tubes. Nucleic acids were precipitated by the addition of 0.1 volumes 3 M Na acetate pH 5.2, 2.5 volumes of ice-cold ethanol and subsequent incubation at -20 °C for 3 h.

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Precipitated nucleic acids were pelleted by centrifugation at 16,300 *g* at 4 °C. Pelleted nucleic acids were resuspended in 2-3 ml sterile ddH_2O. Total RNA was selectively precipitated by the addition of an equal volume of 5 M LiCl and incubation at 4 °C for 12-16 h. Total RNA was pelleted by centrifugation at 16,300 *g* at 4 °C, resuspended in 300 μl sterile ddH_2O and precipitated a second time in the presence of 0.3 M Na acetate by the addition of two volumes of ice-cold ethanol and incubation at -20 °C for 3 h. After centrifugation at 16,000 *g* at 4 °C the pellet was dried and resuspended in 40 μl sterile ddH_2O. Purity and yield of RNA was determined by measuring absorbance at 260 and 280 nm as described previously..

2.3.9. Radiolabelling of DNA probes.

Labelling of DNA with α-[32P} dCTP was by the random primer method (Feinberg and Vogelstein, 1984). 30-50 ng of the DNA fragment to be labelled was isolated and denatured by incubation in a Dri Block for 2-3 min at 100 °C and then immediately cooled on ice. Sterile ddH_2O was added to give a final volume of 16 μl, 5 μl OLB mix (250 mM Tris-Cl, pH 8.0, 25 mM MgCl2, 5 mM ß-mercaptoethanol, 2 mM dATP, 2 mM dTTP, 2 mM dGTP, 1 M HEPES, pH 6.6, 1 mg ml^{-1} oligonucleotides), 1 μl 10 mg ml^{-1} BSA, 1 unit of DNA polymerase I (Klenow, BRL) and 2 μl α-[32P] dCTP was added. Samples were incubated at room temperature for 12-16 h or at 37 °C for 1 h and the reaction stopped by the addition of 200 μl sterile ddH2O. Unincorporated nucleotides were separated from radiolabelled DNA by passing the reaction mix through a 1 ml syringe packed with Sephadex G-50 by centrifugation at 750 *g* for 2 min and collecting the eluate. Samples were then stored at -20 °C until used. Prior to use, radiolabelled DNA was denatured by heating at 100 °C for 2-3 min in a Dri-Block and immediately cooled by being placed on ice.

2.3.10. Northern blot analysis.

5-10 μg total RNA was made up to a volume of 15 μl with sterile ddH_2O and 45 μl RNA denaturing solution was added (64% (v/v) formamide, 8% (v/v) formaldehyde, 6 μl 10x MOPS, 150 ng ml^{-1} ethidium bromide). Samples were heated at 65 °C for 5 mins, size fractionated by electrophoresis thorough a 1.4% (w/v) agarose gel containing 40% (v/v) formaldehyde and 1x MOPS buffer (20 mM MOPS, 5 mM Na acetate pH 7.0, 1 mM EDTA), using 1x MOPS as the

electrophoresis buffer. Transfer of RNA to Hybond-N (Amersham) was carried out using 20X SSPE (3.6 M NaCl, 200 mM Na phosphate pH 7.7, 20 mM EDTA) as the transfer buffer. After overnight transfer the filter was washed briefly in 2x SSPE, left to dry for 2-3 h at room temperature. RNA was fixed to the membrane by irridiation with 0.4 J cm^{-2} using a Hybaid crosslinker (Hybaid, Middlesex). The filter was stored wrapped in Saran wrap (Dow Chemical company) at 4 °C until prehybridisation.

Prehybridisation was carried out by incubating the filter in 5x SSPE, 20% (v/v) formamide, 5x Denhardt's solution, 0.5% (w/v) SDS and 100 μg ml^{-1} denatured herring sperm DNA at 42 °C for 3 h. Hybridisation was carried out overnight under the same conditions with the additional presence of denatured radiolabelled probe. After hybridisation the filter was washed twice with 2x SSPE, 0.1% (w/v) SDS at 42 °C for 15 min and once for 30 min with 1x SSPE, 0.1% (w/v) SDS at 42 °C. The filter was then washed in 0.1x SSPE, 0.1%(w/v) SDS for 15 min at room temperature, blotted dry and sealed in Saranwrap. Autoradiography was carried out in as described in section 2.4.7.

Removal of probe from membranes was achieved by incubating the filter in 5 mM Tris-Cl pH 8.0, 2 mM EDTA, 0.1x Denhardt's solution at 65°C for 1-2 h. Autoradiography was undertaken to determine the extent of probe removal.

2.4. Immunoblot analysis.

2.4.1. Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS PAGE).

SDS PAGE was carried out essentially as described (Laemmli, 1970). Glass plates were cleaned thoroughly and assembled using matching gel spacers and comb. Acrylamide solution (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 made and immediately poured between the plates to about 2-3 cm from top of the plate, H_2O-saturated butanol was overlayed on top of the polymerising acrylamide. The gel was subsequently left to polymerise for 30-60 min. The stacking acrylamide solution was prepared (5% (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) and immediately cast to the top of the cut out plate. The comb was

(100x Denhardt's Solution
2% (w/v) ficoll
2%(w/v)polvinylpyrrolidone
2% (w/v) BSA)

then carefully inserted into the stacking gel solution. After the stacking gel had polymerised the comb was carefully removed and wells straightened using a toothpick. The gel apparatus was then mounted onto the electrophoresis tank.

Tissue was frozen in liquid nitrogen and ground to a powder using a mortar and pestle and transferred to cold corning tubes. The homogenate was prepared by the addition of 1.5 volumes of Laemmli sample buffer (2% (w/v) SDS, 10% (v/v) glycerol, 60 mM Tris-Cl pH 6.8, 100 mM DTT, 0.005% (w/v) bromophenol blue). Samples were vortexed, incubated at 100 °C for 10 min, centrifuged at 2,500 *g* for 5 min at room temperature and the supernatant was stored as 100 μl aliquots at -20°C.

Prior to electrophoresis samples and molecular weight markers were heated at 85 °C for 10 min. Electrophoresis was carried out using 1x Laemmli electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS) at 25 V until the marker dye had migrated to the interface between the stacking gel and resolving gel. The voltage was subsequently increased to 50 V and electrophoresis was carried out until bromophenol blue reached the bottom of the gel.

Fractionated protein was fixed and stained by incubation of the polyacrylamide gel in coomassie stain solution (45% (v/v) methanol, 8% (v/v) glacial acetic acid, 0.05% (w/v) coomassie brilliant blue) for 30-90 min at room temperature. Excess stain was removed by successive incubations in coomassie destain solution (45% (v/v) methanol, 8% (v/v) glacial acetic acid) for 4-16 h at room temperature. The gel was dried onto Whatmann 3 MM paper using a Biorad 583 gel dryer at 80 °C for 1-2 h.

2.4.2. Transfer of protein to Hybond-ECL.

Transfer of size fractionated polypeptides to Hybond-ECL (Amersham), for the detection of MS, ICL and SGAT protein was performed essentially as described by Harlow and Lane (1988). After SDS-PAGE the gel was incubated in transfer buffer (50 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, 20% (v/v) methanol) for 15 min. Hybond-ECL and six sheets of Whatmann 3 MM paper were cut to approximately the same size as the region of gel to be transferred. Hybond-ECL membrane was immersed in distilled water for 10 min and

transferred to transfer buffer for 10-15 min. Ensuring that no bubbles were trapped in the cassette the transfer apparatus was then assembled. Transfer was carried out at 25 V for 4-16 h. After electrophoresis the filter was rinsed 3 times for 5 min with ddH_2O, stored at 4 °C overnight or air dried and stored at room temperature.

Efficiency of transfer was determined by incubating the membrane with ponceau-S solution (2% (w/v) ponceau-S, 30% (w/v) trichloroacetic acid 30% (w/v) sulfosalycilic acid) for 5-10 min at room temperature. The filter was transferred to dH2O and rinsed for 1-2 min with several changes of dH_2O. The position of molecular weight markers was marked using a pencil. Ponceau-S was removed by several washes at room temperature in TBS-Tween (20 mM Tris, 137 mM NaCl, 0.5% (v/v) Tween-20, pH 7.6).

2.4.3. Immunodetection.

All steps for immunodetection were carried out at room temperature on a shaking platform. Solutions were used in sufficient quantity to cover the membrane. Prevention of non-specific binding of antibody was achieved by incubating the filter for 1 h in TBS-Tween containing 5% (w/v) dried milk and washing once for 15 min and then twice for 5 min with TBS-Tween. The filter was then incubated in primary antibody (Table 2.3.) diluted in TBS-Tween for 1-2 h. Unbound primary antibody was removed by washing the filter in TBS-Tween four times for 15 min. Diluted primary antibody solutions were stored at -20 °C and used several times. The membrane was subsequently incubated in an anti-rabbit IgG conjugated with horseradish peroxidase diluted in TBS-Tween to suppliers instructions for 20-30 min. Unbound secondary antibody was removed with four 5 min washes in TBS-Tween.

Detection of antigens was carried out using ECL western detection reagents (Amersham). The membrane was equilibrated in ECL detection solution 2 (final volume 0.0625 ml cm^{-2} of Hybond ECL) for 1-2 min. An equal volume of ECL detection solution 1 was then added, the filter was agitated for 1 min, quickly blotted to remove excess solution, wrapped in Saran wrap and placed in a standard X-ray film holder with the side of the membrane with protein facing upwards. In darkness, a sheet of Hyperfilm-ECL (Amersham) or Cronex-4 X-ray film (Dupont,

Frankfurt) was placed on top of the filter, the cassette closed and detection carried out for 1 min. The film was removed and transferred to another empty cassette and a second sheet of X-ray film was placed on top of the filter. The first sheet of film was then developed ss described. On the basis of the extent of detection of antigens in the first exposure, the second sheet of film was exposed accordingly.

2.4.4. Bradford assay.

Samples were prepared by boiling in 1x Laemmli buffer as described in section 2.3.1. From a 100 μl aliquot, SDS was removed by passing the sample down a 1 ml syringe packed with Sephadex G-50 by centrifugation at 750 *g* for 2 min. The amount of protein was then determined using the Bio-Rad protein determination kit that is based essentially on the Bradford protocol (Bradford, 1976).

The concentrated protein-binding solution was diluted five-fold in distilled water. 100 μl of standards of known concentration and samples were mixed with 5 ml of the diluted protein-binding solution (supplied with kit) and samples incubated at room temperature for 5 min. The absorbance at 595 nm was determined using a Philips PU8625 spectrophotometer. From the values obtained from the samples of known protein concentration the amount of protein in extracts was determined.

2.5. Miscellaneous.

2.5.1. Chemicals.

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

2.5.2. Radiochemicals.

α-[32P] dCTP (3000 Ci mmol $^{-1}$) was purchased from Amersham International Plc.

2.5.3. Autoradiography.

The extent and localisation of radioactivity on Southern and northern filters was determined using a hand-held Gieger-Muller counter. The membrane was

placed in a light-tight X-ray film holder containing Cronex lighting plus intensifying screens. A sheet of Cronex-4 X-ray film was preflashed using a Metz Mecablitz flashgun and placed over the filter and the cassette was placed at -80 °C. Development of the autoradiograph was carried out depending on the amount of radioactivity detected using a Geiger-Muller counter and was typically after 2-7 days.

2.5.4. Antibiotics.

A 100 mg ml^{-1} stock of ampicillin was made using sterile double distilled water and then passed through a 0.2 μm pore size sterile filter into a sterile container. Ampicillin was added to LB or cooled LB agar to a final concentration of 50-100 μg ml^{-1} A stock solution of chloramphenicol was made to a final concentration of 50 mg ml^{-1} in ethanol and was added to a final concentration of 150 μg ml^{-1} to a culture for the amplification of plasmids during a large-scale isolation plasmid DNA. Both stocks of antibiotics were stored at -20 °C.

CHAPTER 3

INDUCTION OF MALATE SYNTHASE AND ISOCITRATE LYASE SYNTHESIS IN SENESCENT CUCUMBER COTYLEDONS.

3.1. Rationale.

The induction of MS and ICL synthesis has been observed in senescent leaves, petals and cotyledons from several plant species (De Bellis *et al.*, 1991; Graham *et al.*, 1992; Pistelli *et al.*, 1991). Although induction of glyoxylate cycle enzyme synthesis during senescence has now been clearly established, the function of MS and ICL and the factors controlling the expression of the genes encoding these enzymes is not clear. This chapter attempts to characterise features of cucumber cotyledons which may provide information concerning the function of MS and ICL during senescence. Changes in cell number, fresh weight, dry weight, chlorophyll, carotenoid, lipid, protein and total RNA were determined at weekly intervals after seed imbibition. Additionally, the changes in the abundance of specific transcripts and proteins was determined. In particular, cDNA clones encoding cucumber MS and ICL and antisera raised against purified preparation of these proteins were used to determine the nature of the induction of these enzymes, which are specific to the glyoxylate cycle, during senescence. Specific changes in components of senescing tissues are well characterised. The main aim of this chapter is to determine how the synthesis of MS and ICL synthesis correlates with some of the observable changes in cucumber cotyledons to test the hypothesis proposed by Gut and Matile (1988) that MS and ICL function in the metabolism of chloroplast lipid during natural senescence.

3.2. Developmental changes in cucumber cotyledons.

3.2.1. Determination of cotyledon cell number.

All the developmental changes observed in cucumber cotyledons during the first 7 days after seed imbibition occur in the absence of cell division (Trelease *et al.*, 1971; Becker *et al.*, 1978). Consequently, data used to describe the developmental changes in cucumber cotyledons during post-germinative growth have been expressed as a function of cotyledon number, that in effect can be expressed on a per cell basis (See Becker *et al.*, 1978; Hill *et al.*, 1992; Hondred *et al.*, 1987; Moran *et al.*, 1990; Smith and Leaver, 1986; Walden and Leaver, 1981, Weir *et al.*, 1980). To confirm

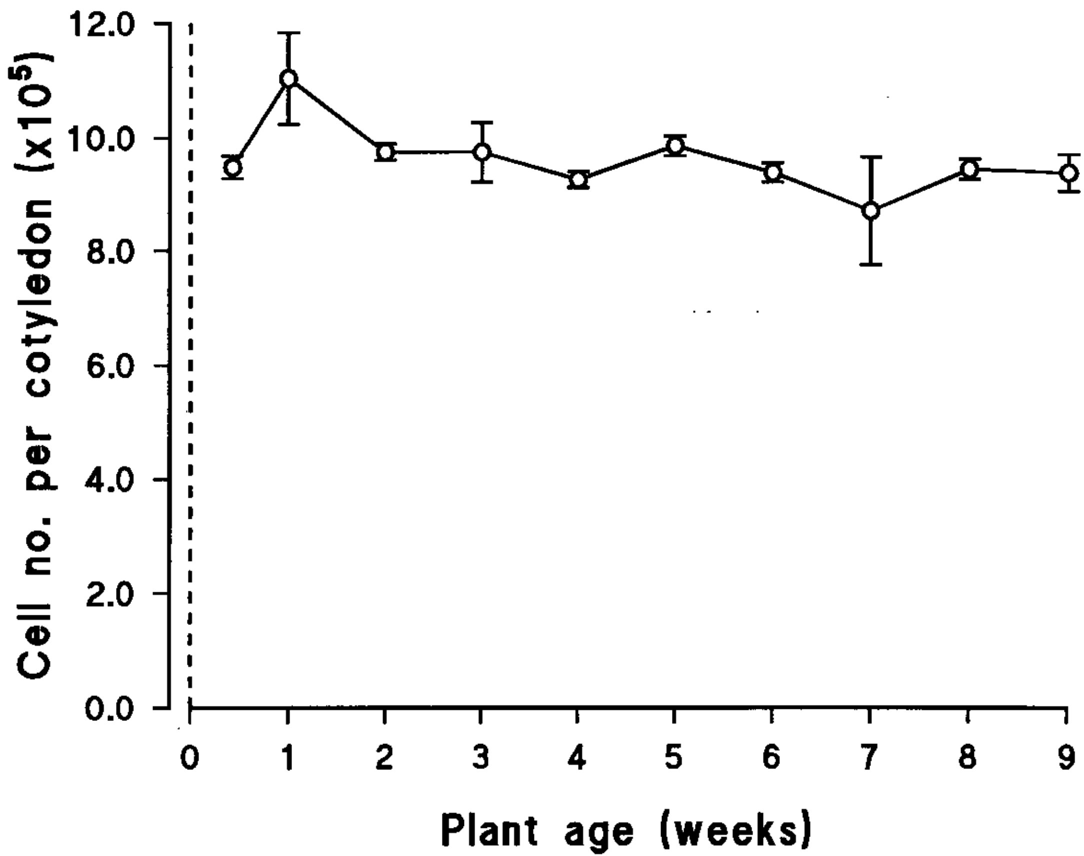


Figure 3.1. Cotyledon cell number during plant growth after seed imbibition. Average cotyledon cell number was determined 3 and 7 days after seed imbibition and at weekly intervals thereafter. Error bars represent the SEM of four replicate samples.

that no cell division occurs in mature and senescent cotyledons, cell number was determined 3 and 7 days after seed imbibition and at weekly intervals thereafter (Figure 3.1.). It is clear that cotyledon cell number does not change throughout the experimental period. Consequently any subsequent data used to describe develpmental changes can be expressed on a per cotyledon basis.

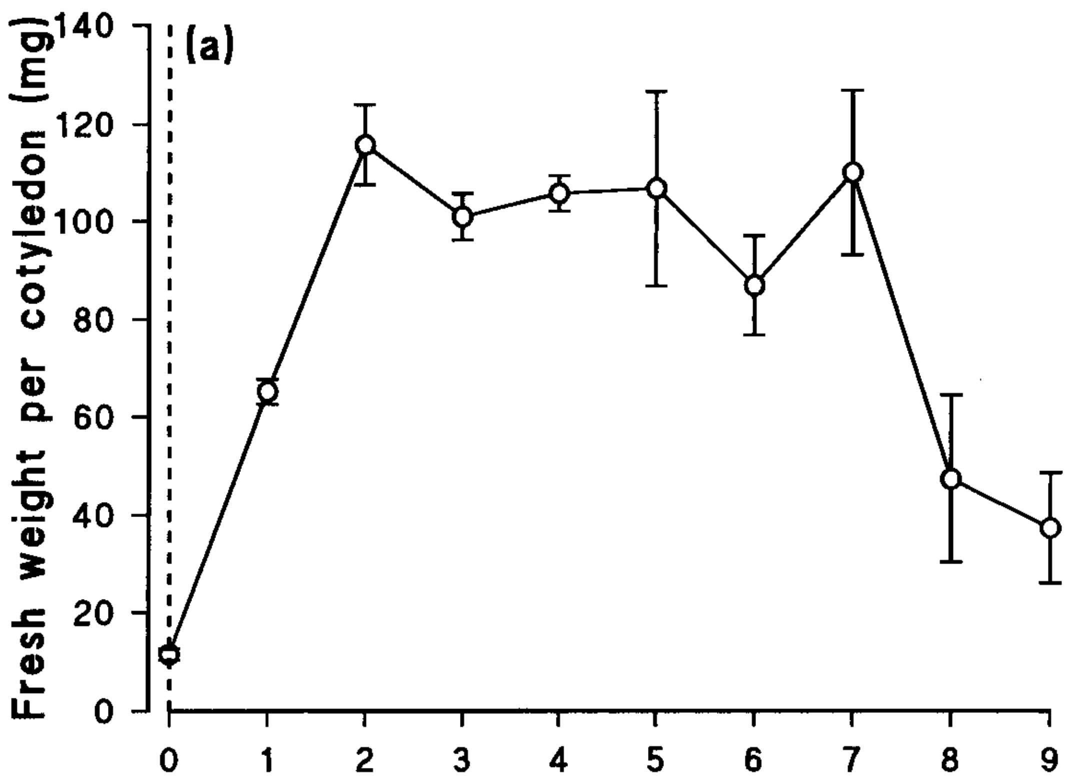
3.2.2. Fresh and dry weight analysis.

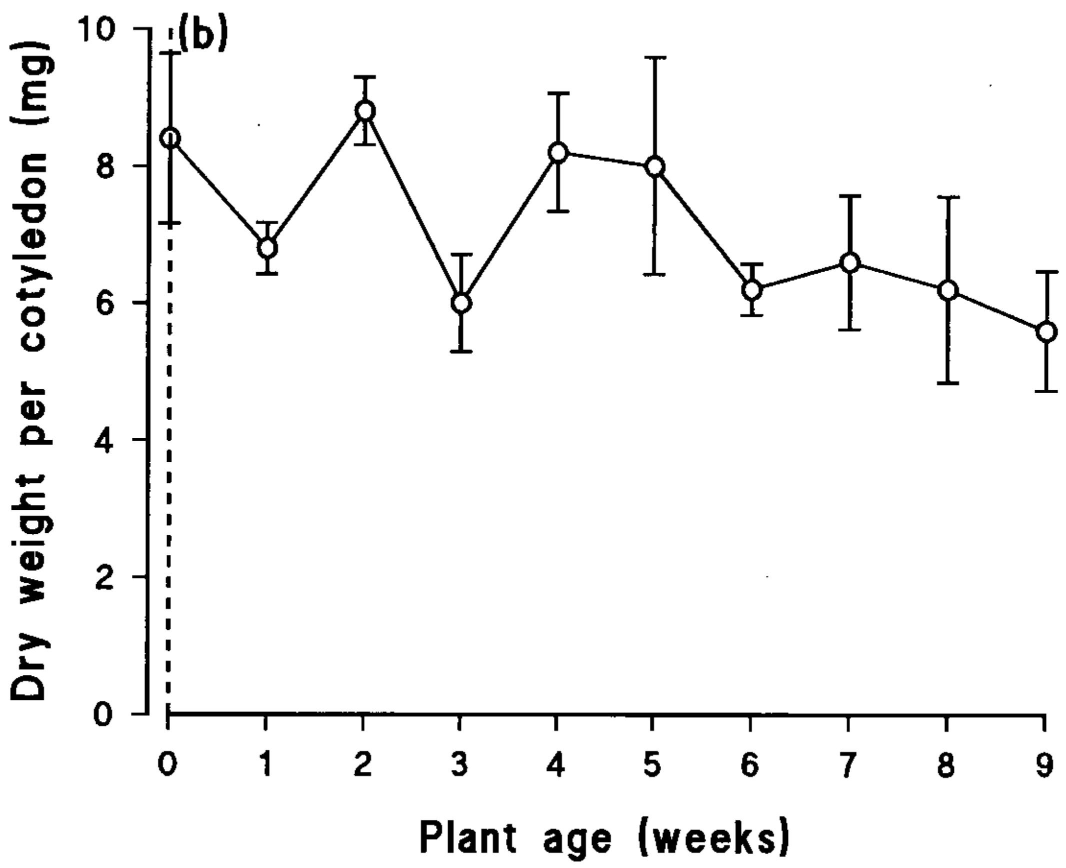
Changes in fresh and dry weight of cucumber cotyledons at weekly intervals after seed imbibition are presented in Figure 3.2. The first 2 weeks of plant growth is associated with a 10-fold increase in cotyledonary fresh weight (Figure 3.2a.). However, no significant change in dry weight occurs during this period (Figure 3.2b.), consequently indicating that increase in fresh weight is due primarily to water uptake that, presumably, supports cell expansion and photosynthesis. Additionally, as no increase in cotyledonary dry weight is observed, any excess photosynthate produced is presumably translocated to other growing parts of the plant and not stored in the organ. The average fresh weight does not appear to change significantly until 7 weeks after seed imbibition although variation between samples is observed at the latter stages of plant development (Figure 3.2a.). After 7 weeks plant growth, cotyledonary fresh weight declines. Throughout the period of study, cotyledonary dry weight remains unchanged indicating that no net accumulation of photosynthate from synthesis in the cotyledons or from import from leaves. t(Figure 3.2b.).

3.2.3. Changes in cotyledonary pigment.

Senescence is most obviously characterised by chlorophyll loss (Thomas and Stoddart, 1980) and was previously correlated with MS and ICL synthesis in detached, dark-incubated barley leaves (Gut and Matile, 1988). Consequently, levels of cotyledonary chlorophyll were determined at weekly intervals after seed imbibition. Carotenoid levels were also determined to provide additional information concerning the decline in chloroplast structure associated with senescence.

Figure 3.2. Developmental changes in cotyledon weight at weekly intervals after seed imbibition. (a) change in fresh weight. (b) change in dry weight. Error bars represent the SEM of five independent replicates.

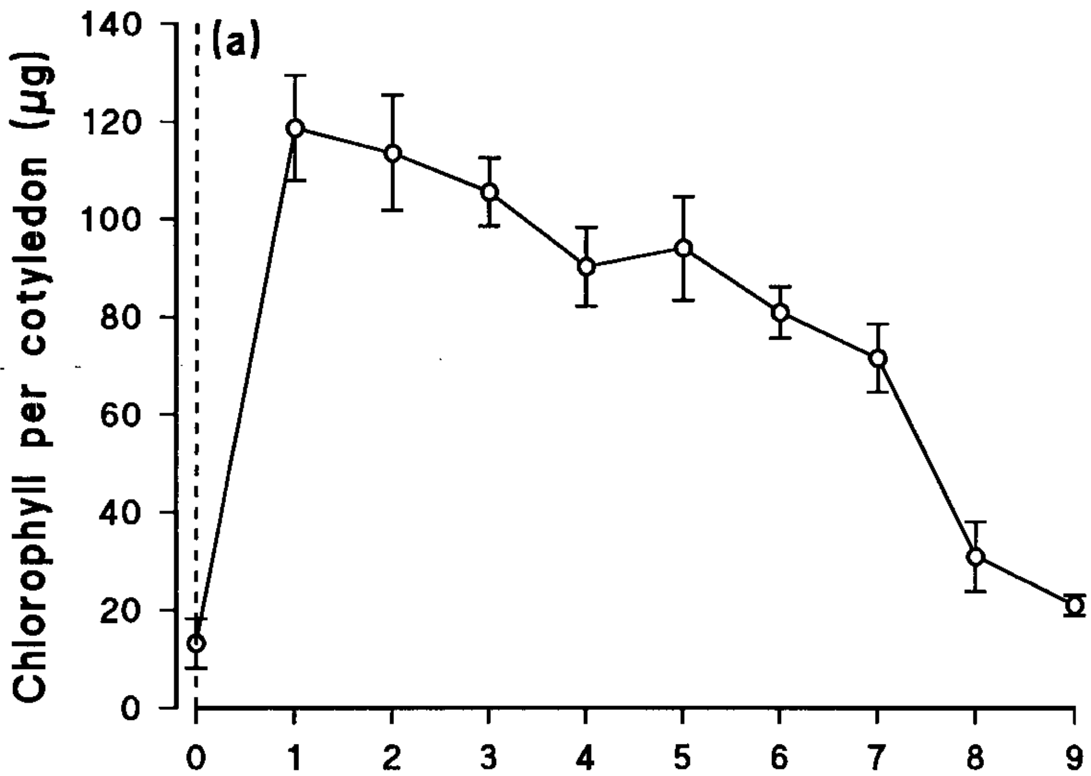

(a)
Fresh weight per cotyledon (mg)
140
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9

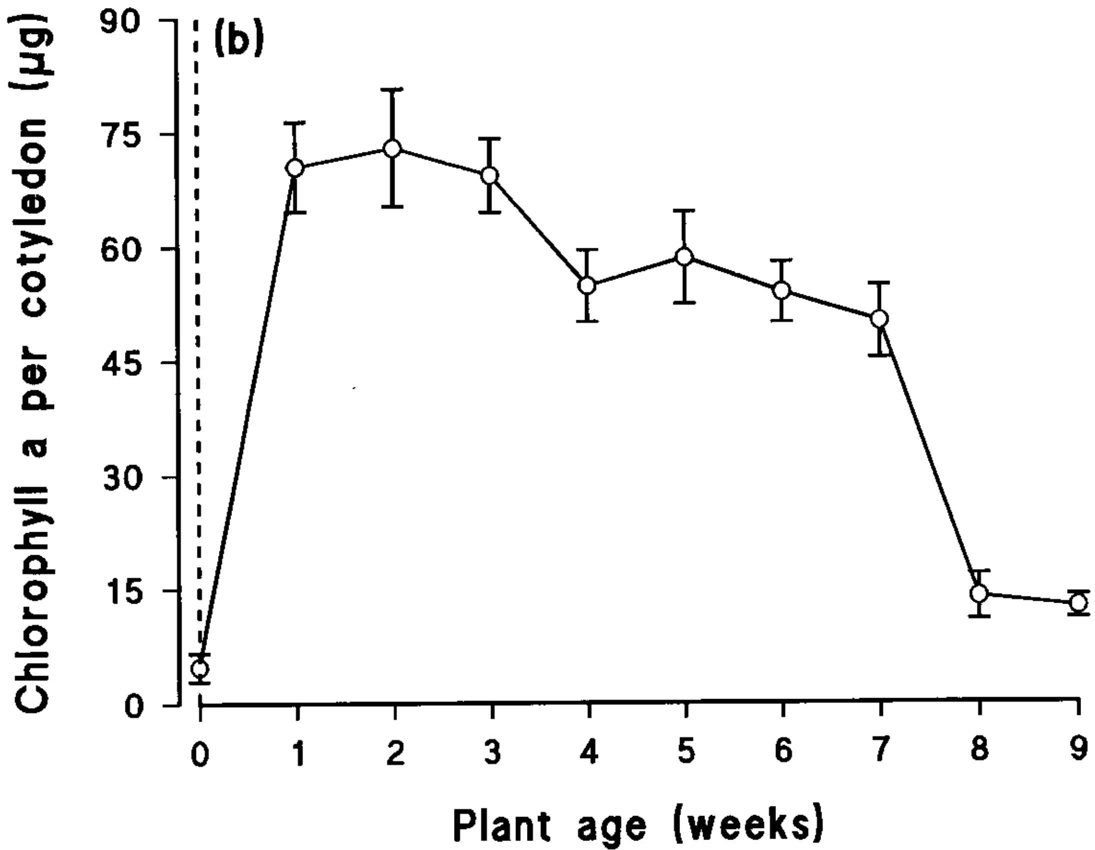

(b)
Dry weight per cotyledon (mg)
10
8
6
4
2
0
0 1 2 3 4 5 6 7 8 9
Plant age (weeks)

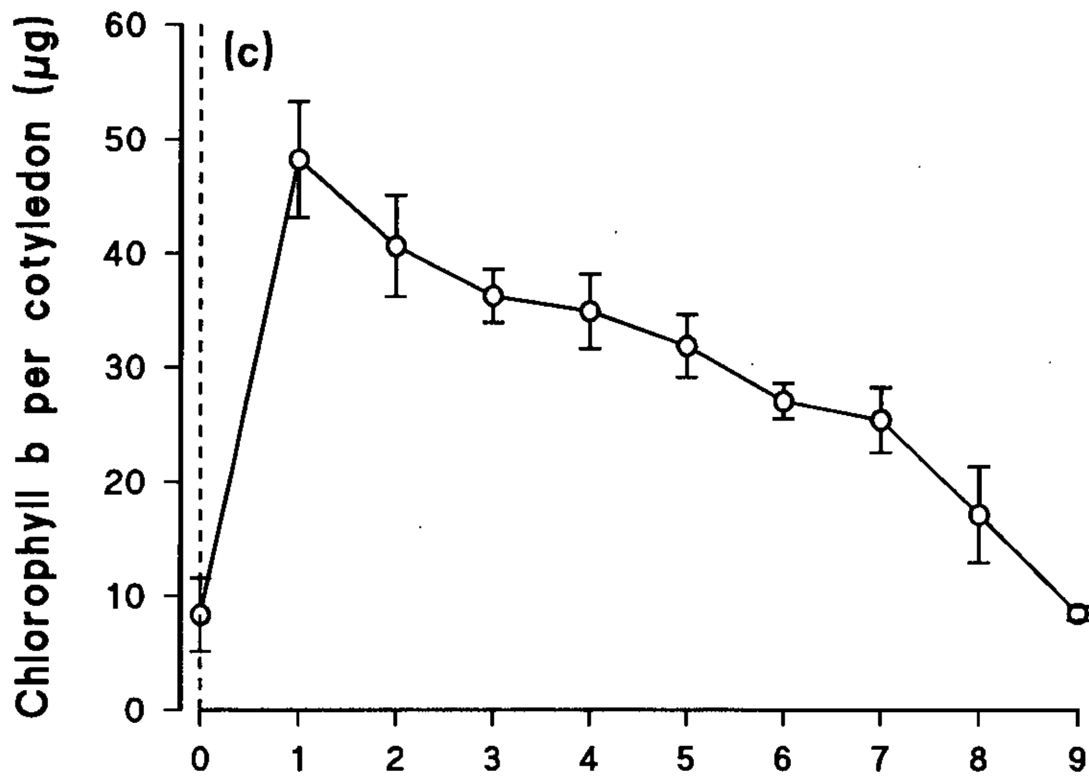
The transition of cucumber cotyledons from a storage to a photosynthetic organ during the first week of plant growth is reflected in a massive accumulation in the amount of total chlorophyll extracted from cotyledons 7 days after seed imbibtion compared to imbibed seed (Figure 3.3a). Thereafter the level of chlorophyll declines from an average value of 118 ± 10.7 μg per cotyledon until the average chlorophyll content 9 weeks after seed imbibition is 21.0 ± 2.1 μg per cotyledon (Figure 3.3a.). Analysis of chlorophyll *a* and chlorophyll *b* content also shows rapid accumulation of pigment in the first 2 weeks of plant growth followed by a decline thereafter (Figure 3.3b.,c.). However, there does appear to be some difference in the relative decline of these pigments. Chlorophyll *a* increases to 70.5 ± 5.9 μg per cotyledon in the first week after seed imbibition. The amount of chlorophyll *a* does not appear to decline during the following 2 weeks after seed imbibtion and a major decline is observed therafter. However, in the period 4-7 weeks after seed imbibition, levels of chlorophyll *a* remain at 69-80% of the maximum value (Figure 3.3c.). A decline in the amount of chlorophyll *a* is observed after 7 weeks plant growth; the average content is 13.8 ± 3.0 μg and 12.5 ± 1.5 μg for cotyledons of 8 and 9 week old plants respectively. The first week of plant growth shows an increase in cotyledonary chlorophyll *b* content to 48.2 ± 5.1 μg in plants from 1 week old plants. Therafter the level of chlorophyll *b* declines. Collectively these data indicate that the earlier stage of plant growth where chlorophyll loss is observed is characterised by a reduction in the amount of chlorophyll *b*, relative to the amounts of chlorophyll *a*. During the latter stages of plant growth loss of chlorophyll is due to a loss of chlorophyll *a* relative to the decline in chlorophyll *b*.

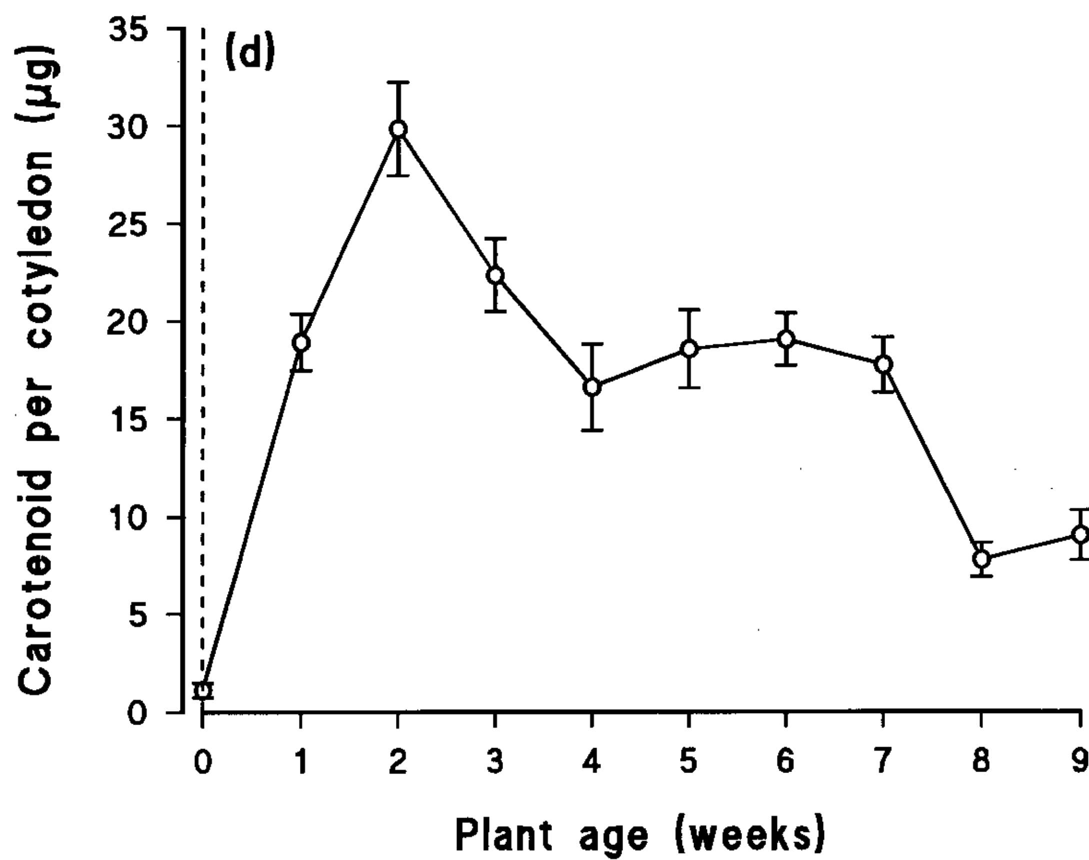
Similarly, the first 2 weeks of plant growth is characterised by a massive increase in carotenoid levels form undetectable amounts to 29.8 ± 3.4 μg per cotyledon (Figure 3.2c.). Thereafter a decline in carotenoid content is observed. However, the amount of carotenoid appears to remain at relatively abundant levels and in cotyledons that are yellow, 9 weeks after seed imbibition, the amount of carotenoid is 25% of the maximun determined value. Consequently, although plant growth is characterised by carotenoid loss this pigment is still present during the latter stages in relatively

Figure 3.3. Developmental changes in the level of chloroplast pigments after seed imbibition, (a) total chlorophyll. (b) chlorophyll *a*. (c) chlorophyll *b*. (d) carotenoids. Error bars represent the SEM of four independent replicate samples.


140
120
100
80
60
40
20
0
(a)
Chlorophyll per cotyledon (µg)
0 1 2 3 4 5 6 7 8 9


90
75
60
45
30
15
0
(b)
Chlorophyll a per cotyledon (µg)
0 1 2 3 4 5 6 7 8 9
Plant age (weeks)


(c)
Chlorophyll b per cotyledon (µg)
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9


(d)
Carotenoid per cotyledon (µg)
35
30
25
20
15
10
5
0
0
1
2
3
4
5
6
7
8
9
Plant age (weeks)

abundant amounts. No attempt was made to investigate the changes in specific types of carotenoids in cotyledons during plant growth.

From these data it can be concluded that cotyledon development is characterised by a transition from a storage organ to photosynthetic organ during early plant growth and this is reflected by a large increase in the amount of chlorophyll and carotenoids. However, these high levels of chlorophyll and carotenoid are not sustained and decline in the amounts of these pigments is observed after 2 weeks plant growth.

3.2.4 Changes in cotyledonary lipid.

The role of the glyoxylate cycle in the mobilisation of storage triacylglycerols during post-germinative growth is well characterised (Huang *et al.*, 1983). And it has been postulated that the glyoxylate cycle may be involved in metabolism of thylakoid lipid during senescence (Gut and Matile, 1988). Consequently, lipid was extracted from cucumber cotyledons at weekly intervals after seed imbibition and the change in lipid composition determined. This allows analysis of MGDG and DGDG abundance in cucumber cotyledons and consequently it will be determined if a correlation exists between decline in galactolipid content and the synthesis of MS and ICL in senescent cotyledons.

Total lipid was extracted from cucumber cotyledons at weekly intervals following seed imbibition and analysed following seperation by TLC (Figure 3.4.). Imbibed seed contain abundant amounts of a neutral lipid class that migrates close to the solvent front. Although this species has not been unambiguously identified, given the abundance and relative mobility on TLC plates, it is likely that this lipid class is triacylglycerol. Lipid extract isolated from cotyledons 1 week after seed imbibtion contain very little of this lipid class and this may indicate that this lipid is mobilised to support seedling growth. In imbibed seed other lipid classes appear to be present in the cotyledons at considerably lower amounts than triacylglycerol (Figure 3.4a,b). The presence of MGDG and DGDG as the major lipids from cotyledon extracts

Figure 3.4. TLC plates of total lipid isolated from cucumber cotyledons at weekly intervals after seed imbibition. Samples were applied to plates on a per cotyledon basis. Actual amounts of lipid applied correspond to 25% (week 0) or 100% (week 1-9) of the lipid content at a given developmental stage. (a) Separation using solvent mix of acetone: benzene: water (91: 30: 8). (b) Separation using chloroform: methanol: water: glacial acetic acid (170: 30: 20: 7). Std. represents the mobility of standard lipids-MGDG, DGDG, PC and PI. Chl/carot, chlorophylls and carotenoids; Anth, putative anthocyanins; O, origin.

Time after seed imbibition (weeks)

Std.

0 1 2 3 4 5 6 7 8 9

Chl/ Carot

MGDG

DGDG

Anth

PC

O

PE

Chl/ Carot

MGDG

Anth

PC

DGDG

PE

O

following 1 week of plant growth indicate the abundance of plastids in these organs. Given that cotyledons 1 week after seed imbibition contain abundant amounts of chlorophyll and carotenoids it would be likely that these plastids are chloroplasts. Other lipid classes identified are PC and PE. The lipid composition of cucumber cotyledons is similiar to that reported previously (Draper, 1969). The amount of MGDG and DGDG present in extracts from cotyledons after 3 weeks plant growth declines to reach low but detectable levels after 8 weeks plant growth. This decline in thylakoid membranes appears similiar to the observed decline in the amount of chlorophyll and carotenoid. Additionally both PC and PE do not appear to decline significantly until after 7 weeks of plant growth. Collectively, these data indicate that cotyledonary sensecence is characterised, initially, by loss of thylakoid localised lipids that occurs after the first 3 weeks of plant growth. The lipids that are localised in extraplastidial membranes are maintained until the latter stages of cotyledon senescence.

3.2.5. Changes in total protein.

From the data presented above chlorophyll loss occurs soon after cotyledon greening has occured. However, although loss of chlorophyll is often considered a reliable criterion in establishing the course of senescence of green tissue it is important to determine the changes in other parameters. Consequently, the relationship between total protein content and cotyledonary age was determined (Figure 3.5a.,b.).

Total protein from cucumber cotyledons was extracted, quantified and is presented in Figure 3.5a. The greatest amount of protein was present in cotyledons from imbibed seed and it is presumed to be cucumber seed storage protein that support growth in the first week of plant development. Consistent with this is the observation that the amount of protein extracted from cotyleons 1 week after seed imbibition has declined approximately 4-fold from 872 $\pm$ 178 μg to 207 $\pm$ 3.0 μg per cotyledon. There appears to be a small increase in the amount of protein isolated from cotyledons 1 week after seed imbibition and this may be associated with the synthesis of new

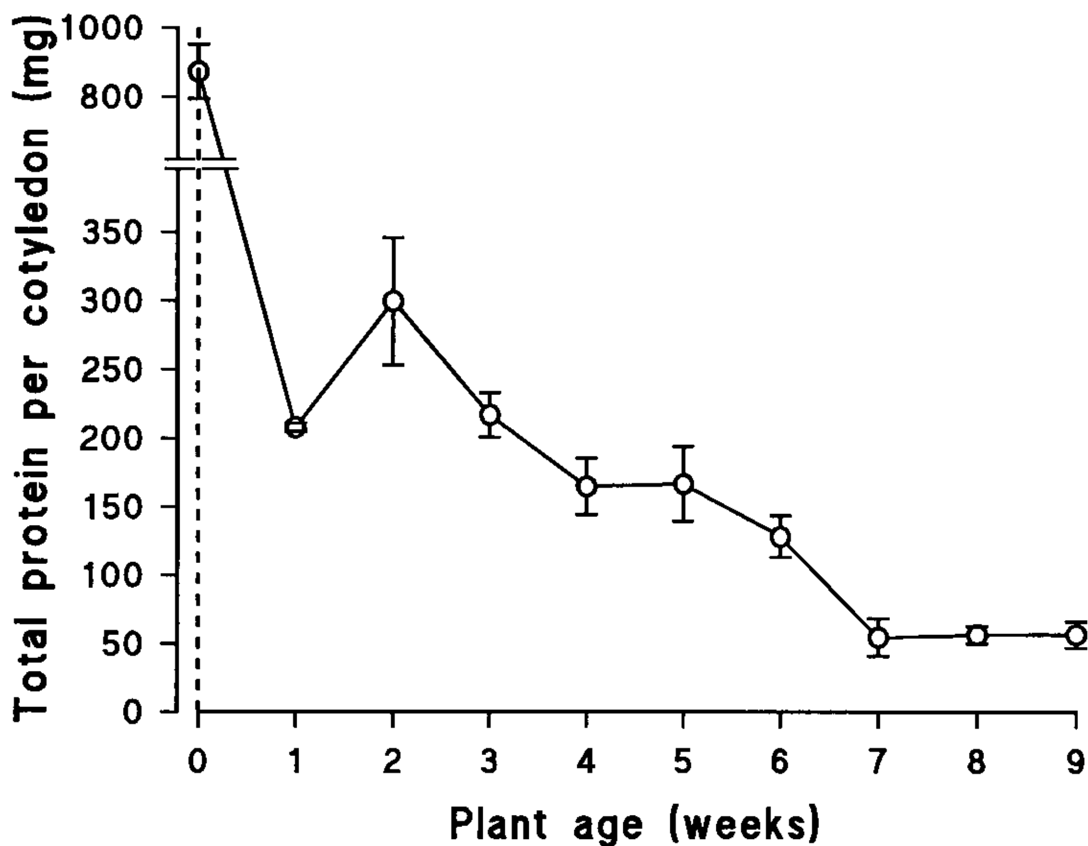


Figure 3.5a. Developmental change in total cotyledonary protein content following seed imbibition. Total protein was extracted from cotyledons at weekly intervals and the average content was determined, error bars represent the SEM of four replicate samples.

polypeptides involved in carbon fixation. However, as plant growth continues thereafter the amount of protein declines and it appears that protein extracted from cucumber cotyledons reaches a constant level of approximately 55 μg per cotyledon 7 weeks after seed imbibition.

SDS-PAGE of cotyledonary protein provides an indication of the changes in the polypeptide composition during plant growth (Figure 3.5b.). Despite the large amount of protein from cotyledons in imbibed seed, there appears to be a cluster of 6 polypeptides with apparent M_r of 20-50,000. These polypeptides were previously identified as putative cucumber seed storage proteins (Becker *et al.*, 1978). Within 2 weeks of plant growth these proteins have declined below detectable levels and it is presumed that they are metabolised to support seedling growth. The polypeptide composition subsequently appears characteristic of a photosynthetic organ; dominant polypeptide with an apparent molecular mass of 55 kDa is likely to be the large subnuit of RuBisCO. As plant growth continues the decline in protein appears to be characterised by a decline in all protein in a coordinate manner; from the level of resolution provided by this analysis there does not appear to be differential accumulation or disappearance of any specific protein during the latter stages of cotyledonary development. Additionally, there does not appear to be any accumlation of any proteins with an apparent molecular weight of 63-64 kDa that may be MS or ICL. However, to unambiguously define the relative rate of decline or increase in the abundance of specific proteins during cotyledonary senescence the use of specifc antiserum is required. However, from the analysis of changes in total protein, further evidence is provided that suggest that senescence of cucumber cotyledons is initiated very soon after attainment of photosynthetic function during the first 2 weeks of plant growth.

3.2.6. Changes in total RNA.

Decline in total RNA is an additional parameter that has been used to characterise senescence (Brady, 1988; Makrides and Goldthwaite, 1981). Consequently the

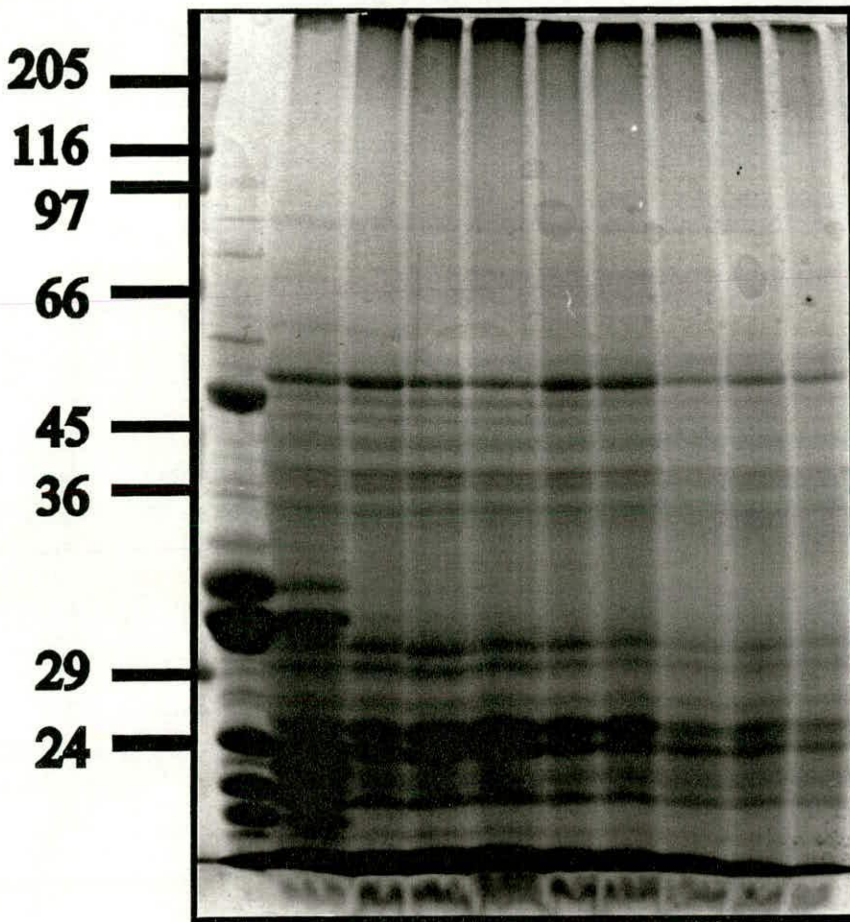


Figure 3.5b. SDS-polyacrylamide gels of total protein isolated from cucumber cotyledons at weekly intervals following seed imbibition. Samples were loaded on a per cotyledon basis onto 12% polyacrylamide gels. Actual amounts of protein applied correspond to 5% (week 0) or 20% (week 1-9) of the total protein content of a cotyledon at a given developmental stage. Molecular weight markers are: myosin (205 kDa), ß-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa) and tyrpsinogen (24 kDa).

amount of total RNA extracted from cotyledons at weekly intervals after seed imbibition was determined (Figure 3.6). The first week of plant growth is associated with a 9-fold increase in cotyledonary total RNA, this probably reflects the increased biosynthetic activity of the cotyledon during the period of post-germinative growth and appears to be very similiar to the changes in RNA content observed during post-germinative development of cucumber cotyledons (Becker *et al.*, 1978). The amount of total RNA extracted from cucumber cotlyledons thereafter declines and reaches a constant basal level of approximately 3.2 μg per cotyledon following 7 weeks plant growth. The amount of phenol-extractable RNA is considered to represent a fraction of the total RNA present in the cell, for example the amount of total RNA extracted from cucumber cotyledons represents values of 1-10% of that reported in cucumber during the period of post-germinative growth (Becker *et al.*, 1978). Consequently, the data presented here are at best semi-quantitative. However it does provide additional evidence that following the period of post-germinative growth a decline in the biosynthetic capacity of the organ is occuring.

From the analysis of the changes in several components of cucumber cotyledons during plant growth and development it is apparent that the biosynthetic capacity of the organ declines very soon after the period of post-germinative growth. The use of antibodies can now be used to determine at what stage in cotyledon development specifc changes occur in the synthesis of leaf-type peroxisomal and glyoxysomal proteins.

3.3. Immunoblot analyses of senescent cucumber cotyledons.

3.4.1 Changes in immunodetectable levels of SGAT.

The use of antisera raised against the leaf-type peroxisomal enzyme SGAT of cucumber (Hondred *et al.*, 1985) would provide information concerning the decline in photosynthetic function of the cotyledon during senescence. Additionally, it would also

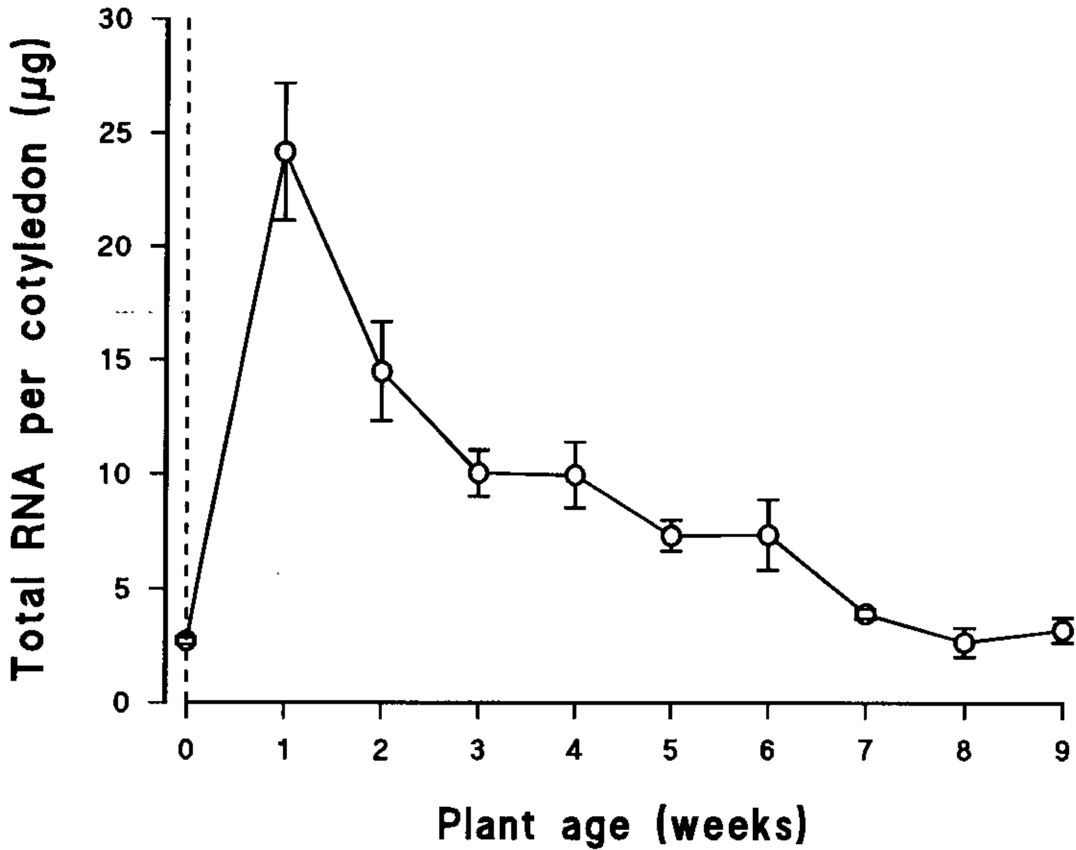


Figure 3.6. Developmental change in total RNA content of cucumber cotyledons after seed imbibition. Average total RNA content was determined for cotyledons at weekly intervals, error bars represent the SEM of four replicate samples.

provide useful information concerning the proposed transition of leaf-type peroxisomes to glyoxysomes during senescence.

Total protein was extracted from cucumber cotyledons at weekly intervals following seed imbibition, and the presence of SGAT was determined, using antibodies raised against the purified enzyme. (Figure 3.7.). Protein extract from cotlyedons in imbibed seed contained no detectable SGAT. After I week of plant growth both isoforms of SGAT are detected in cotyledon protein extract. Surprsingly, no significant difference in the amount of immunodetectble SGAT was observed in protein extracts isolated from cotyledons after 1 week plant growth and both polypeptides are still detectable in cotyledonary extracts isolated 9 weeks after seed imbition. However, despite the observation that the amount of SGAT appears to be constant, relative to total protein amounts, the amount of protein per cotyledon does decline after 2 weeks plant growth. Consequently on a per cell basis this indicates that SGAT does decline and this occurs in parallel with a general decline in total protein. It is of interest to note that despite a decline in SGAT during senescence there are significant amount of both isoforms in yellow cotyledons.

3.3.2. Immunodetection of MS and ICL.

To determine the nature of the induction of MS and ICL synthesis associated with cotyledonary senescence antisera raised against purified enzyme preparations (Lamb *et al.*, 1978; Riezman *et al.*, 1980) were used to detect the presence of both proteins. Total protein was isolated from cotlyedons at weekly intervals after seed imbibition and the presence of MS and ICL determined (Figure 3.8.). No MS or ICL protein is detected in protein samples of cotyledonary extracts until after 7 weeks plant growth. As senescence is characterised by a decline in protein levels (Figure 3.5) it is possible that the actual abundance of MS and ICL does not change throughout the period of the experiment. However, no significant difference in the amount of coyledonary protein is observed during this period of MS and ICL accumulation. Consequently the

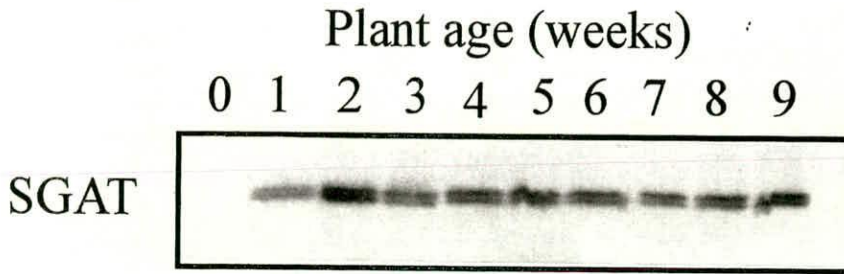


Figure 3.7. Immunoblot analysis of total protein isolated from cotyledons at weekly intervals after seed imbibition. Total protein was extracted and 15 mg protein was separated on 10% SDS-polyacrylamide gels, transferred to "Hybond-ECL" and the presence of both isoforms of SGAT was determined.

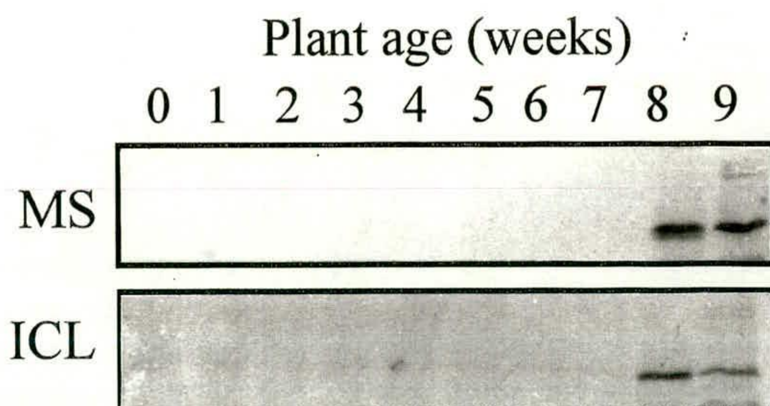


Figure 3.8. Immunoblot analysis of total protein isolated from cotyledons at weekly intervals after seed imbibition. Total protein was extracted and 15 mg protein was separated on 10% SDS-polyacrylamide gels, transferred to "Hybond-ECL" and the presence of MS and ICL was determined.

increase in glyoxysomal MS and ICL during cotyledonary senescence does represent a real increase in both these proteins.

3.4. Discussion.

3.4.1. Post-germinative development of cucumber cotyledons.

The developmental changes observed in cucumber cotyledons associated with germination and post germinative growth have been reported previously (see Becker *et al.*, 1978; Hondred *et al.*, 1987; Hill *et al.*, 1992; Moran *et al.*, 1990). However, a brief discussion of the changes in cucumber cotyledons during the early phase of plant growth is presented.

Cucumber exhibits epigeal germination and consequently the storage cotyledons emerge above soil and become the first photosynthetic organs of the developing seedling. The change in status of cucumber cotyledons from heterotrophy to autotrophy is most obviously demonstrated by the accumulation of chlorophyll and carotenoids (Figure 3.3). During the first two weeks of plant growth the abundance of chlorophyll and carotenoid increase from undetectable or low amounts. Similar data have been reported previously (Becker *et al.* 1978; Moran *et al.*, 1990). This indicates clearly the acquisition of autotrophy as a consequence of epigeal germination.

Similarly, the change in cotyledonary function during germination and post-germinative growth is demonstrated by the analysis of the change in lipid content and composition. Cotyledons from imbibed seed contain abundant deposits of triacylglycerol and other lipid classes appear to constitute a minor component of the total lipid (Figure 3.4). Triacylglycerol is the most common storage lipid in higher plants and is utilised to support seedling growth (Stobart and Stymme, 1987). Decline in the amount of triacylglycerol following 1 week of plant growth indicates that this lipid may be metabolised to support cucumber seedling growth (Figure 3.4.). As triacylglycerol is the most abundant lipid in cotyledons from imbibed seed the complete mobilisation of this

lipid class leads to a decline in the total amount of cotyledonary lipid during the first week of plant growth (Figure 3.4). This supports earlier observations that report a 4-fold decline in lipid content of cucumber cotyledons during the first 7 days following seed imbibition (Becker *et al.*, 1978).

Despite the decline in the amount of storage lipid present in cucumber cotyledons during the first week of plant growth there is an increase in the amount of other lipids. Most notable are the galactolipids MGDG and DGDG (Figure 3.4.). These lipids are confined mainly to plastid membranes (Douce and Joyard, 1980). Consequently an increase in both these lipid classes from undetectable amounts in cotyledons of imbibed seed indicates a rapid proliferation in plastids during post germinative growth (Figure 3.4.). The increase in chlorophyll and carotenoid content (Figure 3.3.) occurs at the same time as the accumulation of MGDG and DGDG and indicates that these lipids are likely to be localised in chloroplasts and demonstrates the autotrophic nature of cucumber cotyledons 1 week after seed imbibition. Other lipid classes are present in extracts isolated from cotyledons following 1 week of plant growth indicates the presence of other membranes including those of mitochondria.. The lipid composition of cotyledons following 1 week plant growth appears qualitatively similar to previous reports analysing the lipid content of cucumber cotyledons (Draper, 1967: Draper and Simon, 1970).

Analysis of the change in protein composition and content also demonstrate the transition exhibited by cucumber cotyledons from heterotrophy to autotrophy during post-germinative growth. In imbibed seed there appears to be a large amount of protein present in the cotyledon that is composed of a limited number of polypeptides most of which have apparent molecular mass in the range of 20-50 kDa (Figure 3.5b.). These proteins have been described as cucumber seed storage proteins (Becker *et al.*, 1978). Following 1 week of plant growth the amount of these putative seed storage proteins declines and following 2 weeks plant growth have decreased below detecable levels (Figure 3.5b.). This is consistent with previous observations that these proteins are indeed metabolised by the seedling to support growth prior to

greening of the cotyledon. In addition to metabolism of these proteins there is a rapid accumulation of many more polypeptides that have considerably more variation in abundance and molecular mass (Figure 3.5b). The accumulation of chlorophyll, carotenoid and galactolipids would imply that many of these proteins are likely to be involved in photosynthesis. Major polypeptides of apparent molecular mass of 55 kDa and 14 kDa may be the large and small subunits of RuBisCO respectively (Figure 3.5b). A second major polypeptide with an apparent *Mr* of 21,000 may be LHCPII. However, unambiguous identification of these proteins may be achieved by the use of antisera raised against purified preparations of these proteins. Previously, two of the major products from *in vitro* translation of RNA isolated from cucumber cotyledons during the first week of plant growth were identified as both subunits of RuBisCO (Walden and Leaver, 1981).

The use of antiserum raised against the leaf-type peroxisomal enzyme SGAT (Hondred *et al.* 1985) does provide reliable information concerning the change in function of cucumber cotyledons during post-germinative growth. Protein isolated from cotyledons in imbibed seed does not appear to contain significant amounts of either isoform of SGAT. In protein extracts isolated from cotyledons from plants following 1 weeks growth the amount of both isoforms has increased (Figure 3.7.). This supports previous data that demonstrate an increase in enzymes involved in photorespiration in cotyledons of light-grown cucumber seedlings during post-germinative growth is brought about primarily by an increase in the availability of mRNAs encoding these proteins. (Hondred *et al.*, 1985; 1987).

3.4.2. Characterisation of cotyledonary senescence.

From the analysis of the changes in a series of different parameters the post-germinative development of cucumber cotyledons can be characterised. Similarly, determination of the changes in protein, lipid, RNA, chlorophyll and carotenoid during senescence will provide a comprehensive characterisation of this process.

Collectively, the analysis of these data indicate that very soon after full greening and expansion of cucumber cotyledons, senescence has been initiated.

3.4.2.1. Cotyledon cell number remains constant.

Cotyledon cell number does not change significantly throughout the period of the experiment indicating that no cell division occurs (Figure 3.1.). Therefore all measurements made subsequently to characterise cotyledonary senescence can be expressed as a function of cotyledon number and therefore can in effect be extrapolated to a per cell basis. Previous microscopical analysis of senescence cotyledons and leaves demonstrate that the plasmalemma remains intact until the final stages of senescence (Barton, 1966: Butler, 1967: Butler and Simon, 1971; Ikeda and Ueda, 1964; Dodge, 1970). Therefore, no significant change in cell number may indicate that the plasmalemma remain intact throughout the period of plant growth even at the latter stages of cotyledonary senescence.

3.4.2.2 Change in cotyledonary dry and fresh weight.

After the initial rise in fresh weight during the period of post-germinative growth, average fresh weight appeared to be similar until 7 weeks plant growth. However considerable variation is observed from the 5th week of plant growth. Similar results were observed previously (Lewington *et al.*, 1967). This is also seen in the data presented in Figure 3.1., after 5 weeks plant growth variation between samples is observed until the final stages of senescence. There does not appear to be any change in cotyledonary dry weight throughout the period of plant growth.

3.4.2.3. Change in chlorophyll content.

Decline in the amount of chlorophyll is one of the most prominent characteristics related to senescence (Thomas and Stoddart, 1980). Consequently the amount of cotyledonary chlorophyll was determined at weekly intervals after seed imbibition (Figure 3.3a.). After the initial rise in the amount of chlorophyll is observed during

the first week of plant growth, there appears to be a decline throughout the period of the experiment. From these data it could be argued that cotyledonary senescence begins very soon after full expansion has occurred and continues for an extensive period of time. Earlier analysis of the senescence of cucumber cotyledons indicated that the optimum values for the amount of chlorophyll present were not maintained for any significant length of time (Lewington et al., 1967). Although chlorophyll loss is often cited as a reliable parameter in chracterisng the course of sensecence, important exceptions are known. The "stay-green" mutants of soybean (Kahanak et al., 1978) and the "non yellowing" mutants of *Festuca pratensis* (Thomas and Stoddart, 1975) have raised questions concerning the validity of employing chlorophyll measurements alone as a determinant of senescence. There appears to be no difference between the time of leaf abscission of the soybean mutant and many of the characteristics of senescence are very similar between the wild type and 'non yellowing' mutant of *F. pratensis*. This indicates that chlorophyll loss is not a primary cause of foliar senescence. However, in most cases it is usually closely associated with this process.

The pathway of chlorophyll degradation has not been determined fully; two putative pathways have been considered (for review, see Hendry *et al.*, 1987). One of the enzymes considered to be involved in chlorophyll degradation is chlorophyase which hydrolyses the phytal ester group (Kuroki *et al.*, 1981). During incubation of chloroplast fragments the addition of chlorophyllase coincided with chlorophyll degradation (Amir-Shapira *et al.*, 1986, 1987). Additionally, a strong correlation was observed between chlorophyll degradation and chlorophyllase activity in maturing citrus fruit (Hirschfiled and Goldsmidth, 1983; Amir-Shapiro *et al.*, 1987). However, the amount of chlorophyllase actually declines in ageing leaves of *Raphanus* (Philips *et al.*, 1969) and in some cases an increase in the activity of this enzyme was associated with chlorophyll synthesis (Shimizu and Tamaki, 1963). A second possible enzymatic pathway involves the action of the enzyme chlorophyll oxidase, the product of this reaction would be chlorophyll *a-1*. In detached dark incubated bean leaves the loss of chlorophyll was associated with a transient increase in chlorophyll *a*-1 (Maunders *et al.*, 1983). The activity of this enzyme appears to be activated by free fatty acids and

it has been proposed that the breakdown of chlorophyll by chlorophyll oxidase is initiated by the hydrolysis of thylakoid membrane lipids (Luthy *et al.*, 1984). A third possible mechanism of chlorophyll degradation may be via a non enzymatic photochemical process (Maunders and Brown, 1983).

3.4.2.4. Decline in the amount of cotyledonary carotenoids.

To obtain additional information concerning changes in chloroplast structure associated with senescence, change in cotyledonary carotenoid content was determined (Figure 3.3b). Following the initial increase in carotenoid abundance, associated with post-germinative growth, the amount begins to decline soon after the peak value. Despite this decline the amount of carotenoids extracted from cucmber cotyledons is still relatively high. In yellow cotyledons that amount of carotenoids present are 25% of the maximum value determined. Initially this would appear inconsistent with the observable decline in chlorophyll and galactolipids. However, it has been reported that coupled with the disassembly of thylakoid membranes during the senescence of a number of plant species there is an accumulation of osmiophillic particles that appear to contain abundant amounts of carotenoids (Butler and Simon, 1971; Dodge, 1970). The significance of this remains to be established. In photosynthetic tissue carotenoids are involved in the quenching of singlet oxygen produced in photosynthetic tissue (Bensasson *et al.*, 1976; Foote, 1970). Associated with senescence is an increase in the levels of free radicals such as singlet oxygen (Knox and Dodge, 1985; Thompson *et al*, 1987) The abundance of carotenoids in yellow cotyledons may be a response to increased amount of singlet oxygen and other free radicals present in senescent tissue: carotenoids may modulate the activity of these highly reactive species. It would be of interest to determine if carotenoids have a specific function during senescence.

3.4.2.5. Decline in cotyledonary lipid content.

Analysis of the changes in the lipid content of cucumber cotyledons after the first week of plant growth also indicate that senescence begins very soon after cotyledon

expansion. From the second week of plant growth there appears to be a general decline in the level of all lipid species. However it can be seen that the decline in MGDG and DGDG occurs at a greater rate and earlier relative to that observed for phospholipids. Ferguson and Draper (1973) observed that loss of MGDG, DGDG and SQ occurred in cucumber cotyledons at approximately the same time as loss of chlorophyll became apparent. However the loss of phospholipids such as PC did not occur until 2-3 weeks after the loss of galactolipid was observed. Previously it was demonstrated that the general decline in lipid content in senescent cucumber cotyledons may be due to a reduction in lipid biosynthesis (Draper and Simon, 1971). Similar data have been observed in many other plant species and generally it would appear that senescence of green tissue is characterised by the loss of galactolipid content prior to the loss of lipid localised in mitochondrial and microsomal membranes (for review see Brown *et al.*, 1991).

Consistent with analysis of the changes in lipid content are ultrastructural studies of senescent green tissues. In cucumber cotyledons the earlier stages of senescence appear to be characterised by loss of thylakoid membrane structure and in the reduction in ribosomes present in the stroma. Other organelles appear to remain intact until later stages of senescence (Butler and Simon, 1967). Similar observations have been made during the senescence of leaves of *Phaseolus* (Barton, 1966), *Betula* (Dodge, 1970), and *Lemna* (Ikeda and Ueda, 1964). Studies of the non-yellowing mutant of *Festuca pratensis* provides evidence that lipid metabolism may play a significant role in the regulation of senescence (Harwood *et al.*, 1982). Loss of galactolipids appeared to be reduced in this mutant. The loss of galactolipid was associated with an increase in lipid rich in linoleic acid which has been found to be an effective activator of thylakoid proteolysis and in the enzyme chlorophyll-oxidase (Thomas, 1982).

3.5.3.6. Change in cotyledon protein content.

Immediately following expansion and greening of the cotyledon there appears to be an increase in the amount of protein present in cucumber cotyledons. This may be due to the increase in the abundance of protein involved in carbon fixation and other related reactions. However despite this modest rise in protein content the amount declines subsequently. This provides further evidence that after 2 weeks plant growth, very soon after full expansion of the cotyledon, senescence has been initiated. SDS-PAGE analysis of total proteins from extracts at weekly intervals after the full expansion of the plant has occurred show the decline in the amount of protein per cotyledon. Additionally it appears, from the resolution provided by this technique, that there is a coordinate decline in the protein content of the cotyledon. However the amount of information gained from the analysis of total protein by standard SDS-PAGE is limited. A more informative approach would be from the analysis of 2D gels. However, it is certainly clear that decline in both subunits of RuBisCO and LHCPII appear very similar and looks to be of the same proportion of the amount of total protein. Additionally, there does not appear to be any newly synthesised proteins appearing with the progression of senescence.

Analysis of the changes of SGAT certainly show a similar relationship. Following the initial increase in immunodetectable levels of both SGAT isoforms associated with post-germinative growth there does not appear to be any significant difference in the abundance of these proteins in extracts isolated form mature and senescent cotyledons. Consequenlty this would indicate that after 1 week plant growth the amount of SGAT appears constant relative to total protein. However as senscence is characterised by protein decline, SGAT levels are declining on a per cell basis. The use of antisera raised against both RuBisCO subunits and LHCPII would also determine the relationship between the decline relative to total protein.

There are many studies that report protein loss associated with senescence (for review see Brady, 1988). In these reports the pattern of protein loss appears not to be

random and there is a selective loss of some proteins. However information on the decline of individual proteins is sparse. The availability of antibodies raised against specific proteins may lead to better characterisation of senescence associated protein loss. Due to the abundance of RuBisCO there is information concerning the decline in this protein. In senescent barley leaves 85% of the soluble protein loss was due to a decline in the amounts of RuBisCO (Friedrich and Huffaker, 1980) which supports earlier observations of senescent bean leaves (Racusen and Foote, 1965). It has also been demonstrated that the amount of RuBisCO declines relative to the amount of soluble protein in a number of other plant species (Makino *et al,* 1983; Wittenbach, 1980). A second well documented group of proteins that exhibit a decline are those localised in thylakoid membranes. The earlier stages of senescence of green tissue is thylakoid disassembly (Butler and Simon, 1970; Cohen *et al.*, 1979). In senescing *Avena sativum* and bean leaves, a loss of cytchrome b_6 and f content precedes any decline in proteins of PSI and PSII (Ben-David *et al.*, 1983; Roberts *et al.*, 1987). This may account for the loss of non cyclic electron transport observed in senescing bean leaves (Jenkins and Woolhouse, 1981).

In general, most estimates indicate that the rate of protein synthesis declines during senescence (for review see Brady, 1988). In expanded leaves of *Perilla*, undetectable amounts of radiolabelled precursors became incorporated into RuBisCO consequently indicatinge that the decline in protein amount is due primarily to cessation of synthesis. This has also been observed in cucumber cotyledons and *Capsicum* leaves (Brady, 1988). The relative rate of synthesis of other chloroplast proteins also decline during senescence of spinach leaves (Silverthorne and Ellis, 1980). However the synthesis of the 32 kDa herbicide-binding polypeptide of PSII remained unchanged. Subsequently it has been observed that it is rapidly synthesised in senescent bean leaves (Roberts *et al.*, 1987). It has been proposed that the continued turnover of this polypeptide indicates a functional chloroplast system for protein synthesis still exists in senescent tissue.

3.4.2.7. Changes in total RNA content.

Following 1 week of plant growth there is an observable decline in the amount of total RNA isolated from cucumber cotyledons and consequently it could be argued that cotyledonary senescence has started very soon after full greening. These data may not be fully quantitative, but they do demonstrate qualitatively the decline in RNA content after cotyledon expansion has occurred. Similar results have been observed during the senescence of leaves of *Triticum aestivum* (Makrides and Goldthwaite, 1981) and *Phaseolus vulgaris* (Bate *et al.*, 1991; Brady and Scott, 1977). In both cases the amount of rRNA declines to a lower level than was observed during leaf expansion. and there appeared to be no difference in the relative amount of chloroplast RNA to cytoplasmic RNA (Makrides and Goldthwaite, 1981, Brady and Scott, 1977). In contrast, during senescence of leaves of cucumber (Callow, 1974), *Perilla* (Callow, 1972) and tobacco (Takegami, 1975) there does appear to be selective loss of chloroplast rRNA.

Loss of RNA can be due to decreases in the rate of synthesis or to an increase in the activity of RNAase. In senescing corn leaves the levels of RNAase increase to such an extent that this tissue has been used as a source of the enzyme (Wilson, 1967). However, it is not clear to what extent RNAases are involved in the degradation of RNA during senescence. This is demonstrated by observations that during senescence of barley leaves no increase in RNAase activity is observed. However, chromatin isolated from senescing barley leaves contained a ten-fold increase in the amount of RNAase associated with it (Srivastava and Ware, 1965; Srivastava, 1968). Additionally during the senescence of soybean cotyledons RNAase levels decline at the same time that RNA declines (Krul, 1974). In contrast, it has been observed that there is an increase in the activity of three RNAases during the senescence of wheat leaves (Blank and McKeon, 1991a; b).

3.4.3. The function and control of MS and ICL during cotyledonary senescence.

From the data presented in Figures 3.6. and 3.8 it is clear that during cotyledonary senescence there is a coordinate induction of MS and ICL synthesis. This supports and extends the observations that demonstrate an induction of MS and ICL synthesis in senescent leaves, petals and cotyledons (Graham *et al.*, 1992; De Bellis *et al.*, 1990; De Bellis *et al.*, 1991; Pistelli *et al.*, 1991) and in detached dark-incubated organs (De Bellis *et al.*, 1990; De Bellis and Nishimura, 1991; Graham *et al.*, 1992; Gut and Matile, 1988; Landolt and Matile, 1990).

During post-germinative growth the control of the regulation of both MS and ICL also appears to be due primarily to change in transcript abundance (Smith and Leaver, 1986; Weir *et al.*, 1980; Reynolds personal communication). Additionally, dark-incubated detached *Nicotiana* leaves genetically transformed with a ß-glucuronidase gene under the control of the cucumber MS promoter accumulated significant amounts of the reporter gene product, indicating that transcription is the primary controlling factor in the synthesis of MS (Graham *et al.*, 1992). However attempts to determine unambigously the changes in the steady-state levels of transcripts encoding MS and ICL during cotyledon senescence were inconclusive. On one occasion, northern blot analysis of total RNA demonstrated that the appearance of transcripts encoding MS and ICL occured between 4-7 weeks after seed imbibtion (data not shown). This is in contrast to the demonstrable increase in MS and ICL 8 and 9 weeks after seed imbibition (Figure 3.8). Consequently western and northern blot analyses of mature and senescent cucumber cotyledons were repeated. Immunoblot analysis demonstrated the appearance of MS and ICL after 7 weeks plant growth (data not shown) and consequently supports the data presented in Figure 3.7. Attempts to analyse the appearance of transcripts were not successful due to degradation of RNA during isolation on several occasions. Previous analysis of senescent cucumber organs and pumpkin petals indicate that transcripts encoding MS do accumulate (Graham *et al.*, 1992; Pistelli *et al.*, 1991). Additionally, transcripts encoding MS and ICL have been shown to be present in total RNA isolated from cotyledons 3 days after seed imbibtion

and during senescence (Dae-Jae Kim, personal communication). Therefore it is clear that senescence is associated with the accumulation of transcripts encoding MS and ICL.

The reason for theappearance of transcripts encoding MS and ICL in cotyledons 4-7 weeks after seed imbibtion in apparently non senescent cucumber cotyledons is not clear (data not shown). However from time to time the appeance of transcripts encoding MS has been observed in apparently healthy green tissue (see Graham *et al.*, 1992, Figure 1, 15 and 21 days after seed imbibtion).

Senescence of cucumber cotyledons is characterised by a loss of protein (Figure 3.5.). Consequently detection of MS and ICL may not be due to any change in the absolute amounts of these molecules per cell, but an increase in the abundance relative to the total protein present in senescent cotyledons. This is unlikely: during the period of increase MS and ICL from undetectable amounts there is no significant change in cotyledon protein content (Figure 3.5a.). Consequently these data indicate that the latter stages of senescence may be characterised by a net accumulation of MS and ICL.

It has been proposed that during senescence of green tissue there is a transition of microbodies from leaf-type peroxisomes to glyoxysomes (De Bellis and Nishimura, 1990; Gut and Matile, 1988). However, the use of specific antibodies raised against cucumber SGAT demonstrate that there are still significant amounts of both isoforms present at the same time as the accumulation of both MS and ICL is evident. Additionally, it would appear that levels of SGAT protein remain at a constant level relative to total protein content. Similarly, during senescence of pumpkin petals it was observed that immunodetectable levels of HPR and glycolate oxidase (GO) also remained at a constant level (De Bellis *et al.*, 1991). Additionally during senescence of pumpkin cotyledons HPR and GO activities were detected in a peroxisomal-enriched fraction at higher levels than MS and ICL (De Bellis and Nishimura, 1991). Glyoxysomes are characterised as microbodies that contain the enzymes of the

glyoxylate cycle (Huang *et al.*, 1983). In senescent organs peroxisomes conform to this criterion, however the observation that leaf-type peroxisomal enzymes may also be present indicate that a problem of nomenclature has to be addressed. Furthermore, it would be of interest to determine if both glyoxysomal and leaf-type peroxoismal enzymes are localised in the same organelle during senescence, in the manner demonstrated during post-germinative development of plants exhibiting epigeal germination (Nishimura *et al.*, 1986; Sautter, 1986; Titus and Becker, 1985).

Attempts to assay the activity of MS and ICL in senescent cucumber cotyledons, leaves and petals were unsuccessful. This was believed to be due to inhibitory factors present in the extracts (data not shown). Similarly, attempts to assay the activity of MS and ICL in extracts isolated from senescent organs also proved difficult (I. Graham personal communication, De Bellis *et al.*, 1990; Pistelli *et al.*, 1991). De Bellis and co-workers (1990) reported inhibition of MS enzyme activity in crude extracts isolated from senescent pumpkin cotyledons. This difficulty was overcome by undertaking further purification of a peroxisome enriched fraction (De Bellis *et al.*, 1990). Similarly, it was observed that ICL enzyme activity was only detectable in a peroxisome enriched fraction isolated from developing watermelon embryos (Fusseder and Theimer, 1976). Consequently to obtain accurate data concerning changes in enzyme activity the purification of an enriched peroxisomal fraction from senescent cotyledons has to be carried out.

Although the extent of any post-translational control of MS and ICL is unknown there has been one report that demonstrates that *in vitro* phosphorylation of MS occurs in endosperm of castor bean during germination (Yang *et al.*, 1988). There is no other report of phosphorylation of any other glyoxylate cycle enzymes in higher plants. However, ICL is phosphorlyated in *Escherichia coli* (Robertson *et al.*, 1988). Therefore it is important to fully characterise the activity of MS and ICL during cotyledonary senescence and determine to what extent the change in immunodetectable abundance of MS and ICL is correlated with changes in enzyme activity.

It has been proposed that MS and ICL are synthesised in detached and senescent organs to metabolise products of chloroplast lipid degradation (Gut and Matile, 1988). However in this study there is no significant correlation with DGDG and MGDG decline and the appearance of glyoxylate cycle enzymes: a significant amount of the thylakoid lipid (Figure 3.4.) present in mature cotyledons has declined prior to any detection of both MS and ICL (Figure 3.10.). Similarly, no correlation is observed between chlorophyll and carotenoid decline and MS and ICL induction. These data are at variance with the results presented by Gut and Matile (1988) that report a significant relationship between an increase in MS and ICL enzyme activity and a decline in MGDG abundance following detachment of barley leaves and incubation in continuous darkness (Gut and Matile, 1988). This apparent discrepancy may be due to a difference between galactolipid degradation in naturally senescent and detached, dark-incubated organs. However, it is clear that during the earlier stages of senescence of cucumber cotyledons, characterised by chloroplast disassembly, the degradation of thylakoid lipids is unlikely to involve the action of glyoxylate cycle enzymes. This does not preclude the possibility that during the latter stages of senescence, characterised by MS and ICL accumulation, glyoxylate cycle enzymes are involved in lipid metabolism. It has been reported that in some cases when the induction of MS and ICL has been observed, no detectable ß-oxidation enzyme activity was detectable (De Bellis *et al.*, 1990; Pistelli *et al.*, 1991). This provides some evidence that MS and ICL may play an alternative or additonal function to the metabolism of lipid.

3.6. Conclusion.

From the data presented in this chapter it is clear that during the latter stages of cotyledonary senescence there is a co-ordinate synthesis of glyoxysomal MS and ICL. However, this induction does not correlate with a decline in chlorophyll, carotenoid, chloroplast lipid, total protein and RNA. Consequently the role of glyoxylate cycle enzymes during senescence remains obscure. In addition to the observation that glyoxysomal enzyme synthesis occurs in naturally senescent organs, MS and ICL can also be experimentally induced by the incubation of detached organs in darkness (De Bellis *et al.*, 1990; De Bellis and Nishimura, 1991; Graham *et al.*, 1992; Gut and Matile, 1988; Landolt and Matile, 1990). It is clear that the validity of analysis of detached, dark incubated organs with respect to natural senescence is limited. However, the incubation of detached cucumber cotyledons in darkness does provide an additional opportunity to further investigate the function of MS and ICL and factors that may regulate the synthesis of these two enzymes.

CHAPTER 4

INDUCTION OF MALATE SYNTHASE AND ISOCITRATE LYASE SYNTHESIS IN DETACHED CUCUMBER COTYLEDONS.

4.1. Rationale.

The results presented and discussed in Chapter 3 demonstrate the synthesis of MS and ICL during senescence of cucumber cotyledons. However, no significant correlation was observed between decline in chlorophyll, carotenoids, lipid, protein and RNA content with the synthesis of MS and ICL. Consequently, these data indicate that the function of the glyoxylate cycle in senescent tissue remains to be established. The first report of glyoxylate cycle enzyme synthesis in non-stroage tissue was following the incubation of detached barley leaves in complete darkness (Gut and Matile, 1988). The increased activity of glyoxylate cycle enzymes appeared to be coupled with a decline in abundance of the thylakoid localised lipid MGDG. Consequently it was argued that the glyoxlate cycle may be involved in the catabolism of thylakoid lipids during senescence. Although informative results have been obtained concerning aspects of the catabolism during senescence from the study of changes in artificially senescent organs, the validity of analysis with respect to natural senescence is limited (See Nooden, 1988: Becker and Apel, 1993). Additionally, from the data presented in Chapter 3 it is clear that there is no strong correlation between galactolipid decline and the synthesis of MS and ICL. However, the induction of MS and ICL has been observed following detachment and dark incubation of cotyledons, leaves and roots in a number of higher plant species (De Bellis *et al.*, 1990; De Bellis and Nishimura, 1991; Graham *et al.*, 1992; Landolt and Matile, 1990). This indicates that incubation of excised organs in darkness does provide a useful experimental system in which the study of the synthesis of glyoxylate cycle enzymes can be undertaken.

In this chapter specific cDNA clones encoding cucumber MS and ICL were used to determine the changes in the steady state levels of the transcripts encoding these proteins. Additionally, specific antibodies were used to characterise the accumulation of MS and ICL in detached cotyledons. However, to determine the role of glyoxylate cycle enzymes, changes in specific components of senescing tissue were also determined. In particular, changes of components of the chloroplast were evaluated. The collective analysis of these data may test the proposal that the glyoxylate cycle is involved in the metabolism of thylakoid membrane lipids (Gut and Matile, 1988).

Cotyledons were detached 14 days after seed imbibtion and incubated in darkness or under a 12 hour photoperiod. At this stage the cotyledon had fully expanded and the first leaf was just visible. Consequently, it was considered that this stage of development the cotyledon is the main source organ of the plant, with its optimum biosynthetic capacity. In addition to being placed in darkness, cotyledons were incubated in light. The incubation of cotyledons in light would provide information concerning the effects wounding and continued photosyntheis may have on the synthesis of MS and ICL.

4.2. Changes in cucumber cotyledons following detachment.

4.2.1. Changes in cotyledon cell number.

Cell number was determined in cucumber cotyledons from plants 14 days after seed imbibition and at two day intervals following detachment and incubation in darkness or under a 12 h photoperiod (Figure 4.1.). From the data it can be seen clearly that cotyledon cell number 14 days after seed imbibition is very similar to that reported in Chapter 3 (Figure 3.1.). Additionally, cell number in detached cotyledons incubated under a 12 h photoperiod remains constant throughout the experimental period. From these data it could be argued that plasmamembranes remain intact in cotyledon cells incubated under a 12 hour photoperiod. This would appear consistent with previous analysis of detached cucumber cotyledons: from ultrastructural analysis of detached light incubated cotyledons the plasmamembrane appeared to remain intact up to 35 days after excision (Butler, 1967). Cotyledon cell number also remained constant in dark-incubated cotyledons until 6 days. Thereafter a 20% decline in cotyledon cell number was observed and may indicate that cell breakdown occurs in detached, dark-incubated cotyledons after 6 days. Alternatively, cell breakdown may have occured during tissue distruption.

4.2.2. Changes in fresh and dry weight.

Cotyledon fresh and dry weight was determined for cucumber cotyledons from plants 14 days after seed imbibition and following detachment and incubation in the

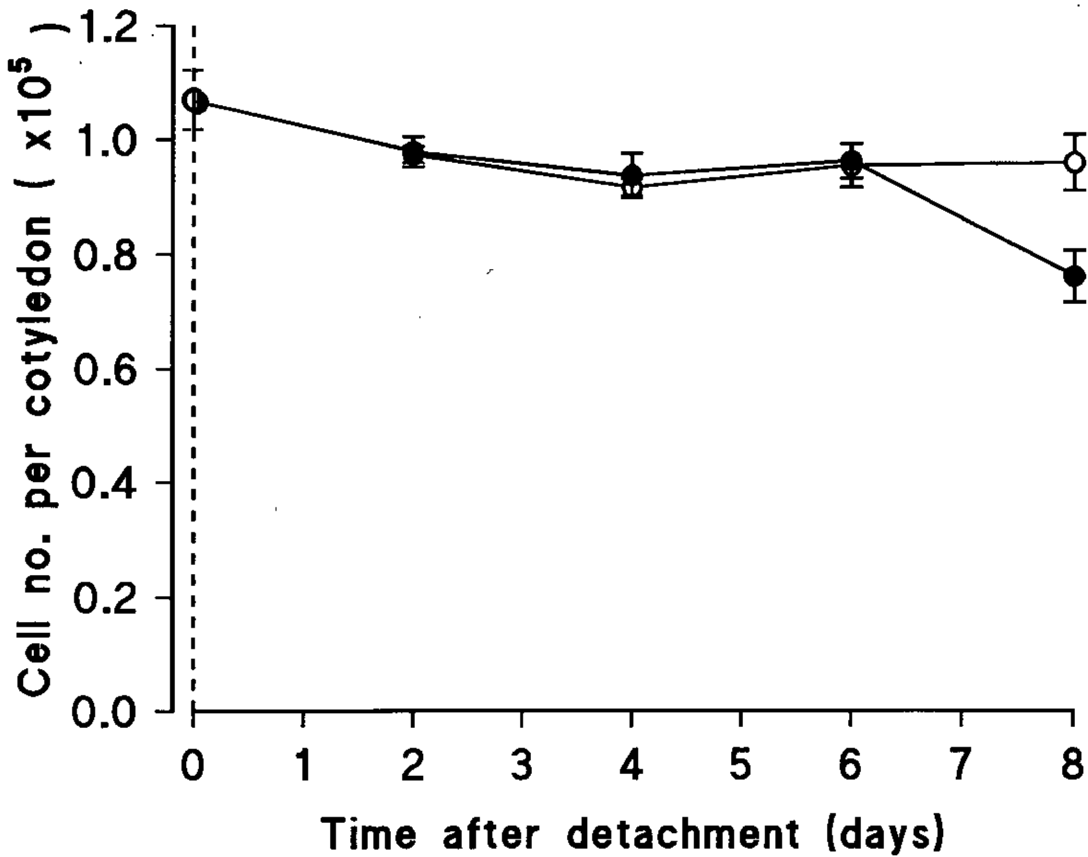


Figure 4.1. Change in cotyledon cell number following detachment. Average total cell number was determined 14 days after seed imbibition (◑) and at 48 h intervals following incubation in light (○) or darkness (●). Error bars represent the SEM of four independent replicate samples.

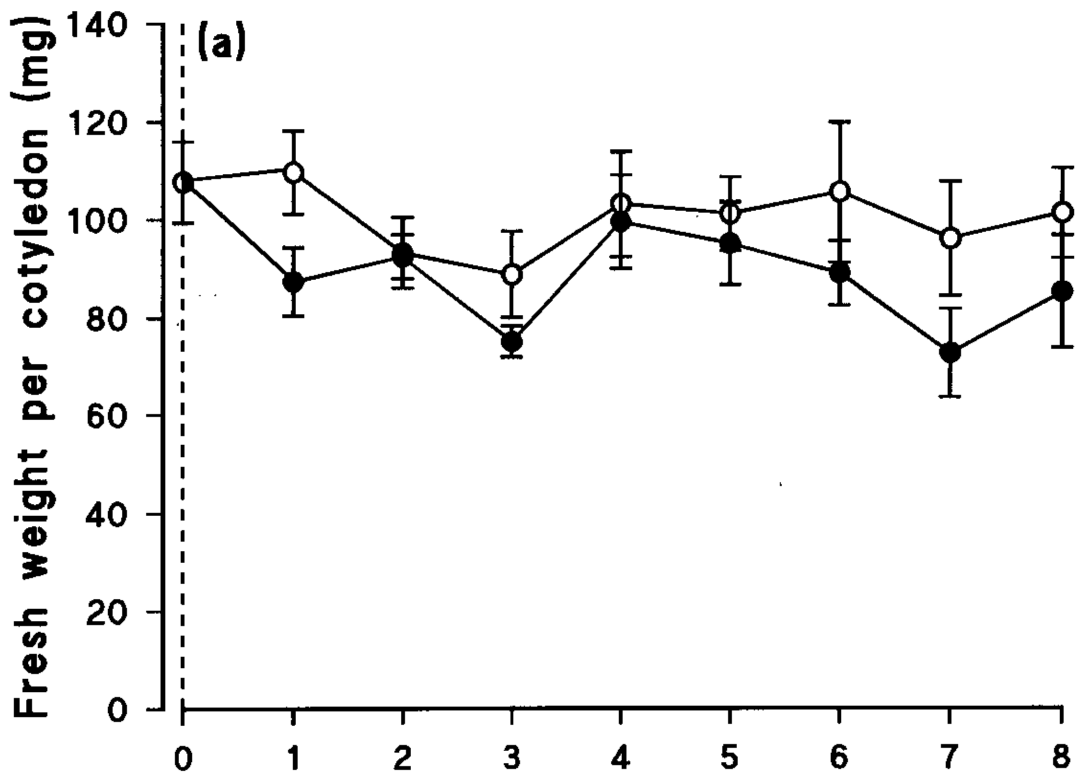
light and dark and is presented in Figure 4.2. No significant change in fresh weight is observed for both dark and light incubated cotyledons (Figure 4.2a.). However, as explant tissue was floated on distilled water, it is not clear to what extent any changes in fresh weight are due to accumulation of water, this is especially the case for dark-treated cotyledons in which cellular breakdown may occur. Cotyledonary dry weight was determined and does indicate a difference in dry weight for cotyledons incubated in the light and dark (Figure 4.2b.). Light incubated cucumber cotyledons appear to show a small increase in dry weight and dark incubated cotyledons exhibit a small decrease throughout the experiment. However no significant difference in dry weight for dark and light-incubated cotyledons is observed until 6 days after detachment (Figure 4.2b.). From these data it would appear that very little cellular breakdown occurs in detached cucumber cotyledons in the light and none is observed for cotyledons until after 6 days.

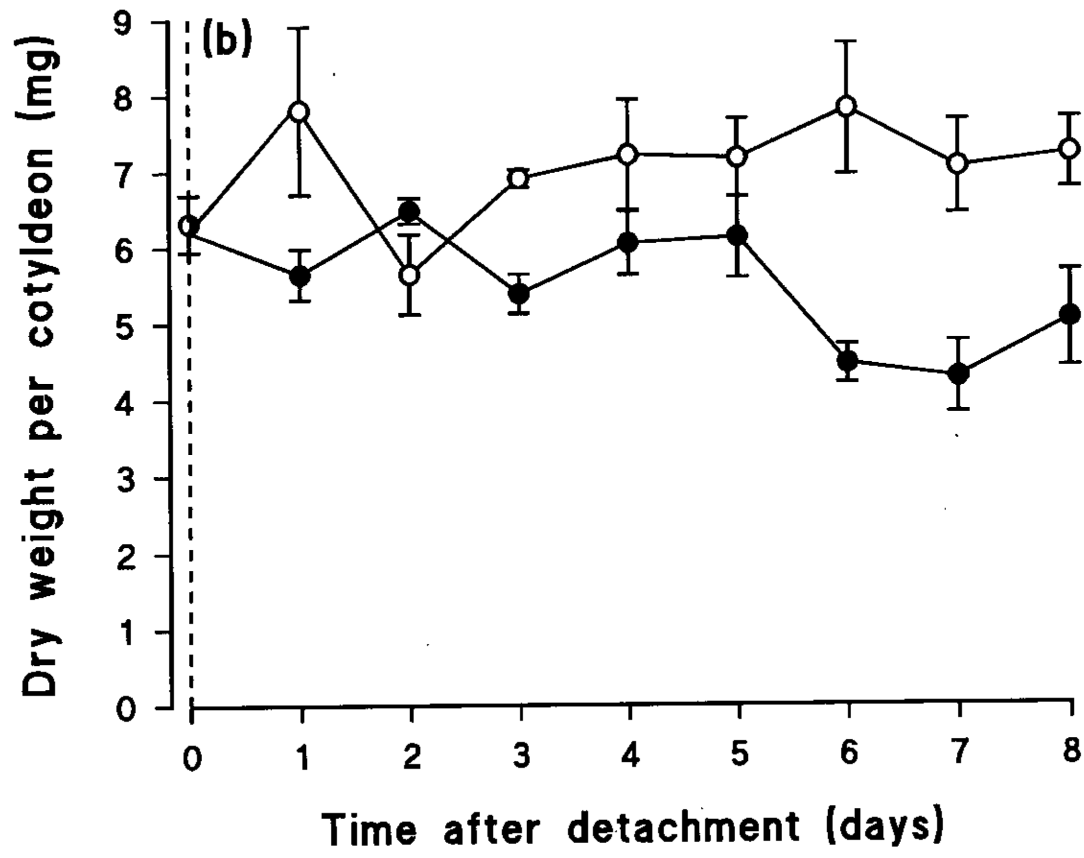
4.2.3. Changes in cotyledonary pigment.

Senescence is most obviously characterised by loss of chlorophyll (Thomas and Stoddart, 1980). Consequently, chlorophyll and carotenoid contents were determined for detached cotyledons incubated in darkness or under a 12 h photoperiod (Figure 4.3.). The amount of chlorophyll extracted from cotyledons incubated under a 12 h photoperiod did not change significantly throughout the period of the experiment. Surprisingly, the level of chlorophyll did not decline in dark-incubated cotyledons until after 4 days incubation. Additionally at the end of the experimental period the level of chlorophyll had only declined to levels of approximately 45% of the value estimated for cotyledons 14 days after seed imbibition. Analysis of chlorophyll *a* and *b* levels also show a similar pattern (data not shown), the level of chlorophylls does not change in light-incubated cotyledons and no significant decline in the amounts of these pigments occurs in dark-treated detached cotyledons until approximately 4-6 days.

The amounts of carotenoids were also determined for cotyledons 14 days after seed imbibition and following detachment (Figure 4.3b). The amount of carotenoids extracted from both light- and dark-treated cotyledons did not appear to

Figure 4.2. Change in cotyledon weight following detachment. Average fresh weight (a) and dry weight (b) was determined for cotyledons 14 days after seed imbibition (◑) and 24 h after following incubation in light (○) and darkness (●). Error bars represent the SEM of five replicate samples.


(a)
Fresh weight per cotyledon (mg)
140
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8

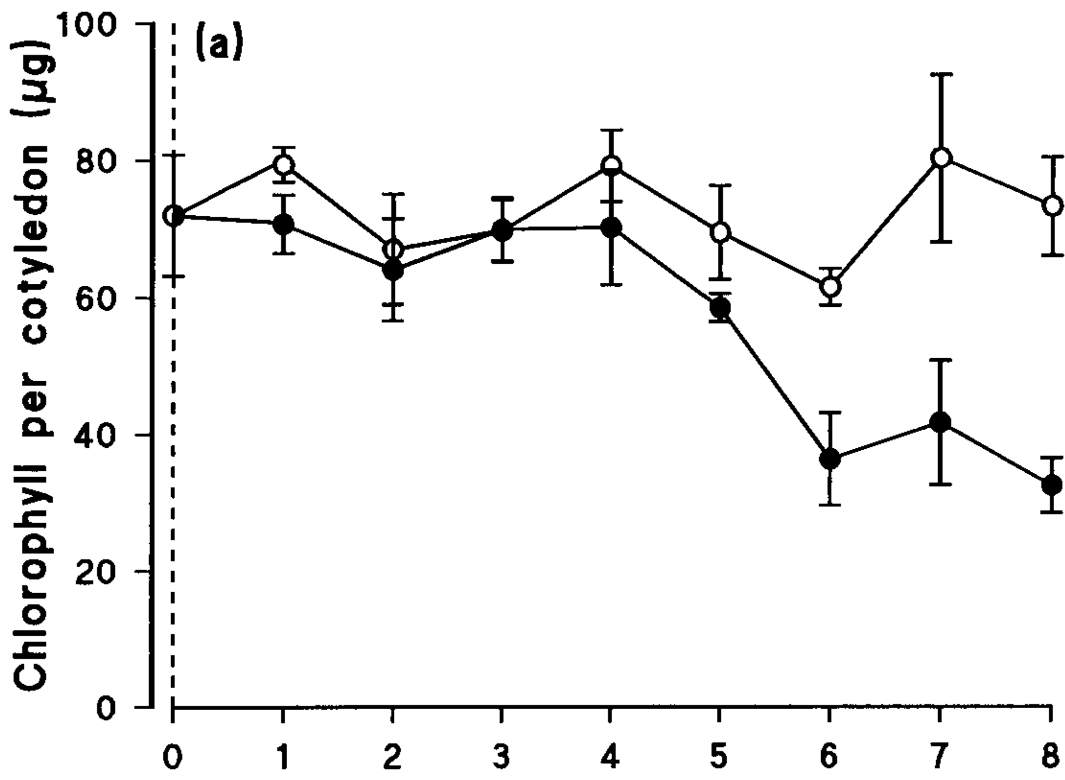

(b)
Dry weight per cotyldeon (mg)
9
8
7
6
5
4
3
2
1
0
0
1
2
3
4
5
6
7
8
Time after detachment (days)

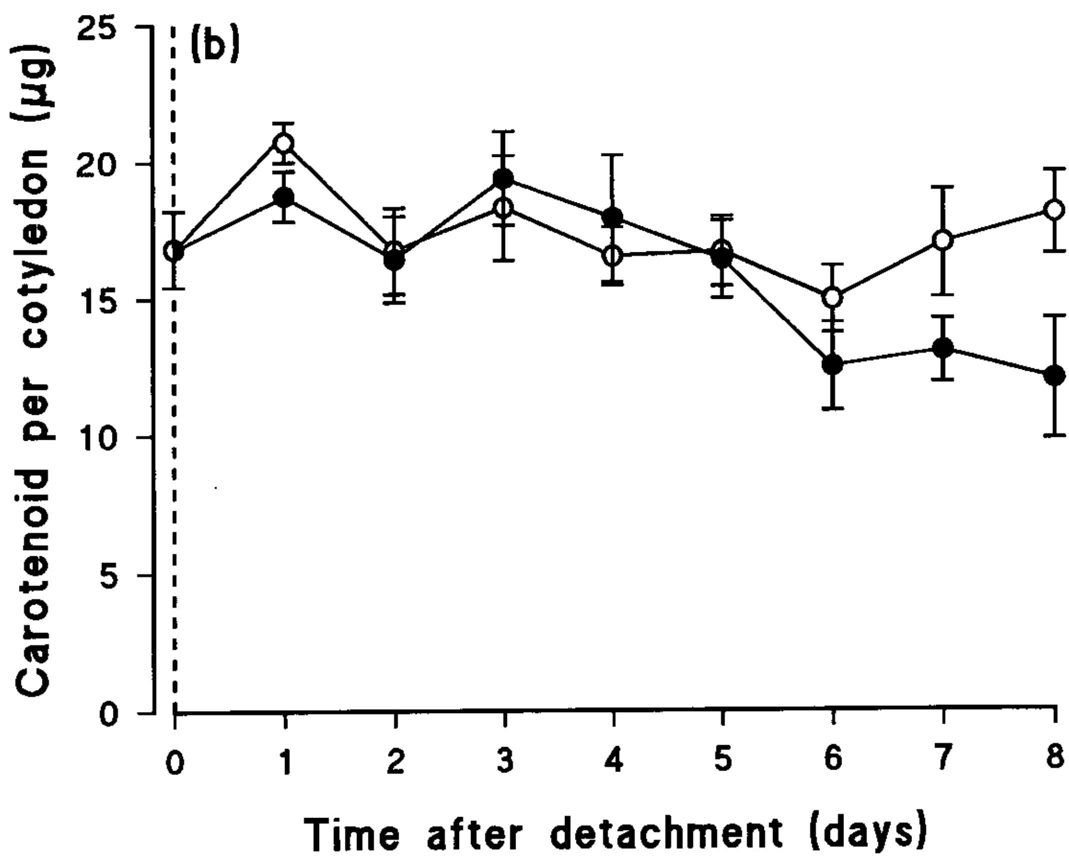
decline significantly throughout the experimental period. At the latter stages of senescence there may be a small decline in the amount of carotenoids in dark-incubated material relative to the amount extracted from cotyledons incubated under a 12 h photoperiod. Collectively these data indicate that no major changes associated with artificially induced senescence occurs in cucumber cotyledons until the latter stages of the experiment; following 6 days dark incubation. However, it is now well established that chlorophyll loss is not necessarily a good parameter to determine that course of artificially induced or natural senescence (see Thomas and Stoddart, 1975, Kahanak *et al.*, 1978). Consequently it is important to determine changes of other parameters associated with detachment of cucumber cotyledons.

4.2.4. Changes in cotyledonary lipid content.

The function of glyoxylate cycle enzymes in natural and artificially-induced senescent organs has not been established. Gut and Matile (1988) reported that an increase in MS and ICL activity in detached, dark incubated barley leaves correlated with loss of the thylakoid lipid MGDG and it was consequently proposed that the glyoxylate cycle is involved in the breakdown of thylakoid lipids during chloroplast disassembly. Therefore, lipid was extracted from detached cotyledons incubated in the light and dark. Lipid was separated by TLC and classes identified by comparison with the migration of known purified standards; MGDG, DGDG, PC and PE (Figure 4.4a, b.). The composition of lipids extracted from cucumber cotyledons 14 days after seed imbibition appears to be qualitatively similar to that reported previously: the major lipids present are the galactolipids MGDG and DGDG and the most abundant phospholipid classes identified are PC and PE (Draper, 1967; Draper and Simon, 1970). Following detachment and incubation of cotyledons under a 12 h photoperiod, no difference in the lipid abundance or composition was observed throughout the experimental period. Similarly, following detachment and incubation of cotyledons in darkness there appears to be no difference in the lipid composition of extracts when compared to cotyledons 14 days after seed imbibition. However, lipid extracted from cotyledons incubated in darkness for 8 days is characterised by a greatly reduced amount of all lipid classes.

Figure 4.3. Change in chloroplast pigments following detachment of cotyledons from seedling 14 days after seed imbibition. Average chlorophyll (a) and carotenoid (b) content was determined for cotyledons 14 days after seed imbibition (○) and at 24 h intervals following incubation in light (○) or darkness (●). Error bars represent the SEM of four replicates.


(a)
Chlorophyll per cotyledon (μg)
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8


(b)
Carotenoid per cotyledon (μg)
25
20
15
10
5
0
0
1
2
3
4
5
6
7
8
Time after detachment (days)

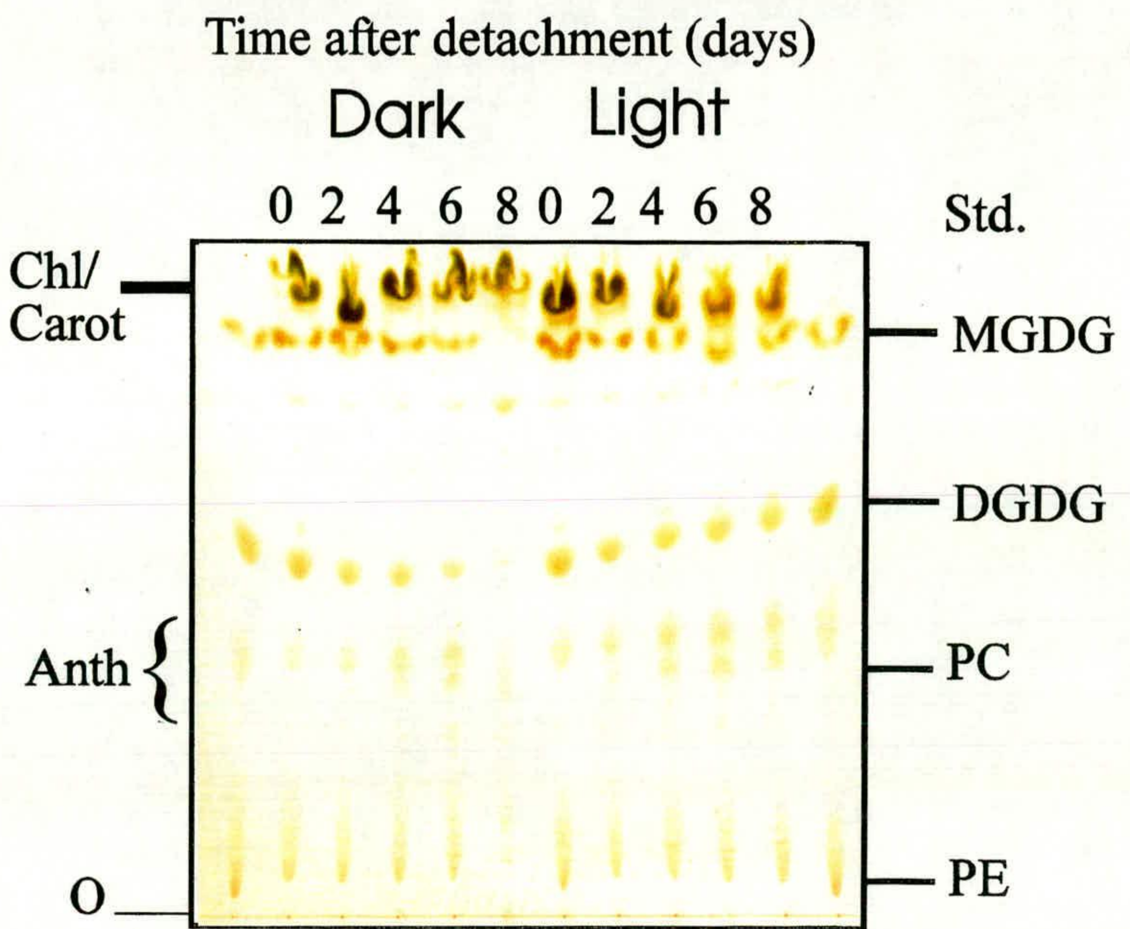
In lipid extracts isolated from both dark- and light-incubated detached cotyledons red pigments appears on TLC plates (Figure 4.4a,b). The identity of these pigments has not been determined unambigously. The best characterised red pigments in higher plants are anthocyanins. The observation that these pigment appears in both dark- and light-incubated cotyledons may indicate that this pigment may be synthesised in a response to wounding associated with detachment. However, it is important to fully determine the nature of these pigments prior to making any firm conclusions.

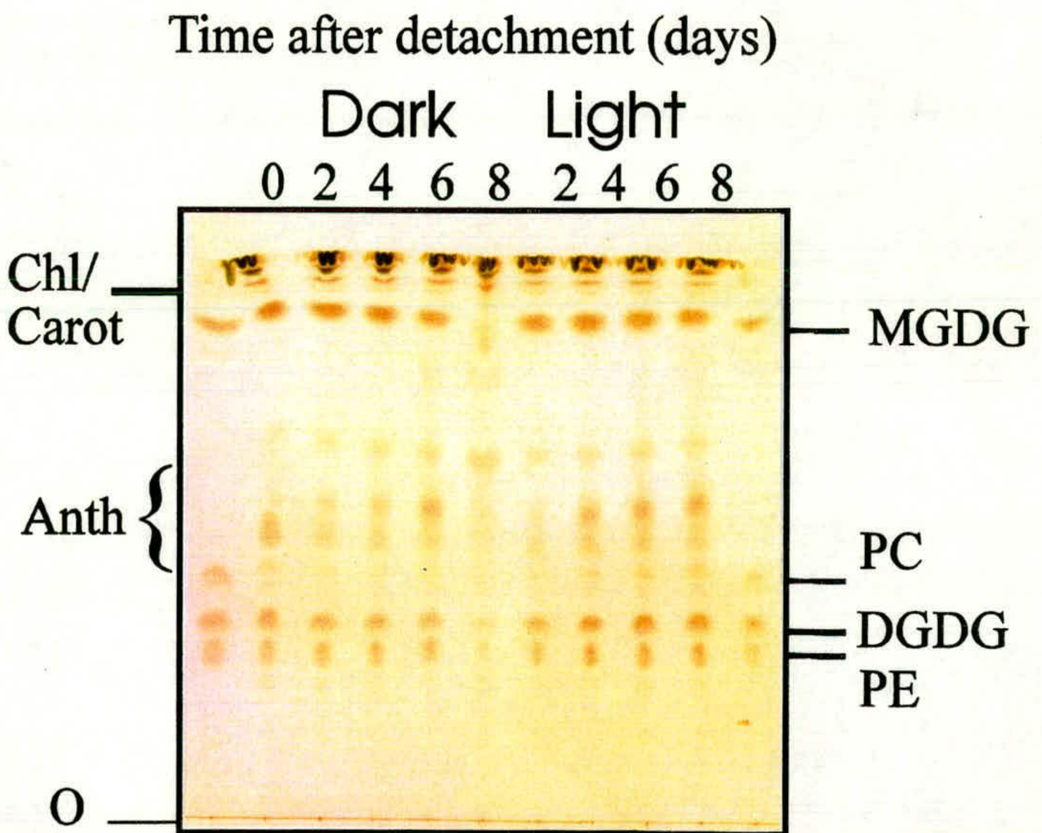
4.2.5. Changes in cotyledonary protein.

Total protein was extracted and the amount determined in cotyledons 14 days after seed imbibition and following incubation in darkness or under a 12 h photoperiod and is presented in Figure 4.5a. The amount of protein extracted from cucumber cotyledons 14 days after seed imbibition is similar to that reported previously in Chapter 3 (Figure 3.5a). The amount of protein extracted from light-incubated cotyledons does not appear to change significantly within the time course of the experiment. Surprisingly, the amount of protein extracted from detached dark-incubated cotyledons appears to be very similar to light incubated cotyledons. Consequently it can be seen that following detachment of cucumber cotyledons from seedlings 14 days after seed imbibition no decline in total protein content is observed for up to eight days. Similar results were obtained from the analysis of detached, dark-incubated pumpkin cotyledons in which very little decline in the amount of protein was reported until 10 days after detachment (De Bellis and Nishimura, 1991). Consequently if the experimental period was extended beyond 8 days a decline in the level of protein extracted from cotyledons may be have been observed.

In a separate experiment, protein was isolated from detached cotyledons and subjected to SDS-PAGE analysis (Figure 4.5b). It is clear that no significant loss of protein occurs during the experimental period and confirms the results presented in Figure 4.5a. Additionally, the polypeptide composition of cotyledons 14 days after seed imbibition appears very similar to that observed for light-incubated cotyledons. The presence of major polypeptides with apparent molecular mass of

Figure 4.4. TLC plates of total lipid isolated from cucumber cotyledons following detachment and at 48 h intervals thereafter. Total lipid equivalent to 1 cotyledon was spotted onto the plate. (a) Separation using solvent mix of acetone: benzene: water (91: 30: 8). (b) Separation using chloroform: methanol: water: glacial acetic acid (170: 30: 20: 7). Std. represents the mobility of standard lipids-MGDG, DGDG, PC and PI. Chl/carot, chlorophylls and carotenoids; Anth, putative anthocyanins; O, origin.


Time after detachment (days)
Dark
Light
0 2 4 6 8 0 2 4 6 8
Std.
Chl/
Carot
MGDG
DGDG
Anth
PC
PE
O


Time after detachment (days)
Dark
Light
0 2 4 6 8 2 4 6 8
Chl/
Carot
MGDG
Anth
PC
DGDG
PE
O

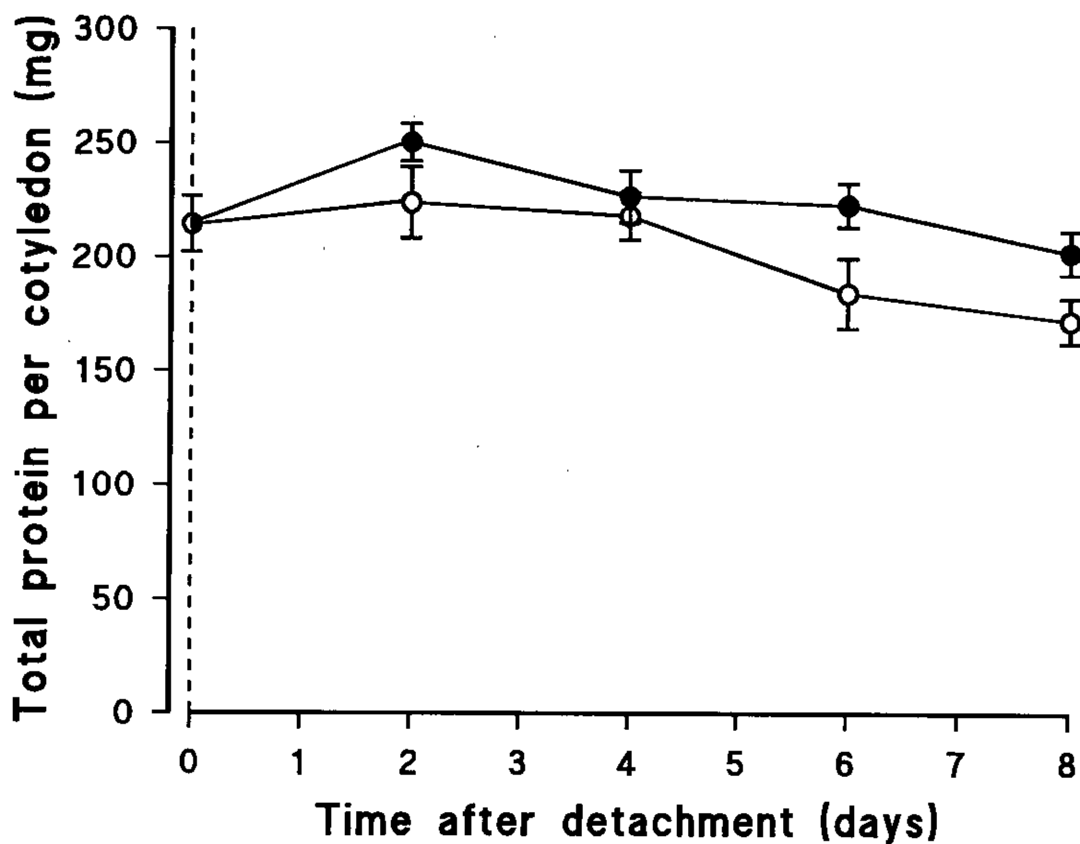


Figure 4.5a. Cotyledonary protein levels in detached cucumber cotyledons. Average total protein content was determined for cotyledons 14 days after seed imbibition (◑) and at 48 h intervals following incubation in light (○) or darkness (●). Error bars represent the SEM of four independent replicates.

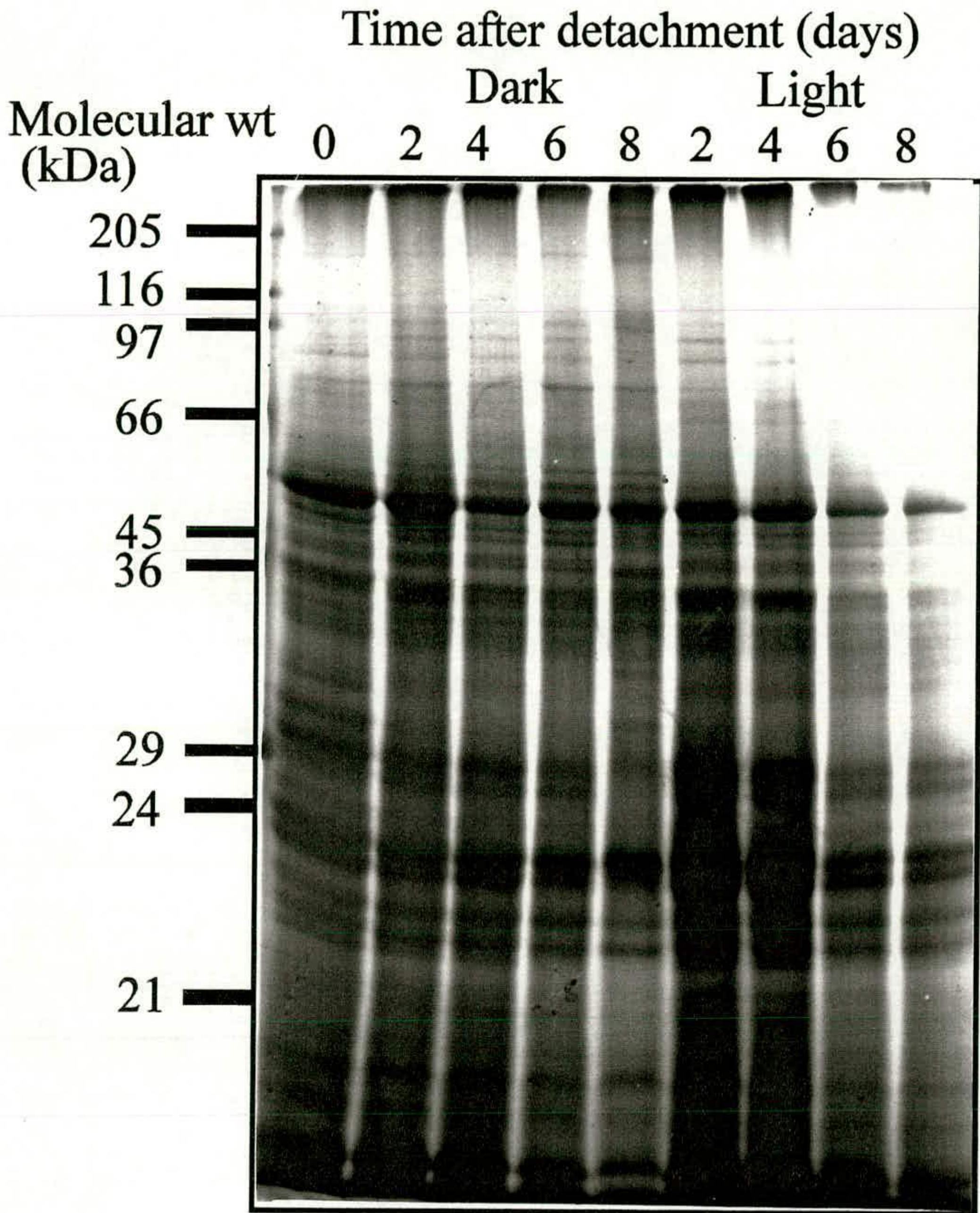


Figure 4.5.b. SDS-polyacrylamide gel of total protein isolated from cucumber cotyledons at 48 h intervals following detachment. Samples were loaded on a per cotyledon basis onto 12% (w/v) polyacrylamide gels. Actual amounts of protein applied correspond to 40% of total protein content of cotyledon. Molecular weight markers are: myosin (205 kDa), ß-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa) and trypsin inhibitor (21 kDa).

55 is likely to be the large subunit of RuBisCO. However, unambiguous identification of these proteins may made with the use of specific antibodies raised against these proteins. Similarly, the polypeptide composition of dark-incubated cotyledons does not appear to change during the first 6 days of the experiment. However, protein extracts isolated from cotyledons incubated in continual darkness for 8 days are characterised by the presence of additional polypeptides with apparent molecular masses of 34, 60 and 95 kDa. The identity of these proteins is not known, however ethylene-induced senescence of cucumber cotyledons is associated with an accumulation of peroxidases with apparent M_r of 34,000 and 60,000 (Abeles *et al.*, 1988). However, the identification of these proteins requires further analysis before any firm conclusions on their function can be established.

4.2.6. Changes in phenol-extractable total RNA.

Total RNA was extracted and quantified from cucumber cotyledons 14 days after seed imbibition and at 48 h intervals after detachment and incubation under a 12 h photoperiod or in continuous darkness (Figure 4.6). Although the amount of phenol-extractable RNA may not represent accurate quantitative data concerning the absolute amount of RNA present, it may provide qualitative data concerning the changes following detachment. The amount of RNA isolated from cucumber cotyledons 14 days after seed imbibition is similar to that observed in a separate experiment described in Chapter 3 (Figure 3.6.). The levels of total RNA extracted from detached cucumber cotyledons appear to decline a little in both dark- and light-treated explants indicating that no significant degeneration of the tissue or loss of cellular integrity.

4.3. Changes in steady-state levels of specific transcripts following detachment of cucumber cotyledons.

4.3.1 Changes in the steady-state levels of transcripts encoding HPR.

A cDNA clone encoding cucumber HPR (Greenler *et al.*, 1989) was used to provide data concerning the changes in the levels of transcripts that encode proteins

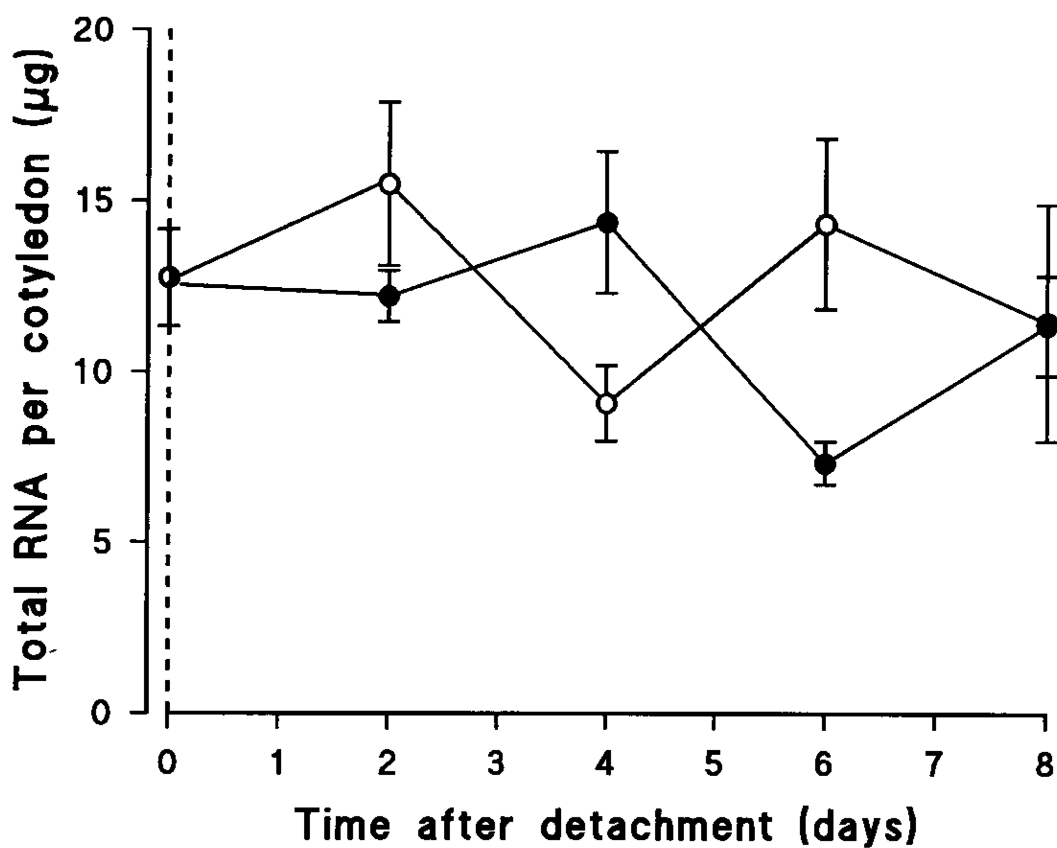


Figure 4.6. Change in total RNA content for cotyledons 14 days after seed imbibition and following detachment. Average total RNA content was determined for cotyledons 14 days after seed imbibition (◑) and at 48 h intervals following incubation in light (○) or darkness (●). Error bars represent the SEM of four independent replicate samples.

involved in carbon fixation. Additionally, this cDNA clone was used to provide information concerning the postulated transition from leaf-type peroxisome to glyoxysome that may occur during senescence. Total RNA isolated from cotyledons 14 days after seed imbibition contained abundant transcripts encoding HPR (Figure 4.7.). Following detachment and incubation of cotyledons under a 12 h photoperiod there appeared to be no change in the abundance of these transcripts. The steady-state level of mRNA encoding HPR remains at similarly abundant levels in detached cotyledons incubated under a 12 h photoperiod for up to 15 days (data not shown). Consequently these data indicate that any wound response associated with excision does not have any direct effect on the steady-state levels of transcripts encoding cucumber HPR. Following detachment of cucumber cotyledons and subsequent incubation of explant tissue in continual darkness the levels of transcripts encoding HPR decreased to low levels within four days. Collectively, these data appear consistent with the demonstrable role light plays in the regulation of HPR transcript abundance in cucumber (Hondred *et al.*, 1987; Greenler and Becker, 1990) and the decline in peroxisomal protein and enzyme activity (De Bellis and Nishimura, 1991) in detached, dark-incubated pumpkin cotyledons.

cDNA clones encoding the small subunit of RuBisCO and LHCPII (Greenland *et al.*, 1987) were also used in northern blot experiments (data not shown). Similarly, there was no significant decline in the steady-state levels of transcripts encoding RbcS and LHCPII following the incubation of cotyledons under a 12 hour photoperiod. The incubation of cotyledons in darkness lead to a decline in the abundance of mRNA encoding RbcS and LHCPII. Collectively, these data are consistent with the well documented role light plays in controlling the expression of genes that encode photosynthetic proteins (Thompson and White, 1991).

4.3.2. Changes in the steady-state levels of transcripts encoding cucumber MS and ICL.

cDNA clones encoding cucumber MS (Graham *et al.*, 1989) and ICL (Gift from Wayne Becker) were used in northern blot analysis to determine changes in steady-state levels of the transcripts encoding these proteins (Figure 4.8.).

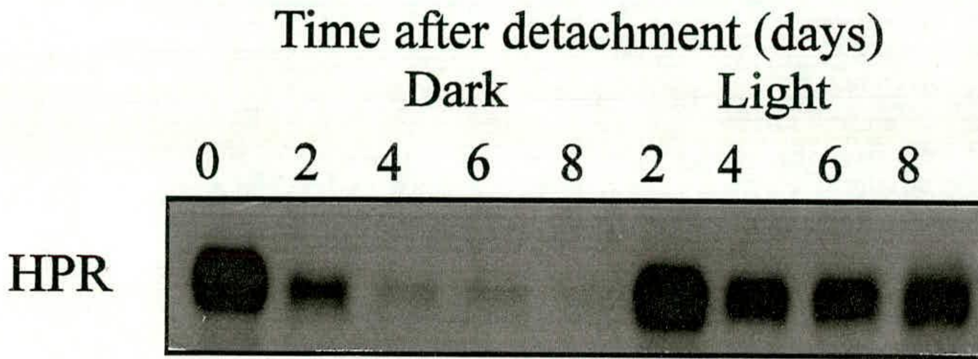


Figure 4.7. Northern blot analysis of total RNA isolated from cucumber cotyledons following detachment. RNA was extracted from cotyledons 14 days after seed imbibition (0) and at two-day intervals thereafter following incubation in darkness or under a 12 hour photoperiod. 10 μg total RNA was size fractionated, transferred to "Hybond-N" and the presence of transcripts encoding HPR was determined.

Cotyledons from 14 day-old seedlings were detached and incubated in darkness or under a 12 h photoperiod. Total RNA was isolated from detached cotyledons at 48 h intervals and the presence of MS and ICL mRNA determined. RNA gel blot analysis demonstrate that an increase in the steady-state levels of transcripts that encode MS and ICL is demonstrated within 2 days incubation of cotyledons in darkness and the levels of these transcripts continues to increase for eight days following detachment. Cotyledons incubated under a 12 h photoperiod exhibited no significant increase in the abundance of transcripts encoding MS and ICL, initially. However, at the later stages of the experiment a small increase was observed. The observation that transcripts encoding MS and ICL increase following the detachment and the dark-incubation of cucumber cotyledons support and extends previous reports of the accumulation of MS mRNA in detached cotyledons (Graham *et al.*, 1992).

4.4. Changes in the abundance of peroxisomal proteins following detachment of cucumber cotyledons.

4.4.1. Changes in immunodetectable SGAT.

Antisera raised against the leaf-type peroxisomal enzyme SGAT of cucumber (Hondred *et al.*, 1985) was used to provide useful information concerning changes in the levels of proteins involved in carbon fixation and also provide additional data regarding the postulated leaf-type peroxisome to glyoxysome transition associated with senescence. Antiserum raised against SGAT recognises two isoforms of the protein (Hondred *et al.*, 1985) and both isoforms of SGAT were detected in protein extract isolated form cotyledons 14 days after seed imbibition (Figure 4.9.). Following detachment and incubation of cotyledons in the light there does not appear to be any difference in the abundance of SGAT (Figure 4.9.). In the dark the amount of SGAT appears to remain constant during the earlier period of dark incubation and no decline in these proteins is detected until after 6 days when it declines to undetectable levels (Figure 4.9.). This supports previous observations that report a decline in the immunodetectable levels of GOX and the activity of HPR and GO in artificially induced senescent pumpkin cotyledons (De

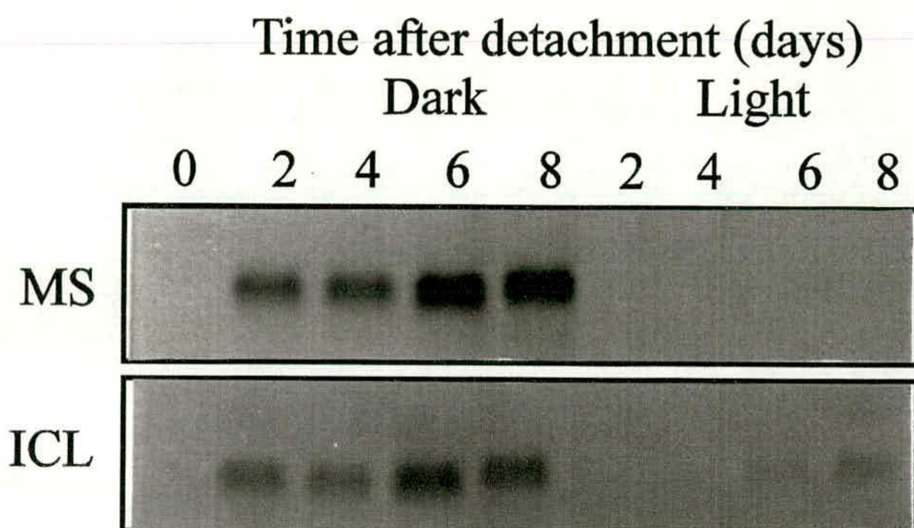


Figure 4.8. Northern blot analyses of total RNA isolated from cucumber cotyledons following detachment. RNA was extracted from cotyledons 14 days after seed imbibition (0) and at two-day intervals thereafter following incubation in darkness or under a 12 hour photoperiod. 10 μg total RNA was size fractionated, transferred to "Hybond-N" and the presence of transcripts encoding MS and ICL was determined.

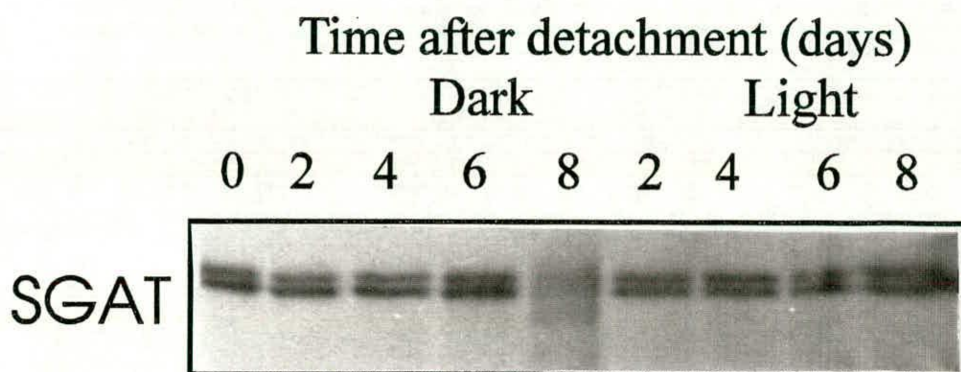


Figure 4.9. Immunoblot analysis of total protein isolated from cotyledons following detachment. Total protein was extracted from cotyledons 14 days after seed imbibition (0) and at two day intervals thereafter following incubation in light or dark conditions. 15 mg protein was separated on 10% SDS-polyacrylamide gels, transferred to "Hybond-ECL" and the presence of both isoforms of SGAT was determined.

Bellis and Nishimura, 1991). Additionally, these data are consistent with the role light plays in the accunmulation of SGAT (Hondred *et al.*, 1985, 1987).

4.4.2 Changes in immunodetectable levels of MS and ICL.

Antisera raised against purified preparations of cucumber MS (Riezman *et al.*, 1980) and ICL (Lamb *et al.*, 1978) were used to detect changes in the abundance of these proteins following detachment. MS and ICL were undetectable in protein extracts isolated from cotyledons 14 days after seed imbibition (Figure 4.10). Following detachment and incubation of cotyledons in continuous darkness, immunodetectable MS and ICL appear after 2 days and increase thereafter (Figure 4.10.). MS and ICL were not detected in light-incubated cotyledons. The accumulation of MS and ICL in the dark is essentially the same as the induction of transcripts encoding these proteins (Figure 4.7). Additionally, the observation that no MS and ICL accumulates in light-incubated cotyledons would support the proposal that wounding does not lead to the accumulation of glyoxysomal enzymes. These data are consistent with the observation that MS protein accumulates in detached dark-incubated leaves and cotyledons of both cucumber (Graham *et al.*, 1992) and pumpkin (De Bellis and Nishimura, 1991)

4.5. Discussion.

4.5.1. Characterisation of changes following detachment.

Changes associated with dark incubation of green tissue have been reported in a number of species (for review see Nooden, 1988). However, it is important in an attempt to define a role for glyoxylate cycle enzymes in detached organs to determine changes in cotyledonary protein, RNA, lipid, chlorophyll, carotenoid, cell number and dry weight. Collectively, the analysis of the changes in these cotyledonary constituents indicates that senescence-like changes associated with dark-incubation do not appear to occur until 4-6 days incubation. It is anticipated that the determination of changes in these ,and other, parameters and subsequent correlation with the induction of glyoxylate cycle enzymes may provide

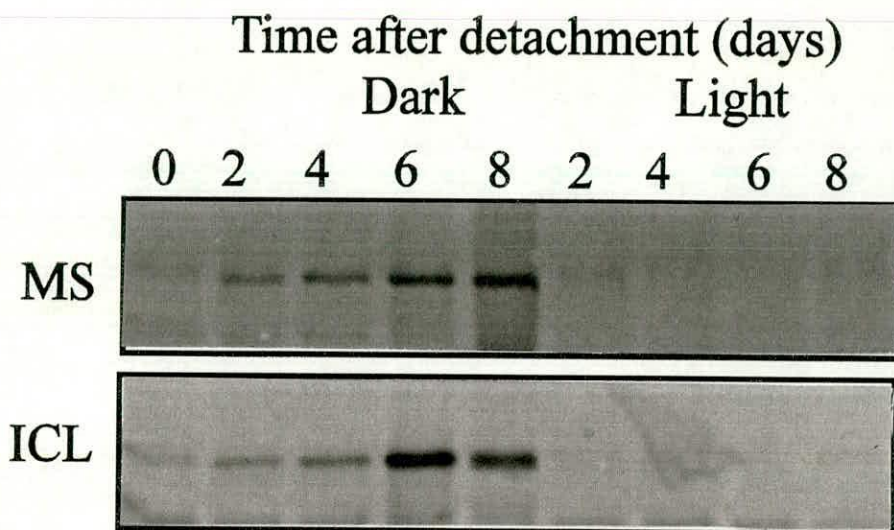


Figure 4.10. Immunoblot analyses of total protein isolated from cotyledons following detachment. Total protein was extracted from cotyledons 14 days after seed imbibition (0) and at two day intervals thereafter following incubation in light or dark conditions. 15 mg protein was separated on 10% SDS-polyacrylamide gels, transferred to "Hybond-ECL" and the presence of MS and ICL was determined.

information concerning a possible function for the glyoxylate cycle in dark-induced senescent organs.

4.5.1.1. Changes in cotyledon cell number.

Cotyledon cell number does not change significantly in light-incubated cotyledons and this appears to support microscopical analysis of the ultrastructral changes in detached cucumber cotyledons incubated in the light (Butler, 1967). In that study it was observed that the plasma membrane remained intact in light-incubated cotyledons for an extensive period of time (Butler, 1967). Similarly, in dark-incubated cotyledons there was no change in cell number indicating that no cellular breakdown had occurred. However, after 8 days there appears to be a 20% decline in cotyledonary cell number. However, as senescence is associated with membrane deterioration, the addition of chromic acid may have lead to the breakdown of cells and consequently lead to a false determination of cell number. Consequently alternative methods, such as isolation of protoplasts, of determining cell number may have to be employed to confirm the data presented in Figure 4.1.

4.5.1.2. Change in chloroplast structure.

The collective analysis of the changes in chlorophyll (Figure 4.3a), carotenoids (Figure 4.3b) and galactolipdis (Figure 4.4a,b) give a measure of the change in chloroplast structure following detachment of cucumber cotyledons. The amounts of chlorophyll, carotenoids and both MGDG and DGDG do not appear to decline significantly in detached, light-incubated cotyledons from the amounts determined in cotyledons 14 days after seed imbibition. Consequently this would indicate that detachment and subsequent incubation of cucumber cotyledons under a 12 h photoperiod has no demonstrable effect on chloroplast structure for up to 8 days. This is compatible with previous microscopical analysis of changes in the ultrastructure of attached and detached cucumber cotyledons in which little change was observed during the first 7 days of incubation (Butler, 1967). Dark incubated cotyledons, however, demonstrate a decline in the amount of chlorophyll and galactolipid. The levels of both chlorophyll and galactolipids appear to decline following 4 and 6 days incubation respectively (Figure 4.3a and Figure 4.4.).

Consequently the changes in both these compounds would appear to demonstrate a deterioration in thylakoid membrane structure associated with dark-incubation of cucumber cotyledons. These data appear consistent with previous reports detailing ultrastructural changes following detachment and incubation of photosynthetic organs in darkness (for review see Butler and Simon, 1971). It is important to note that differences appear to exist between the temporal decline in chlorophyll and galactolipids in cucumber and other plants. No significant decline in these parameters appears detectable until after 4 to 6 days, this is in contrast to similar experiments with barley leaf segments (Gut and Matile, 1988; Wanner *et al.*, 1990) and radish cotyledons (Kawakami and Watanabe, 1988a) in which a detectable decline in MGDG and chlorophyll has been reported very soon after detachment. For instance in detached barley leaves, MGDG declined to only 15% within 4 days (Gut and Matile, 1989). Clearly this does not occur in detached cucumber cotyledons, but MS and ICL are synthesised. Consequently, the role of the glyoxylate cycle in the metabolism of thylakoid lipids during senesecence requires further analysis.

It is interesting to note that carotenoids are still present in amounts very similar to those determined in cotyledons 14 days after seed imbibition (Figure 4.3c). Initially, this would appear inconsistent with the observable decline in chlorophyll and galactolipids. However, it has been reported that coupled with the disassembly of thylakoid membranes during the senescence of a number of plant species there is an accumulation of osmiophillic particles in the stroma that appear to contain abundant amounts of carotenoids (Butler and Simon, 1971; Dodge, 1970). The significance of this remains to be established. In photosynthetic tissue carotenoids are involved in the quenching of singlet oxygen produced in photosynthetic tissue (Bensasson *et al.*, 1976; Foote, 1970). Associated with artificially induced and natural senescence is an increase in the levels of free radicals such as singlet oxygen (Knox and Dodge, 1985; Thompson *et al.*, 1987) The presence of carotenoids in detached, dark incubated cotyledons may be a response to increased amount of singlet oxygen and other free radicals present in senescent tissue and carotenoids may modulate the activity of these highly reactive species.

4.5.1.3 Change in phospholipid content.

In addition to the demonstrable changes in the galactolipids MGDG and DGDG, TLC analysis of lipid extracts also provides information concerning the changes in abundance of phospholipids PC and PE (Figure 4.4.). In light-incubated cucumber cotyledons there does not appear to be any major decline in the abundance of PC and PE compared with cotyledonary lipid extracts isolated from seedlings 14 days after seed imbibition, and this may indicate that detachment and incubation of cucumber cotyledons under a 12 h photoperiod does not lead to any major changes in the lipid composition of extraplastidial membranes. Similarly, there does not appear to be any change in the amount of PC and PE in lipid extracted from dark-incubated cucumber cotyledons until after 6 days incubation. Cotyledons incubated in complete darkness for 8 days are characterised by a decline in the amounts of both PC and PE. This indicates that the deterioration of microsomal and mitochondrial membranes has occurred. It is of interest to note that phospholipids are still present in cotyledons following 8 days incubation in detectable amounts whereas galactolipids are not detectable. These data are in accord with previous analysis of the changes in lipid composition of detached cucumber cotyledons that demonstrated that galactolipids declined prior to the decline in phospholipids (Draper and Simon, 1970).

4.5.1.4. Changes in protein content and composition.

Analysis of protein indicates that no major decline in protein content is observed in cucumber cotyledons incubated under a 12 h photoperiod or in complete darkness for 8 days. Although this was initially considered a surprise, similar results have been reported during the dark incubation of detached pumpkin cotyledons where very little decline was observed during the first 8 days of the experiment (De Bellis and Nishimura, 1991). Consequently it would be anticipated that if the experimental period was extended beyond 8 days incubation in darkness, that loss of protein would have been observed.

SDS-PAGE analysis of protein extracted form light-incubated cotyledons does not appear to show any change in the polypeptide composition (Figure 4.5b).

Similarly, the polypeptide composition for cotyledons incubated in darkness does not appear to change significantly during the initial period of the experiment. However the amount of information obtained from the analysis of one dimensional SDS PAGE gels of total protein extracts is limited. A more informative approach would be to isolate soluble and membrane-bound protein extracts and use these samples in 2-dimensional gels. This approach has been undertaken for the analysis of the changes inproducts of translatable mRNA in *in vitro* translation assays (Becker and Apel, 1993; Kawakami and Watanabe, 1988a,b; Malik. 1987).

Protein extracts isolated from cotyledons following 8 days dark-incubation appear to be characterised by the accumulation of polypeptides with apparent molecular mass of 34, 60 and 90 kDa. It has been reported that following ethylene treatment of cucumber cotyledons an induction of peroxidases with apparent molecular mass of 33 and 60 kDa is observed (Abeles *et al.*, 1988). As peroxidases have been implicated in the degradation of chlorophyll (Matile, 1980; Yamauchi and Minamide, 1985) the possibility that these enzymes are peroxidases does appear attractive. However, in an earlier study of detached, light incubated cucumber cotyledons it was observed that there was a 7-fold increase in peroxidase activity within 4 days after detachment (Lewington *et al.*, 1967). No information concerning changes in dark-incubated excised cotyledons was presented. Consequently if these polypeptides were peroxidases they may be observed in protein extracts isolated from light incubated cotyledons. It is important to identify these proteins to aid our understanding of the senscence process. This may achieved by using antibodies raised against peroxidases or from further purification and subsequent analysis of amino acid sequence.

4.5.1.4. Changes in transcripts encoding cucumber LHCPII, RuBisCO small subunit and HPR.

Northern blot analysis of total RNA isolated from cotyledons 14 days after seed imbibition and following detachment, using cDNA clones encoding the small subunit of RuBisCO, LHCPII and HPR all demonstrate similar patterns of transcript abundance. Following detachment and subsequent dark treatment the levels of all transcripts appear to decline to undetectable levels within four days.

Furthermore, no significant decline in the level of all three transcripts is observed in light-incubated cotyledons and indicates that it is unlikely that a wound response, as a consequence of detachment, contributes to any decline in transcripts observed in dark-treated organs. This is consistent with previous data that analysed the change in steady-state levels of transcripts encoding cucumber HPR (Hondred *et al.*, 1987) and LHCPII (Greenland *et al.*, 1987) in cucumber cotyledons during the first 7 days following seed imbibition. Transcripts were only detected in the cotyledons of light-grown seedlings: no significant accumulation of either species of mRNA was detected in the cotyledons of dark grown plants. Additionally, following the transfer of light-grown cucumber plants to darkness the levels of transcripts encoding HPR declined 20-fold within four days (Greenler and Becker, 1990). This has also been observed for transcripts encoding RbcS in soybean, pea and *Lemna* leaves (Tobin and Silverthorne, 1985), and glutamine synthase in pea leaves (Edwards and Coruzzi, 1989). Analysis of the changes in transcripts encoding LHCPII following detachment appears identical to that reported for transcripts encoding RbcS and HPR, indicating that light was the primary factor in controlling the accumulation of this transcript. However, quantitative dot-blot analysis of RNA isolated from both dark- and light-grown cucumber plants (Greenland *et al.*, 1987) demonstrated that significant amounts of transcripts encoding RbcS accumulated in the dark (approximately 20-40% of the levels observed in light grown plants). Consequently, from these data it was concluded that an additional, light-independent factor was involved in the accumulation of transcripts encoding RbcS in cucumber. Generally, the decline in the steady-state levels of transcripts encoding HPR, RbcS and LHCPII in detached cotyledons incubated in darkness is compatible with the general observation that light plays a primary role in controlling the abundance of such mRNA species.

4.5.1.6. Changes in the levels of immunodetectable amounts of SGAT.

Antibodies raised against purified preparations of cucumber SGAT were used to determine the decline in both leaf-peroxisomal protein and to characterise the decline in photosynthetic function of detached cucumber cotyledons. In extracts isolated from cotyledons 14 days after seed imbibition both isoforms of SGAT were detected at levels comparable to those demonstrated in Chapter 3. Following

detachment and incubation of cotyledons under a 12 h photoperiod no change in the immunodetectable levels of both isoforms is observed. No significant decline in the levels of SGAT was observed in cotyledons incubated in darkness until after 6 days when the amount of SGAT declined to levels below detectable limits. This appears consistent with the observable pattern of SGAT protein accumulation in cotyledons during post-germinative growth of cucumber seedlings: no significant accumulation of SGAT is observed in dark-grown plants (Hondred *et al.*, 1987). Additionally, these data support other reports that have demonstrated a decline in both the amount of immunodetectable glycolate oxidase and in the activities of HPR and GO in detached dark-incubated pumpkin cotyledons (De Bellis and Nishimura, 1991).

4.5.1.7. The peroxisome glyoxysome transition

The observation that the decline in HPR transcripts and SGAT protein is coupled with an increase in glyoxylate cycle enzymes would appear to support the proposal that during senescence of photosynthetic organs a transition of the microbody population from leaf-type peroxisome to glyoxysome is evident (Gut and Matile, 1988; De Bellis and Nishimura, 1990). However, given the demonstrable effect light plays in the accumulation of SGAT protein and transcripts encoding HPR (Hondred *et al.*, 1985, 1987; Greenler and Becker, 1990) it would appear that such a decline is due primarily to the incubation of cotyledons in dark. During natural senescence of cucumber cotyledons it is evident that SGAT protein (Figure 3.7) remain at detectable levels in cotyledons during periods of MS and ICL accumulation (Figures 3.8. and Figures 3.9).. Consequently it would appear that the proposed transition of leaf type peroxisomes to glyoxysomes is unlikely to be aphenomenon associated with senescence *per se* but also occurs following the incubation of explants in darkness. However, it would be of interest to determine if such a transition conforms to the one-population model observed in microbodies during post-germinative growth of plants exhibiting epigeal germination (Nishimura *et al.*, 1986; Sautter, 1986; Titus and Becker, 1985).

4.5.2. Control of synthesis of glyoxylate cycle enzymes in detached cucumber cotyledons.

The data presented in this chapter demonstrate clearly that associated with incubation of detached cucumber cotyledons in darkness there is a coordinate synthesis of MS and ICL. These data are in accordance with previous reports demonstrating the synthesis of both MS and ICL in detached dark incubated organs (De Bellis *et al.*, 1990; De Bellis and Nishimura, 1991; Graham *et al.*, 1992; Gut and Matile, 1988; Landolt and Matile, 1990).

The results presented demonstrate that the induction of MS and ICL protein synthesis occurs in detached, dark-incubated cucumber cotyledons and that this occurs primarily through increases in the steady-state levels of the transcripts encoding these proteins (Figures 4.8 and 4.10). This supports the earlier observation that detached, dark-incubated leaves from *Nicotiana plumbaginifolia* plants genetically transformed with a ß-glucuronidase gene under the control of a cucumber MS promoter accumulated significant amounts of the reporter gene product, indicating that transcription is the primary controlling factor in the expression of MS (Graham *et al.*, 1992). Similarly, during post-germinative growth of cucumber, the coordinate synthesis of MS and ICL appears to be brought about primarily by increase in the steady-state levels of transcripts encoding these proteins (Smith and Leaver, 1986; Weir *et al.*, 1980). During embryogenesis and post-germinative growth in *Brassica napus* the synthesis of MS and ICL is brought about by an increase in the level of transcription (Comai *et al.*, 1989).

Northern blot experiments indicate that in total RNA extracted from cucumber cotyledons incubated under a 12 h photoperiod for 8 days there appears to be a low but detectable amount of transcript encoding ICL (Figure 4.8). However, no ICL protein was detected in extracts isolated from cotyledons incubated in the light (Figure 4.10). The significance of this observation remains to be established. Although it has been demonstrated that transcripts encoding cucumber HPR, RbcS and LHCPII remain at similarly abundant levels in detached cotyledons incubated

under a 12 h photoperiod for up to 15 days (data not shown), senescence-like changes will occur (Butler, 1967; Lewington *et al.*, 1967). Consequently, an increase in the steady state levels of transcripts encoding ICL may occur in light-incubated cotyledons. The additional observation that there appears to be no immunodetectable ICL in light-incubated cotyledons may be due to a lag between gene transcription and mRNA translation. Allen and co-workers reported a lag between appearance of transcripts encoding ICL and the accumulation of the protein during post-germinative growth of sunflower (Allen *et al.*, 1988). Additionally, it has been observed that small amounts of transcripts encoding MS have been detected in total RNA isolated from green, intact cucumber cotyledons (Graham *et al.*, 1992). This observation may indicate that MS and ICL may be involved in additional processes that are not associated with post germinative growth or senescence. However, further analysis of the synthesis of MS and ICL has to be undertaken before any firm conclusions on the role of these enzymes can be made.

Attempts to assay the activity of MS and ICL in crude homogenates isolated from detached cucumber cotyledons was unsuccessful. This was presumably due to inhibitory factors present in the homogenate used in the assay (data not shown). Similarly, attempts to assay the activity of MS and ICL in crude extracts isolated form detached leaves and cotyledons were also unsuccessful (I. Graham, personal communication, De Bellis *et al.*, 1990; Pistelli *et al.*, 1991). Attempts to assay ICL activity in extracts isolated from developing embryos of cucumber and sunflower have also proved to be problematical (Frevert *et al.*, 1980; Fusseder and Theimer, 1984; Theimer, 1976). De Bellis *et al.* (1990) observed that activity of MS was only detectable in a peroxisomal-enriched fraction isolated from artificially-induced and naturally senescent pumpkin leaves. Similarly, it was observed that ICL enzyme activity was only detected during embryogenesis following purification of a peroxisome-enriched fraction (Fusseder and Theimer, 1976).

It has been reported that MS from endosperm of germinating *Ricinus communis* is phosphorylated in a calcium dependent manner indicating that the

activity of MS may be controlled by a poorly characterised, post-translational mechanism (Yang *et al.*, 1988). During dark-induced senescence of pumpkin cotyledons the pattern of MS enzyme activity and accumulation of the protein was very similar, indicating in this instance, that post-translational control of MS was not a significant controlling factor (De Bellis and Nishimura, 1991). However, it is important to assay the activity of MS and ICL in detached cucumber cotyledons, to fully characterise the induction of glyoxylate cycle enzymes.

4.5.3. Function of MS and ICL in detached cucumber cotyledons.

It has been proposed that MS and ICL are synthesised in detached leaves to metabolise the products of lipid breakdown (Gut and Matile, 1988). However it has been demonstrated here that no significant decline in the amount of MGDG, DGDG or any other lipid (Figure 4.4a,b.) occurs during the initial period of MS and ICL accumulation (Figure 4.9. and 4.11.). This does not preclude the possibility that the glyoxylate cycle is involved in thylakoid lipid metabolism during the latter stages of artificiality induced senescence but does provide some evidence that MS and ICL may be involved in an additional or alternative metabolic pathway prior to lipid breakdown.

As the activity of MS and ICL was not demonstrated it may be possible that an inactive form of MS and ICL may accumulate prior to lipid breakdown and post-translational mechanism may lead to activation subsequently during periods of lipid breakdown (eg. by protein phosphorylation, Yang *et al.*, 1988). In pumpkin it has been demonstrated that MS and ICL activity is very similar to the pattern of protein accumulation in detached, dark-incubated cotyledons (De Bellis and Nishimura, 1991). This would indicate that the activity of glyoxylate cycle enzymes does not appear to be controlled by any post-translational modification. However, studies must be undertaken to confirm the activity of MS and ICL enzyme activity in detached cucumber cotyledons.

It has been proposed that MS may have alternative roles during purine catabolism (Rodriguez *et al.*, 1990), citric acid biosynthesis (Miernyk and

Trelease, 1981) and glycine metabolism (Dieuaide *et al.*, 1992). However the coordinate expression of MS and ICL in detached cotyledons indicate that these pathways may not be involved in this case. Additionally no significant difference in the decline in levels of total RNA is observed between dark and light-incubated cotyledons (Figure 4c) indicating that MS and ICL may not be involved in breakdown of purines from polynucleotides. More rigorous analysis is required to determine fully the relationship between purine degradation and glyoxylate cycle enzymes during senescence and following detachment. It is important to note that although MS and ICL are synthesised prior to any significant decline in cotyledonary lipid the glyoxylate cycle may still be involved in gluconeogenesis at a later stage.

4.6. Conclusion.

It is clear from the results presented in this chapter that there is a coordinate synthesis of MS and ICL in detached dark-incubated cucumber cotyledons. However, there appears to be no correlation between the accumulation of MS and ICL and decline in cotyledoary lipid, cholrophyll, carotenoid, protein and RNA. The collective analysis of the accumulation of MS and ICL in dark-induced and naturally senescent cotyledons and the changes in cotyledon components would indicate that the role of the glyoxylate cycle still remains to be established.

From the collective analysis of data on the synthesis of MS and ICL during germination, senescence and in suspension cultures of higher plant cells, it has been proposed that the glyoxylate cycle may be repressed by the presence of sugars and activated by the products of lipid breakdown (Graham *et al.*, 1992). Detached cucumber cotyledons provide a useful experimental system in which the synthesis of glyoxylate cycle enzymes can be readily induced. Additionally, metabolites that may potentially control the synthesis of glyoxylate cycle enzymes may be introduced to determine the effect these molecules have on the synthesis of MS and ICL. Also, endogenous metabolites may also be measured in detached cotyledons following various treatments. The aim of subsequent experiments was to determine if exogenous sugars and other metabolites could be employed to regulate

the synthesis of MS and ICL, and to establish an experimental system to investigate the metabloic regulation of gene expression.

CHAPTER 5

EFFECT OF SUCROSE ON THE SYNTHESIS OF MALATE SYNTHASE AND ISOCITRATE LYASE IN PROTOPLASTS AND DETACHED CUCUMBER COTYLEDONS.

5.1. Rationale.

From the analysis of MS and ICL enzyme synthesis in higher plants, it has been proposed that the synthesis of glyoxylate cycle enzyme is repressed by sugars and that products of lipid breakdown (acetate or acetyl-CoA are candidate molecules) induce expression (Graham *et al.*, 1992). Additionally, there is some evidence that glyoxylate cycle enzymes may be involved in purine degradation (Rodriguez *et al.*, 1990). The induction of glyoxylate cycle enzyme synthesis following the incubation of detached cotyledons in darkness provides a useful, easily manipulated experimental system in which the effect of the introduction of metabolites, that may regulate the synthesis of MS and ICL, can be investigated. Additionally, protoplasts provide a potentially superior experimental system in which to study metabolic regulation of gene expression, since metabolites can be more effectively delivered to single cells than to whole organs (Loake *et al.*, 1990; Sheen, 1989). This chapter describes the effect the addition of sucrose has on the synthesis of MS and ICL under conditions where glyoxylate cycle enzyme synthesis occurs.

5.2. The role of sucrose in controlling the synthesis of MS and ICL in detached cucumber cotyledons.

5.2.1. Change in endogenous sucrose content in detached cucumber cotyledons.

Given that the synthesis of MS and ICL is readily induced in detached, dark incubated cucumber cotyledons (Chapter 4), it would be of interest to determine if a correlation exists between glyoxylate cycle enzyme synthesis and change in endogenous sucrose content. Cucumber cotyledons were detached from seedlings 14 days after seed imbibition and incubated under a 12 h photoperiod or in darkness. Sucrose content was determined at 48 h intervals following detachment (Figure 5.1.). Within 2 days after detachment there is a 4 to 5-fold increase in the amount of sucrose of light-incubated cotyledons. Thereafter, the amount of sucrose declines, but remains at significantly higher levels than dark-incubated cotyledons. Surprisingly, the level of sucrose remains at similar levels for that determined for light-grown cotyledons 14 days after seed imbibition.

The accumulation of sucrose in light-incubated cotyledons indicates that detachment prevents translocation of photosynthate that is continued to be produced after excision. This is consistent with the observation that levels of

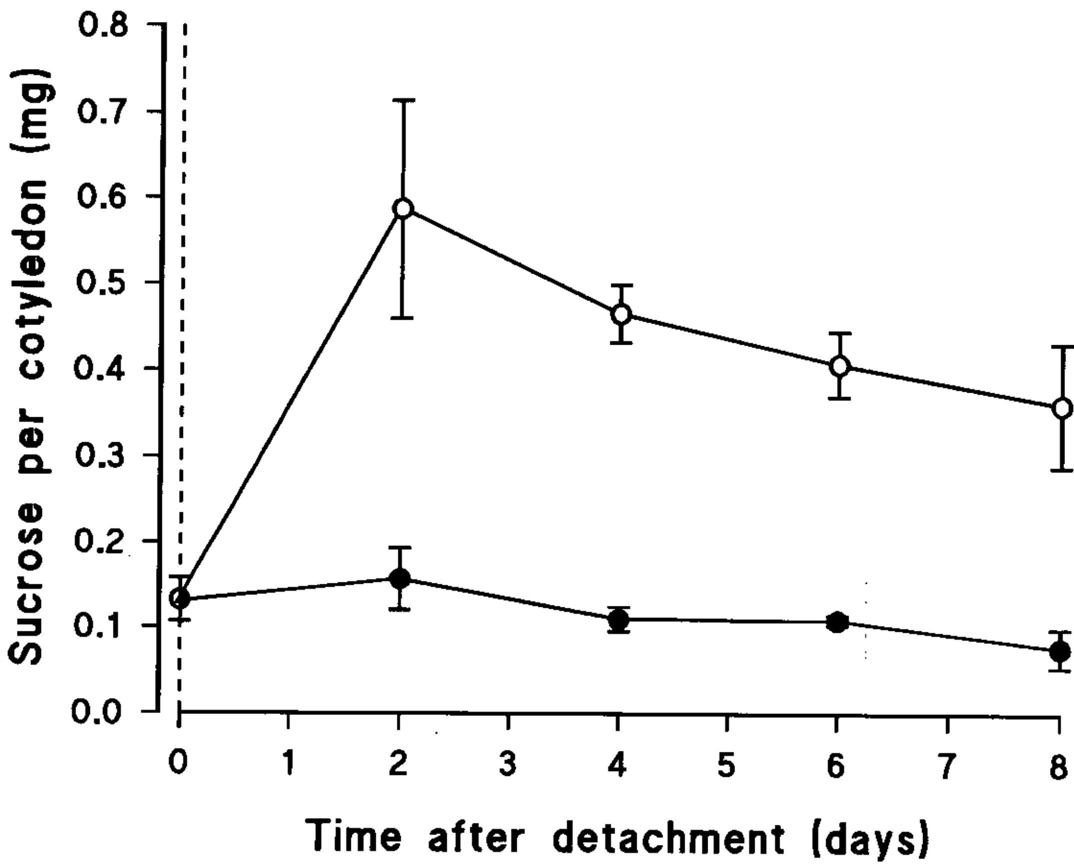


Figure 5.1. Change in sucrose content of cucumber cotyledons following detachment. Sucrose content was determined for cotyledons 14 days after seed imbibition (◑) and at 48 h intervals after detachment and incubation in light (○) and darkness (●) error bars represent the SEM of four replicate samples.

immunodetectable SGAT and transcripts encoding RbcS, LHCPII and HPR remain constant following detachment and incubation of cotyledons under a 12 h photoperiod (Chapter 4). Translocation of photosynthate from cotyledons would lead to the maintenance of sucrose at low levels similar to the amount determined for dark incubated cotyledons for up to 8 days. Given the demonstrable pattern of glyoxylate enzyme synthesis in detached cucumber cotyledons and the observation that sucrose levels do not change following incubation in darkness, this would indicate that decline in sucrose may not be directly involved in derepression of the synthesis of MS and ICL gene expression. However, the observation that sucrose levels increase in light-incubated cotyledons may indicate that increased sucrose levels may inhibit the accumulation of MS and ICL.

5.2.2. Exogenous sucrose represses glyoxylate cycle enzyme synthesis in detached cucumber cotyledons.

Synthesis of MS and ICL in detached, dark-incubated cotyledons is detectable within 48 h (Chapter 4). Consequently, the effect of the addition of sucrose on the synthesis of MS and ICL was determined. Cotyledons were detached from seedlings 14 days after seed imbibition, incubated in darkness for 48 h in the presence of 25 mM sucrose, 25 mM mannitol or water. Northern blot analysis (Figure 5.2a.) indicates that the presence of 25 mM sucrose prevents an increase in the steady-state levels of transcripts encoding MS and ICL, relative to cotyledons incubated in water and 25 mM mannitol. Similarly, western blot analysis (Figure 5.2b.) indicates that the presence of 25 mM sucrose completely reduces the amount of immunodetectable MS and ICL. Therefore, it would appear that the presence of exogenous sucrose inhibits the accumulation of MS and ICL and that this inhibition is brought about primarily through preventing an increase in the steady-state levels of the transcripts encoding these proteins.

5.2.3. The effect of exogenous sucrose on the accumulation of MS and ICL in cucumber protoplasts.

Protoplasts provide an additional experimental system in which to examine the effect of exogenous metabolites on the synthesis of MS and ICL. Cucumber leaf protoplasts synthesise MS and ICL when incubated in the dark in the absence of sucrose (Ian Graham, personal communication). Therefore, to inhibit the synthesis of MS and ICL, cotyledon protoplast isolation was carried out in the presence of 25

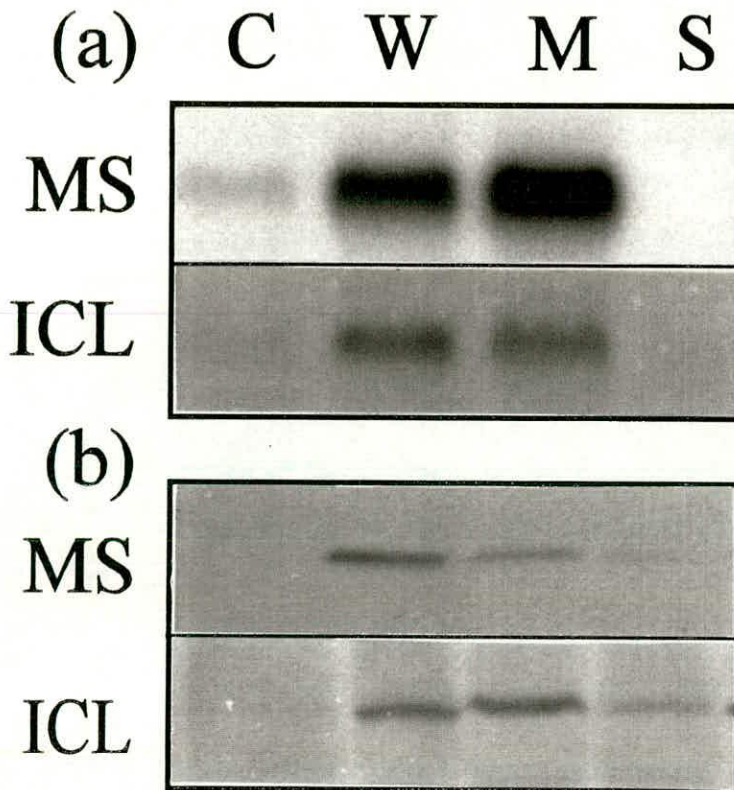


Figure 5.2. Endogenous sucrose prevents the synthesis of MS and ICL in detached, dark-incubated cotyledons. (a) total RNA was isolated from cotyledons 14 days after seed imbibition (C) and 48 h after incubation in water (W), mannitol (M) and sucrose (S). 10 μg total RNA was size fractionated, transferred to "Hybond-N" and the presence of transcripts encoding MS and ICL was determined. (b) total protein was isolated from cotyledons 14 days after seed imbibition (C) and 48 h after incubation in water (W), mannitol (M) and sucrose (S). 15 mg protein was separated on 10% SDS-polyacrylamide gels, transferred to "Hybond-ECL" and the presence of MS and ICL was determined.

mM sucrose in addition to 475 mM mannitol. Following isolation, protoplasts were incubated in darkness in the presence of mannitol or sucrose and mannitol for 48 h. Total protein was subsequently isolated and the presence of MS and ICL determined (Figure 5.3.). Following isolation of protoplasts in the presence of 25 mM sucrose and 475 mM mannitol there appeared to be no accumulation of MS and ICL. Following transfer of protoplasts to media contain 500 mM mannitol there was an increase in the amounts of immunodetectable MS and ICL. These data indicate further that sucrose can inhibit the accumulation of MS and ICL.

5.3. Discussion.

5.3.1. Regulation of MS and ICL gene expression by sucrose.

The results presented in this chapter are consistent with previous reports that indicate that the addition of sugars can inhibit the synthesis of MS and ICL (Kudiekla and Theimer, 1983a, b; Slack *et al.*, 1977;) and it would appear to support the model proposed by Graham *et al.* (1992). However, the observation that sucrose can be maintained at low levels and the synthesis of MS and ICL is repressed would indicate that a decline in sucrose content does not lead to the synthesis of MS and ICL. This is supported by the observation that glucose can also repress the synthesis of glyoxylate cycle enzyme synthesis (Longo and Longo, 1970; Lado *et al.*, 1968). It would be more likely to assume that the repressor compound is produced during photosynthesis and as a consequence of sugar metabolism.

These experiments indicate the potential usefulness of directly assaying changes in compounds that may control the synthesis of MS and ICL in detached cotyledons. This may lead to a positive correlation between MS and ICL synthesis and a decline in a specific metabolite. Additionally, once a correlation is established, the effect of these potential repressor compounds on the synthesis of MS and ICL in detached cotyledons and protoplasts may be directly determined. However, the concentration of metabolites in specifc subcellular comprtments is very difficult or impossible to measure. Addtionally, the fate of exogenous metabolites once taken by the cells is unknown.

Possible molecules that may lead to an induction of the synthesis of MS and ICL include acetate, acetyl-CoA, allantoin and PEP. However, data obtained from experiments where these potential activators are added to protoplasts and detached

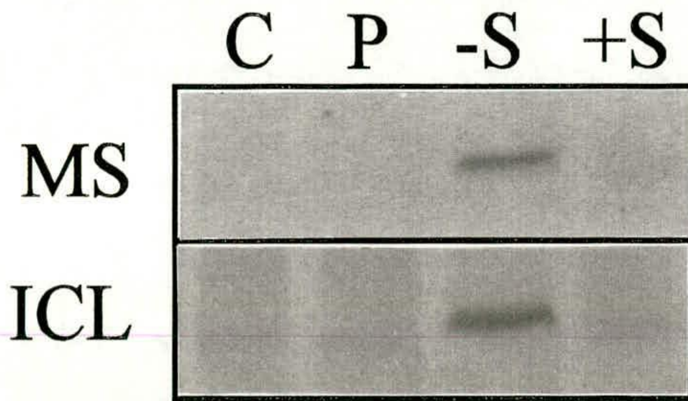


Figure 5.3. Endogenous sucrose prevents the accumulation of MS and ICL in dark incubated cucumber cotyledon protoplasts. Total protein was isolated from cotyledons 14 days after seed imbibition (C), freshly isolated protoplasts isolated in the prescence of 25 mM sucrose (P), protoplasts incubated in 25 mM sucrose for 48 h (+S) and protoplasts incubated in the absensce of sucrose for 48 h (-S). Total protein was size fractionated through a 10% polyacrylamide gel, transferred to "Hybond-ECL" and the presence of MS and ICL was determined.

cotyledons must be carefully interpreted. Data presented here indicate that MS and ICL synthesis is associated with cotyledonary senescence. The addition of some compounds may indirectly lead to an increase in the synthesis of MS and ICL by accelerating senescence-like processes. For example, the addition of 25 mM acetate to detached cucumber cotyledons lead to an increase in the steady-state levels of transcripts encoding MS and ICL relative to tissue incubated in water (data not shown). In a second experiment the addition of 3 mM acetate to protoplasts appeared to have no effect on the accumulation of MS and ICL, relative to protoplasts incubated in the presence of mannitol only (data not shown). This may indicate that at a physiological concentration (3 mM) acetate has no effect on the synthesis of MS and ICL and that the observed effect at 25 mM may be due a toxic effect, that indirectly leads to MS and ICL synthesis. This conclusion is based on preliminary data and consequently requires more rigourous analysis before any significant conclusion can be made.

The derepression of MS and ICL synthesis appears to be mediated primarily by the availability of transcripts. This would appear similar to other instances where the synthesis of MS and ICL has been observed in cucumber (Graham *et al., 1992*; Smith and Leaver, 1986; Weir *et al.*, 1980). However, although the addition of sucrose is associated with no detectable accumulation of MS and ICL, transcripts encoding these proteins are present at low levels. This may indicate that although MS and ICL synthesis is regulated primarily by the prevalence of transcripts, post-transcriptional processes may also be involved. Analysis of rates of transcription of genes encoding MS and ICL in *Brassica napus* indicate that post-transcriptional processes may play a secondary role in controlling the synthesis of MS and ICL (Comai *et al.*, 1989; Ettinger and Harada, 1990).

CHAPTER 6

CONCLUSIONS AND FUTURE WORK.

6.1. Function of glyoxylate cycle enzymes in non storage tissue.

The results presented in this thesis appear consistent with and extend previous reports that demonstrate the synthesis of glyoxylate cycle enzymes in senescent and detached organs (De Bellis *et al.*, 1990: De Bellis and Nishimura, 1991; Graham *et al.*, 1992; Gut and Matile, 1989; Landolt and Matile, 1990; Pistelli *et al.*, 1991). However, there does not appear to be any strong relationship between the synthesis of MS and ICL and the decline in the levels of the galactolipids MGDG and DGDG. These data are at variance to those reported by Gut and Matile (1988) in which an increase in the activity of MS and ICL in dark-incubated barley leaves is coupled with a decline in the amount of MGDG. Consequently, this indicates that there may be an alternative role for the glyoxylate cycle in detached and senescent organs.

6.1.1. Alternative roles for MS and ICL in senescent and detached cotyledons.

The observation that both MS and ICL are coordinately synthesised in detached and senescent cotyledons would indicate that acetyl-CoA is generated and the entire glyoxylate cycle is likely to be active. However, in some instances where the synthesis of MS and ICL has been observed, the activity of ß-oxidation enzymes could not be detected (De Bellis *et al.*, 1990; Pistelli *et al.*, 1991). Therefore, it is important to fully characterise the activity of enzymes involved in fatty acid ß-oxidation in senescent and detached organs These data could then be subsequently compared to the demonstrated increase in MS and ICL synthesis in detached and senescent cucumber cotyledons. Recently a cDNA clone encoding cucumber thiolase has been isolated (Preiseig-Muller and Kindl, 1993). Northern blot analysis of RNA isolated from senescent and detached cucumber cotyledons using this clone as a probe would provide useful data concerning the changes in the synthesis of enzymes involved in fatty acid ß-oxidation.

Further information concerning the activity of fatty acid ß-oxidation may be obtained from determining the fate of radiolabelled galactolipids, fatty acids and acetyl-CoA following detachment of cucumber cotyledons. Data obtained from this analysis could determine the relationship between galactolipid decline, fatty acid ß-oxidation and the synthesis of the enzymes of the glyoxylate cycle. Similarly, the affect of the addition of these compounds to detached cucumber

cotyledons, protoplasts or cells in culture on the synthesis of MS and ICL would also be determined. This would then provide useful information concerning the relationship between fatty acid degradation and the role of the glyoxylate cycle.

Pathways that may lead to the generation of acetyl-CoA in addition to ß-oxidation of fatty acids are poorly characterised. A source of substrate for fatty acid oxidation in non-fatty tissue can result from the degradation of branched chain amino acids leucine, isoleucine and valine in the course of steady state turnover in amino acids. It has been demonstrated that peroxisomes isolated from mung bean (*Vigna radiata* L.) hypocotyls could oxidise branched chain fatty acids which are produced from the transamination of leucine, isoleucine and valine (Gerbling and Gerhardt, 1988). Although there appears to be no correlation between protein decline and the synthesis of MS and ICL, detachment and senescence may be associated with a greater turnover of amino acids, that may precede the decline in protein content. Change in isoleucine, leucine and valine content of detached and senescent cotyledons may provide some information concerning the relationship between amino acid metabolism and the synthesis of MS and ICL. Additionally, the effect of the addition of branched chain amino acids on the synthesis of MS and ICL may also provide information concerning the pathways that are capable of degrading these compounds in senescent and detached cotyledons.

A common feature of situations where the synthesis of MS and ICL has been observed in non-fatty tissue appears to be carbon limitation. The consequences of carbohydrate starvation have been studied in a number of plant species. Sucrose starvation of sycamore cells in culture leads firstly to a decline in sucrose and starch and this is followed by the utilisation of fatty acids derived from membrane lipids as a source of ATP (Dorne *et al.*, 1987; Journet *et al.*, 1986; Roby *et al.*, 1987). Similar data were obtained from the analysis of maize root tips during glucose starvation (Brouquisse *et al.*, 1991). Recently, it has been demonstrated that glucose starvation leads to an increase in the ß-oxidation of radiolabelled palmitic acid to acetyl-CoA and increase in activity of MS and fatty acid ß-oxidation enzymes (Dieuaide *et al.*, 1992). Therefore, it would appear that the changes observed in detached and senescent cucumber cotyledons may be similar to those demonstrated during carbohydrate starvation of maize root tips and sycamore cells in culture.

A second well documented change associated with sucrose starvation is a decline in protein content, this is coupled with an increase in the amino acid asparagine which serves as a nitrogen store. It was proposed that amino acids produced during protein degradation would be utilised by the mitochondria in the synthesis of ATP (Genix *et al.*, 1990). However, no significant relationship was observed between MS and ICL synthesis and the degradation of protein in detached and senescent cucumber cotyledons. It would appear that the glyoxylate cycle is not directly involved in protein degradation in senescent and detached cucumber cotyledons.

It has been proposed that MS may play a role in degradation of purines during senescence (Rodriguez *et al.*, 1990). However, it is not clear if ICL is similarly involved in purine degradation: in higher plants the breakdown of purines leads to the formation of glyoxylate that would preclude the activity of ICL (Beevers, 1976). However, this remains to be firmly established in detached and senescent organs. Additionally, it has been demonstrated that two enzymes involved in purine degradation, uricase and allantoinase are present in the glyoxysomes of several higher plants (Theimer and Beevers, 1971). Consequently, it may be of interest to determine fully the relationship between purine degradation and the synthesis of glyoxylate cycle enzymes in detached and senescent organs. In some instances MS activity has been observed in the absence of any detectable ICL enzyme activity and it was proposed that MS was involved in glycine and citric acid metabolism (Dieuaide *et al.*, 1992; Miernyk and Trelease, 1981). However, as MS and ICL synthesis is observed, it is unlikely that the metabolism of glycine and citrate is important in detached and senescent cucumber cotyledons.

6.1.2. The use of "antisense" in reducing MS and ICL synthesis.

The employment of molecular biological techniques in the construction of transgenic plants containing "antisense" copies of a given cDNA sequence provides a potentially useful system in which the role of any given enzyme or protein can be determined . The effect of the specific reduction of MS and ICL synthesis could provide valuable information concerning the role of the glyoxylate cycle and the contribution this pathway plays in carbon partitioning during senescence. This technique could also be used in reducing the activity of enzymes involved in fatty acid ß-oxidation and reverse glycolysis.

Careful use of promoters that would direct the synthesis of antisense MS or ICL RNA is required. Although the role of MS and ICL is not fully understood during embryogenesis, a reduction in the synthesis of these enzymes at this important developmental stage may lead to the production of non viable seed. Similarly, the synthesis of antisense MS and ICL RNA may be undesirable during post germinative growth. Consequently, the use of MS or ICL promoters would not be desirable in specifically altering the expression of MS and ICL during senescence. Similarly, the use of the CaMV 35S promoter, that would direct the synthesis of abundant amounts of antisense RNA in cotyledon tissue, may be of little use in specifically altering the synthesis of MS and ICL at a given stage of development. The use of a promoter that directs the synthesis of antisense MS and ICL mRNA during senescence would be desirable. Other than MS and ICL very few promoters are available that would confer the expression of antisense genes during senescence.

One exception are the genes that encode 1-aminocyclpropane-1-carboxylase synthase (ACC synthase) from pumpkin (Sato and Theologis, 1989; Sato *et al.*, 1991). ACC synthase catalyses the rate limiting step in ethylene production in several plant species (for review, see Yang and Hoffman, 1984). The synthesis of ACC synthase occurs through changes in transcription of the gene encoding this protein. The transcription of these genes is increased by the addition of exogenous ethylene. Consequently, the promoter that directs the transcription of genes that encode ACC synthase may be useful in directing the synthesis of antisense MS and ICL RNA during senescence or following detachment. The application of exogenous ethylene provide a convenient means of inducing the synthesis of antisense MS and ICL RNA and initiating senescence concurrently. Additionally, wounding associated with excision may be sufficient to bring about the synthesis of ethylene in detached cucumber cotyledons that could direct the synthesis of antisense MS and ICL RNA.

Genetic transformation of cucumber has only been reported in a number of occasions and is far from routine. Additionally, it is not known if antisense cucumber MS and ICL RNA could lead to a reduction of MS and ICL synthesis in a heterologous host. Therefore, cDNA clones encoding MS and ICL are being cloned from potato and *Nicotiana*. These will subsequently used in the generation of transgenic plants containing reduced amounts of MS and ICL.

6.2. Metabolic and genetic regulation of the synthesis of MS and ICL.

The availability of cDNA and genomic clones that encode MS and ICL in cucumber provide a useful opportunity to study the genetic and metabolic factors that regulate the expression of genes of the glyoxylate cycle. Given that the control of MS and ICL synthesis appears to be primarily due to an increase in the steady-state level of the transcripts encoding these proteins, a study of the factors that regulate transcription of these genes will provide an understanding of the factors that control the synthesis of glyoxylate cycle enzymes.

The data presented in Chapter 5 demonstrate that the presence of 25 mM sucrose can inhibit the synthesis of MS and ICL. This supports previous reports that sucrose and glucose can prevent the synthesis of MS and ICL during post germinative growth and in culture (Kudielka and Theimer, 1983b; Lado *et al.*, 1968; Longo and Longo, 1970; Slack *et al.*, 1977). Additionally, this indicates that detached cucumber cotyledons and protoplasts could be used to determine which molecules may play an important role in regulating the synthesis of MS and ICL. However, very careful interpretation of such data is required. For instance, exogenous compounds may not be effectively taken up by the cells. Additionally, exogenously applied may be toxic and lead to an indirect increase in the synthesis of MS and ICL. Consequently, it must be determined that added metabolites are effectively taken up by cells and are supplied at concentrations that are non toxic to the cells. In bacteria the growth of cells on acetate is associated with the synthesis of MS and ICL. However, the addition of this metabolite can be toxic to higher plant cells and is not effectively taken up. The use of added metabolites on the synthesis of MS and ICL may not provide any meaningful data. However, the availability of cloned DNA sequences that encode MS and ICL provides an excellent opportunity to study the genetic regulation of MS and ICL gene expression. This may then provide useful data that may in turn be interpreted in terms of metabolic control.

As the synthesis of MS and ICL appears to be coordinate, sequence analysis of the promoter regions of these genes may reveal sequence motifs that may control the expression of these genes. However, to determine precisely the important elements of the 5' region of the genes that encode MS and ICL, a series of truncated promoter fragments fused to reporter genes such as GUS can be used in

the generation of transgenic plants. From the analysis of GUS activity of these transgenic plants the regions of the promoters that control the expression of genes encoding MS and ICL can be determined. Previously, it was reported that 1,098 bp of the 5' flanking region of MS was sufficient to confer spatial and temporal expression of GUS in transgenic tobacco and petunia plants (Graham *et al.*, 1990). Currently, truncated fragments of this promoter and a 2,000 bp 5' fragment of the gene encoding ICL are being analysed to establish important *cis*-acting elements of these genes in transgenic plants. Once important 5' flanking regions are determined, DNase I protection and DNA mobility shift assays could be employed in determining whether these regions of MS and ICL genes interact specifically with DNA binding factors in nuclear extracts isolated from cucumber. This approach may eventually lead to the determination of the signals that may induce the expression of MS and ICL.

The study of gene regulation using transgenic plants can be time consuming and can only be applied to a limited number of plants that are routinely genetically transformed. In contrast, transient expression in protoplasts can provide useful information relatively quickly in cells of cucumber. To date, transient expression assays have been used to study gene regulation by metabolites (Sheen, 1990), UV. light (Lipphardt *et al.*, 1988), heat shock (Callis *et al.*, 1988) abscisic acid (Marcotte *et al.*, 1988), anaerobic stress (Walker *et al.*, 1987) and intermediates of phenylpropanoid pathway (Loake *et al.*, 1990). However, it is important that the results of transient assays are interpreted very carefully. Following electroporation, the introduced plasmid is present in the cytoplasm at very abundant levels. Chromatin structure, transport and turnover of mRNA may play an important role in the expression of genes and the presence of abundant amounts of an introduced plasmid may perturb these factors. Ideally, results obtained from analysis of gene promoters using transient assays should also be presented with data obtained from transgenic plants containing the same constructs,

One major advantage of transient assays of gene regulation is the relative ease that metabolites can be delivered to the cells. If it has been established that a given promoter does confer the correct spatial and temporal expression of the reporter gene in transgenic plants, this may be of use in determining important metabolites that may control the regulation of genes encoding MS and ICL in cucumber. The results obtained from the analysis of the affects of added metabolites in a transient

assay can also be confirmed further by analysis of the changes of the endogenous genes in protoplasts and cells in culture. The integration of such analysis may then aid our understanding of the metabolites that may control the synthesis of MS and ICL.

In conclusion, the availability of genomic and cDNA clones encoding MS and ICL provides an ideal opportunity to study the function and regulation of MS and ICL in cucumber. Peroxisomes appear to be ubiquitous in all eukaroytic cells. However, the precise function of these organelles is unknown to a large extent. It now appears that glyoxysomes, in addition to being involved in mobilisation of storage lipid, may play other roles during plant development.

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